Effect of diet and physical activity on the markers of oxidative stress

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September, 2004

Acknowledgements

I would like to thank all volunteers, who took time to participate in this study.

Thank you to **Chris Whyburd** and **Yan Wang** for their help in making my everyday laboratory work efficient by solving some little (and sometimes big) technical problems.

A special thanks to **Roger Whiting** for his cooperation and willingness to discuss and share some of my concerns and uncertainty.

Thanks to **Michelle Cumin** for sharing, discussing and analysing the information throughout the study period, for her compassion and great spirit.

Thanks to **Lynn Ferguson,** my co-supervisor for her help and encouragement.

I would like to acknowledge the generous support of Glycoscience Research Center for allowing to use their Freezer for storing the precious volunteer plasma samples.

Thank you to all the administrative staff at AUT including **Heather Stonyer**, **Benneth Gilbert** and **Annette Tiaiti** for their help and support**.** A special thanks to the **Zespri International** for the supply of the kiwifruit used in this study.

The most important and special thanks to my supervisor **Elaine Rush** for always being there for me, for her generosity and patience, for her advices and support, for her encouragement and compassion, for always pushing me for perfection and for her special role in my professional development.

Statement of originality

‘I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgement is made in the acknowledgements’.

Signed ……

Date…….

Abstract

Lifestyle diseases such as cardiovascular diseases, hypertension, cancer, type 2 diabetes are the major causes of mortality and morbidity worldwide. The prevalence of these diseases is high in New Zealand as well. It is believed that promoting a healthy diet and increased physical activity can make beneficial changes and extend the healthy life expectancy.

This twelve week study with follow-up at 52 weeks was designed to demonstrate if a diet and physical activity group intervention in the workplace would result in changes in risk factors for antioxidant damage and therefore reduce the risk for lifestyle diseases. The effect of the addition of kiwifruit to the diet on markers of oxidative stress was also measured in a crossover substudy within this study.

Fifty two healthy subjects (male 24, female 28, mean age 46), completed the 12 week study with measurements points being at 0, 3, 6, 9 and 12 weeks. Thirty eight subjects presented for another set of measurements at 52 weeks. The intervention started at week 3 and the kiwifruit crossover treatment was launched between weeks 6 and 12.

Ferric reducing ability of plasma (FRAP) assay was utilized to measure the changes in plasma antioxidant activity(AOA) and thiobarbituric acid reactive substances (TBARS) assay to measure the changes in plasma malondialdehyde (MDA), as a marker of lipid peroxidation (LP) at each measurement point. Since almost all participants had a normal range of baseline measurements of plasma AOA and LP (plasma MDA), they were categorized as relatively low and high AOA (1200micromol/L cut off point) and LP groups (1.70mmol/L cut off point), as well as divided into male and female groups.

The effect of changed diet and increased physical activity during the 12 week study period resulted in a significant increase ($P<0.05$) in plasma AOA. The changes were much higher in low AOA group ($P=0.005$) and in male subjects ($P<0.005$), while no changes were observed in subjects with already high AOA at baseline. The increased plasma AOA level

was maintained and increased even more over the year. No changes were observed in LP (plasma MDA). The effect of kiwifruit on the markers of oxidative stress was modest, the 3-week daily kiwifruit consumption (2-3 kiwifruit per day) resulted in significant increase (P=0.01) in plasma AOA only in female subjects within the low AOA group.

However, the precision and validity of the measurements were limited by a possible loss of vitamin C due to storage of the plasma samples rather than analysis when fresh. Given that kiwifruit is particularly high in vitamin C the effect of the addition of kiwifruit to the diet might not have been detected.

In this study it was shown that a group diet and physical activity intervention within the workplace can increase the level of plasma antioxidant activity and thereby reduce the risk for oxidative stress and related lifestyle diseases

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Abbreviations

AOA	Antioxidant activity
AA	Ascorbic acid
BIA	Bioelectrical impedance analysis
BMI	Body mass index
CVD	Cardiovascular diseases
FRAP	Ferric reducing ability of plasma
FFA	Free fatty acids
FFQ	Food frequency questionnaire
FFM	Fat free mass
GPX	Glutathione peroxidase
HPLC	High performance liquid chromatography
HOMA	Homeostasis model assessment
HDL	High density lipoprotein
LDL	Low density lipoprotein
MDA	Malondialdehyde
ORAC	Oxygen radical absorbing capacity
PUFA	Polyunsaturated fatty acid
PAQ	Physical activity questionnaire
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
TBW	Total body water
TPTZ	Tripiridyltriazine
TBA	Thiobarbituric acid
WHR	Waist to hip ratio

CHAPTER 1

Introduction

1.1 The problem

Lifestyle diseases such as cardiovascular diseases (CVD), hypertension, Type 2 diabetes, cancer, etc, are the major causes of mortality and morbidity worldwide (Murray *et al.,* 1997) measured by deaths and by disability. The Global Burden of Disease Study estimated and selected the 25 major risk factors for death and disability in which high blood pressure was ranked third place, obesity – fourth, high blood cholesterol – seventh, low fruit and vegetable intake – twelfth, and physical inactivity ranked the fourteenth place (Ezzati *et al.,* 2002). Therefore, diet and activity intervention studies are gaining the importance for the reduction of the risk for lifestyle diseases and for increasing healthy life expectancy.

Mortality and Demographic Data 1999 (classified according to the Ninth revision, Clinical Modification of the International Classification on Diseases) showed that malignant neoplasms (27.2%), ischaemic heart diseases (23.3%) and cerebrovascular diseases (10.0%) are the major cause of death in New Zealand (Statistics New Zealand). The prevalence of obesity is 17%, which is predicted to rise to 29% by the year 2011. About 82,000 New Zealanders are affected by Type 2 diabetes, which is estimated to cost the government $170 million a year, and the prevalence is also predicted to double to 7.7% by 2011. The relative increase is forecast to be greater for Maori and Pacific people than for Europeans (Diabetes New Zealand). Therefore, by promoting lifestyle changes such as healthy diet and increase physical activity the current rate may be reduced.

A pilot study "Effect of diet and physical activity intervention on the markers of oxidative stress" (commenced in 2002) was initiated at the Auckland University of Technology (AUT) – Body Composition and Metabolic Research Center, which was supervised by Associate Professor Elaine Rush. The study intended to establish an

evidence-based intervention protocol to design intervention strategies to address the problem of lifestyle diseases in New Zealand. The overall question asked in this study and reported in this thesis was "Does the increased fruit, vegetable and whole grain intake and the increased physical activity will decrease the oxidative stress, and therefore decrease the risk of lifestyle diseases?"

1.2 Etiology and predictors of lifestyle diseases

There is substantial evidence available to link oxidative stress to the development of lifestyle diseases. The following sections explain the etiology of oxidative stress, how it is related to the lifestyle diseases and how making changes in everyday diet and physical activity can modify the effects of oxidative stress.

1.2.1 Oxidative stress – the imbalance between reactive oxygen species and the antioxidant defence system

Reactive oxygen species are the source of oxidative stress of the body. Reactive oxygen species are free radicals i.e. molecules that have an unpaired electron in their outer atomic orbit. The presence of an unpaired electron makes the atom or molecule more reactive by increasing their chemical reactivity. They are able to donate an electron to other molecules, therefore behaving as oxidants. Free radicals contain oxygen, such as superoxide anion (O_2^-), hydroxyl radical ($OH*$), non-radical hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), thus they are called the **reactive oxygen species (ROS)** (Halliwell, 1994a).

Although ROS generated in our body are part of normal cell metabolism and play important roles in both immune-mediated defence and as cell-signalling molecules, at high concentrations ROS can modify and damage DNA, lipids, and proteins (Fenster *et al.,* 2002, Rosen *et al.,* 2001).

The source of ROS is an incomplete reduction of O_2 to water in a variety of physiologic as well as potentially pathologic processes (Mak *et al.,* 2001). The complete reduction of O_2 (especially in lung cells) requires four electrons. This process occurs in the mitochondrial electron transport chain and is catalyzed by cytochrome oxidase. However, there is a constant "leak" of a few electrons and partially reduced intermediates, such as ROS, are produced (Young *et al.,* 2001).

As well as the normal physiological "leak" of electrons, exogenous environmental factors can also promote free radical formation. Ultraviolet light can lead to the formation of ROS in the skin; atmospheric pollutants such as ozone, oxides of nitrogen, tobacco smoke, and motor vehicle exhaust can generate free radicals and cause cell damage (Jacob *et al.,* 1996).

Some of the main representatives of ROS are discussed below:

Superoxide anion, O_2^-, is not usually very reactive, but it can generate more toxic products. The O_2^- vigorously seeks to strip an electron from biological molecules to pair up the lone electron in its outer orbit, thus it is able to attack, for example, sugars, proteins, polyunsaturated fatty acids (PUFA) and DNA (Alessio *et al.,* 1997). The O_2^- is produced by the activated phagocytes such as neutrophils, monocytes, macrophages, and eosinophils to kill the pathogen organisms. However, this normal protective mechanism may cause damage to the cells (Halliwell, 1994_b, Rosen *et al.,* 2001)

The **hydroxyl radical, OH*** is extremely reactive and probably is the final mediator of most free radical induced tissue damage (Okizie, 1999). It attacks all proteins, DNA, PUFA, and almost any biological molecules it touches. The hydroxyl radical is an initiator of lipid peroxidation: OH* removes a hydrogen atom from the unsaturated fatty acids (LH) of membrane phospholipids resulting in the formation of free lipid radical (L*)

$$LH + OH^* \rightarrow L^* + H_2O$$

The lipid radical, in turn, reacts with molecular oxygen and forms a lipid peroxide radical (LOO*)

$$L^* + O_2 \rightarrow LOO^*$$

Like OH* the lipid peroxide radical can function as an initiator of oxidation by removing another hydrogen atom from a second unsaturated fatty acid

$$LOO^* + LH \rightarrow LOOH + L^*$$

Thus a chain reaction is initiated…
Lipid monohydroperoxidases (LOOH) formed in this reaction can be used as a marker of lipid peroxidation. The result is destruction of the unsaturated fatty acids of phospholipids, loss of membrane integrity and cell death.

Hydroxyl radicals can also modify cell membrane proteins by formation of disulfide (S-S) bonds resulting in aggregation of membrane proteins, forming ion channels and finally disruption of membrane structure and function (Farber, 1997). Hydroxyl radicals can interact with DNA and inhibit its replication. They can attack the purine and pyrimidine bases resulting in mutation (Halliwell, 1994_b).

Hydrogen peroxide (H_2O_2) is not a radical as it is electrically neutral. It resembles water in its molecular structure and is very diffusible within and between cells. Hydrogen peroxide has low reactivity but in excess it can attack certain cellular targets, blocking glycolysis and interfering with cell energy metabolism (production of ATP). The H_2O_2 molecule can also damage DNA. Free iron and copper ions can decompose H_2O_2 to OH*; therefore, the "sequestration" of metal ions (bound to transport or storage proteins) is an important body defence mechanism (Halliwell, 1994_b).

Nitric oxide radical (NO*) is synthesized by vascular endothelial cells, phagocytes, and by the certain cells in brain. Nitric oxide has physiological importance as a vasodilator agent and a neurotransmitter. However, in excess and in combination with oxygen it can be cytotoxic. **Peroxynitrite** ($ONOO^-$) is the product of NO* and O_2^-. Peroxynitrite can cause direct biological damage by oxidizing the thiol (-SH) groups of proteins, can be a powerful initiator of lipid peroxidation (Halliwell, 1994_a), can deplete antioxidants such as glutathione and vitamin C, and can inactivate α_1-untiproteinaze, a major inhibitor of proteolyses enzymes in plasma (Okizie, 1999). The $ONOO^-$ complex can inhibit the mitochondrial respiratory chain leading to energy failure and ultimately cell death.

In healthy individuals the **antioxidant system of the body** defends tissue against free radical attack. Antioxidants can offer electrons that stabilize radical molecules or regulate reactions that convert ROS into less destructive forms (Alessio *et al.,* 1997, Balch, 1999).

Antioxidants can be divided into three classes:

- *Primary antioxidants* – prevent formation of new ROS. This includes antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and metal binding proteins such as ceruloplasmin (Cu binding protein), transferrin (Fe binding protein), and ferritin.

- *Secondary antioxidants* – also called the chain breaking antioxidants, include vitamin E, ascorbic acid, vitamin A, uric acid, and albumin. These antioxidants remove newly formed free radicals before they can initiate a chain reaction.

- *Tertiary antioxidants* – include repair enzymes as they repair cell structures damaged by free radicals.

The O_2^- anions are removed by **superoxide dismutase (SOD),** which is one of the most important cellular antioxidants. SOD is present in cell cytoplasm (copper-zinc enzyme) and in mitochondria (manganese enzyme) and is the main scavenger of O_2^- (Farber, 1997)

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

The conversion of O_2^- to H_2O_2 is a useful temporary fix, but removal of H_2O_2 is critical for cell survival, since although it is a weak oxidative agent, H_2O_2 can cross cell membranes (unlike O_2^-) and directly damage proteins and enzymes containing reactive thiol groups (Young *et al.,* 2001).

Glutathione peroxidase (GP), a mitochondrial enzyme, is another cellular antioxidant. GP uses reduced glutathione (GSH) as a cofactor to reduce H_2O_2 to produce water and 2 molecules of oxidized glutathione (GSSG) (Farber 1997).

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

GP requires the presence of selenium at the active site and is the main scavenger of H_2O_2 (Young *et al.,* 2001).

Catalase, a heme containing protein, is another free radical trapping enzyme. It acts like GP by reducing H_2O_2 into water and can also detoxify the O_2^- anion (Alessio *et al.,* 1997). Catalase is largely located within cells, in peroxisomes which are organelles involved in the nonmitochondrial oxidation of fatty acids and amino acids; thus it plays an important role in the inhibition of lipid peroxidation and preventing damage to DNA and RNA (Adams *et al.,* 2002).

Free metal ions such as Fe^{+3}, Cu^{+2} are required for partial reduction of oxygen species to injure cells (Farber 1997). Cells obtain iron from the plasma as ferric iron (Fe^{+3}) bound to **transferrin**. It is delivered to the cytoplasm where an acidic environment releases free ferric iron for the synthesis of hemoproteins and then is stored as a ferritin. However, free ferric iron can be reduced by superoxide anions to ferrous iron (Fe^{+2})

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

Ferrous iron then reacts with the H_2O_2 to produce OH* (Fenton reaction):

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

Chain breaking antioxidants are crucial in preventing lipid peroxidation. **Vitamin E** (α tocopherol) is the most abundant fat-soluble antioxidant. It is absorbed from the gut with the aid of fats and is stored in the body (Haslett *et al.,* 1999).

Vitamin E is one of the body's primary defences against oxidation. It donates an electron to the radical (Alessio *et al.,* 1997), thus blocks the chain reaction and minimizes formation of secondary radicals. Ascorbic acid (AA, vitamin C) or reduced glutathione (GSH) can than regenerate the tocopheroxyl radical (T*) formed in this process:

$$T^* + AA \rightarrow TH + AA^*$$

Therefore, vitamin E is one of the most efficient chain breaking antioxidants available. Also, vitamin E contains several precursors for the production of adrenal steroids and male/female hormones; it reduces muscle fatigue during exercise and inflammation by supporting the production of prostaglandins (Haslett *et al.,* 1999).

Vitamin C (ascorbic acid, AA) is the most abundant water-soluble antioxidant found in food but it is not stored in the body; therefore it is needed in the daily diet. Ascorbic acid is important in collagen synthesis and wound healing, in the absorption of iron from the gut and immune functions are also under the influence of AA (Farber, 1997). Ascorbate is also involved in the production of several amino acids and hormones (Balch *et al.,* 1999).

More than 80% of the vitamin C in the Western diet comes from fruits and vegetables and 70-90% of the dietary intake is absorbed from the small intestine (Will *et al.,* 1996). However, at a low concentration, especially in the presence of transition metals, vitamin C may become a pro-oxidant (Alessio *et al.,* 1997).

Vitamin C is a powerful reducing agent and essential cofactor for several enzymes. It is a scavenger of superoxide, hydrogen peroxide, and the hydroxyl radical (Young *et al.,* 2001). Ascorbate may help to detoxify inhaled oxidizing air pollutants in the respiratory tract (Halliwell 1994_b), inhibit in vivo formation of carcinogenic nitrosamines (Jacobs *et al.,* 1996), assists α tocopherol by recycling the tocopherol radicals (Sies *et al.,* 1995). Studies have shown copper induces inhibition of low density lipoprotein (LDL) oxidation by vitamin C (Retsky *et al.,* 1999). Intracellular enzymes then reduce oxidized ascorbate using NADH or GSH.

Vitamin A (β carotene) is a lipid soluble antioxidant. Diet derived β carotene is absorbed from the gut and stored in the liver (Champe *et al.,* 1997). Carotenoids contain a number of conjugated double bonds responsible for their antioxidant activity (Sies *et al.,* 1995). They can trap the peroxyl radical and prevent lipid peroxidation (Alessio *et al.,* 1997). The efficiency is as great as that of α tocopherol.

Vitamin A is a component of the visual pigment and is essential for supporting spermatogenesis, thus carotene is important in maintaining reproduction and in the

visual cycle. Maintenance of epithelial cells is also under the influence of vitamin A (Champe *et al.,* 1997).

Uric Acid, the end product of purine metabolism, is the largest contributor to the trapping of the peroxyl radicals (Jacob *et al.,* 1996). It is a direct free radical scavenger and part of its antioxidant effect is attributed to the formation of stable non-reactive complexes with iron (Young *et al.,* 2001).

Albumin, the predominant plasma protein, can function as a chain breaking antioxidant by donating an electron to neutralize free radicals. Albumin neutralizes peroxyl radicals due to the presence of 17 disulphide bridges (S-S) (Young *et al.,* 2001), and traps free copper ions (Halliwell, 1994 $_{b}$).

Under normal circumstances the production of ROS and antioxidants defences are in dynamic equilibrium and thus homeostasis is maintained within the human body. However it is easy to tip this balance in favour of the ROS and create a situation of oxidative stress. So, oxidative stress can result from either the depletion of antioxidants (e.g. in malnutrition) or excess production of ROS (e.g. in chronic inflammatory diseases).

For example, the expression of proteins within a cell can be altered by oxidative stress. The sequence of the bases in deoxyribose nucleic acid (DNA) found within most cells determines the code for the proteins that determine the function of that cell. The bases of DNA can be altered either spontaneously or as a result of environmental insult and this occurs regularly. Luckily, cells are remarkably efficient at repairing the damage done to their DNA. The **DNA repair enzymes** recognize the lesion, remove the damaged section of the DNA strand, and use the sister strand as a template to fill in the gap left by the excision of the abnormal DNA. For example, specific **endonucleases** recognize that a base is missing and initiate the process of excision and gap filling; abnormal bases are recognized by specific **glycosidases** that hydrolytically cleave the base from the strand. In rare genetic diseases the cells cannot repair the damaged DNA, resulting in extensive accumulation of mutations and, consequently, for example, skin cancer (Champe *et al.,* 1997).

"**Oxidative stress"** has been defined as the imbalance between the generation of reactive oxygen species and the activity of the antioxidant defences (Okizie, 1999). Oxidative stress leads to the damage of a wide range of molecular species including lipids, proteins, and nucleic acids, which in turn may lead to cellular dysfunction and contribute to the pathophysiology of a wide variety of chronic diseases such as cardiovascular diseases, hypertension, Type 2 diabetes, cancer, etc.

The next section discusses the relation between changes in oxidative stress levels and the development of lifestyle diseases.

1.2.2 Oxidative stress and diseases

Deficiencies in the antioxidant system can activate ROS and, as a result, the risk of free-radical-mediated disease increases. The aetiology of cardiovascular disease (e.g. atherosclerosis, hypertension and thrombus formation), Type 2 diabetes, obesity and cancer, in relation to ROS, are discussed here.

Oxidative stress is related to the development of **cardiovascular diseases (CVD)** particularly via oxidation of low density lipoproteins (LDL) and nitrous oxide. Clinical studies have provided evidence that: patients with CVD have a greater level of markers of lipid peroxidation than those without CVD (Usberti *et al.,* 2002), oxidative LDL are present within the atherosclerotic lesions (Berliner *et al.,* 2002), ischemia/reperfusion of tissues results in an outburst of ROS which will overwhelm the body's antioxidant defences (Farber, 1997).

Oxidized LDL can:

- Injure vascular smooth muscles and endothelial cells (Farquharson *et al.,* 2002);
- Up-regulate expression of cellular adhesion molecules, facilitate leukocyte binding, stimulate production of chemotactic factors and cytokines (Fenster *et al.,* 2002);
- Stimulate smooth muscle cell proliferation by stimulating production of growth factor (Menendez *et al.,* 2002);
- Induce production of autoantibodies against oxidized LDL (Young *et al.,* 2001).

The oxidation hypothesis proposed by Young and colleagues in 2001 for the development of atherosclerosis suggests that the oxidized LDL in the vessel wall plays a key role in the formation of atherosclerotic lesions.

The formation of foam cells in the arterial wall from macrophages is not caused by native LDL, but only after modification by oxidation of LDL (Berliner, 2002). Several mechanisms are likely to be involved including transition metal ion mediated generation of hydroxyl radicals, the production of ROS by enzymes such as myeloperoxidase and lipoxygenases, and direct modification by reactive nitrogen species (Young *et al.,* 2001).

Since the oxidation of LDL is inhibited by antioxidants, antioxidant depletion in the body may be an important risk factor for developing of CVD and atherosclerosis. Vitamin E and carotenoids scavenge free radicals to prevent or limit oxidative damage. The level of α tocopherol in plasma increases the oxidative resistance of LDL (Franz, 1998).

Nitric oxide (NO) is a cell-signalling molecule generated by the endothelial cells in response to several stimuli such as acetylcholine, bradykinin, oestrogen, insulin and lipids.
A key role of NO is to regulate vasomotor tone in large arteries and therefore it regulates blood flow. NO induces vasodilation, inhibits platelet activation and adhesiveness, and regulates inflammatory responses (Farber 1997). NO may also up-regulate the production of glutathione and the expression of SOD (Fenster *et al.,* 2002).

An increase in ROS results in a decrease in NO bioactivity, which is attributed to the development of **hypertension**. Excess O_2^- radical converts NO into the potent oxidizing $ONOO^-$ radical, which inactivates the ability of NO to initiate vasodilation (Fenster *et al.,* 2002).

Oxidized LDL in arterial endothelial cells may alter their function and contribute to the decreased NO production and consequently induce vasospasm in diseased vessels. In addition, fatty acids are precursors of prostaglandins, which act as potent antiaggregators and vasodilators. They also inhibit the vasoconstrictor thromboxane A

(Champe *et al.,* 1997). Therefore, oxidation of LDL may contribute to hypertension in many ways.

Supplementation of dietary antioxidants might ameliorate vascular injury. For example, it has been shown (Frenoux *et al.,* 2001) that the consumption of fish oil and poly unsaturated fatty acids (PUFA) have protective effect against CVD and hypertension, as they are the precursors for prostaglandins that are antiaggregators and vasodilators.

Oxidative stress is also believed to play a major role in the development of obesity-related disorders, including **Type 2 diabetes**.

The majority of cases of Type 2 diabetes are multifactorial in origin with the interaction of environmental and genetic factors. Obesity probably acts as a diabetogenic factor (through increasing resistance to insulin action) in those genetically predisposed to develop Type 2 diabetes (Haslett *et al.,* 1999).

Several hypotheses have been suggested to explain the associations between **obesity** and increased markers of oxidative stress. For example, accumulation of intracellular triglycerides can elevate O_2^- production within the electron transport chain, and the excessive adiposity per se could be the source of increased oxidative stress, since adipocytes are source of inflammatory cytokines that promote production of ROS (Fenster *et al.,* 2002). This is supported by a study (VanGaal *et al.,* 1998) that showed that *in vitro* oxidizability of LDL is significantly increased in obese, non-diabetic subjects and is related to increased body weight and triglyceride levels. Chronic hyperglycemia promotes nonenzymatic glycation of proteins, which can trigger ROS production by activating monocytes or by directly releasing O_2^- and H_2O_2 (Fenster *et al.,* 2002)

The antioxidant defence system is also compromised in Type 2 diabetes. Reviewing the studies of the vitamin C status of people with Type 2 diabetes, Will *et al.,* (1996) concluded that people with Type 2 diabetes have at least 30% less plasma ascorbic acid concentration compared with people without Type 2 diabetes.

One reason that water-soluble ascorbic acid is a potent antioxidant is that glutathione and ascorbic acid function as a redox couple. Reduced glutathione enzymatically

regenerates oxidized ascorbic acid at the cellular level, and the ascorbic acid regenerates another major antioxidant, α tocopherol. A case-control study (Tessier *et al.,* 1999) compared older subjects with Type 2 diabetes with normal controls and showed diminished intragranulocytic level of ascorbic acid, increased oxidized glutathione (GSSG), and decreased GSH/GSSG ratio after the glucose challenge in diabetic patients.

Oxidative stress has also been linked to insulin resistance, a precursor of Type 2 diabetes (Evans *et al.,* 2002, Rosen *et al.,* 2001). The insidious elevation of glucose and free fatty acids (FFA) in the development of Type 2 diabetes induces oxidative stress, which causes insulin resistance and β cell dysfunction. For example, FFA have an adverse effect on mitochondrial function resulting in the generation of ROS as well as impairing the endogenous antioxidant defence by decreasing intracellular glutathione. The β cells are particular susceptible to the damage caused by oxidative stress as they are low in ROS quenching enzymes. This lack of enzymes could contribute to the β cells reduced ability to survive under condition of oxidative stress such as poor diet. Increasing the strength of oxidative defences and maintaining the "normal" body weight may be important in modulating the consequences of hyperglycemia.

Free radicals can cause oxidative damage to DNA. If the damage is not repaired, a permanent mutation can result in a number of deleterious effects, including loss of control over the proliferation of the mutant cell, leading to **cancer** (Champe *et al.,* 1997). Although at a steady-state level the oxidative DNA damage may be one modified base per 10^5 unmodified purines and pyrimidines, over the long human lifespan it can be a significant contributor to the age-related development of major cancers such as those of the colon, prostate, rectum, and breast (Halliwell, 2002).

The level of oxidative DNA damage is increased prior to cancer development, however, Halliwell suggests that oxidative DNA base damage is not a biomarker of subsequent cancer development as the other factors, such as the status of repair enzymes, accelerated cell proliferation caused by ROS, should be also taken into account (Halliwell, 2002).

In conclusion, since the oxidative stress is implicated in the pathophysiology of the chronic diseases listed here, it can be hypothesized that by stimulating the antioxidant defence system the risk of those chronic diseases could be decreased.
The effect of physical activity on the antioxidant status of the body is discussed next.

1.2.3. Antioxidants and physical activity

The blood-flow redistribution to the working muscles and increased oxygen consumption during exercise may create a hypoxic environment in different organs, for example liver, kidney and gut. The ischemia-reperfusion of these organs can activate xantine oxidase, a membrane-bound enzyme, which produces superoxide and hydrogen peroxide (Adams *et al.,* 2002, Vina *et al.,* 2000). Studies (Alessio *et al.,* 1997, Alessio *et al.,* 2000) report evidence of oxidative stress during strenuous exercise. Increased energy demand and heat production can strain the electron transport system in mitochondria that allows the reduction of oxygen to water. Neutrophil activation related to tissue injury in the post-exercise period can be another source of free radicals. A decreased reduced to oxidized glutathione ratio (GSH/GSSG) during exercise is a measure of the body's compromised antioxidant defence capacity (Ji, 1996).

However, it seems that the degree of oxidative stress and muscle damage does not depend on the intensity of exercise, but can be related to the degree of exhaustion of the person performing the exercise. Regular exercise can initiate adaptations to enhance the enzymatic defence system against free radical activity. Regular, moderate-intensity exercise increases the number and the size of mitochondria (Ginsburg *et al.,* 1996), and the amount of mitochondrial enzymes GPX, SOD (Adams *et al.,* 2002), and cytochromes (Alessio *et al.,* 1997).

These adaptations have been demonstrated in trained athletes. It appears that the susceptibility of plasma lipids to peroxidation following exercise is reduced in this group (Ginsburg *et al.,* 1996). This means that trained athlets have a more efficient electron transport system function. Surprisingly, athletes not using vitamin E had a greater decrease in susceptibility of lipids to peroxidation than athletes using vitamin E (This will be discussed later in this chapter). Another study (Shern-Brewer *et al.,* 1998) has also showed that regular aerobic stress for an overall shorter time span creates a

more oxidative environment in the body and increases the susceptibility for lipid peroxidation, whereas chronic exercise over several months or years decreases the susceptibility of lipids to undergo oxidation.

An increase in NO production during exercise can be another protective adaptation that leads to long-term vascular protection (Fenster *et al.,* 2002). The well-established cardiovascular effect of chronic exercise can be attributed to those adaptations. In addition to increasing the oxidative capacity, exercise training improves immune function and mediators involved in muscle adaptations (Jacob *et al.,* 1996).

Therefore, regular physical activity may be crucial to naturally promote the function of the endogenous antioxidant defence and reduce the risk of ROS mediated diseases. However, increased physical activity is not the only way to modify the effects of the oxidative stress. The antioxidant defence system of the body depends significantly on the exogenous antioxidants such as vitamins and trace minerals. Therefore, implementing the healthy diet, rich in fruit, vegetables, oily fish, whole grain, etc, that naturally contain antioxidants and other important nutrients, in everyday diet, could increase the antioxidant defences and decrease the risk for lifestyle diseases. This concept is discussed in the next section.

1.2.4. Dietary food or supplements?

Diet is an important factor in the development of many chronic degenerative diseases such as cardiovascular and heart diseases, hypertension, Type 2 diabetes, cirrhosis of the liver, gallstones, carcinomas of stomach and the large bowel. (Haslett *et al.,* 1999).

Antioxidants in the diet are a vital requirement that play a major role in protecting us from our internal and external environment. Although vitamins are necessary in trace amounts for normal metabolic functions, by definition they are not synthesized endogenously; therefore the body totally depends on the dietary sources (Farber *et al.,* 1997). The normal body antioxidant system also depends on the integrity of an enzymatic system that requires regular, adequate intake of trace minerals (selenium, copper, zinc, manganese, etc.), and on the adequate plasma concentration of the antioxidant vitamins E, C and A (Allard *et al.,* 1998). Since an increased level of free radicals has been implicated in many chronic diseases this has lead to suggestions that the dietary supplementation of antioxidants as therapeutic or prophylactic agents might have health benefits. Vitamin E, vitamin C and β carotene are most widely studied antioxidants.

Paradoxically, the attempts to use micronutrient (vitamins and trace minerals) supplements therapeutically have, so far, demonstrated marginal or no therapeutic efficiency (Young *et al.,* 2001; Mak *et al.,* 2001). Studies demonstrated thickening of arteries in subjects consuming vitamin C daily and another study showed no protection of arteries in young smokers taking vitamin C daily (Balsh *et al.,* 1999). A clinical intervention trial in Finland (Preziosi *et al.,* 1998) has reported a negative effect of β carotene supplementation on the lung cancer incidence rate in high-risk subjects. It seems that the evidence that higher consumption of vitamin supplements provides a health benefit, are not sufficiently convincing (Jacob *et al.,* 1996), therefore these authors conclude that supplements should not be used routinely.

But dietary supplements do cure deficiencies. They have proven benefits in certain high risk groups such as woman during pregnancy and lactation, total vegetarians, elderly patients in intensive care units or long term nursing homes, alcoholics, and individuals eating less that 1000 calories a day (Franz, 1998).

However, many epidemiological studies demonstrated that diets high in antioxidants such as fruit, vegetables, grains, etc, were associated with a decreased risk of chronic diseases (Adams *et al.,* 2002; Jacob *et al.,* 1996). Therefore, the failed clinical trials have raised questions concerning the utility of antioxidant therapy, rather than the role of oxidative stress in the pathogenesis of chronic diseases.

The complex mixture of natural antioxidant micronutrients (vitamins, trace minerals) found in diets might be more effective than large doses of vitamins in supplements. For example, Collins *et al.,* (2001) showed that consumption of kiwifruit (particularly rich in vitamin C) extract resulted in a 54% decrease in oxidative DNA damage compared to a 24% decrease with equivalent concentrations of vitamin C.

Antioxidants could have a ***cumulative effect*** on preventing the oxidative damage. Nutrients in whole foods may act in synthesis whether by synergy or antagonism for their specific effects. For example, vitamin E absorption can be decreased by high doses of β carotene; blood and the adipose tissue level of γ- tocopherol is depressed when α-tocopherol is supplemented; intestinal conversion of β carotene to vitamin A is less efficient at high doses and the transport and storage of vitamin E depends on selenium. "Therefore, independent, high doses of particular nutrients, not naturally endemic, might well upset redox relationships and therefore interact with normal cellular mechanisms and signalling in ways not yet known" (Wheatley *et al.,* 1998). In addition, metabolism and utilization of micronutrients is dependent on an individual's nutritional status and is integrated with other nutrients, hormones, and physiological factors (Franz, 1998). Thus it is difficult to determine individual requirements for micronutrients.

Despite the glowing advertisements for micronutrient supplements, manufacturers or companies do not provide guarantees for the purity or safety of their products. Some supplements can interfere with drugs or food combinations. Alternatively, nutrients that occur naturally in food can boost our defences and reduce the risk for lifestyle disease if the diet is chosen wisely.

Many epidemiological studies support this concept but the epidemiological studies do not provide causality. Therefore, there is an increased interest to provide significant evidence of the effect of diet (and physical activities) on health and in particular for this thesis the effectiveness in helping to reduce oxidative stress over time.

Particular interest has been taken to the addition of kiwifruit to the diet in this study. Kiwifruit is rich natural source of vitamin C and other potentially active phytochemicals, which can provide a dual protection against oxidative damage by enhancing antioxidant level and by stimulating DNA repair (Collins *et al.*, 2003).

1.3 Aim

The general aim of this thesis was to demonstrate the beneficial effect of lifestyle changes such as a healthy diet and increased physical activity on antioxidant status of the body.

The specific questions to be answered were:

a. Does diet and activity intervention have a measurable effect on the antioxidant activity of the body?

b. Does kiwi fruit addition to the diet increase the antioxidant activity of the body?

c. How are changes in antioxidant activity associated with the blood biochemical parameters?

I hypothesized that the diet rich in fruit, vegetables, whole grains, and oily fish and increased physical activity will increase the antioxidant status of the body, measured by the antioxidant activity (AOA) of plasma. Furthermore, adding the kiwifruit to the diet will also reduce the risk of oxidative stress.

1.4 Measurement of antioxidant status

The total antioxidant capacity of plasma is not a simple sum of the activity of various antioxidative substances. Each antioxidant works in cooperation and synergy to provide greater protection against oxidative stress. There is no "gold standard" method capable of measuring the total antioxidant activity of plasma (Koracevic *et al.,* 2001); therefore variable and comparable methods can be used to detect different components of antioxidants activity in plasma.

For example, Randox antioxidant Kits, Ransel and Ransod, measure enzymatic antioxidant defences such as Glutathione Peroxide and Superoxide dismutase respectively (www. Randox. Com), Oxygen radical absorbing capacity (ORAC) assay and Trolox equivalent antioxidant capacity (TEAC) assay measure serum protein antioxidant such as albumin (Cao *et al.,* 1998), Ferric reducing ability of plasma (FRAP) assay measure combined antioxidative effect of the nonenzymatic defences in biological fluids such as ascorbic acid, α tocoferol, uric acid, bilirubin and albumin (Benzie *et al.,* 1996), Thiobarbituric acid reactive substances (TBARS) assay measures lipid peroxidation (Koracevic *et al.,* 2001).

Two methods, FRAP assay and TBARS assay, were elected for this thesis to assess the changes in antioxidant defence system in subjects undergone the intervention study. The basic principles of FRAP and TBARS assay are outlined next

1.4.1. The principles of FRAP assay

The ferric reducing ability of plasma (FRAP) assay was used to measure the combined antioxidative effect of the nonenzymatic defenses in biological fluids. The method is based on reduction of ferric-tripiridyltriazine (Fe^{+3}-TPTZ) complex to the ferrous (Fe^{+2}) form at low pH by the plasma antioxidants.

$$\mathbf{Fe^{+3}\text{-}TPTZ} \xrightarrow[\text{pH 3.6}]{\text{antioxidant}} \mathbf{Fe^{+2}\text{-}TPTZ}\ \text{(blue color)}$$

Reduced Fe^{+2}-TPTZ forms an intense blue color, with an absorption maximum at 593nm. The absorption changes proportional to the antioxidant concentration (Benzie *et al.,* 1996).

The FRAP assay was elected for several reasons: it directly measures antioxidants in plasma samples, it is fast and easy to use, and the method is known to be inexpensive and highly reproducible. However, the limitation of the FRAP assay is that it does not measure the thiol (SH-) containing antioxidants (Benzie *et al.,* 1996; Cao *et al.,* 1998).

1.4.2. The principles of TBARS assay

The most specific assays of lipid peroxidation (also the most difficult to do) involve high performance liquid chromatography (HPLC), gas chromatography, mass spectrophotometer, or antibody-based determination of such individual products as isoprostanes or peroxides. The thiobarbituric acid reactive substances (TBARS) assay was elected to use because it is simple to use and is cheap.

Lipid peroxidation results in production of lipid peroxides and their by products such as aldehydes. Malondialdehyde (MDA) is the most abundant aldehyde derived from the breakdown of polyunsaturated fatty acids (PUFA) with more than two double bonds (Li *et al.,* 1994). Therefore, MDA can be used as a marker of lipid peroxidation. Measurement of MDA is considered an important contributor to the assessment of oxidative stress and therefore is the most widely used test for measuring the extent of lipid peroxidation (Sim *et al.,* 2002).

The basic principle of the TBARS assay is the reaction of MDA and thiobarbituric acid (TBA) to form a red MDA-TBA complex, which can be quantitated spectrophotometrically (at 532nm).

$$MDA + 2TBA \longrightarrow \text{TBA-chromogen}$$

TBARS assay measures the ability of plasma to suppress the production of MDA (Koracevic *et al.,* 2001); therefore, the inhibition of color development is defined as the antioxidant activity (AOA) of plasma.

The limitation of this method is that it lack of specificity, since other TBA-reactive substances including sugars and other aldehydes could interfere with the MDA-TBA reaction. However, the TBA test is applicable for comparing samples of a single material at different states of oxidation.

CHAPTER 2

Study Design and Methods

This study, "Effect of diet and physical activity intervention on the markers of oxidative stress", was part of a pilot study, which was designed to test whether a group work-based dietary and activity intervention would decrease the risk of oxidative stress and make beneficial changes to other risk factors for lifestyle diseases. The study was approved by the Auckland University of Technology Ethics Committee on February 20, 2002 (Appendix 1). Part of this study, the efficacy of diet and activity intervention on risk factors for lifestyle diseases such as blood lipids, glucose and insulin, body fatness, blood pressure and anthropometric measurements, has been published as a Masters thesis (Cumin, 2004). Intervention effect on DNA fragility measured by Comet assay was assessed by Elaine Rush and will be reported separately.

The effect of diet and activity intervention on the level of plasma antioxidants was measured by me using two specific methods; ferric reducing ability of plasma (FRAP) assay for measuring the changes in total antioxidant activity of plasma and thiobarbituric acid reactive substances (TBARS) assay for measuring the changes in lipid peroxidation (i.e. plasma malondialdehyde (MDA)). Furthermore, a crossover study design was added to the overall study design to measure the specific effect of kiwifruit on plasma antioxidants.

2.1 Study design

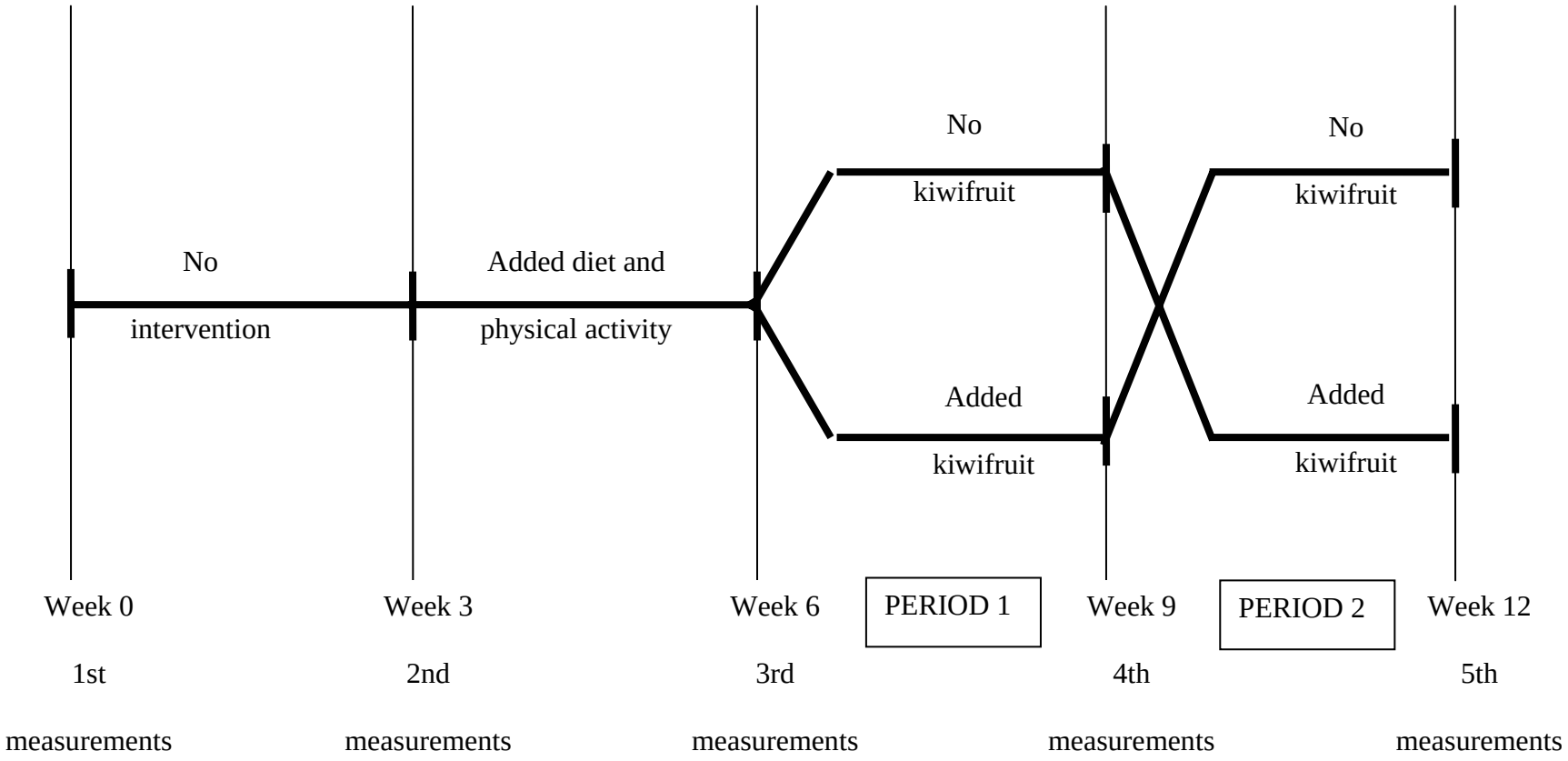
The study commenced on February 2002. The study period of 12 weeks was divided into 4 phases of 3 weeks each (Figure 2.1). During the first phase participants reported in the morning in a fasting state to the research centre for baseline measurements (week 0). After 3 weeks these measurements were repeated (week 3).

In the second phase the diet and activity plan was introduced by Cumin. During the session at week 3, diet and physical activity "shake-up", the participants were informed about the changes they could make to reduce their risk of lifestyle diseases. The dietary advice was based on 50-60% energy from carbohydrates, 15-18% energy from protein and 24-27% energy from fat. Dietary changes concentrated on an increase in dietary fibre by increasing fruit, vegetables and whole grains, an increase in omega-3 fatty acid mainly from consumption of oily fish, and a decrease in saturated fats and dietary sodium. Two booklets were handed out at the shake-up session: one booklet containing partially individualized dietary information, hints for improving diet and physical activity, and goal sheets to help motivate participants. The other booklet was a book of recipes to help implement dietary changes e.g. lower fat, the use of less used ingredients like legumes, beans, and oily fish (Cumin 2004). The activity advice was based on regular and gradual increase in physical activities that are more easy to maintain and build for a long term. Pedometers (Digiwalker SW-700) were issued to the participants to help motivate them increase their physical activity. It was recommended that they aim to do a minimum of 10,000 steps per day.

The third set of measurements was made at week 6. At the beginning of phase 3 the subjects were randomized into two groups, one asked to consume kiwifruit (2-3/day), and others asked to abstain from kiwifruit consumption. At the week 9 subjects who had not consumed kiwifruit were asked to consume the prescribed fruit and the other group were asked to abstain from kiwifruit consumption. The measurements 4 and 5 were made at the middle (week 9) and at the end (week 12) of crossover period.

The diagram (Figure 2.1) below outlines the structure of the study design.

Figure 2.1 Diagrammatic representation of the study design over the first 12 weeks.



The follow up measurements were made at week 52.

The next sections describe the participants and the measurements that were made during the study period to assess the changes in risk factors for lifestyle diseases as a result of diet and activity intervention.

2.1.1. Subjects

Fifty-two healthy subjects, male and female, aged 30 years or more were recruited from staff at AUT and University of Auckland by advertisement and personal contact. It was expected that in this age range it would be more likely to find risk factors for lifestyle diseases. Exclusion criteria were diabetes mellitus, known hypertension, or use of medication for elevated cholesterol. Following the initial contact, the subjects were given an information sheet about the research and were required to sign the consent form after being given an opportunity to ask questions related to the study.

The procedures involved in this study required the full cooperation of the volunteers over the period of 12 weeks. At the week 12 measurement point subjects asked if they could be contacted for further measurements at one year, pending ethical approval being extended. This extension was approved by the ethics committee (10 March 2003).

2.1.2. Measurements

Five sets of measurements were made during the 12 week study period, and a sixth set of measurements was made at week 52 (follow up measurements). Each set of measurements included:

- Anthropometric measurements and body composition assessment
- Blood tests
- Blood pressure
- Dietary and physical activity questionnaire.

All measurements were made at the Body Composition and Metabolic Centre at AUT.

Anthropometric measurements included measurements of body weight, height, girths

(waist circumference) and skinfolds (biceps, triceps, subscapular, suprailiac) which were done by trained and experienced measurers Elaine Rush and Vishnu Chandu. The waist to hip ratio (WHR) and waist circumference, as a measure of central fat distribution (Taylor *et al.*, 1998) was used to assess the risk factors for lifestyle diseases, as well as body mass index (BMI) (weight in kg/[height in m^2]) as a measure of total body fat mass.

Body composition was measured by the Impedimed (IMP5 single frequency bioimpedance (BIA), Impedimed Pty Ltd, Australia). The BIA measures total body water (TBW) and fat free mass (FFM) (Kushner *et al.,* 1996) that was used to assess the changes in the fat content of subjects during the study.

Systolic and diastolic blood pressure was measured at each of the measurement dates using a mercury sphygmomanometer (AC Cosser and Son (Surgical) Ltd, London).

Blood tests included fasting plasma glucose, insulin and plasma lipids such as total cholesterol, high density lipoproteins (HDL) cholesterol, low density lipoproteins (LDL) cholesterol, total cholesterol/HDL ratio and triglycerides. The venous blood was taken by an experienced phlebotomist from Diagnostic Medlab Ltd. Fresh blood was analysed for glucose and lipids at Diagnostic Medlab Ltd, Ellerslie, Auckland, and for insulin by A Plus Laboratories (Lab +, Auckland). The venous blood samples were also collected in EDTA-coated sterile tubes to be measured later for Comet assay and for antioxidant activity. The antioxidant samples were immediately centrifuged at 2500rpm for 7 min, then the plasma was transferred into the Eppendorf tubes and stored in a freezer at -85^0C before use.
Glucose was measured by the Roche-Hitachi glucose oxidase method; insulin by the Abbot IMx Insulin assay method; lipids by the standard Roche-Hitachi methodology, and HDL by direct assay. The homeostasis model assessment (HOMA) method was utilized to measure insulin resistance (HOMA-B%) and insulin sensitivity (HOMA-S%). This calculation requires the input of fasting insulin and glucose levels (Hermans *et al.,* 1999).

The dietary and physical activity questionnaire was created and assessed by Michelle

Cumin (Cumin, 2004). At each measuring session the participants completed the food frequency questionnaire (FFQ) and the physical activity questionnaire (PAQ).The FFQ gave a semiquantitative daily measure of the food intake categorized into fruit, vegetables, dairy, meat, fat and bread and average weekly measure of oily fish intake. The PAQ was used to assess the changes in mild/moderate activities (e.g. climbing some stairs instead of taking the elevator, walking instead of driving a short distance, etc), and changes to more strenuous activities (e.g. jogging, or running, swimming, exercise in a gym, playing strenuous sports (rugby, tennis, basketball, rowing, etc).

I joined the study in July 2002 and was actively involved in collecting the follow up period (week 52) data which included anthropometric measurements, bioimpedance measurements, handling blood samples (centrifugation) and storing and sorting the volunteers' plasma samples in the -85^{0}C freezer at the Glycoscience Research Centre.

2.2 Methods

This section describes two specific analyses that were carried out by the author: the FRAP assay and the TBARS assay, which were used, respectively, to measure plasma antioxidant activity and lipid peroxidation in the volunteer plasma samples. Each method will be discussed separately. Each includes an explanation of the principle of the analysis, the procedure for the preparation of materials and equipment, the chemicals used and the experimental procedure.

2.2.1. The ferric reducing ability of plasma (FRAP) assay

The FRAP assay was used to measure the combined antioxidant effect of the nonenzymatic defences in plasma samples. At a low pH antioxidants present in plasma reduce ferric 2,4,6-tripyridyl-s-triazine (Fe^{+3}-TPTZ) to the ferrous form (Fe^{+2}-TPTZ), which has an intense blue colour with absorbance maximum at 593nm.

$$\mathbf{Fe^{3+}\text{-}TPTZ} \xrightarrow[\text{pH 3.6}]{\text{antioxidants}} \mathbf{Fe^{2+}\text{-}TPTZ}\ \text{(blue color)}$$

The unit of measurement is the **FRAP value** (or antioxidant activities-AOA)**,** measured in micromol/L. The FRAP value was calculated by comparing the amount of plasma antioxidants with the amount of ferrous ions (Fe^{2+}) required to give the same absorbance change (ΔA).

The absorbance of the products of the FRAP reaction was measured on an Ultrospec 2100pro (Amersham Pharmacia, Biosciences, England) at a wavelength of 593nm. Eppendorf cuvettes (volume 50-2000microL, 10mm optical path length) were used for FRAP assay as only 340microL solution was used for absorption readings.

Samples

Plasma samples were obtained from 52 subjects at week 0, 3, 6, 9 and 12. Their venous blood was taken after an overnight fast into EDTA coated tubes. Plasma was separated by centrifugation at 2500rpm for 7 min and stored in a freezer at – 85^0C before use.

Reagents

The chemicals used in the FRAP assay included the FRAP working reagent and the ferrous sulphate standard solutions. Preparation of the FRAP working reagent is detailed in table 2.1.

Table 2.1. Preparation of FRAP working reagent

FRAP working reagent ingredients*	**Preparation**
1. 25 ml 0.3 M Acetate buffer pH 3.6	3.1g of sodium acetate ($C_2H_3O_2Na*3H_2O$) was mixed with 16ml of glacial acetic acid ($C_2H_4O_2$) and diluted with deionized water (DI) water to 1L. The pH was adjusted to 3.6
2. 2.5ml 10 mmol/L TPTZ solution	TPTZ (2,4,6-tripyridyl-s-triazine) solution: 0.156g of TPTZ was mixed with 2ml of HCl (1M) and diluted to 50ml.
3. 2.5ml 20mmol/L ferric chloride solution	Ferric chloride solution: 0.54g of $FeCl_3*6H_2O$ was dissolved in DI water and diluted to 100ml.

* the order of mixing the solutions 1, 2 and 3 is important

Calibrations

The working standard of 10mmol/L ferrous ion solution was used to prepare 0, 300, 600, 900,1200 and 1500micromol/L concentrations of Fe^{+2} standard solutions. This range covered the normal adult FRAP value ranging from 600-1600micromol/L (Benzie et.al 1996).

Experimental procedures

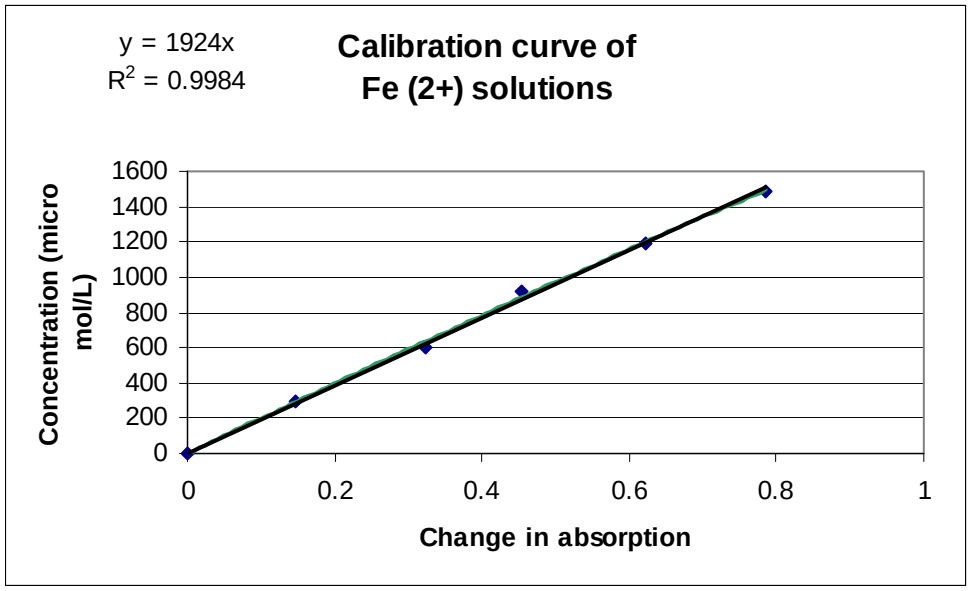
- 30ml freshly prepared FRAP reagent was warmed in a 37^{o}C water bath;
- 300microL of FRAP reagent was transferred to a cuvette and a reagent blank absorption reading (A_1) was taken at 593nm;
- 10 microL plasma samples was added to the FRAP reagent in the cuvette along

with 30 microL of deionized water (DI) and absorbance reading (A) was recorded after 5min reaction time;

- The change in absorption (ΔA) between the final reading (A) and the reagent blank reading (A_1) was calculated for each sample ($\Delta A = A - A_1$) and related to ΔA of a Fe^{+2} standard solutions tested in parallel.

The calibration curve of the six Fe^{+2} standard solutions was made before each set of sample measurements. Example of a typical calibration curve is shown in Figure 2.2 and details of standard preparation are given in Appendix 2.

Figure 2.2 Relationship between the concentration of Fe^{+2} standard solutions and the changes in absorption (at 593nm)



A calibration curve of a Fe^{+2} standard solutions was used to demonstrate the linear relationship between the concentration of Fe^{+2} and the change in absorbance ΔA (the formula from calibration curve was obtained by forcing the linear regression line through zero) and was used to calculate the plasma antioxidant activities (AOA).

An example of working spreadsheet for FRAP assay results is attached (appendix 2).

2.2.2. Thiobarbituric acid reactive substances (TBAR) assay

The TBARS assay was used to measure the extent of lipid peroxidation in plasma samples. The basic principle of the assay is the ability of plasma to suppress the production of malondialdehyde (MDA), a by-product of lipid peroxidation.

The reactions involved in a TBARS assay are as followed:

A standardized solution of Fe^{+2}-EDTA complex reacts with hydrogen peroxide (H_2O_2) by a Fenton-type reaction, leading to the formation of hydroxyl radicals (OH*).

$$\mathbf{2Fe^{2+}\text{-}EDTA + H_2O_2 \longrightarrow 2Fe^{3+}\text{-}EDTA + 2OH^*}$$

The hydroxyl radical degrades benzoate releasing malondialdehyde (MDA)

$$\mathbf{2OH^* + benzoate \longrightarrow MDA}$$

MDA reacts with tiobarbituric acid (TBA) producing the TBA-chromogen

$$\mathbf{MDA + TBA} \xrightarrow{\text{Heat}} \mathbf{TBA\text{-}chromogen}$$

(-)

↑

plasma antioxidants

Plasma antioxidants interfere with TBA-chromogen production. The antioxidant activity (AOA) of plasma is defined as the inhibition of colour development by the plasma antioxidants. In other words, less colour development is related to the decreased lipid peroxidation (i.e. plasma MDA) and related to the increased plasma AOA.

Chemicals:

Procedure for preparation of the working solutions used for TBARS assay are listed in Table 2.2.

Table 2.2 Preparation of working solutions for TBARS assay

Solutions	**Preparation**
1. Sodium phosphate buffer pH 7.4 100mmol/L	3.8g of sodium phosphate (Na_3PO_4*$12H_2O$) was mixed with DI water and diluted to 100ml in volumetric flask
2. Sodium benzoate – 10mmol/L Solutions 1and 2 were kept in a refrigerator (0-4^oC)	0.144g of sodium benzoate ($C_7H_5NaO_2$) was mixed with DI water and diluted to 100ml
3. Uric acid: 1mmol/L in 5mmol/L NaOH was kept in freezer (-15 ^oC)	0.0168g of uric acid ($C_5H_4N_4O_3$) (99%) was mixed with 5mmol/L NaOH and diluted to 100ml
Solutions prepared immediately before use at each run	**Preparation**
1.EDTA 2mmol/L	0.074g of EDTA ($C_{10}H_{16}N_2O_8$*$2H_2O$) was mixed with sodium phosphate buffer and diluted to 100ml
2. Ammonium iron (+2) sulphate hexahydrate 2mmol/L Equal amounts of solutions 1 and 2 were mixed and left to stand for 60 min.	0.078g of Fe[$(NH_4)_2(SO_4)_2$]*$6H_2O$ was mixed with DI water and diluted to 100ml
3. Hydrogen peroxide 10mmol/L	0.113ml of hydrogen peroxide (H_2O_2) (88%) was dissolved in DI water and diluted to 100ml.
4. Acetic acid 20%	
5. Thiobarbituric acid (4,6-dihydroxy-2-mercaptopyrimidine) 0.8% (wt/vol)	0.8g of thiobarbituric acid ($C_4H_4N_2O_2S$) was mixed with 50mmol/L NaOH and diluted to 100ml

Experimental procedures:

Each sample absorption(A_1) had its own control absorption(A_0). The 1mmol/L uric acid absorption (UA_1 and its own control UA_0) was used as a standard, instead of plasma. For each series of analysis a negative control absorption K_1 (without plasma or uric

acid) and K_0 its own control absorption was used. The samples and the working solutions were pipetted into tubes. The order of pipetting the solutions in to the tubes are detailed in table 2.3.

Table 2.3 The steps (1-9) of pipetting solutions (in milliliters).

Steps	**Solutions**	**A_1** (Run1,2,3,4, 5,6, int st.)	**A_0** (Run1,2,3,4, 5,6, int st.)	**K_1** (in triplicates)	**K_0** (in triplicates)	**UA_1**	**UA_0**
1	**Serum (ml)**	0.01	0.01	-	-	-	-
2	**Uric acid (ml)**	-	-	-	-	0.01	0.01
3	**Buffer (ml)**	0.49	0.49	0.50	0.50	0.49	0.49
4	**Na-benzoate(ml)**	0.50	0.50	0.50	0.50	0.50	0.50
5	**Acetic acid (ml)**	-	1.00	-	1.00	-	1.00
6	**Fe-EDTA (ml)**	0.20	0.20	0.20	0.20	0.20	0.20
7	**H_2O_2 (ml)**	0.20	0.20	0.20	0.20	0.20	0.20

int.std = internal standard

The tubes were incubated at 37^0C for 60 min.

Then the following were added:

8	**Acetic acid (ml)**	1.00	-	1.00	-	1.00	-
9	**TBA (ml)**	1.00	1.00	1.00	1.00	1.00	1.00

The tubes were incubated in a boiling water bath for 10 min and then cooled in an ice bath. The absorbance readings were made at 532nm against DI water.

The antioxidant activity (AOA) was calculated by using the formula (Koracevic *et al.*, 2001)

AOA=Cua x (A contr.- A sample) / (A contr. – A ua)

Where

Cua is a concentration of uric acid. Cua = 1 mmol/L

A contr. = $K_1 – K_0$

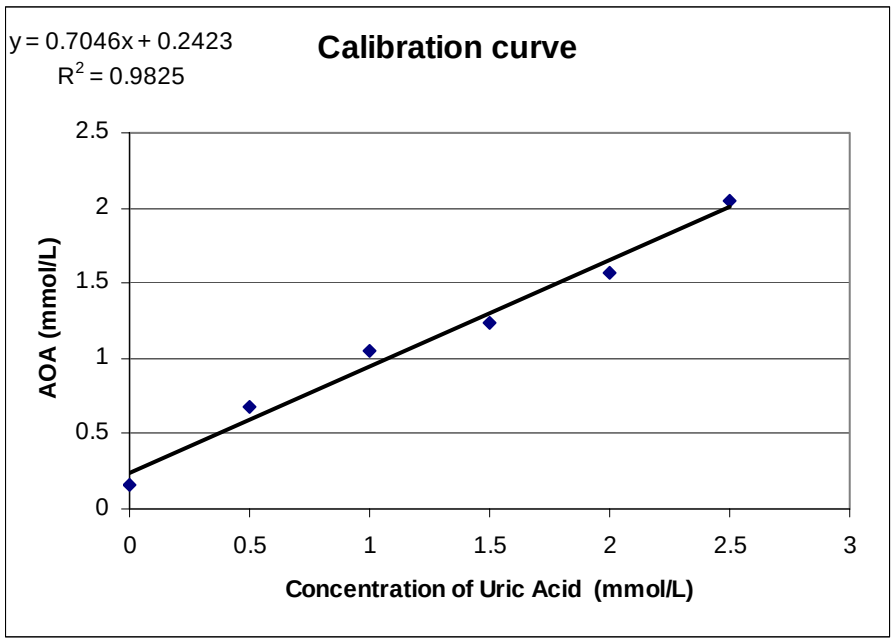
A sample = $A_1 – A_0$

A ua = $UA_1 – UA_0$

Uric acid working solutions (0.5-2.5mmol/L) were used to construct a curve and to demonstrate the linearity between the concentration of uric acid and the calculated AOA.

Example of a typical curve and regression equation is shown in Figure 2.3.

Figure 2.3 Relationship between uric acid standard solutions and AOA (at 532nm)



An example of working spreadsheet for TBARS assay results is attached as Appendix 3.

Quality control of data

A number of quality control measures were used in the measurement of the plasma antioxidant status for both the Ferric Reducing Ability of Plasma (FRAP) and Thiobarbituric Acid Reactive Substances (TBARS) assays. These included measurements of technical precision as described below:

a. Triplicate FRAP and duplicate TBAR measurements of each plasma sample were made. By calculating, using one way analysis of variance for the within sample variation; the standard deviation of the differences within the same sample measurements was used to calculate the within sample coefficient of variation (Bland, p265, 1995). For the FRAP assay within sample coefficient of variation was 26% and for the TBARS assay was 27% when absorbance measurements for 100 plasma samples were examined for each assay.
b. My own blood plasma was used as an internal standard (the ethical approval was obtained; Appendix 4). Fifteen millilitres of blood was drawn in to EDTA tubes, centrifuged to separate the plasma and the well mixed plasma frozen in one millilitre aliquots at -85ºC. Before each day's measurement run this reference plasma sample was measured to ensure that all reagents were working. The coefficient of variation for 24 measurements of this sample was 14% for TBARS and for 22 measurements 19% for FRAP.

The FRAP method used for this thesis was adopted from Benzie but carried out manually for every step. Benzie (Benzie *et al.,* 1996) had the advantage of being able to automate the test and her reported between-run CV was 3% compared to my 19%. Errors due to the manual technique include both measurement of reagents and the timing (5min) of the reactions before measurement of absorbance.

The other method, thiobarbituric acid reactive substances (TBARS) assay, was developed by Koracevic (Koracevic *et al.,* 2001), was adopted to measure the ability of

plasma antioxidants to suppress the production of malondialdehyde (MDA), the product of lipid peroxidation. The reproducibility of the method based on 12 measurements done by Koracevic was 5.4% between-run. Much higher coefficient of variance (CV=27%) in my method could possibly related to the instability of the working solutions that had to be prepared immediately before use. However, the time required to perform the tests makes it impractical as for each plasma sample there were six tubes to be tested, each in duplicate, and for one person six plasma samples for six measurements. Furthermore, there were seven solutions to be added to the tubes. Therefore, the working solutions were standing on the bench at least two-to-three hours.

The other quality control measures that were included in the design of the testing procedure were:

c. Preparation of a series of standard solutions and sample solutions were made by gravimetric serial dilution to check for linearity and accuracy;
d. The quantity of liquid delivered by the automatic pipettes (Labsystems Oy, Helsinki, Finland) was gravimetrically checked daily and manually adjusted so that the quantity of distilled water delivered was within 0.0001 g of the desired amount.
e. Sets of cuvettes were checked by placing distilled water in them and comparing absorbance measured. If the absorbance varied by more than 0.01 the cuvette was not used.
f. The quantity of working solutions, reagents and plasma samples was carefully measured each time when added to the tubes; exact required amounts were always gravimetrically measured, e.g 0.0084 g uric acid exactly was dissolved in a Grade A 100 mL volumetric flask.
g. Calibration curves of standard solutions were constructed each time before testing the volunteers' plasma samples and the regression equation slope (with zero intercept) from that day's calibration curve was used to calculate concentration. The R^2 value from these 6 point lines was always greater 0.98.

2.3 Statistical analysis

Statistical analysis was carried out using the Microsoft Excel 97™, SigmaPlot 4.0™ and SigmaStat™ software package for Windows (2.03). Statistical significance was set at $P<0.05$.

- One Way Repeated Measures ANOVA and paired t-test was used to assess the effect of diet and activity intervention on the AOA of plasma and lipid perxidation before and after diet and physical activity intervention;
- One Way ANOVA and unpaired t-test was used to investigate the changes of AOA of plasma and lipid peroxidation in males and females;
- Pearson correlation was utilized to demonstrate relationship between the changes in AOA of plasma, lipid peroxidation and blood biochemical parameters;
- The crossover analysis for kiwifruit was analyzed using unpaired t tests following the Hills and Armitage method (1979). This method compares the effects of different treatments (kiwifruit included in the diet and the diet without kiwifruit consumption) on the same subjects during different treatment period (Period 1, between week 6 and 9 and Period 2, between week 9 and 12). The analysis of the crossover includes the treatment effect (differences between the week 12 and week 9), the period effect (difference between added and no kiwifruit in the diet), and the interaction between the treatment and the period (week 9+week 12) to test for residual effects of the first treatment, or the general level of response within the participant. The two-sample t-test was used to test the significance of the treatment effect, period effect and the interaction between the treatments. The level of significance was set at $P<0.05$.
- The normality of data was tested with One Sample Kolmogorov-Smirnov test.

All data in the text are expressed as means ± standard deviation (SD).

CHAPTER 3

Results

The initial 12-week study "Effect of diet and physical activity intervention on markers of oxidative stress" was followed up with measurements 52 weeks later. Fifty-two participants (male 24, female 28) presented at 5 measurement points during the12 week study period, while only 38 participants returned measurement at week 52. The ethnicity of 52 participants presented at the beginning of the study is shown in Table 3.1.

Table 3.1 . Ethnicity of participants by self identification

Ethnicity	Number
New Zealand European	38
British	6
Maori	3
Tongan	2
Samoan	2
Indian	1
Total	52

Most participants were self reported "health conscious" individuals and were aware of the importance of healthy diet and physical activity according to their answers to the food frequency and physical activity questionnaires at the beginning of the study. However, after the baseline measurements were made at week 0, a variety of biological risk factors were identified in 52 participants (Table 3.2. obtained from Cumin 2004).

Table 3.2. Pre-existing risk factors determined by measurement of participants at start of study.

Risk factor	Number of participants n	Percent %
Systolic BP >140 mmHg	4	8 %
Diastolic BP >90mmHg	4	8 %
Fasting plasma glucose >6.0 mmol/L	2	4 %
Insulin >7.1μU/mL	8	15 %
Total cholesterol >5.0 mmol/L	37	70 %
HDL cholesterol <1.0 mmol/L	1	2 %
LDL cholesterol >3.0 mmol/L	38	72 %
Triglyceride >2.0 mmol/L	5	9 %
Total/HDL ratio >4.5 mmol/L	21	40 %
%body fat by BIA* >25% male;>30% female	39	74 %

*BIA – by single frequency bioimpedance analysis

Almost 70 percent of participants were classified as being either overweight or obese using body mass index (BMI) cut off values and adjusted to ethnic differences (Cumin, 2004).

Changes in diet and physical activity by the participants were assessed by the food frequency (FFQ) and physical activity (PAQ) questionnaires (Cumin 2004). The intake of the average daily number of fruit, vegetables and fruit and vegetable servings, average weekly number of oily fish servings and the average daily physical activity over the period of the study were recorded as daily scores. e.g intake of 5 fruits in one day was scored as 5. The physical activity questionnaire asked how many times in the previous week the participant had been involved in physical activity such as climbing some stairs for exercise instead of taking the elevator, walking instead of driving a short distance, parking away from their destination, walking during their launch hour or after dinner, getting off at a bus stop which is not the one nearest to their destination and walking, or other mild/moderate physical activity. The more strenuous activities such as

jogging or running, playing tennis, rugby or soccer, swimming, riding a bicycle, exercising in a gym, etc, were assessed as a number of minutes the participant was involved in it. A significant increase in the number of participants who reported consuming fruit at a rate of 2 or more portions per day, vegetables 3 or more per day, fruit+vegetable 5+ per day and oily fish 3 times a week. The number of participants reporting that they were undertaking at least 30 minutes or more per day strenuous activity increased. The number of participants consuming the average daily number of fruit, vegetable and fruit and vegetable servings, average weekly number of oily fish servings and were involved in more average daily physical activity (excluding pedometer readings) increased over the period of the study (Table 3.3). The biggest changes were in the increase in reported fruit consumption and having oily fish in the diet.

Table 3.3 The percentage of participants reporting the recommended average numbers of fruit, vegetable and oily fish servings, and time spent in strenous physical activity

	Week0 N=52	**Week3 N=52**	**Week6 N=52**	**Week9 N=52**	**Week12 N=52**	**Week12* N=38**	**Week52 N=38**
Fruit 2+per day	47%	62%	67%	77%	62%	64%	75%
Vegetable 3+per day	81%	75%	83%	83%	83%	86%	83%
Fruit+veg5+per day	64%	79%	85%	85%	72%	72%	78%
Oily fish 3x/week	14%	11%	23%	32%	38%	39%	42%
Strenuous Physical activities (≥ 30 min.day^{-1})	9%	9%	9%	19%	11%	11%	11%

* week 12 reported with only those 38 participants that returned for measurements in week 52

The study succeeded in decreasing the risk factors for cardiovascular diseases. The most significant changes were found in plasma lipid profile namely decrease in total cholesterol, triglycerides and total cholesterol to high density lipoprotein (tot chol/HDL) ratio. The level of decreased triglycerides was maintained over the year. A positive trend were observed in risk factors of type 2 diabetes, namely plasma insulin and glucose decreased, insulin resistance (measured as HOMA B%) decreased and insulin sensitivity (measured as HOMA S%) increased over the first 12 week of diet and activity intervention. Plasma insulin and insulin sensitivity decreased at week 52 and insulin resistance increased but they were still much positive than at the start of the study (Table 3.4 adopted from Cumin 2004).

The association of short (12 week) and long (52 week) term lifestyle changes with changes in plasma antioxidant activity (AOA) and lipid peroxidation (LP) were measured by the ferric reducing ability of plasma (FRAP) and the thiobarbituric acid reactive substances (TBARS) assays, respectively, are discussed next.

Table 3.4 Mean changes in risk factors for cardiovascular diseases and type 2 diabetes

Blood biochemical analysis	Week 0 n=52	Week 3 n=52	Week 6 n=52	Week 9 n=52	Week 12 n=52	p* RMANOVA	Week12‡ n=38	Week 52 n=38	t test
Total chol (mmol/L)	5.6±1.1[abc]	5.4±1.0[cde]	5.3±1.0[be]	5.5±1.0[f]	5.3±1.0[adf]	<0.001**	5.3±1.1	5.3±1.0	0.982
HDL (mmol/l)	1.5±0.31[cd]	1.4±0.31[bd]	1.4±0.3[acef]	1.5±0.31[e]	1.5±0.31[ab]	<0.001**	1.4±0.34	1.5±0.31	0.833
LDL (mmol/l)	3.5±0.97[ab]	3.4±0.9	3.4±0.94	3.4±0.91[b]	3.3±0.94[ac]	<0.001**	3.4±0.99	3.3±0.94	0.536
Trigl (mmol/l)	1.3±0.7	1.3±0.7[c]	1.2±0.7[b]	1.4±0.8[abc]	1.2±0.6[a]	<0.001**	1.1±0.4	1.3±0.5	0.023**
tot/hdl	4.0±1.0[c]	4.0±1.1[b]	4.0±1.1[a]	3.9±1.0[d]	3.7±0.9[abcd]	<0.001**	3.8±0.97	3.8±0.1	0.849
Mean gluc (mmol/L)	5.2±0.1[ab]	5.1±0.1	5.1±0.1[c]	5.0±01[ad]	5.1±0.1	0.003**	5.1±0.4	5.0±0.4	0.207
Mean insulin (pm)	58.42±28.93	58.62±29.33	53.73±25.55	57.94±27.55	55.31±29.72	0.369	50.54±21.50	49.65±24.07	0.869
Mean HOMA B%	92.3±28.5	94.1±27.7	92.1±26.8	97.9±27.8	90.8±27.8	0.285	86.0±25.8	89.9±27.3	0.779
Mean HOMA S%	109.0±45.5	112.9±53.6	119.8±52.5	116.5±65.0	118.0±54.4	0.063	125.7±56.3	127.3±50.3	0.957

[a,b,c,d,e] P<0.050 for weeks in the same category with the same letters

p* rmANOVA weeks 0,3,6,9,12 ** P<0.05

t test weeks 12 and 52(for the 38 participants measured at both weeks only)

‡participants measured at week 52

3.1 Plasma Antioxidant changes (AOA) measured by the FRAP assay

3.1.1 Twelve week changes in plasma antioxidant activity

At the baseline (week 0) mean plasma antioxidant activity (AOA) measured by the FRAP assay ranged from 571mmol/L to 2706mmol/L (mean 1227; ±420). It was thought that the participants with relatively lower plasma AOA at baseline were more likely to be affected by the intervention then the participants starting with relatively higher plasma AOA. Therefore, the 28 participants with initial plasma AOA lower than 1200mmol/L were assigned to a "low AOA" group and the 24 participants with plasma AOA higher than 1200mmol/L were assigned to a "high AOA" group.

The data for the participants was divided into groups: full data (12 week), low AOA, high AOA and the completed 52 week group was also divided as low AOA and high AOA groups. The statistical analysis with One Way RM ANOVA – Pairwise Multiple Comparison Procedure (Tukey test) was utilized to demonstrate the statistically significant differences among the 5 measurements in different groups (Table 3.5). The results showed statistically significant differences among the 5 measurements in full data group ($P=0.05$) and low AOA group ($P<0.001$) during the 12 week intervention period and in completed 52 week group ($P=0.05$) and low AOA group ($P=0.007$) during the one year intervention period.

Table 3.5 Mean antioxidant activity (AOA) of plasma (in micromol/L) measured by FRAP assay over 12 (n=52) and 52 weeks (n=38)

Category by AOA status	Size	Week 0	Week 3	Week 6	Week 9	Week 12	Week 52	P RMANOVA
Full data 12 week	N=52	1213±420$^{\eta}$	1250±422	1285±474	1338±445	1337±501		0.05*
Low AOA	N=28	933 ±181$^{\delta}$	1022±297	1030±261	1148±389	1135±433		<0.001*
High AOA	N=24	1533 ±381	1531±377	1580±489	1558±408	1550±498		0.975
Completed 52 week	N=38	1216 ±422	1257±401	1314±447	1382±455	1356±242	1335±373	0.05*
low AOA	N=22	965 ±171$^{\gamma}$	1014±276	1040±239	1176±385	1132±425	1150±297	0.007*
High AOA	N=16	1565 ±421	1597±285	1698±379	1669±403	1656±519	1558±361	0.668

* the differences are significant – measured by One Way RM ANOVA, Posthoc Tukey Test

η – significantly different from week 12

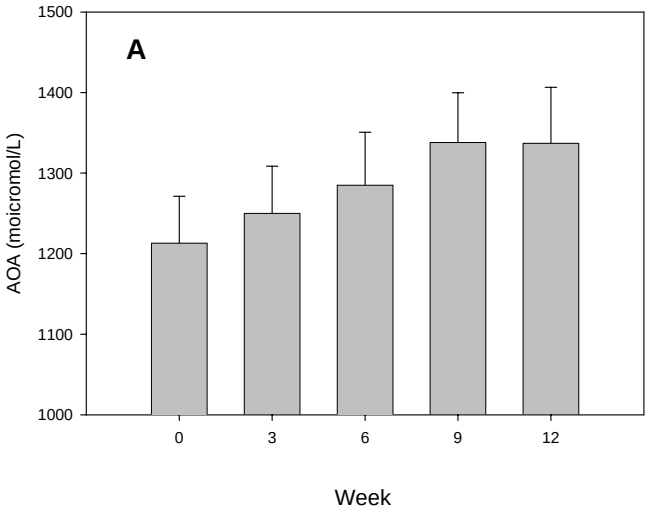
δ – significantly different from week 9 and week 12

γ – significantly different from week 12 and week 52

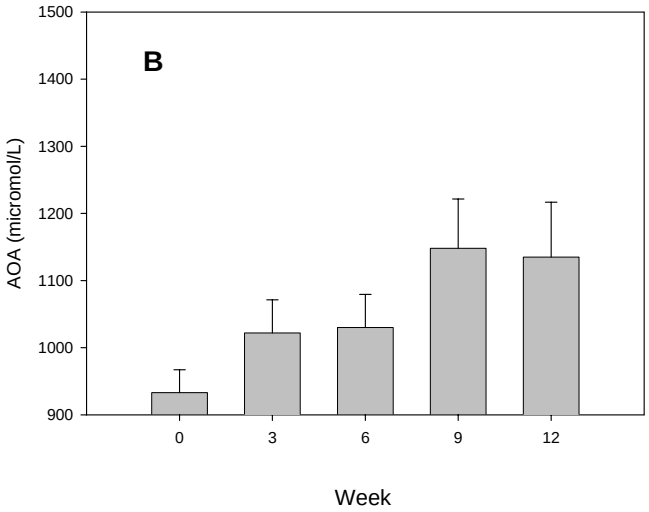
During the 12 week period plasma AOA tended to increase as a result of the diet and activity intervention. Although, in the full data group, the average increase of 124mmol/L in plasma AOA between week 0 and week 12 was significant (from 1213±420 to 1337±501mmol/L, N=52, P=0.047) the increase was even higher, 202mmol/L (from 933±181 to 1135±433, P<0.001), in the participants with lower AOA at baseline, whereas no statistically significant changes were shown over the year for the 16 participants with higher AOA at baseline (1533±381 to 1550±498, N=24; P=0.859) (Figure 3.1).

Figure 3.1 Antioxidant activity (AOA) measured at week 0, 3, 6, 9 and 12 in A. All participants, B. 28 participants with low AOA C. 24 participants with high AOA at baseline

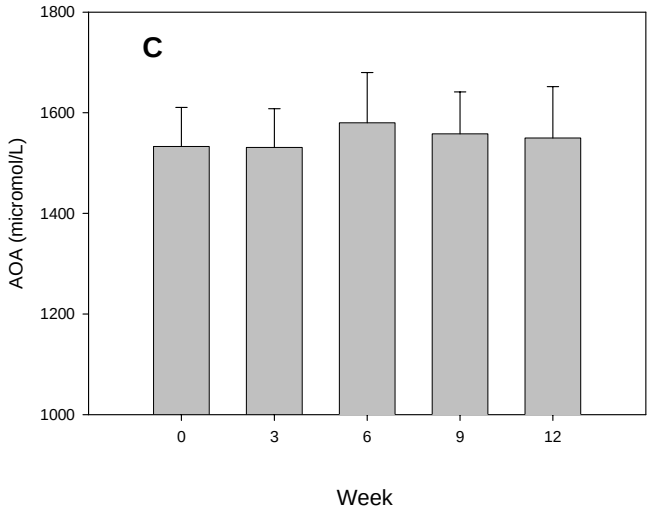
A. Full AOA, N=52 (12 weeks)



B. Low AOA, N=28 (12 weeks)



C. High AOA, N=24 (12 weeks)



Furthermore, changes in plasma AOA (assessed by paired t-test) during the12-week intervention period were affected by the sex. A statistically significant increase in AOA of 349micromol/L was found in males (N=12, 886±210 to 1235±500; P=0.006) with lower AOA at baseline but no significant changes were found in females with lower AOA of the same group (N=16; 967±146 to 1067±360; Δ100micromol/L, P=0.243) Table 3.6.

Table 3.6 Changes in plasma AOA (in micromol/L) between week 0 and week 12 – all participants and divided by sex and AOA activity level

Participants	Size	Plasma AOA Week 0	Plasma AOA Week 12	P- value
Full data	N-52	1213±420	1337±502	0.047*
Male	N=24	1238±516	1389±490	0.104
Female	N=28	1184±314	1277±512	0.253
Low AOA	N=28	933±181	1135±433	0.005*
Male	N=12	886±210	1235±500	0.006*
Female	N=16	967±146	1067±360	0.243
High AOA	N=24	1532±380	1550±498	0.859
Male	N=12	1590±493	1543±447	0.708
Female	N=12	1474±228	1556±464	0.602

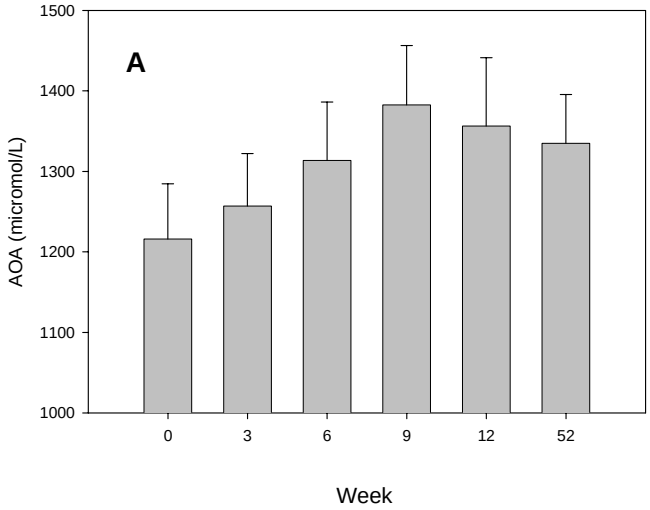
* Statistically significant difference (P<0.05) measured by paired t test

3.1.2 The one year effect of diet and activity intervention on plasma antioxidant activity (AOA) measured by FRAP assay

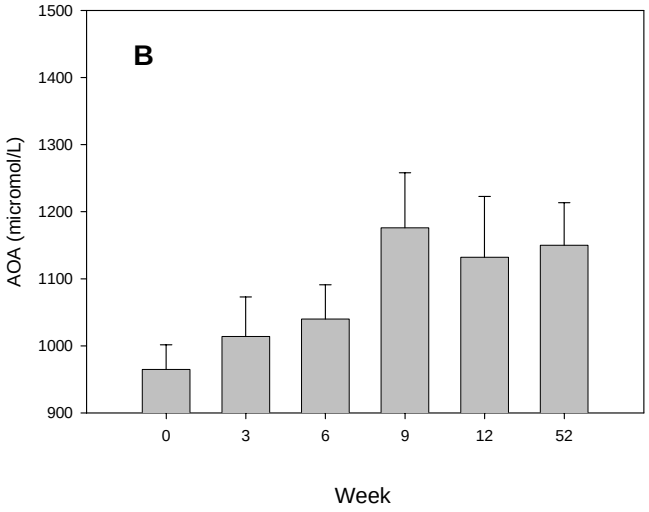
The long term effect of healthy diet and increased physical activity was measured by the differences in AOA between week 0 and week 52 in the thirty-eight participants who presented at all 6 measurement points (Table 3.5). One Way RM ANOVA demonstrated that although average plasma AOA at week 52 was decreased a little compared to the previous measurement made at week 12, the overall increase of AOA was statistically significant (119micromol/L increase) compared to the baseline measurements made at week 0 (1216±423 to 1335±373, P=0.014, N=38). As was expected, more significant changes (185micromol/L increase) in AOA were observed in 22 participants with lower AOA at baseline (965±171 to 1150±297, P= 0.004) whereas no significant changes were found in the 16 participants with higher AOA at baseline after one year (1565±421 to 1558±361, P=0.924) (Figure 3.2).

Figure 3.2 Antioxidant activity (AOA) measured at week 0, 3, 6, 9, 12 and 52 in A. 38 participants who compete measurements Over the one year, B. 22 participants with low AOA at baseline and C. 24 participants with high AOA at baseline

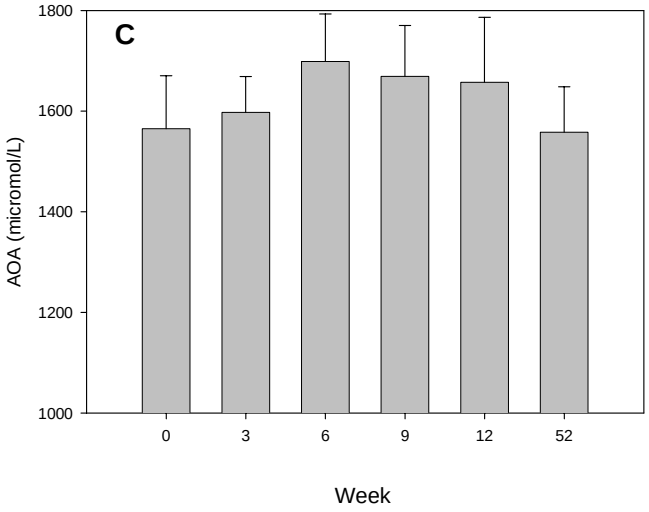
A. Completed AOA, N=38 (52 weeks)



B. Completed, low AOA, N=22 (52 weeks)



C. Completed, high AOA, N=16 (52 weeks)



As for the short term, 12 week, effect of intervention, the changes in plasma AOA after one year were statistically significant (297micromol/L increase) in male participants with lower AOA at baseline (883±205 to 1180±376, P=0.008, N=11), whereas no statistically significant changes were observed in female participants (1042±51 to 1111±180, P=0.206, N=11, Table 3.7).

Table 3.7 Statistically significant changes in plasma AOA (in micromol/L) between week 0 and week 52

Participants	N	Plasma AOA Week 0	Plasma AOA Week 52	P- value
Full data	N-38	1216±423	1335±373	0.014*
Male	N=21	1260±541	1394±441	0.074
Female	N=17	1161±201	1226±267	0.241
Low AOA	N=22	965±171	1150±297	0.004*
Male	N=11	883±205	1180±376	0.008*
Female	N=11	1042±51	1111±180	0.206
High AOA	N=16	1564±420	1558±361	0.924
Male	N=10	1656±518	1625±399	0.744
Female	N=6	1412±59	1446±283	0.776

* Statistically significant difference (P<0.05) by paired t test

3.1.3 Effect of kiwifruit on plasma AOA

After the 3rd measurement made at week 6 the participants were randomized into two equal groups. The first 26 participants were asked to consume kiwifruit and the second 26 participants were asked to abstain from kiwifruit consumption. The participants were crossed over at week 9. The measurements were made at week 9 and at week 12. The effect of kiwifruit on plasma AOA, on the same participants, during the two treatment periods was analyzed according to Hills and Armitages' "The Two-period Cross-over Clinical Trial" method (Hills *et al.,* 1979).

The analysis of the crossover period included the treatment effect (differences between the week 12 and week 9), the period effect (week (12-9)*-1), and the interaction between the treatment and the period (week9+week12) such as residual effect of the first treatment, or the general level of response within the participant. The two-sample t-test was used to test the significance of the treatment effect, period effect and the interaction between the treatments. The level of significance was set at $P<0.05$. The participants were classified as full data (N=52, 28 female, 24 male), low AOA (N=28,16 female, 12 male) and high AOA (N=24, 12 female, 12 male).

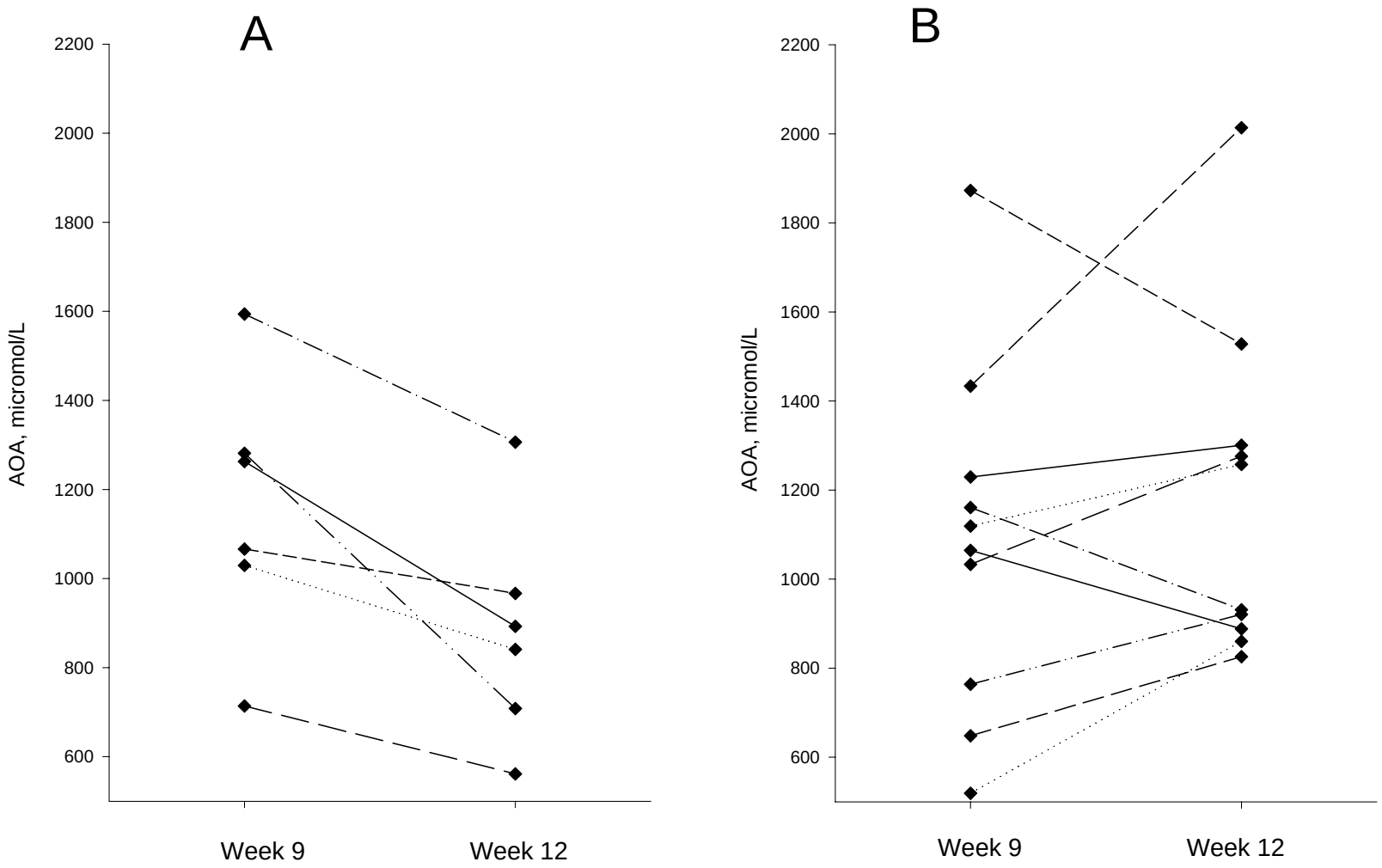
The introduction of kiwifruit to the diet showed no statistically significant difference (P=0.079) in plasma AOA in the fifty two participants who completed the 12 weeks study period and when divided by antioxidant status participants with lower plasma AOA at baseline (N=28; P=0.116) and participants with higher AOA (N=24, P=0.358) also showed no changes (Table 3.8). However, when categorized by sex, kiwifruit consumption increased plasma AOA in female participants but the significance level was reached only in female participants with lower plasma AOA at baseline (N=16; P=0.01) (Figure 3.3), while in male participants, kiwifruit decreased the plasma AOA but did not reached the significant level in any male groups.

Table 3.8 Crossover trial of effect of kiwifruit consumption on AOA of plasma measured by FRAP assay

Group	Size	Period 1 Kiwifruit first		Size	Period 2 Kiwifruit second		P value[£]
		Week 9	Week 12		Week 9	Week 12	
Full data	N=26	1379±446	1385±579	N=26	1294±440	1272±410	0.079
Female	N=14	1431±478	1412±539	N=14	1148±360	1241±367	0.19
Male	N=12	1318±417	1397±418	N=12	1465±477	1307±471	0.054
Low AOA	N=14	1174±256	1080±367	N=14	1123±471	1190±477	0.116
Female	N=6	1158±296	879±253	N=10	1084±392	1180±378	0.01*
Male	N=8	1150±248	1246±347	N=4	1283±717	1212±795	0.233
High AOA	N=12	1642±483	1732±610	N=12	1474±315	1368±274	0.358
Female	N=8	1636±500	1637±656	N=4	1308±231	1396±332	0.813
Male	N=4	1655±519	1920±535	N=8	1557±331	1355±264	0.106

[£] t-test for comparison of the two groups for changes from baseline over the two periods
* significant change (P<0.05)
No significant period or treatment*period effects were observed (P>0.05)

Figure 3.3 Effect of kiwifruit on plasma AOA. A. Period 1 – kiwifruit consumed between week 6 and 9,
B. Period 2 – kiwifruit consumed between week 9 and 12



3.2 Lipid peroxidation changes measured by TBARS assay

3.2.1 The 12 week effect of diet and activity intervention on lipid peroxidation (LP) measured by the TBARS assay

At the baseline (week 0) the lipid peroxidation measured by TBARS assay in the fifty two participants ranged from 1.07mmol/L to 2.3mmol/L (mean 1.79± 0.24). It was thought that the participants with relatively higher LP at baseline were more likely to be affected by the study (e.g. intervention will decrease the LP); therefore, the participants were divided into relatively higher initial LP group (N=34, LP>1.788mmol/L) and relatively lower initial LP group (N=18, LP<1.788mmol/L).

Five measurements were made during the 12 weeks diet and activity intervention study period. No significant changes analyzed by the One Way RM ANOVA, Posthoc Tukey test were seen in lipid peroxidation among the five measurements for all participants or when divided by lipid peroxidation status groups.

The table 3.9 summarizes the changes in LP for 12 weeks and over 52 weeks. The mean (± SD) of plasma LP of at the six measurement points is shown in table 3.8. The participants are divided by the groups: full data (12 week), low LP, high LP and completed 52 week group also divided as high LP and low LP groups.

Table 3.9 Lipid peroxidation (LP) measured by TBARS assay

Participants	Size	Week 0	Week 3	Week 6	Week 9	Week 12	Week 52	P RMANOVA
Full data 12 week	N=52	1.76±0.24	1.74±0.24	1.76±0.23	1.76±0.25	1.78±0.24		0.152
High LP	N=34	1.92±0.11	1.90±0.13	1.89±0.15	1.91±0.13	1.93±0.14		0.167
Low LP	N=18	1.54±0.22	1.53±0.21	1.58±0.19	1.57±0.25	1.57±0.22		0.064
Completed 52 week	N=38	1.80±0.24	1.80±0.22	1.77±0.22	1.79±0.24	1.82±0.23	1.83±0.25	0.127
High PL	N=26	1.91±0.09	1.90±0.11	1.89±0.14	1.90±0.12	1.93±0.13	1.94±0.28	0.208
Low LP	N=12	1.54±0.26	1.55±0.23	1.60±0.22	1.56±0.26	1.60±0.25	1.595±0.29	0.213

The lipid peroxidation did not change between week 0 and week 12 (1.757±0.24 to 1.775±0.24, P=0.208, N=52). No changes in LP were found in participants with relatively higher LP at baseline (1.922±0.11 to 1.929±0.22, P=0.675, N=34) as well as in participants with relatively lower LP at baseline (1.535±0.22 to 1.573±0.22, P=0.157, N=18).

Furthermore, the changes in LP during the 12 week study period were not affected by the sex (P=0.643) measured by paired t-test. The effect of diet and activity intervention on LP between week 0 and week 12 categorized by sex is summarized in table 3.10.

Table 3.10 Changes in lipid peroxidation (LP) between week 0 and week 12

Participants	Size	LP Week 0	LP Week12	P- value
Full LP	N=52	1.76±0.24	1.78±0.24	0.208
Male	N=24	1.85±0.19	1.88±0.21	0.239
Female	N=28	1.74±0.27	1.75±0.26	0.548
High LP	N=34	1.92±0.11	1.93±0.14	0.675
Male	N=19	1.92±0.09	1.95±0.13	0.157
Female	N=15	1.92±0.12	1.90±0.14	0.495
Low LP	N=18	1.54±0.22	1.57±0.22	0.157
Male	N=5	1.57±0.23	1.59±0.18	0.858
Female	N=13	1.52±0.23	1.57±0.24	0.069

3.2.2 The one year effect of diet and activity intervention on lipid peroxidation (LP) measured by the TBARS assay

The 38 participants were measured for lipid peroxidation at week 52. The One Way RM Anova was used to find the long term (between week 0 and week 52) effect of changed diet and physical activity on plasma PL.

No changes in LP were found in the 38 participants after one year (1.797±0.24 to 1.828±0.25, P=0.139), as well as in the 26 participants with relatively higher LP at baseline (1.914±0.09 to 1.935±0.13, P=0.35) and in the 12 participants with relatively lower initial LP (1.544±0.26 to 1.595±0.29, P=0.264) (Table 3.9).
The LP was not affected by the sex as measured by the paired t-test (P=0.156). The changes in LP between week 0 and week 52, categorized by sex, are summarized in table 3.11.

Table 3.11 Changes in lipid peroxidation (LP) between week 0 and week 52

Participants	Size	LP Week 0	LP Week12	P- value
Full data	N=38	1.80±0.24	1.828±0.25	0.139
Male	N=20	1.86±0.19	1.88±0.21	0.486
Female	N=18	1.73±0.27	1.77±0.28	0.167
High LP	N=26	1.91±0.09	1.94±0.13	0.35
Male	N=16	1.93±0.1	1.95±0.15	0.556
Female	N=10	1.90±0.07	1.92±0.14	0.376
Low LP	N=12	1.54±0.26	1.60±0.29	0.264
Male	N=4	1.60±0.25	1.62±0.26	0.752
Female	N=8	1.52±0.27	1.58±0.32	0.303

3.2.3 Effect of kiwifruit on lipid peroxidation

The effect of kiwifruit on lipid peroxidation was analyzed by the two-tailed T-test according to the Hills and Armitage method (Hills *et al.,* 1979). The participants were classified as full data (N=52, 28 female, 24 male), high initial LP (N=34, 15 female, 19 male) and low LP (N=18, 13 female, 5 male).

Changes in LP as a result of kiwifruit introduction to the diet of the 52 participants were not significant (P=0.472). This lack of measurable change was still demonstrated in the subgroup of 34 participants with higher LP at baseline (P= 0.71) and in the 18 participants with lower initial LP (P=0.304).

Although there was no significant period (P>0.05) or interaction (P>0.05) effects observed during the analysis of the treatment (kiwifruit) effect, the results showed a minimal increase, though not significant, in LP in full data (Δ0.150mmol/L) and in high LP groups (Δ0.027mmol/L), in participants after they stopped eating kiwifruit (Table 3.12, Period 1). Kiwifruit consumption was found to be associated with a minimal increase in LP in participants of all groups who consumed kiwifruit second (Period 2) (see the table 3.12 for details).

Table 3.12 Crossover trial of effect of kiwifruit consumption on lipid peroxidation measured by TBARS assay

Group	Size	Period 1 Kiwifruit First		Size	Period 2 Kiwifruit Second		P value†
		Week 9	Week 12		Week 9	Week 12	
Full data	N=26	1.77±0.24	1.92±0.14	N=26	1.81±0.25	1.83±0.22	0.472
Female	N=14	1.7±0.27	1.69±0.26	N=14	1.78±0.27	1.80±0.26	0.324
Male	N=12	1.86±0.22	1.89±0.18	N=12	1.84±0.22	1.87±0.18	0.993
High LP	N=14	1.95±0.14	1.97±0.13	N=20	1.88±0.12	1.90±0.13	0.71
Female	N=6	1.84±0.04	1.84±0.11	N=9	1.92±0.16	1.94±0.14	0.586
Male	N=8	1.97±0.13	2.01±0.14	N=11	1.89±0.14	1.91±0.12	0.571
Low LP	N=12	1.61±0.25	1.59±0.24	N=6	1.49±0.249	1.52±0.24	0.304
Female	N=8	1.59±0.28	1.58±0.26	N=5	1.53±0.25	1.58±0.22	0.494
Male	N=4	1.63±0.19	1.62±0.18	N=1	1.28*	1.41*	-

† Unpaired t-test for comparison of the two groups for changes from baseline over the two periods

No significant period or treatment x period effects was observed (P>0.05). * only one participant

3.3 Associations among blood lipids, glucose and insulin, plasma antioxidant activities (AOA) and lipid peroxidation (LP)

The effect of diet and physical activity on blood biochemical parameters (Table 3.4) such as fasting plasma glucose, insulin, insulin sensitivity and resistance, plasma lipids – total cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL), total cholesterol:HDL ratio, triglycerides, was assessed by Cumin (Cumin 2004). The Pearson Product Moment Correlation was used to test the association between the changes in blood biochemical parameters and the changes in plasma AOA and lipid peroxidation as a result of the intervention.

The correlation between the changes in AOA and LP and the changes in blood biochemical parameters were tested in different categorical groups such as Full data (AOA), Low AOA, High AOA, Full data (LP), High LP and Low LP (each group were also categorized by sex). The short term (12 week) and long term (52 week) correlations were tested separately.

Unexpected positive associations were found between plasma AOA and the total cholesterol. During the 12 week intervention period the positive relationship (particularly in female participants) were found between the AOA and total cholesterol, which means that increased plasma AOA was associated with increased total cholesterol. The significant correlation was found in full data (AOA), female group ($r=0.477$, $P=0.01$), high AOA, female group ($r=0.637$, $P=0.026$) and in high AOA group ($r=0.455$, $P=0.015$) (Table 3.13 and Figure 3.4).

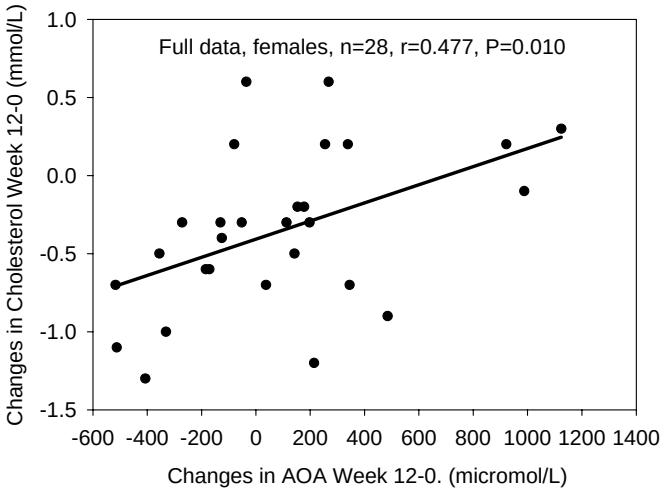
Table 3.13 Association between AOA and total cholesterol

Participants	Size	Correlation between	r	P
Full data (AOA), female	N=28	AOA and total cholesterol	0.477	0.01*
High AOA, female	N=12	AOA and total cholesterol	0.637	0.026*
High AOA	N=24	AOA and total cholesterol	0.455	0.015*

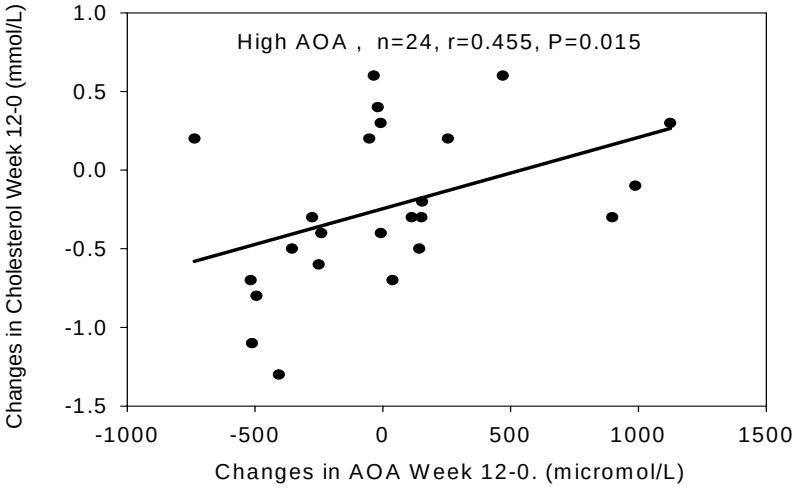
*significant correlation ($P<0.05$), Pearson moment correlation

Figure 3.4 Associations of changes in AOA and total cholesterol before and after intervention in A. Full data, Female, n=28 B. High AOA, n=24 and C. High AOA, Female, n=12

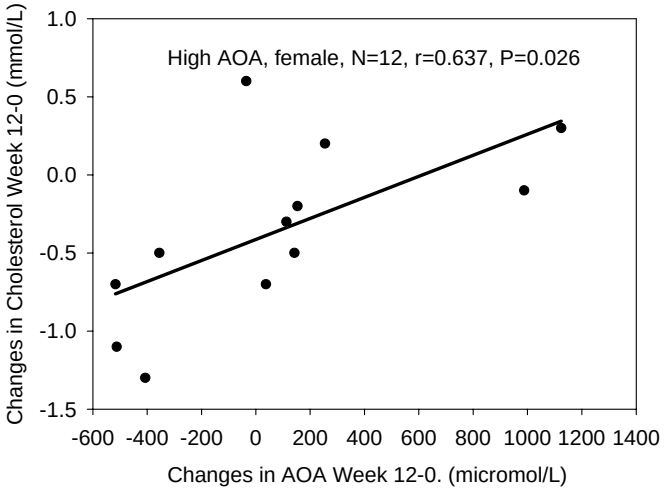
A



B



C



Lipid peroxidation was also found to be related to the total cholesterol during the 12 week study period. However, the relationship was positive which means that a decrease in LP was associated with a decrease in total cholesterol. A significant correlation was found in the full data (LP) group (r=0.354, P=0.01), the full data (LP) female group (r=0.425, P=0.024 and in the high LP group (r=0.394, P=0.021, Table 3.14 and Figure 3.5).

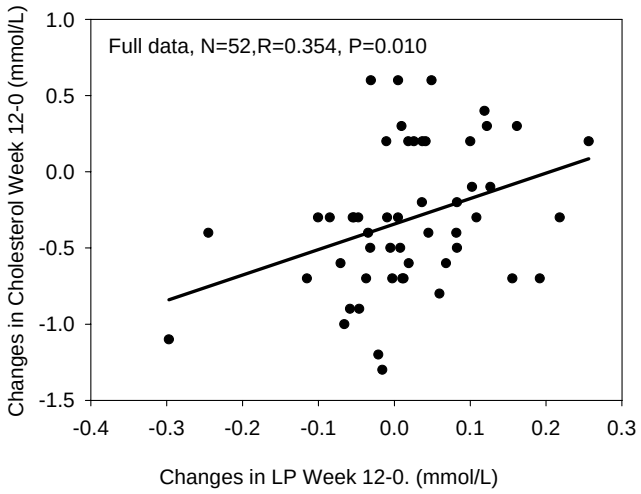
Table 3.14 Association between lipid peroxidation and total cholesterol

Participants	Size	Correlation between	r	P
Full data (LP)	N=52	LP and total cholesterol	0.354	0.01*
Full data (LP), female	N=28	LP and total cholesterol	0.425	0.024*
High LP	N=34	LP and total cholesterol	0.394	0.021*

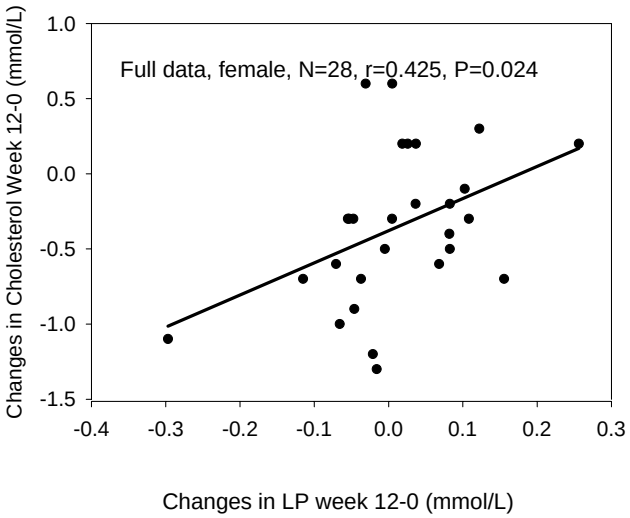
*significant correlation (P<0.05), Pearson moment correlation

Figure 3.5 Associations of changes in LP and total cholesterol before and after intervention in A. Full data, n= 52, B. Full data, female, n=28

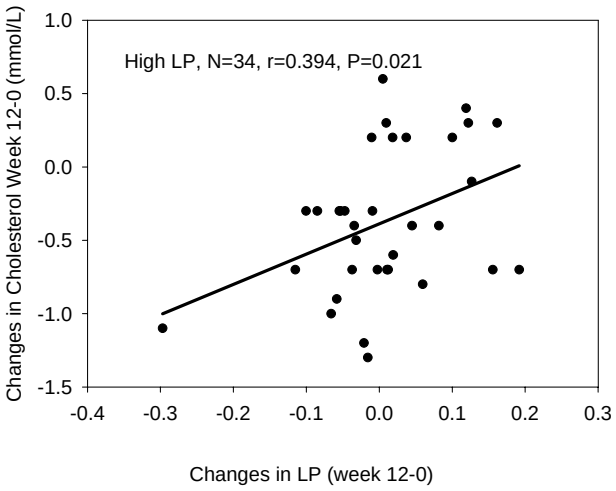
A.



B.



C.



Other significant correlations were found between lipid peroxidation and plasma triglyceride level as well as between AOA and triglycerides. The changes in lipid peroxidation were positively correlated to the changes in triglycerides during the 12 week period (N=52, r=0.275, P=0.048). More strong negative associations were found between AOA and triglycerides after one year in female participants with relatively low AOA at baseline (N=11, r=-0.722, P= 0.018) (Table 3.15 and Figure 3.6). The relationship was negative, which means that an increase in plasma AOA was associated with a decrease in plasma triglyceride level, however, the strong correlation might be related to the smaller sample size and relatively extreme values.

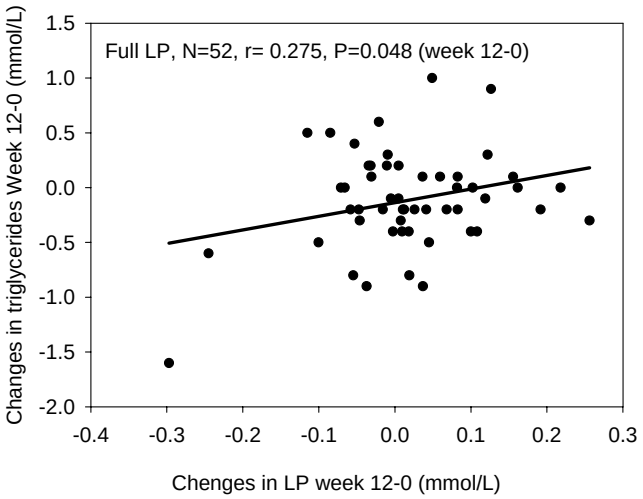
Table 3.15 Correlation between LP and triglycerides and AOA and triglycerides

Participants	**Size**	**Correlation between**	**r**	**P**
Full data (LP) (12 week)	N=52	LP and triglycerides	0.275	0.048*
Low AOA, female (52 week)	N=11	AOA and triglycerides	-0.722	0.018*

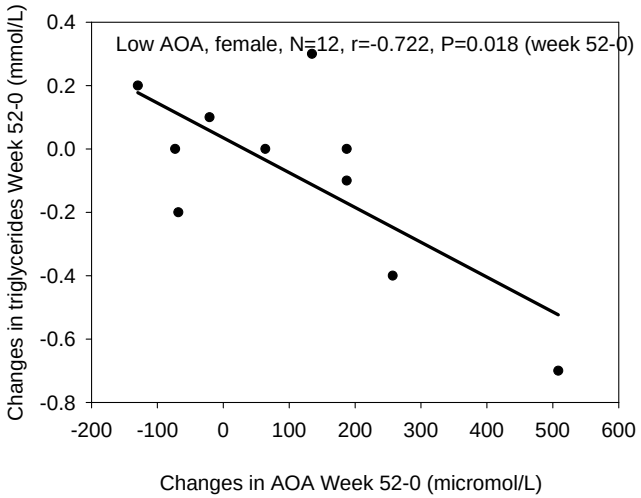
*significant correlation (P<0.05), measured by Pearson moment correlation

Figure 3.6 Associations between changes in LP and triglycerides (A) and between changes in AOA and triglycerides (B)

A.



B.



Conflicting associations at 12 weeks and 52 weeks were also found between the AOA, insulin, insulin resistance and sensitivity (Table 3.16 and Figure 3.7). During the 12 week period AOA were found to be positively associated with insulin resistance in male participants with initial high AOA (N=12, r=0.576, P=0.05) but after one year in the 22 participants with lower AOA at baseline an increase in AOA was correlated with a decrease in insulin sensitivity (r=-0.533, P=0.01). Furthermore, AOA was found to be positively associated with plasma insulin level in female participants with low initial AOA at week 52 (N=16, r= 0.505, P= 0.046). However, during the 12 week study period, increases in AOA were correlated with decreases in plasma glucose level in the 24 participants with high AOA group (r=-529, P=0.007) and in the 12 male participants in the same group (r=-617, P=0.033).

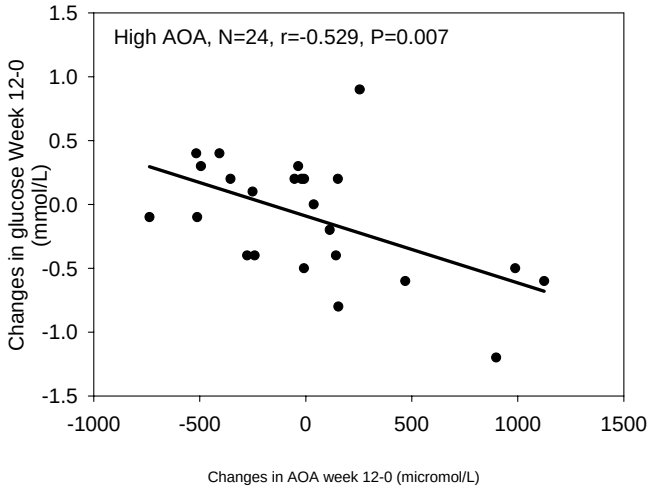
Table 3.16 Correlation between changes in AOA and blood glucose, insulin resistance and insulin sensitivity

Participants	Size	Correlation between	r	P
High AOA (12 week)	N=24	AOA and glucose	-0.529	0.007*
High AOA, male (12 week)	N=12	AOA and glucose	-0. 617	0.033*
High AOA, male (12 week)	N=12	AOA and insulin resistance	0.576	0.05*
Low AOA, (52 week)	N=22	AOA and insulin sensitivity	-0.533	0.01*
Low AOA, female (52 week)	N=16	AOA and insulin	0.505	0.046*

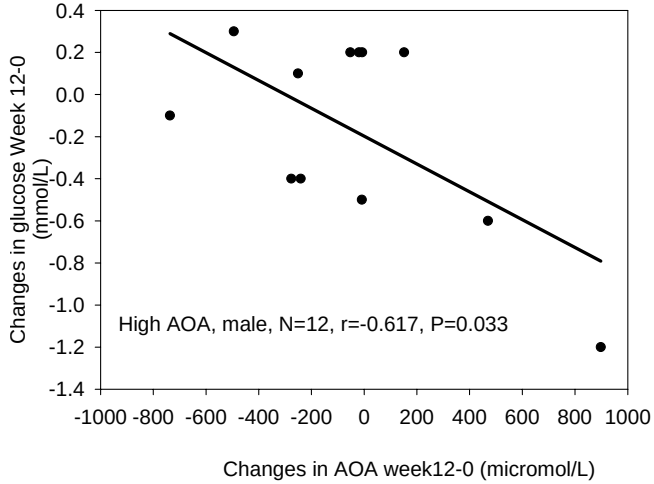
*significant correlation (P<0.05), measured by Pearson moment correlation
Systematic Pearson Correlation analysis of the associations between the changes in blood biochemical parameters and the changes in plasma AOA and lipid peroxidation as a result of the intervention are presented in Appendix 5.

Figure 3.7 Correlations between changes in AOA and blood glucose (A, B), insulin resistance (C) and insulin sensitivity (D)

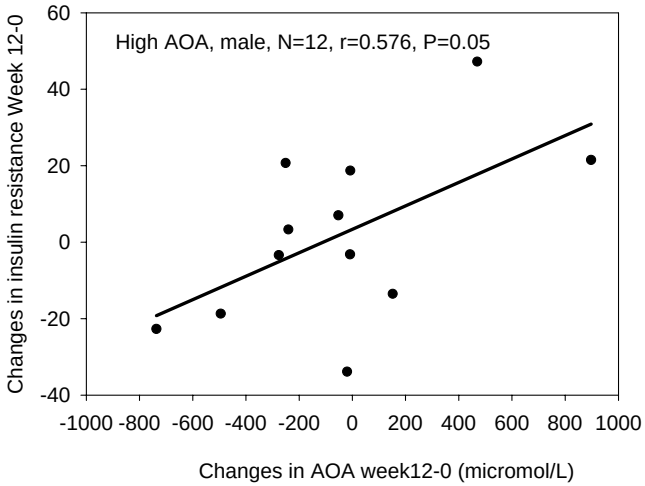
A.



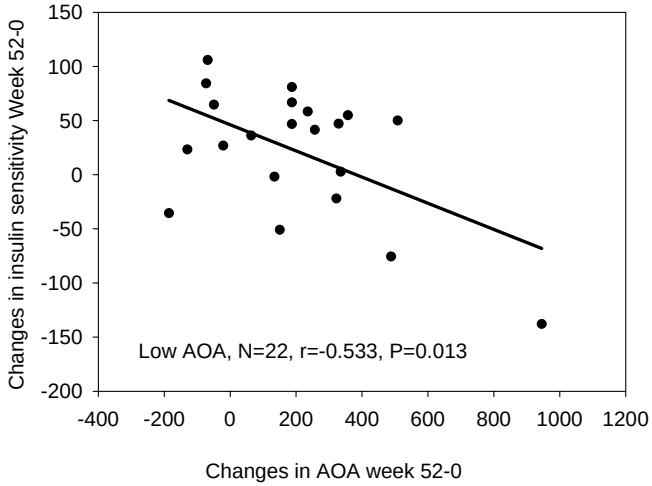
B.



C.



D.



CHAPTER 4

Discussion and conclusion

4.1 Interpretation of the results

This study of the "effect of diet and physical activity intervention on the markers of oxidative stress" was designed to demonstrate that a group work-based dietary and activity intervention can increase the level of antioxidants in the blood over a short period and in the long term and at the same time make beneficial changes to risk factors for lifestyle diseases such as cardiovascular diseases (CVD), hypertension, type 2 diabetes and cancer. The overall question asked in this study was " will the increased daily fruit, vegetable, whole grain and oily fish intake and increased physical activities decrease the oxidative stress and therefore, decrease the risk of lifestyle diseases?"

The answer is yes. The changes in diet and increased physical activities during the 12 week study period significantly improved the plasma soluble antioxidant activity (AOA) in 52 volunteers and therefore potentially decreased the level of oxidative injuries, but did not change the markers of lipid peroxidation (LP). Increased plasma soluble antioxidant levels were maintained by thirty-eight participants after one year.

4.1.1. Effect of diet and activity intervention on the markers of oxidative stress

Almost all participants had initial AOA (47/52) and LP (46/52) measured as plasma malondialdehyde (MDA) in the normal range (600-1600micromol/L (Benzie *et al.,* 1996) and 1.63-2.44mmol/L (Koracevic *et al.,* 2001) respectively). As was expected, as a result of continued lifestyle changes made since starting the study, significant changes in plasma soluble antioxidants (plasma AOA) were found in subjects with relatively lower initial AOA (AOA<1200micromol/L cut off point), at both, at 12 week and 52 weeks. In individuals with already higher antioxidant status no changes were observed. No statistically significant changes were found in markers of lipid peroxidation (plasma MDA), however, it was discovered in a subsequent search of the literature that plasma

MDA was not a good choice of method for assessment the effect of intervention on LP (see limitations section 4.2.1).

Similar findings to this study were found in a 25-day diet intervention study in Denmark (Dragsted *et al.,* 2004). With complete control of dietary intake of fruit and vegetables forty three healthy men and women were randomly assigned to 3 groups having a basic diet which was without fruit and vegetables: the "Fruveg" group received 600g fruit and vegetable in addition to basic diet, the supplement group received pills with vitamins and supplements corresponding to those present in 600g of fruit and vegetables, and the placebo group received placebo pills. Plasma AOA was measured by automated assays for Trolox equivalent antioxidant capacity (TEAC) and Ferric reducing ability of plasma (FRAP). Plasma MDA and urine 8-isoprostane $F_{2\alpha}$ were utilized as a markers of respectively *in vitro* and *in vivo* lipid peroxidation and two markers, specific for low density lipoproteins (LDL) oxidation, namely direct determination of MDA in LDL and plasma lipoprotein resistance (LDL-oxidation lag time) were used as a markers of LDL resistance to oxidation. Plasma AOA measured by FRAP and TEAC tended to increase in the Fruveg and supplement groups, although this increase was not statistically significant. However, the significant effect of sex on the FRAP results, observed in this study, was consistent with my findings. In both my study and that of Dragsted changes in plasma AOA measured by FRAP were higher in men than in women. It is unclear why men responded better to dietary interventions than women and how this higher response to dietary antioxidants is related to the fact that men have been reported to have significantly higher ($P<0.005$) "total antioxidants power" than women (Benzie *et al.,* 1999). As in my results the Dragsted study gave no evidence that the intervention changed the overall oxidative damage to lipids measured by plasma MDA (and urinary isoprostanes). However, the Dragsted study reported that changes in lipoprotein resistance (LDL-oxidation lag time) were significantly increased in both the Fruveg and supplements group. Furthermore, changes in lipoprotein resistance were significantly higher in the Fruveg group compared with the supplement group. This evidence, once again, underlines the preference of dietary antioxidants over the vitamin supplements.

Findings of another study of the effect of dietary patterns on markers of oxidative stress and conducted in Baltimore (Miller *et al.,* 1998), is also related to this research. One hundred and twenty three healthy individuals (mean age 48.5 years) were fed with a control diet low in fruit and vegetables with 37% of calories from fat. After a three week

"run-in" period subjects were randomized to 3 groups: the control group received diet low in fruit and vegetable, fruit and vegetable group received similar diet as control group but high in fruit and vegetables, and the combination group were fed with diet rich in fruit and vegetables and reduced in fats such as saturated fat, total fat and cholesterol. The eight week intervention effect was assessed by measurements including: Oxygen radical absorbing capacity (ORAC) assay for assessment serum AOA, plasma MDA (thiobarbituric acid adduct) for measuring *in vitro* LP and breath ethane (end product of N-3 polyunsaturated fatty acid (PUFA) oxidation) for measuring *in vivo* LP. The increase in fruit and vegetable consumption for 8 week intervention period resulted in a significant increase in serum AOA in both the fruit and vegetable and the combination diet group, while AOA decreased in control group. Similar to my results, *in vitro* LP measured as serum MDA increased between in-run and intervention in all dietary groups but, as in my results, did not reach the statistical significance. By contrast, *in vivo* LP (breath ethane test) increased in control group, but no changes were observed in fruit and vegetable and in combination group. However, "median regression analysis adjusted for baseline ethane measurements" showed statistically significant increases *in vivo* LP in the control group compared to the fruit and vegetable (P=0.04) and in the combination group (P=0.005). This increase was associated with a decrease in saturated (S) fat intake compared to polyunsaturated (P) fat in the combination diet group (P/S ratio was 1.3 in combination group and 0.5 and 0.6 in control and fruit and vegetable group respectively). The explanation of the increase in *in vivo* LP put forward in the paper was that "such a difference in the P/S ratio may have a major influence on the MDA assay, potentially overwhelming the effects of increased dietary intake of antioxidants" (Miller *et al.,* 1998).

Polyunsaturated fatty acids (PUFA) are well known to lower the plasma lipid level (especially LDL) and are clinically useful hypolipidemic agents. The hypolipidemic effect of PUFA is related to their ability to reduce the production of apolipoprotein B100 (Apo B 100) secretion from the hepatocytes and therefore reduce the release of very low density lipoproteins (VLDL) and low density lipoproteins (LDL) from the liver (Pan *et al.,* 2004). Pan demonstrated that LP and oxidant stress within "primary cultured hepatocytes" plays a central role in the physiological control of Apo B100 lipoprotein secretion and degradation; namely Apo B100 degradation in hepatocytes is directly and positively related to the degree of polyunsaturation of the supplied fatty acids and the ability of PUFA to increase cellular oxidative stress (i.e. increase in LP

products and decrease in antioxidants) (Pan *et al.,* 2004). This means that the potential benefits of antioxidant activities might be counteracted by potentially harmful alterations in ApoB100 metabolism within the liver.

The TBARS assay measures plasma MDA which actually is an *in vitro* estimate of PUFA peroxidation. Therefore, in dietary interventions with high PUFA product use advice can potentially complicate the interpretation of results in changes in LP. In this study dietary advice was to increase the use of canola oil, olive oil and oily fish as sources of PUFA and to reduce the consumption of saturated fat in the diet. The number of participants who regularly consumed oily fish (≥3x per week) increased during the study period and was maintained over the year (Cumin 2004) which was potentially related (together with other dietary and physical activity changes) to the significant lowering in the cardiovascular disease (CVD) risk factors; total cholesterol and triglycerides. However, the TBARS assay can not discriminate between MDA (PUFA product) absorbed from dietary sources and MDA endogenously produced due to cellular oxidative events (Thompson *et al.,* 1999). Therefore, a little increase in LP (plasma MDA) in this study, as well as in Millers' study (the combination diet group) can be potentially related to the increased dietary PUFA i.e. exogenous MDA rather than endogenous MDA. Furthermore, the TBARS assay measures the ability of plasma antioxidants to suppress the production of MDA, however "the TBARS assay is intrinsically nonspecific for MDA:nonlipid-related materials "and can offer, at least, a narrow and somewhat empiric window on the complex process of lipid peroxidation" (Janero 1990).

For the reasons discussed above it was thought in retrospect by this author that plasma MDA measured by TBARS assay was not a good choice for measuring the effect of changed diet and increased physical activities on the lipid peroxidation.

4.1.2. Effect of kiwifruit on the markers of oxidative stress

Some fruits or vegetables are particularly rich with antioxidants vitamins and phytochemicals, thus, it is tempting to assume that the health benefits associated with the antioxidant properties of that particular fruit or vegetable can be achieved and can potentially replace dietary supplementation e.g. Vitamin C is commonly used in clinical

practice for prophylactic or for treatment purposes. In this regard the effect of kiwifruit on the markers of oxidative stress was tested as a part of this intervention study and in a cross over design.

The water soluble vitamins such as vitamin C and 8 vitamin Bs are not stored in the body (Franz 1998) so they need to be eaten daily. Kiwifruit contains very significant amount of antioxidants such as ascorbic acid, folate and flavonoids. The antioxidant properties of kiwifruit have been demonstrated to protect against oxidative DNA damage (Collins *et al.,* 2001) and to stimulated DNA repair (Collins *et al.,* 2003) by promoting DNA repair enzymes. Flavonoids present in kiwifruit may also have antioxidant as well as anti-thrombotic properties. It has recently been reported that consuming 2 or 3 kiwifruit per day for 28 days reduces platelet aggregation and decreases plasma triglyceride levels (Duttaroy *et al.,* 2004). Both these responses are favourable for reduction of risk factors for CVD. However, individual variations in digestion and absorption of compounds found in kiwifruit could be further compromised by the laxative effect of kiwifruit (Rush 2002).

In this study the effects of kiwifruit on the markers of oxidative stress and DNA fragility were tested. The preliminary results showed a decrease in DNA fragility as a result of kiwifruit consumption (Rush personal communication, 2002). However, the effect of kiwifruit on the markers of oxidative stress was rather more modest than was expected. Namely, the 3-week daily kiwifruit consumption (2-3 kiwifruit per day) resulted in a significant increase in plasma AOA, measured by FRAP, only in female participants, but did not change markers of LP measures as plasma MDA in any group of participants.

These findings of AOA changes but not LP in response to kiwifruit could be compared with another intervention study that measured the effect of carotenoid-rich vegetable products (tomato-rich in lycopene, carrot-rich in α- and β-carotene and spinach-rich in lutein) on the markers of serum antioxidant status (Bub *et al.,* 2000). The effect of an 8 week intervention was that plasma MDA (as determined with TBARS assay) significantly reduced after treatment and the resistance of LDL to oxidation (determined as LDL-oxidation lag time) increased significantly while no changes were observed in water-soluble serum antioxidant level (measured as FRAP assay). The authors suggest that “lipid-soluble carotenoids from vegetable products do not substantially influence

water-soluble antioxidants, antioxidant power and antioxidant enzyme activities in healthy humans" (Bub *et al.,* 2000).

It seems that different methods for measuring different markers for oxidative stress do not correlate with each other – they are not measuring the same thing. Therefore, it is essential to avoid a misleading impression of the antioxidant defences to understand the relative contribution of individual antioxidants to the antioxidant defences, the nature of the oxidative insult examined with a particular analysis and the experimental conditions e.g. how the samples or the chemical have been treated prior to choosing the method for assessment of antioxidant status of biological fluids. A problem with the storage of the plasma samples may have interfered with the assessment of the effect of kiwifruit on the markers of oxidative stress in plasma samples. It appears that ascorbic acid in plasma is stable for less than 6 hours if stored at 4^0C and vitamin C can virtually disappear after 72 hours after being stored at 4^0C (Benzie *et al.,* 1995). The plasma samples in this study were stored at -85^0C for approximately 6 months before were tested for antioxidant defences. Because of the effect of cold on vitamin C it could be possible that the AOA measured during the 3 week kiwifruit treatment measured do not relate fully to the increased vitamin C (as shown by Collins *et al*, 2001) and do not fully represent the potential antioxidant effect of kiwifruit on the markers of oxidative stress.

4.1.3 Associations among the markers of oxidative stress and the markers of lifestyle diseases

The main aim of this study was to demonstrate associations between markers of oxidative stress and the markers of lifestyle diseases. Changes in lipid profile such as total cholesterol, HDL, LDL, triglycerides and total cholesterol/HDL ratio were measured to assess the changes in risk factors of cardiovascular diseases as a result of dietary and physical activity intervention, and the changes in fasting plasma glucose and insulin, insulin resistance and insulin sensitivity were measured as changes in risk markers for type 2 diabetes. It has been shown in this same study group (Cumin 2004) that a group dietary and physical activity intervention succeeded in decreasing the level of risk factors for cardiovascular disease. The most significant changes were found in plasma lipid profile namely a decrease in total cholesterol, triglycerides and total cholesterol/HDL ratio. The level of decreased triglycerides was maintained over the year. A positive trend was observed in risk factors of type 2 diabetes.

Some beneficial associations between markers of oxidative stress and the markers of lifestyle diseases were found in this study such as the changes in plasma LP being positively associated to the plasma triglyceride level during the 12 week intervention period as well as changes in plasma AOA, which were strongly and negatively related (r=-0.722) to the changes in plasma triglyceride level during the 52 week intervention period. Plus a positive correlation was found between the changes in LP and the changes in total cholesterol in the 12 week study period, and an increase in AOA was found to be related to the decrease in fasting glucose. However, some conflicting associations complicated the interpretation of the results. For example, it was found that the plasma AOA was positively and significantly associated with increases in total cholesterol (Table 3.13). The possible explanation, as was discussed previously (4.1.1), could be that the oxidative stress in the hepatocytes regulate the degradation of Apo B 100 and in the presence of increased antioxidant activities the Apo B100 secretion is increased that elevate the plasma level of VLDL and LDL (Pan *et al.,* 2004). It seems that more studies are needed to demonstrate the beneficial effect of antioxidants on the plasma total cholesterol as other studies have also shown an attenuated effect of antioxidant supplements on lipid-lowering drug therapy (Simvastatin-Niacin therapy) (Brown *et al.*, 2001, Cheung *et al.,* 2001), although this effect was restricted to the HDL cholesterol only.

Other conflicting associations were found between plasma AOA and markers of type 2 diabetes. There is considerable evidence (Evans *et al.,* 2002, Rosen *et al.,* 2001) that hyperglycemia results in generation of ROS, furthermore, the β-cells of the pancreas are particularly susceptible to the effects of ROS as they are low in antioxidant enzymes and have a limited ability to survive under conditions of oxidative stress. Therefore, oxidative stress has been linked to the pathogenesis of type 2 diabetes. For these reasons it was expected that the increased plasma AOA would decrease the markers of type 2 diabetes. However, it was found that the increases in plasma AOA were positively associated with increases in insulin resistance during the 12 week intervention period and negatively related to the insulin sensitivity over the one year intervention (Table 3.16). Some studies (Chen *et al.,* 2003) suggest that elevated intracellular antioxidant enzymes can cause insulin resistance. For example, it has been shown on mice that the overexpression of glutathione peroxidase (GPX) enzyme in the liver and soleus muscle results in hyperglycemia, hyperinsulinemia and insulin resistance (McClung *et al.,*

2004). These authors suggest that "the increased GPX activity may interfere with insulin function by overquenching intracellular ROS require for insulin sensitizing" (McClung *et al.,* 2004). Similar observations were made in normal pregnant women from 16 weeks of pregnancy. Namely significant ($P<0.01$) positive associations between increases in erythrocyte GPX activity and the levels of insulin resistance were associated with pregnancy, which is promotes insulin resistance. These results (and the associations found in this research) clearly contradict the notion that the incidence of insulin resistance and type 2 diabetes inversely related to the tissue antioxidant activities.

4.1.4 Why diet and activity interventions should be applied throughout the lifecycle

The risk for lifestyle diseases and their complications increases with age. Age cannot be changed by intervention. There were no apparent relationships of antioxidant status with age but the age range of the subjects was relatively small (from 37 to 56years) and this study was not designed to measure the effect on age on risk factors. But it is well documented (Wei *et al.,* 2002, Sohal 2002) that reactive oxygen species (ROS) and other free radicals are associated with the aging process as the ability of older tissues to cope with oxidation products decreases and results in an increase in the rate of generation of ROS and accumulation of oxidative damage. Pathogenesis of diseases such as cardiovascular diseases (CVD), hypertension, cancer and type 2 diabetes is associated with declining ability to cope with oxidative damage.

The antioxidants reserve capacity in most tissues is marginal (Banerjee *et al.,* 2003). There is a consistent behaviour in the antioxidant defences related to ageing and age-related diseases, namely a decrease in the nonenzymatic antioxidants such as vitamin E, vitamin C, vitamin A and total thiol groups which are supplied through the diet, and at the same time a relative increase in enzymatic antioxidants such as plasma and red blood cell superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (Mecocci *et al.,* 2000, Sraymen *et al.,* 2003, Polidori *et al.,* 2001). However, if the increased generation of ROS could be perfectly balanced by the antioxidant defences there would not be increased risk for age-related diseases in population.

Therefore, awareness of the importance of a healthy diet and increased physical activity is crucial to modulate the effect of oxidative stress and to increase the healthy life expectancy. Dietary antioxidants can provide the nonenzymatic antioxidants (vitamins and other phytochemicals), while increased physical activities can boost the enzymatic antioxidant defences. The mechanism by which antioxidant enzymes can be upregulated in response to physical exercise is largely unknown (Ji *et al.,* 1998) but these adaptational changes have been demonstrated in different studies (Adams *et al.,* 2002, Alessio *et al.,* 1997).

There is no one perfect recipe for a healthy diet or lifestyle. The ongoing debates on how to balance healthy diet (i.e. comparing the low-fat and low-carbohydrate diets), or the relative contribution of each of the macronutrients on total body energy and metabolism (Atkins 1999, Grundy *et al.,* 2002, Howard 2002, Bravats *et al.,* 2003, Hung *et al.,* 2003, Jequir *et al.,* 2002, Saris 2003) have not reached concensus but it is generally agreed that throughout the lifecycle health may be improved by a nutrient dense diet and maintenance of physical activity.

4.2 Limitation and suggestions for future improvements

The aim of this study was to demonstrate that the changes in diet and increased physical activity can reduce the risk for lifestyle diseases in an *ad libitum* population, therefore, using the "healthy" volunteers to increase their awareness for healthy lifestyle and minimizing the risk factors for diseases was justified but also meant that there were more variables to be considered and errors in the assessments of change.

Using "healthy" individuals for this intervention study potentially affected the compliance of participants as they may have thought that there was no immediate threat to their health. However, the motivation of the participants was high as they volunteered to participate in this research which means they were interested in their health and in learning more about how to stay healthy. Evidence for this was that they made some lifestyle changes between the first and second measurement points (week 0 and week 3), prior to the discussion and booklets aimed at helping them to achieve the goals. However, as was reported participants compliance to the study was not good, "the questions relating to the goal sheets and the answers given in the summary were also not very congruent. The accuracy of the Food Frequency Questionnaires (including physical

activity)(FFQ) and Goal sheet questionnaires pose serious questions when they are compared to each other" (Cumin 2004).

Although this study succeeded in demonstrating the beneficial effect of changed diet and increased physical activity intervention on the plasma antioxidant activity and therefore, on the risk markers of lifestyle diseases, several factors were identified that limited the effect of intervention on the study results. Those limitations were associated with different aspects of the study, namely with the choice of the study methods for assessing the markers of oxidative stress, as well as the technical problems. These limitations are discussed next.

4.2.1. Study limitations

The limitation in the budget for this Masters' thesis research restricted the choice of analyses able to be used. The choice to use FRAP and TBARS assay for measuring the plasma AOA and plasma MDA respectively was made because these methods were cheap, relatively easy to perform manually and the spectrophotometer was available. Ideally at least two methods for assessing the change in each marker of oxidative stress would be appropriate for this research. As a result of intervention the measured changes in plasma AOA were found to be significant, however, measuring the the changes in antioxidant enzymes as well would potentially add more power to the analysis of the effect of intervention. The FRAP method was limited because it only detects the non-enzymatic, water soluble antioxidants in the biological fluids. The TBARS assay was chosen to measure the changes in LP because it is the most commonly reported and used method for assessment of LP *in vitro*. However, methods more specific to LDL oxidation such as LDL-oxidation lag time, marker of LDL resistance to oxidation, or direct determination of MDA in LDL could be the better choice in assessing the effect of changed diet and increased physical activity on the lipid peroxidation, especially, if the dietary intake of PUFA was increased in the diet, which, as was discussed previously, could potentially attenuate the effect of increased plasma antioxidant defences (at least the effect of increased non-enzymatic plasma antioxidants).

The assessment of the effect of adding kiwifruit to the diet on markers of oxidative stress was particularly disappointing as the deteriorating effect of storing the plasma

samples at –85^{0}C on the activity of ascorbic acid had not been considered. This fact considerably limited the potential findings for a kiwifruit effect. For the future studies on kiwifruit effect on the markers of oxidative stress it is strongly suggested that the plasma antioxidant defences to be measured at less than 6 hours after collecting the blood. It is also advisable to measure the plasma level of ascorbic acid at the same time to assess agreement between the methods.

The best choice to measure kiwifruit effect on markers of lipid peroxidation could be the resistance of LDL to oxidation, because it has been shown that ascorbic acid is able to inhibit LP in LDL by preventing the initiation of LP in LDL and by terminating the LP in partially oxidized LDL (Retsky *et al.,* 1995, Retsky *et al.,* 1999).

The other limitation in the kiwifruit treatment period was that there was no wash out period for those participants who received kiwifruit. The residual effect of treatment might be greater for the subjects with high responses that could potentially affect the results.

4.2.2 Technical limitations

Some technical limitations were also identified in this study. Despite the number of quality control measures that have been considered (Chapter 2, section 2.3) the coefficients of variation of repeat measurements and for the internal standard were high for both FRAP and TBARS methods. The FRAP assay that was adopted from Benzie (Benzie *et al.,* 1996) was carried out manually for every step whereas she had the advantage of being able to automate the test. Errors due to the manual technique could include the accuracy in measurement of reagents, as very small amounts of solutions (in the range of 10-300microL) were added to the cuvettes, as well as the accuracy in timing (5 min reaction time) of the reaction before measuring the absorbance, as the absorbance was changing with reaction time. An automated method would be better to use in this case or, if the budget allows, to use validated antioxidant measuring kits.

For the lipid peroxidation, TBARS, assay, it is possible that that the high coefficient of variation of measurement was related to the instability of working solutions. Five of the nine working solutions were recommended by Koracevic (Koracevic *et al.*, 2002) to be

prepared immediately before use. However as described in the method, for each plasma sample a series of thirteen tubes were used (Table 2.3); duplicate plasma samples (A_1), one for control (A_0), triplicate negative control without plasma or uric acid (K_1) and triplicate of its own control (K_0), and duplicate uric acid (UA_1) and duplicate of its own control (UA_0). Therefore for one subject there were 6 plasma samples (in duplicate) to be measured (for week 0, 3, 6, 9, 12 and 52). At least 5 individuals' plasma samples were measured on each working day. In addition, 9 solutions were added to each tube before measuring the absorbance. This process was time consuming and that time delay and variability would affect the stability of the working solutions and the precision of the results. In the future it is recommended that as for the FRAP assay an automated or kit method be used so that the complexity and potential for errors is reduced.

4.3 Conclusion

What has been shown in this study is that a diet and physical activity intervention can modulate the level of plasma soluble antioxidants in the blood demonstrating that the macro- and micronutrients in whole food act in synergy and have cumulative effect on body metabolism. The metabolism and utilisation of nutrients depends on an individual's nutritional status, sex and is integrated with other physiological factors. Therefore, balance (everything in moderation) and variety in combination with physical activity allow improvement in risk factors and are key concepts in nutritional metabolism and health advice.

The main message of this study is that even minimal changes in diet and physical activities can improve the body's antioxidant defences and reduce the risk for lifestyle diseases. The addition of a "prescribed" fruit to the diet is also a model that might be adopted instead of the prescription of supplements. The changes made were apparently easy to achieve and maintain for a year and therefore potentially a lifetime.

References

Adams AK, Best TM. The role of antioxidants in exercise and disease prevention. (2002), *The physician and sportsmedicine* May, 2002

Alessio HM and Blasi ER. (1997), Physical activity as a natural antioxidant booster and its effect on a healthy life span. *Res Q Exerc Sport,* **68,** 292-302.

Alessio HM, Hagerman AE, Fulkerson BK, Ambrose J, Rice RE and Wiley RL. (2000), Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. *Med Sci Sports Exerc,* **32,** 1576-1581.

Allard JP, Aghdassi E, Chau J, Salit I and Walmsley S. (1998), Oxidative stress and plasma antioxidant micronutrients in humans with HIV infection. *Am J Clin Nutr,* **67,** 143-147.

Atkins R.C. Dr. Atkin's new diet revolution. Revised and updated. HarperCollins Publishers Inc. NY 1999

Balch JF. (1999), How do antioxidants protect you? *Total Health; Woodland Hills;* Nov/Dec 1999.

Banerjee AK, Mandal A, Chanda D and Chakraborti S. (2003), Oxidant, antioxidant and physical exercise. *Mol Cell Biochem,* **253,** 307-312.

Benzie IF and Strain JJ. (1995), The effect of ascorbic acid on the measurement of total cholesterol and triglycerides: possible artefactual lowering in individuals with high plasma concentration of ascorbic acid. *Clin Chim Acta,* **239,** 185-190.

Benzie IF and Strain JJ. (1996), The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem,* **239,** 70-76.

Benzie IF and Chung WY. (1999), Total antioxidant power of plasma: male-female differences and effect of anticoagulant used. *Ann Clin Biochem,* **36 (Pt 1),** 104-106.

Berliner J. Lipid oxidation products and atherosclerosis. (2002), *Vascular Pharmacology,* 38; 4:187-191.

Bland M. An introduction to medical statistics. Oxford University Press Inc., New York.1995. Page 35.

Bravata D. M. Sanders L. Huang J. Olkin I. Gardner C.D. Efficacy and safety of low-carbohydrate diets. *JAMA* 2003; **289**. No14.1837-50.

Brown BG, Zhao XQ, Chait A, Fisher LD, Cheung MC, Morse JS, Dowdy AA, et al. (2001), Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease. *N Engl J Med,* **345,** 1583-1592.

Bub A, Watzl B, Abrahamse L, Delincee H, Adam S, Wever J, Muller H, et al. (2000), Moderate intervention with carotenoid-rich vegetable products reduces lipid peroxidation in men. *J Nutr,* **130,** 2200-2206.

Cao G and Prior RL. (1998), Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin Chem,* **44,** 1309-1315.

Champe P.C. Harvey R.A. *Boichemistry* J.B.Lippincott Company. 1997

Chen X, Scholl TO, Leskiw MJ, Donaldson MR and Stein TP. (2003), Association of glutathione peroxidase activity with insulin resistance and dietary fat intake during normal pregnancy. *J Clin Endocrinol Metab,* **88,** 5963-5968.

Cheung MC, Zhao XQ, Chait A, Alberts JJ, Brown BG. Antioxidant supplements block the responcse of HDL to Simvastatin-Niacin therapy in patients with coronary artery disease and low HDL. (2001), *Atherosclerosis, Thrombosis and Vascular Biology.* Aug 2001; 21.8; 1320-1326.

Collins BH, Horska A, Hotten PM, Riddoch C and Collins AR. (2001), Kiwifruit protects against oxidative DNA damage in human cells and in vitro. *Nutr Cancer,* **39,** 148-153.

Collins AR, Harrington V, Drew J and Melvin R. (2003), Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis,* **24,** 511-515.

Cumin MB, (2004) Reduction of risk for lifestyle diseases: Group diet and physical activity intervention in the workplace. Master Thesis.

Diabetes News Zealand. Models and Forecasts. 1996-2011. www.moh.govt.nz (date of access March, 2004).

Dragsted LO, Pedersen A, Hermetter A, Basu S, Hansen M, Haren GR, Kall M, et al. (2004), The 6-a-day study: effects of fruit and vegetables on markers of oxidative stress and antioxidative defense in healthy nonsmokers. *Am J Clin Nutr,* **79,** 1060-1072.

Duttaroy AK and Jorgensen A. (2004), Effects of kiwi fruit consumption on platelet aggregation and plasma lipids in healthy human volunteers. *Platelets,* **15,** 287-292.

Evans JL, Goldfine ID, Maddux BA and Grodsky GM. (2002), Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr Rev,* **23,** 599-622.

Ezzati M, Lopez AD, Rodgers A, Vander Hoorn S and Murray CJ. (2002), Selected major risk factors and global and regional burden of disease. *Lancet,* **360,** 1347-1360.

Farber E, Rubin E. Pathology, 2nd edition. J.B. Lippincott Company, 1998

Farquharson CA, Butler R, Hill A, Belch JJ and Struthers AD. (2002), Allopurinol improves endothelial dysfunction in chronic heart failure. *Circulation,* **106,** 221-226.

Fenster CP, Weinsier RL, Darley-Usmar VM and Patel RP. (2002), Obesity, aerobic exercise, and vascular disease: the role of oxidant stress. *Obes Res,* **10,** 964-968.

Franz MJ. (1997), Protein: metabolism and effect on blood glucose levels. *Diabetes Educ,* **23,** 643-646, 648, 650-641.

Frenoux JM, Prost ED, Belleville JL and Prost JL. (2001), A polyunsaturated fatty acid diet lowers blood pressure and improves antioxidant status in spontaneously hypertensive rats. *J Nutr,* **131,** 39-45.

Ginsburg GS, Agil A, O'Toole M, Rimm E, Douglas PS and Rifai N. (1996), Effects of a single bout of ultraendurance exercise on lipid levels and susceptibility of lipids to peroxidation in triathletes. *Jama,* **276,** 221-225.

Grundy SM, Abate N and Chandalia M. (2002), Diet composition and the metabolic syndrome: what is the optimal fat intake? *Am J Med,* **113 Suppl 9B,** 25S-29S.

Halliwell B. (1994a), Free radicals and antioxidants: a personal view. *Nutr Rev,* **52,** 253-265.

Halliwell B. (1994b), Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet,* **344,** 721-724.

Halliwell B. (2002), Effect of diet on cancer development: is oxidative DNA damage a biomarker? *Free Radic Biol Med,* **32,** 968-974.

Haslett C, Chilvers ER, Hunter JAA, Boon NA, Davidson's Principles and Practice of Medicine. 18th edition, 1999. Curchill Liwingstone

Hermans M, Levy J, Morris R, Turner R. (1999), Comparison of insulin sensitivity tests across a range of glucose tolerance from normal to diabetes. *Diabetologia*; **42**: 678-687.

Hills M and Armitage P. (1979), The two-period cross-over clinical trial. *Br J Clin Pharmacol,* **8,** 7-20.

Howard B.V. Dietary fat and diabetes: a consensus view. *The American Journal of Medicine*. 2002; vol 113 (9B)

Hung T, Sievenpiper JL, Marchie A, Kendall CW, Jenkins DJ. Fat versus carbohydrate in insulin resistance, obesity, diabetes and cardiovascular disease**.** *Curr Opin Clin Nutr Metab Care.* 2003 Mar;6(2):165-76

Jacob RA and Burri BJ. (1996), Oxidative damage and defense. *Am J Clin Nutr,* **63,** 985S-990S.

Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Review article (1990), *Free radical biology and medicine*. 9(6):515-540

Jequier E, Bray GA. Low-fat diets are preferred. *Am J Med*. 2002 Dec 30;113 Suppl 9B:41S-46S.

Ji LL. (1996), Exercise, oxidative stress, and antioxidants. *Am J Sports Med,* **24,** S20-24.

Ji LL, Leeuwenburgh C, Leichtweis S, Gore M, Fiebig R, Hollander J and Bejma J. (1998), Oxidative stress and aging. Role of exercise and its influences on antioxidant systems. *Ann N Y Acad Sci,* **854,** 102-117.

Koracevic D, Koracevic G, Djordjevic V, Andrejevic S and Cosic V. (2001), Method for the measurement of antioxidant activity in human fluids. *J Clin Pathol,* **54,** 356-361.

Kushner RF, Gudivaka R, Schoeller DA. (1996), Clinical characteristics influencing bioelectrical impedance analysis measurements. *Am J Clin Nutr*; **64**(3 Suppl): 423S-427S.

Mak S and Newton GE. (2001), The oxidative stress hypothesis of congestive heart failure: radical thoughts. *Chest,* **120,** 2035-2046.

McClung JP, Roneker CA, Mu W, Lisk DJ, Langlais P, Liu F and Lei XG. (2004), Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. *Proc Natl Acad Sci U S A,* **101,** 8852-8857.

Mecocci P, Polidori MC, Troiano L, Cherubini A, Cecchetti R, Pini G, Straatman M, et al. (2000), Plasma antioxidants and longevity: a study on healthy centenarians. *Free Radic Biol Med,* **28,** 1243-1248.

Menendez R, Mas R, Armor AM, Ledon N. (2002), Inhibition of rat lipid peroxidation by the oral administration of D003, a mixture of very long-chain saturated fatty acids. *Canadian Journal of Physiology and Pharmacology;* Jan 2002.

Miller ER, 3rd, Appel LJ and Risby TH. (1998), Effect of dietary patterns on measures of lipid peroxidation: results from a randomized clinical trial. *Circulation,* **98,** 2390-2395.

Murray CJ and Lopez AD. (1997), Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet,* **349,** 1269-1276.

Okezie IA. Free radicals, antioxidants and international nutrition. Review Article. (1999), *Asia Pacific J Clin Nutr.* 8(1):53-63

Pan M, Cederbaum AI, Zhang YL, Ginsberg HN, Williams KJ and Fisher EA. (2004), Lipid peroxidation and oxidant stress regulate hepatic apolipoprotein B degradation and VLDL production. *J Clin Invest,* **113,** 1277-1287.

Polidori MC, Cherubini A, Senin U and Mecocci P. (2001), Peripheral non-enzymatic antioxidant changes with human aging: a selective status report. *Biogerontology,* **2,** 99-104.

Preziosi P, Galan P, Herbeth B, Valeix P, Roussel AM, Malvy D, Paul-Dauphin A, et al. (1998), Effects of supplementation with a combination of antioxidant vitamins and trace elements, at nutritional doses, on biochemical indicators and markers of the antioxidant system in adult subjects. *J Am Coll Nutr,* **17,** 244-249.

Retsky KL, Frei B. (1995), Vitamin C prevents metal ion-dependent initiation and propagation of lipid peroxidation in human low-density lipoprotein. *Biochim Biophys Acta*.1257(3):279-87.

Retsky KL, Chen K, Frei B. (1999) Inhibition of copper-induced LDL oxidation by vitamin C is associated with decreased copper-binding to LDL and 2-oxo-histidine formation. *Free Radic Biol Med*. 26(1-2):90-8.

Rosen P, Nawroth PP, King G, Moller W, Packer L. (2001) The role of oxidative stress in the onset and progression of diabetes and its complication: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes/Metabolism research and Rwviews.* 17:189-212.

Rush EC, Patel M, Plank LD and Ferguson LR. (2002), Kiwifruit promotes laxation in the elderly. *Asia Pac J Clin Nutr,* **11,** 164-168.

Saraymen R, Kilic E, Yazar S and Cetin M. (2003), Influence of sex and age on the activity of antioxidant enzymes of polymorphonuclear leukocytes in healthy subjects. *Yonsei Med J,* **44,** 9-14.

Saris W.H.M. Glycemic carbohydrate and body weight regulation *Nutrition Reviews*; 2003; 61; 5 ; S10-S16

Shern-Brewer R, Santanam N, Wetzstein C, White-Welkley J and Parthasarathy S. (1998), Exercise and cardiovascular disease: a new perspective. *Arterioscler Thromb Vasc Biol,* **18,** 1181-1187.

Sies H and Stahl W. (1995), Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. *Am J Clin Nutr,* **62,** 1315S-1321S.

Sim AS, Salonikas C, Naidoo D and Wilcken DE. (2003), Improved method for plasma malondialdehyde measurement by high-performance liquid chromatography using methyl malondialdehyde as an internal standard. *J Chromatogr B Analyt Technol Biomed Life Sci,* **785,** 337-344.

Sohal RS. (2002), Role of oxidative stress and protein oxidation in the aging process. *Free Radic Biol Med,* **33,** 37-44.

Statistics New Zealand. www.nzhis.govt.nz/stats/mortstats.html (date of access March, 2004)

Taylor RW, Keil D, Gold EJ, Williams SM and Goulding A. (1998), Body mass index, waist girth, and waist-to-hip ratio as indexes of total and regional adiposity in women: evaluation using receiver operating characteristic curves. *Am J Clin Nutr,* **67,** 44-49.

Tessier D, Khalil A and Fulop T. (1999), Effects of an oral glucose challenge on free radicals/antioxidants balance in an older population with type II diabetes. *J Gerontol A Biol Sci Med Sci,* **54,** M541-545.

Thompson HJ, Heimendinger J, Haegele A, Sedlacek SM, Gillette C, O'Neill C, Wolfe P, et al. (1999), Effect of increased vegetable and fruit consumption on markers of oxidative cellular damage. *Carcinogenesis,* **20,** 2261-2266.

Usberti M, Gerardi GM, Gazzotti RM, Benedini S, Archetti S, Sugherini L, Valentini M, et al. (2002), Oxidative stress and cardiovascular disease in dialyzed patients. *Nephron,* **91,** 25-33.

Van Gaal FL, Vertommen J, De Leeuw IH, The in vitro oxidizability of lipoprotein particles in obese and non-obese subjects. *Free radical biology and medicine.* Vol 23, Issue 3, 1997, 361-366

Vina J, Gomez-Cabrera MC, Lloret A, Marquez R, Minana JB, Pallardo FV and Sastre J. (2000), Free radicals in exhaustive physical exercise: mechanism of production, and protection by antioxidants. *IUBMB Life,* **50,** 271-277.

Wei YH and Lee HC. (2002), Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. *Exp Biol Med (Maywood),* **227,** 671-682.

Wheatley C. (1997), Vitamin trials and cancer. *Lancet,* **349,** 1844-1845.

Will JC and Byers T. (1996), Does diabetes mellitus increase the requirement for vitamin C? *Nutr Rev,* **54,** 193-202.

Wolever TM. (2003), Carbohydrate and the regulation of blood glucose and metabolism. *Nutr Rev*, **61,** S40-48.

Young IS. (2001a), Measurement of total antioxidant capacity. *J Clin Pathol*, **54,** 339.

Young IS and Woodside JV. (2001b), Antioxidants in health and disease. *J Clin Pathol*, **54,** 176-186.

Appendices

A p p e n d i x 1

M E M O R A N D U M

Academic Registry – Academic Services

To: Elaine Rush

From: **Madeline Banda**

Date: 20 February 2002

Subject: 02/08 Reduction of risk for lifestyle diseases

Dear Elaine

Your application for ethics approval was considered by AUTEC at their meeting on 11 February 2002.

Your application was approved for a period of two years until February 2004.

You are required to submit the following to AUTEC:

- A brief annual progress report indicating compliance with the ethical approval given.
- A brief statement on the status of the project at the end of the period of approval or on completion of the project, whichever comes sooner.
- A request for renewal of approval if the project has not been completed by the end of the period of approval.

The Committee wishes you well with your research.

Please include the application number and study title in all correspondence and telephone queries.

Yours sincerely

Madeline Banda
Executive Secretary
AUTEC

From the desk of …
9999

Private Bag 92006, Auckland 1020

Tel: 64 9 917

Madeline Banda
Academic Services
Student Services Group

New Zealand
E-mail: madeline.banda@aut.ac.nz

ext 8044
Fax: 64 9 917 9812

Appendix 2. Working spreadsheet for FRAP assay

Changes in antioxidant concentrations in plasma following a lifestyle intervention

Date 5/4/2004
Signature Lela Migriauli

Standard preparation:

0.278 g FeSO4.7H2O in 100 mL

		mmol/L
Actual amount weighed (g)	0.2779	9.995684

[C] = 10 millimol/L

Calibration curve:

(300 FRAP, 30 water, 10 Fe+2)

Series of standard solutions (0 - 1500 micromol/L)

Dilution factor **0.029412**

	Fe2+ (g)	Water (g)	Concentration (micromol/L)
1	0	5	0
2	0.1497	4.8765	297.7108
3	0.3015	4.7278	597.1449
4	0.453	4.5532	904.4874
5	0.6025	4.3988	1204.167
6	0.7552	4.2495	1508.33

Standards:

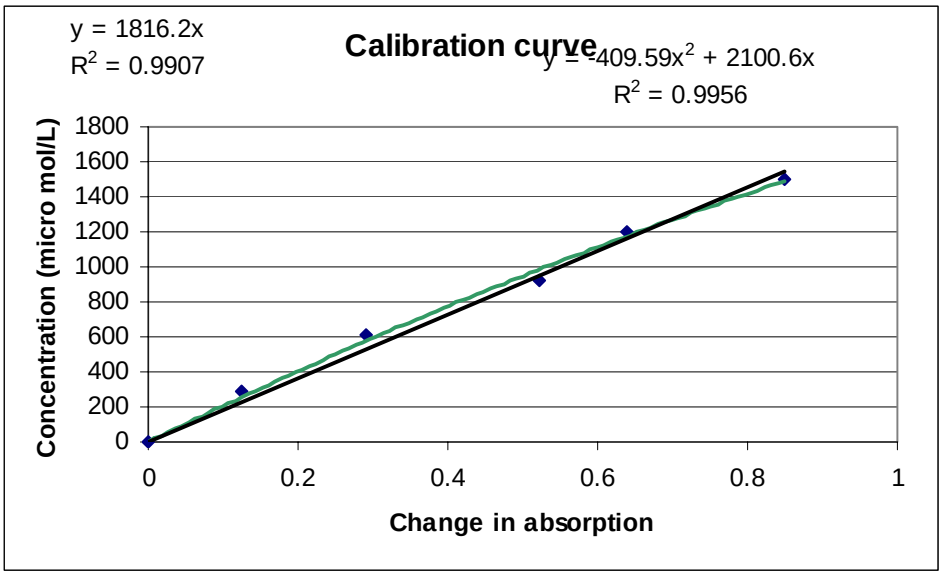
	Standard	Water	FRAP	Diln factor	Change in absorption	Actual Concentrationn
1	0	0	0.2966	0	0	0
2	0.0099	0.03	0.3016	0.02899	0.124	293.4391
3	0.0102	0.0305	0.3011	0.029842	0.292	605.8802
4	0.0102	0.0301	0.3011	0.029877	0.521	918.7939
5	0.01	0.0307	0.3007	0.029291	0.64	1199.229
6	0.01	0.031	0.3016	0.029189	0.85	1496.883

Internal standards:

300 FRAP, 10 plasma, 30 water **factor** **1816.2**

	Plasma	Water	FRAP	Actual Diln	Change in absorption	Concentration
	0	0	0.3065		0	
1	0.0107	0.03	0.3027	0.031159	0.423	768.2526
2	0.0109	0.03	0.302	0.031788	0.48	871.776
3	0.0108	0.0296	0.3025	0.031496	0.478	868.1436
4						
5						
6						
					mean	836.0574

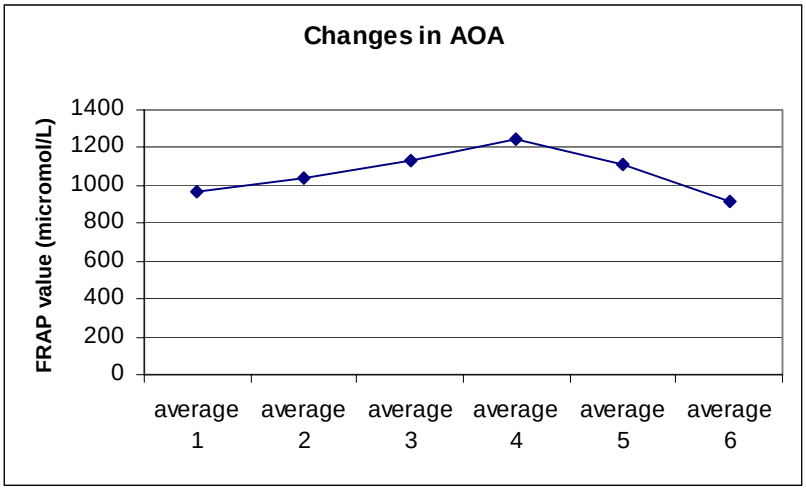
sd	58.74876
cv	7.026881



Samples: INT44

Week 0	**Plasma**	**Water**	**FRAP**	**Actual Diln**	**Change in absorption**		**Concentration**
	1	0.0109	0.03	0.2994	0.032031	0.514	933.5268
	2	0.0108	0.0305	0.3025	0.031414	0.345	
	3	0.0109	0.0302	0.3042	0.031567	0.552	1002.542
Week 3	**Plasma**	**Water**	**FRAP**	**Actual Diln**	**Change in absorption**		
	1	0.0114	0.03	0.3015	0.033246	0.592	1075.19
	2	0.0107	0.0303	0.3024	0.031159	0.301	
	3	0.0103	0.0304	0.3035	0.029924	0.554	1006.175
Week 6	**Plasma**	**Water**	**FRAP**	**Actual Diln**	**Change in absorption**		
	1	0.0104	0.0303	0.3026	0.030294	0.599	1087.904
	2	0.01	0.0308	0.3011	0.029248	0.253	
	3	0.0104	0.0303	0.3025	0.030303	0.647	1175.081
Week 9	**Plasma**	**Water**	**FRAP**	**Actual Diln**	**Change in absorption**		
	1	0.01	0.031	0.3065	0.028777	0.676	1227.751
	2	0.0107	0.0307	0.3052	0.030871	0.699	1269.524
	3	0.0096	0.0304	0.3046	0.027858	0.954	
Week 12	**Plasma**	**Water**	**FRAP**	**Actual Diln**	**Change in absorption**		
	1	0.01	0.0299	0.3035	0.029121	0.544	
	2	0.0105	0.0299	0.3027	0.030603	0.619	1124.228
	3	0.0107	0.03	0.3034	0.031096	0.603	1095.169

Week 52	Plasma	Water	FRAP	Actual Diln	Change in absorption	
1	0.012	0.0305	0.3037	0.034662	0.508	922.6296
2	0.0109	0.0298	0.2994	0.032049	0.504	915.3648
3	0.0108	0.0302	0.3056	0.03116	0.899	



Appendix 3. Working spreadsheet for TBARS assay

Antioxidant Activity of human plasma
Lipid peroxidation - TBARS assay

Date:
Signature: Lela Migriauli

AOA=C ua*(A contr.- A sample) / (A contr. – A ua)

AOA - antioxidant activity
C ua - concentration of uric acid
A contr. – absorption of control
A sample - absorption of sample
A ua - absorption of uric acid

C ua = 1 mmol/L
A contr. = K1 – K0
A sample = A1 – A0
A ua = UA1 – UA0

range 1.42-2.44mmlo/L

14/07/04

INT 02	A1 (mean)	A0	K1	K0	UA1	UA0	K1-K0	A1-A0	UA1-UA0	AOA
Week 0	0.283	0.069	0.815	0.047	0.413	0.048	0.732	0.214	0.365	1.411444
Week 3	0.29	0.064	0.756	0.047				0.226		1.378747
Week 6	0.239	0.034	0.765	0.046				0.205		1.435967
Week 9	0.251	0.031	0.778667	0.046667				0.22		1.395095
Week 12	0.23	0.041						0.189		1.479564
Week 52	0.277	0.031						0.246		1.324251

A p p e n d i x 4

M E M O R A N D U M

Student Services Group - Academic Services

To: Elaine Rush
From: **Madeline Banda**
Date: 15 August 2003
Subject: 02/08 Reduction of risk for lifestyle diseases

Dear Elaine

Your application to allow student researcher L Migriauli to use her own blood for measuring antioxidant status to test methods prior to using volunteer samples was considered by AUTEC at their meeting on 11 August 2003.

Your application was approved. This project currently has approval until 10/03/05.

Please include the application number and study title in all correspondence and telephone queries.

Yours sincerely

Madeline Banda
Executive Secretary
AUTEC
Cc: 9714747 Lela Migriauli

From the desk of ... 9999
Private Bag 92006, Auckland 1020
Tel: 64 9 917
Madeline Banda
Academic Services
Student Services Group
New Zealand
E-mail: madeline.banda@aut.ac.nz
ext 8044
Fax: 64 9 917 9812

Appendix 5. The associations between changes in markers of oxidative stress and blood biochemical parameters

AOA

FRAP (week 12-0)		**Glucose**	**Insulin**	**HOMA %B**	**HOMA %S**	**Tot.cholest.**	**HDL**	**IDL**	**Triglyc.**	**Tot.chol/HDL**
Full data	R	-0.202	0.08	0.116	0.083	0.205	0.182	0.025	0.146	-0.134
N=52	P value	0.15	0.564	0.415	0.558	0.145	0.196	0.858	0.302	0.344
Male	R	-0.23	0.128	0.177	-0.08	-0.17	0.197	-0.38	0.182	-0.37
N=24	P value	0.279	0.55	0.409	0.709	0.428	0.357	0.067	0.395	0.075
Female	R	-0.174	0.043	0.042	0.247	0.477	0.158	0.172	0.105	0.139
N=28	P value	0.376	0.827	0.834	0.205	0.01*	0.42	0.382	0.595	0.482
Low AOA	R	0.143	0.248	0.07	-0.044	0.06	0.264	-0.027	-0.151	-0.372
N=28	P value	0.468	0.202	0.722	0.822	0.936	0.174	0.89	0.443	0.051
Male	R	-0.181	-0.153	-0.002	0.137	-0.319	0.108	-0.464	-0.105	-0.448
N=12	P value	0.574	0.636	0.993	0.671	0.311	0.739	0.128	0.746	0.144
Female	R	0.487	0.505	0.188	-0.336	0.277	0.279	0.222	-0.288	-0.119
N=16	P value	**0.055**	0.046*	0.486	0.203	0.305	0.296	0.408	0.279	0.661
High AOA	R	-0.529	-0.144	0.179	0.215	0.425	0.171	0.212	0.331	0.098
N=24	P value	0.007*	0.501	0.402	0.314	0.038*	0.426	0.575	0.114	0.647
Male	R	-0.617	0.304	0.576	-0.189	0.223	0.341	-0.113	0.41	-0.166
N=12	P value	0.033*	0.338	0.05*	0.556	0.485	0.276	0.728	0.186	0.606
Female	R	-0.52	-0.416	-0.07	0.468	0.637	0.094	0.167	0.306	0.299
N=12	P value	0.08	0.179	0.823	0.125	0.026*	0.769	0.604	0.333	0.345

AOA

FRAP (week 52-0)		**Glucose**	**Insulin**	**HOMA %B**	**HOMA %S**	**Tot.cholest.**	**HDL**	**IDL**	**Triglyc.**	**Tot.hol/HDL**
Full data	R	0.222	0.005	-0.091	0.271	0.065	0.076	0.133	-0.012	-0.068
N=38	P value	0.193	0.977	0.596	0.11	0.702	0.66	0.438	0.458	0.691
Male	R	0.439	0.085	-0.093	-0.414	0.088	0.069	0.185	-0.175	0.011
N=21	P value	0.052	0.721	0.694	0.069	0.711	0.772	0.435	0.46	0.962
Female	R	-0.206	-0.329	-0.188	0.222	0.002	0.068	0.007	-0.029	-0.274
N=17	P value	0.444	0.213	0.485	0.409	0.994	0.802	0.979	0.914	0.304
Low AOA	R	0.21	0.165	0.075	-0.533	-0.59	0.01	0.064	-0.28	-0.108
N=22	P value	0.36	0.475	0.746	0.012*	0.798	0.963	0.78	0.218	0.641
Male	R	0.242	0.072	-0.006	-0.552	-0.87	-0.089	0.039	-0.255	0.89
N=11	P value	0.474	0.831	0.986	0.078	0.798	0.793	0.908	0.449	0.794
Female	R	0.079	-0.155	-0.245	-0.087	-0.162	-0.039	0.11	-0.722	-0.369
N=11	P value	0.827	0.67	0.495	0.811	0.656	0.913	0.763	0.018*	0.293
High AOA	R	0.061	-0.241	-0.101	0.043	0.363	0.415	0.248	0.05	-0.048
N=16	P value	0.829	0.396	0.721	0.877	0.184	0.124	0.372	0.858	0.863
Male	R	0.3	0.004	-0.064	-0.163	0.353	0.416	0.445	-0.28	-0.054
N=10	P value	0.433	0.992	0.869	0.675	0.352	0.265	0.23	0.466	0.889
Female	R	-0.449	-0.572	-0.041	0.637	0.433	0.401	-0.251	0.806	-0.212
N=6	P value	0.371	0.236	0.938	0.174	0.391	0.431	0.631	0.053	0.687

Lipid peroxidation (LP)

TBARS (week12-0)		**Glucose**	**Insulin**	**HOMA %B**	**HOMA %S**	**Tot.cholest.**	**HDL**	**IDL**	**Triglyc.**	**Tot.chol/HDL**
Full data	R	-0.146	-0.046	0.012	0.206	0.354	0.245	0.015	0.275	-0.071
N=52	P value	0.303	0.741	0.929	0.143	0.01*	0.079	0.913	0.048*	0.617
Male	R	-0.123	-24	0.053	0.16	0.248	0.327	-0.017	0.209	0.331
N=24	P value	0.568	0.908	0.803	0.455	0.244	0.119	0.936	0.328	0.143
Female	R	0.173	-0.069	-0.037	0.25	0.425	0.177	0.036	0.32	-0.006
N=28	P value	0.379	0.724	0.851	0.199	0.024*	0.367	0.854	0.096	0.975
High LP	R	0.124	0.052	-0.028	0.13	0.394	0.258	0.009	0.318	-0.112
N=34	P value	0.486	0.769	0.873	0.463	0.021*	0.141	0.959	0.066	0.53
Male	R	0.23	0.199	0.084	-0.015	0.302	0.425	0.13	0.095	-0.177
N=19	P value	0.343	0.415	0.73	0.951	0.208	0.07	0.595	0.853	0.468
Female	R	0.055	-0.034	-0.187	0.284	0.428	0.117	0.022	0.466	-0.005
N=15	P value	0.844	0.904	0.505	0.305	0.111	0.678	0.937	0.08	0.984
Low LP	R	-0.531	-0.3	0.034	0.327	0.202	0.075	0.186	-0.045	0.072
N=18	P value	0.023*	0.227	0.892	0.186	0.421	0.766	0.461	0.857	0.776
Male	R	-0.799	-0.532	0.036	0.566	0.233	0.274	-0.513	0.461	0.005
N=5	P value	0.105	0.356	0.954	0.32	0.706	0.655	0.377	0.435	0.992
Female	R	-0.286	-0.08	0.09	0.163	0.304	0.318	0.35	-0.361	-0.24
N=13	P value	0.343	0.793	0.762	0.595	0.312	0.29	0.241	0.226	0.429

Lipid peroxidation (LP)

TBARS (week52-0)		Glucose	Insulin	HOMA %B	HOMA %S	Tot.cholest.	HDL	IDL	Triglyc.	Tot.chol/HDL
Full data	R	-0.014	0.055	0.004	-0.062	0.067	-0.112	-0.023	0.227	0.217
N=38	P value	0.934	0.745	0.978	0.713	0.991	0.509	0.891	0.177	0.197
Male	R	-0.091	0.068	0.036	-0.003	-0.097	-0.319	-0.207	0.365	0.222
N=21	P value	0.701	0.774	0.879	0.988	0.681	0.171	0.381	0.114	0.347
Female	R	0.082	0.103	-0.013	-0.254	0.304	0.096	0.289	0.031	0.2
N=17	P value	0.753	0.693	0.96	0.324	0.236	0.713	0.261	0.905	0.442
High LP	R	0.127	0.134	-0.016	-0.043	-0.181	-0.147	-0.299	0.379	0.092
N=26	P value	0.545	0.524	0.937	0.837	0.386	0.483	0.147	0.061	0.661
Male	R	0.123	0.088	-0.039	-0.045	-0.179	-0.42	-0.248	0.45	0.191
N=16	P value	0.649	0.745	0.885	0.869	0.506	0.105	-0.354	0.08	0.479
Female	R	0.204	0.307	-0.008	0.119	-0.281	0.393	-0.754	0.147	-0.319
N=10	P value	0.599	0.421	0.982	0.761	0.465	0.296	0.019*	0.706	0.402
Low LP	R	-0.208	-0.18	0.058	-0.283	0.338	-0.092	0.276	0.067	0.39
N=18	P value	0.516	0.575	0.858	0.372	0.282	0.774	0.385	0.834	0.21
Male	R	-0.754	-0.164	0.466	0.458	0.687	-0.018	-0.096	0.262	0.415
N=4	P value	0.246	0.836	0.534	0.542	0.313	0.982	0.904	0.738	0.585
Female	R	-0.0003	-0.147	-0.047	-0.499	0.343	-0.108	0.439	-0.084	0.453
N=8	P value	0.999	0.729	0.912	0.208	0.405	0.799	0.276	0.843	0.259