Australian Government

Grape and Wine Research and Development Corporation

Reduction of damage to low density lipoproteins (LDL) and deoxyribonucleic acid (DNA) from oxidative free radicals by regular and moderate consumption of wine



FINAL REPORT to The Grape and Wine Research and Development Corporation

Project Number: **CSN 97/02**

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Date: **August 2006**

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1. Abstract

A Mediterranean-style diet, which is high in certain fruits, grains and vegetables, is observed to reduce the risk of cardiovascular disease (CVD) and certain cancers. Wine is part of a traditional Mediterranean-style diet and some epidemiological studies suggest that wine-derived phenolic compounds may supplement the cardioprotective and cancer-protective effects of an already high plant-derived phenolic diet, and counters the harmful effects of a high fat diet on haemostasis, endothelial function and lipid oxidation as well as on DNA. The extent to which wine-derived phenolic compounds supplement the effects of a low plant-derived phenolic but not high fat diet is unknown.

The three aims of the study were, therefore, to test the hypothesis that regular and moderate consumption of wine:

- elevates the plasma concentration of phenolic compounds;
- protects against oxidative damage to DNA, a risk factor for cancer in humans; and
- protects against oxidative damage to LDL, a risk factor for cardiovascular disease.

To investigate the protective effect of wine against oxidative damage to DNA, an initial *in vitro* plasma spiking was undertaken to determine which compounds in wine are actually responsible for antioxidant properties was undertaken. The relative contribution of ethanol, glycerol, tartaric acid, catechin and caffeic acid mixture, a mixture of all of these and phenolic-stripped white wine at *in vivo* relevant concentrations on spontaneous and oxidative stress induced DNA damage was evaluated by the cytokinesis-block micronucleus assay. The results showed that only ethanol significantly increases spontaneous DNA damage, but this effect is eliminated when ethanol is included in a mixture of all the other wine components ($P<0.05$). Also, a significant protective effect against hydrogen peroxide-induced DNA damage was observed for the catechin and caffeic acid mixture ($P<0.05$). While all compounds tested were significantly protective against ionising radiation-induced DNA damage in a dose dependent manner, the strongest protection was again observed for the catechin and caffeic acid mixture, and a mixture of all components ($P < 0.0001$).

Since little is known of the bioavailability and bioefficiency of wine components, a crossover intervention was then used to assess the *in vivo* effects of acute moderate consumption. Healthy non-smoking males (n=6) 23.3 ± 0.2 years of age adhered to a diet low in plant-derived phenolic compounds for 48 hours. Volunteers then drank 300 mL of red wine, de-alcoholised red wine or an ethanol solution. Blood samples were taken prior to clearance and drinking and 30, 60 and 120 min after consumption. Radiation induced MNed cell frequency increased in a time dependent manner after consumption of the ethanol solution, but in contrast decreased after consumption of the dealcoholised wine ($P<0.001$). A difference of differences analysis of MNed cell frequency between treatments indicated that red wine and dealcoholised red wine were significantly different ($P<0.05$) from the ethanol solution at all times post-consumption. The results of the subsequent *ex vivo* challenge of whole blood showed that both red wine and dealcoholised red wine consumption significantly reduced gamma-radiation-induced micronucleated cell frequency when compared to consumption of the ethanol solution. This reached significance for both drinks at 1 and 2 hours post-consumption ($p<0.05$). The most significant reduction (44±2.0%, $p<0.001$) was observed 2 hours after consumption of the dealcoholised red wine. Consumption of the ethanol solution clearly increased induction of micronucleated cells in a time responsive manner post-consumption ($P=0.0002$) while

consumption of the red wine produced an intermediate effect. The data from these studies are particularly interesting because they clearly demonstrate the potential beneficial effects of wine-derived phenolic compounds in counteracting the harmful effects of ionising radiation, which are mediated by oxidative free radicals.

To investigate the effect of moderate red wine consumption coupled with a low plant-derived phenolic diet on LDL and other biomarkers of cardiovascular risk, a randomized, controlled dietary crossover intervention was undertaken. Healthy non-smoking males (n=57) aged 41–57 years were placed on a diet low in plant-derived phenolic compounds and followed a set drinking protocol of three 4-week periods:

1. alcohol-free washout;
2. 200 mL red wine per day; and
3. either 200 mL white wine or 200 mL dealcoholised red wine or 200mL 12% alcohol solution per day.

The first four weeks of the study required subjects to remain alcohol-free as a washout period. At the beginning of the intervention and after each protocol period, a blood sample was collected, and blood pressure was measured using the HDI/Pulse Wave TM CR-2000 Research Cardiovascular Profiling System.

The results from the randomized, controlled dietary crossover intervention showed that apart from an increase in the plasma concentration of HDL cholesterol, which could be attributed to the ethanol component of the wine, there were no statistically significant differences in the other plasma lipids, that is, total and LDL cholesterol and triglyceride. Overall, the wine had a positive impact on the ratio of LDL:HDL, resulting in a more protective lipid profile. No effects on vascular compliance were observed.

No significant changes were observed in markers of oxidative stress. This lack of effect could to be due to the fact that the intervention was conducted against a background diet low in plant-derived phenolic compounds. From analysis of the data for sequential visits to the clinic irrespective of the type of intervention, it appeared that there was a trend towards a reduction in the plasma concentration of catechin as a result of the low plant-derived phneolic compound diet, that was not able to be countered by consumption of wine-derived phenolic compounds until the end of the study. In this same analysis, the concentration of F2alpha isoprostane in plasma appeared to be inversely correlated with that of catechin, implying that catechin may protect against oxidative damage, further implying that catechin-rich wine has the potential to be protective against the risk of cardiovascular disease.

Overall, the results indicate that studies examining the effects of wine consumption on risk factors for cardiovascular disease should take into account the phenolic content of the background diet.

2. Executive summary

In Australia, cardiovascular diseases accounts for approximately 40% of all deaths. Numerous epidemiological studies have suggested that the moderate intake of alcoholic beverages may have a cardioprotective effect. The regular moderate consumption of alcoholic beverages has been observed to decrease the risk of cardiovascular disease by up to 40%, and regular moderate consumers of alcohol have a longer life span in comparison to that of both abstainers and excessive consumers of alcohol. The cardioprotective effects of alcohol apply to both men and women, different age groups from approximately 40 years of age onwards, different racial populations and different geographical regions of the world.

While there is evidence that the ethyl alcohol content common to all alcoholic beverages has certain cardioprotective effects, this evidence does not fully explain all the protective mechanisms observed following wine consumption. Statistical analyses of data generated by the World Health Organisation and Organization for Economic Cooperation and Development suggest that, independent of the cardioprotective effects of the ethyl alcohol or ethanol content, the consumption of wine confers additional cardioprotective effects.

One mechanism proposed for the cardioprotective effect is a reduction in the concentration of low density lipoprotein (LDL) cholesterol as well as a reduction in its oxidation. Plasma cholesterol concentration and cholesterol carried in the circulation by LDL particles are risk factors for cardiovascular disease. In particular, it is the concentration of LDL cholesterol, which can be oxidized by oxidative free radicals that has been implicated in the development of atherosclerosis—the accumulation of plaque on the artery wall**.** It is postulated that when the protective antioxidant compounds present in the LDL particle are depleted, oxidation of cholesterol occurs. A high plasma concentration of antioxidants such as α-tocopherol (vitamin E), has been observed in those populations with a low incidence of cardiovascular disease.

Grapes and wines contain antioxidant phenolic compounds which are classified as either flavonoid or non-flavonoid compounds. The average total phenolic component of white table wines is 200 mg/L and of red table wines is 1200 mg/L. Most research has focussed on flavonoid antioxidants and the concentration of these is significantly greater in red wine than in white wine. The consumption of two 100 mL glasses of red wine per day increases the flavonoid content of the average diet by approximately 40%, and these may increase antioxidant levels in the LDL particles.

Therefore, any additional cardioprotective effects of wine, compared to other alcoholic beverages, may be due to the endogenous phenolic compounds. At least one clinical study in humans complementing an experimental study in animals, concluded that wine, and in particular red wine, is the most potent alcoholic beverage conferring protection against atherosclerosis and thus cardiovascular disease, by modifying the established risk factors, such as the concentration of LDL. These wine-derived phenolic compounds are observed, *in vitro*, to be more protective against the oxidation of LDL than ascorbic acid (vitamin C) and α-tocopherol; they have collectively and individually 10 to 20-fold more antioxidant activity. Wine also contains ascorbic acid and glycerol, which may contribute to the cardioprotective effects observed.

In Australia, cancer accounts for approximately 25–30% of all deaths. Oxidative free radicals also cause mutation of DNA sequences and breakage of DNA strands, which are the underlying causes of the initiation and progression of cancer. Epidemiological evidence indicates that a diet comprising of fruits and vegetables, which contain phenolic compounds, may decrease the incidence of cancer by protecting DNA from oxidative damage. Furthermore, one epidemiological study reported a 22% reduction in the risk of death from all cancers in subjects consuming up to approximately two standards drinks per day (21 g ethanol/day) compared to abstainers; 82% was consumed as wine. It has thus been suggested that wine-derived phenolic compounds may also be protective against the mutation of DNA sequences (cancer-protective). However, beyond two drinks per day the risk for cancer starts to increase. In addition, a common link between cardiovascular disease and cancer is the observation that an elevated micronucleus frequency, which is a marker of DNA damage and hence cancer, is also associated with increased severity of cardiovascular disease. No cellular mechanism of action has, however, been determined, but individual wine-derived phenolic compounds appear capable of inhibiting each of the three steps involved in the development of cancer—initiation, promotion and progression.

The aims of the study were, therefore, to test the hypothesis that regular and moderate consumption of wine:

- elevates the plasma concentration of phenolic compounds;
- protects against oxidative damage to DNA, a risk factor for cancer in humans; and
- protects against oxidative damage to LDL, a risk factor for cardiovascular disease.

The results from the human intervention study showed that apart from an increase in the plasma concentration of HDL cholesterol, which could be attributed to the ethanol component of the wine, there were no statistically significant differences in the other plasma lipids, that is, total and LDL cholesterol and triglyceride. Overall, the wine had a positive impact on the ratio of LDL:HDL, resulting in a more protective lipid profile.

No significant changes were observed in markers of oxidative stress such as LDL oxidation, FRAP, ORAC or nitrate/nitrite ratio following the consumption of red wine or the control beverages. As the efficacy of the phenolic compounds is dose-dependent, this suggests that if the subjects had not been on a low plant phenolic compound diet, a beneficial effect of catechin on lipid oxidation and the other biomarkers may have been observed. The phenolic content of the wine was insufficient to counter the reduction in dietary phenolic compounds. This needs to be confirmed by further investigation.

The concentration of F2alpha ispoprostane in plasma, which is an indicator of *in vivo* oxidation of arachidonic acid, was inversely correlated with that of catechin, suggesting that catechin can protect against oxidative damage, thus implying that catechin-rich wine has the potential to be protective against the risk pf cardiovascular disease.

Another marker of cardiovascular health is the elasticity (compliance) of blood vessels. In an early report of our work, using a tonometric technique for measurement of arterial compliance, a significant increase in small artery elasticity was observed after consumption of red wine. However, when the full complement of volunteers for this section of the intervention was included the result lost significance. The absence of changes in nitric oxide activity (measured as nitrate/nitrite ratio) may well be related to the lack of arterial compliance. Nitric oxide production is known to influence flow-related vascular dilatation, and since arterial elasticity is related to the degree of vessel relaxation,

it could be conceived that no evident change in nitric oxide activity, results in no change in vascular compliance. Related to this is the lack of change in the concentration of plasma prostaglandin the product of cyclooxygenase activity (quantified as 6-keto PGFalpha, the stable end product of prostacyclin) which could account for the lack of effect on blood pressure since prostaglandins are known to modulate blood pressure.

These results do not account for other factors that influence the dynamic characteristics of the peripheral vascular tree, and further work is necessary to determine the mechanism of the relationship between nitric oxide and prostaglandin production and vascular elasticity.

Overall, the results indicate that studies examining the effects of wine consumption on risk factors for cardiovascular disease should take into account the phenolic content of the background diet.

The results from the *in vitro* 'spiking' studies showed significant reductions in gamma-ray-induced DNA damage in those blood samples that contained catechin and caffeic acid, ethanol, stripped wine and the mixture at both 2.5% and the 10% concentration of that found in wine. Similar experiments with isolated lymphocytes showed a protective effect of catechin and caffeic acid against hydrogen peroxide-induced DNA damage. The observed increase in induction of cell death following oxidative challenge by the genome protective components of wine suggested that these compounds may lower the threshold for the elimination of DNA damaged cells by necrotic cell death. This may occur when the NAD/NADH ratio and ADP concentration is reduced in response to increased induction of polyADPribose polymerase (PARP), which is the initial DNA damage response element.

The results from the ex vivo study showed that alcohol on its own aggravated the level of gamma-ray-induced DNA damage observed in lymphocytes but the de-alcoholised red wine significantly reduced the observed DNA damage relative to controls. Whole red wine gave an intermediate effect with a non-significant trend towards protection and significantly better than alcohol. A more intensive time response study to better assess the duration of the post-consumption protective effect, and an investigation with dealcoholised wine would be a most valuable continuation of this work.

These data demonstrate the potential beneficial effects of wine-derived phenolic compounds in counteracting the harmful effects of ionising radiation, a ubiquitous source of DNA damage. They also demonstrate that the potentially exacerbating effects of alcohol in an oxidative environment may be neutralised by other wine components.

The mechanism by which wine-derived phenolic compounds protect against DNA damage induced by ionising radiation remains unclear. Studies of the distribution of wine-derived phenolic compounds within cells, and effects of gene expression and chromatin structure, would help to resolve this question.

3. Background

3.1 Introduction

Damage to critical molecules such as DNA, lipids and proteins by reactive oxygen species (oxidative free radicals) has been implicated in accelerated aging, as well as degenerative diseases including cancer (Ames *et al.*, 1995), cardiovascular disease (Steinbrecher *et al.*, 1990) and Alzheimer's disease (Smith *et al.*, 1996). These diseases of old age are expected to increase significantly over the next few decades as more people begin to survive beyond the age of 80 years (Kelner and Marx, 1996). Consequently there is a great interest in identifying life-style factors and molecular mechanisms that can minimise risk of these debilitating conditions.

Oxygen is necessary for immediate survival but it is becoming increasingly evident that the toxicity of oxygen is hazardous over the long-term. Molecular oxygen is a bi-radical. Upon single electron additions in the mitochondria, it generates the reactive oxygen species superoxide, hydrogen peroxide and the hydroxyl radical, which generate additional reactive oxygen metabolites and cause extensive oxidative damage to biological macromolecules (Sohal and Weindruch, 1996). Similarly, air pollutants, carcinogens, cigarette smoke, excessive exercise and radiation have all been identified as sources or promoters of oxidative stress.

A high level of oxidative stress leads to: (a) the carbonyl modification of proteins observed in neural tissue of Alzheimer's disease patients (Smith *et al.*, 1996); (b) mutation of DNA sequence and DNA strand breakage, which are among the main underlying causes of cancer initiation and progression (Ames *et al.*, 1995); and (c) the peroxidation of lipoproteins that is thought to be one of the main initiating steps in atherosclerosis or the accumulation of plaque on the artery wall**,** the progression of which leads to cardiovascular disease (Steinbrecher *et al.*, 1990).

Since these reactive oxygen species are ever present, a complex system of native enzymatic and dietary antioxidants regulates the delicate oxidative balance (Halliwell and Gutteridge, 1995). Plant-derived phenolic compounds in foods and beverages are emerging as potential preventive and therapeutic agents in oxidative free radical-mediated disease, postulated as inhibitors of oxidative damage. One class of phenolic compounds, the flavonoids, has been shown to be more protective *in vitro* than vitamin C (ascorbate) or vitamin E (α-tocopherol) (Rice-Evans *et al.*, 1996), and an association between reduced mortality from coronary heart disease and dietary flavonoid consumption has been reported (Hertog *et al.*, 1993). Wine is one of the richest sources of plant-derived phenolic compounds in the diet, contributing up to 70 g of flavonoids annually per person in France or Italy (Singleton, 1992).

Ethno-ecological population studies have revealed that populations consuming large quantities of fresh fruit and vegetables are less prone to certain types of cancer (Ames *et al.*, 1993). It is an extension of the Mediterranean dietary and life-style model followed by people in the grape and olive growing regions of southern Europe that include plentiful fresh fruit, vegetables and cereals, liberal amounts of olive oil, and regular, moderate consumption of red wine from lunch-time through to the evening meal (Trichopoulou *et al.*, 1997).

In 1979, St. Leger and colleagues demonstrated a negative association between moderate wine consumption and coronary heart disease morbidity and mortality in 18 western world countries. The phenomenon, now known as "the French paradox" following publication of a paper by Renaud and de Lorgeril in 1992, describes the low incidence of coronary heart disease despite a high prevalence of risk factors for coronary heart disease. A reduced risk of coronary heart disease for moderate wine consumers was further demonstrated by Boffetta and Garfinkel (1990), who established relationships between risk of mortality from different causes and alcohol consumption. A pronounced reduction in risk of mortality from all causes was observed in people consuming up to 36 g of alcohol per day, predominantly due to a reduction in risk of mortality from coronary heart disease. Handa *et al* (1990) have also demonstrated that moderate alcohol consumption may protect against severe coronary atherosclerosis.

Furthermore, epidemiological research examining alcohol consumption and all cause mortality have indicated a reduced relative risk of 22% for cancer in men consuming moderately (between approximately 10 g and 20 g alcohol/day), the majority of which was in the form of wine (>80%), when compared to abstainers (Renaud *et al.*, 1998).

Phenolic compounds are found in especially high concentration in red wine (>1200 mg/l, Singleton, 1992). Wine also consists of alcohol, glycerol and ascorbate, which may all contribute to the potential of wine as an important life-style and dietary factor in the prevention or promotion of damage by reactive oxygen metabolites (Ames *et al.*, 1995; Boffetta and Garfinkel, 1990).

Two potential measures of the health benefits of moderate wine consumption are a reduction in markers of genetic damage (and hence cancer), and a reduction in markers for cardiovascular disease.

3.2 Reduction in markers of genetic damage

Genetic damage is a risk factor for the formation and progression of cancer. A number of factors may contribute to this damage, including chemical genotoxins, lifestyle factors (diet, exercise and environment), and medical therapies including radiotherapy and cytotoxic drugs. Oxidising agents such as hydrogen peroxide and ionising radiation cause chromosome breakage and loss, as well as cell death (Fenech *et al.*, 1999a, Fenech *et al.*, 1999b). It has been recognised that the antioxidant phenolic compounds present in wine may potentially reduce the damage associated with cancer initiation or progression by quenching or reducing oxidative agents (Ames *et al.*, 1995). Clifford *et al* (1996) have shown that red wine solids, rich in polyphenols, delayed tumor onset when fed to transgenic mice that spontaneously develop externally visible tumors.

A pilot study in our laboratory examining the potential benefits of wine consumption on genetic damage as induced by hydrogen peroxide treatment (an exogenous source of oxidative free radicals in plasma) indicated a reduction in the level of damage up to three hours post consumption of 300 mL wine, with strongest inhibition (>70%) at the one hour post-consumption time point (Fenech *et al.*, 1997). *In vitro* experiments in the same sequence of work showed that lymphocytes incubated in either 12% ethanol solution, or phenolic-stripped white wine prior to hydrogen peroxide challenge resulted in a statistically significant 57–87 % reduction ($p=0.0354$ and $p=0.0053$, respectively) in micronucleated cell frequency. Micronuclei are formed following whole chromosome loss

events or when portions of broken chromosomes are not packaged as normal into daughter nuclei during nuclear division (***Figure I*** *Micronucleus formation.*).

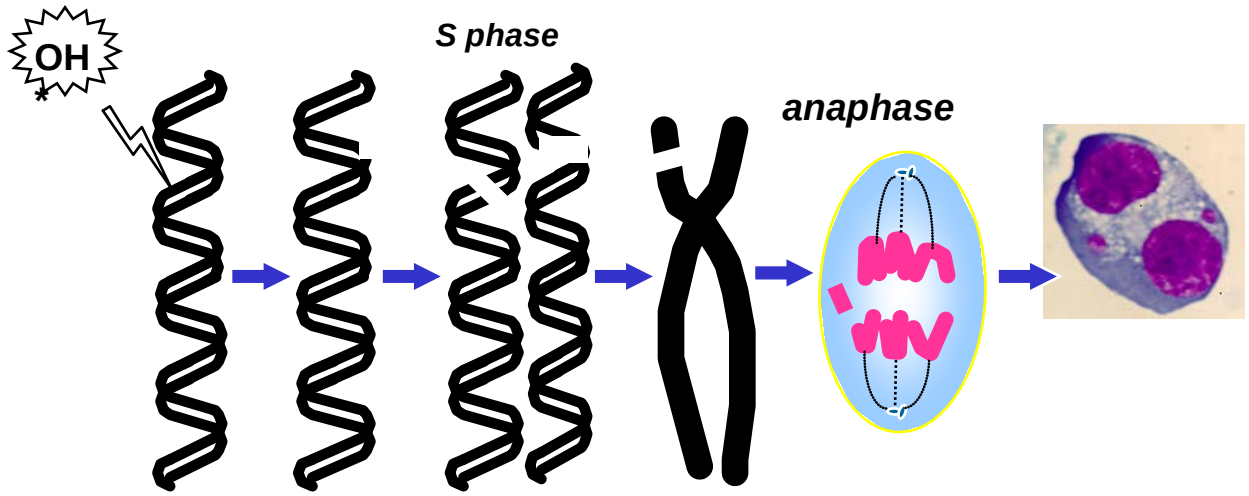


Figure I - Micronucleus formation. Oxidative radicals may cause damage to DNA by base modification or single strand breakage. On synthesis during S-phase, these are converted to double strand breaks and subsequently chromosome fragments. During chromosome pairing at anaphase, prior to movement into daughter cell nuclei, these fragments are unable to pair and micronuclei result. Similarly, during a whole chromosome loss event, perhaps due to defect in the kinetochore region, a replicated chromosome is unable to be drawn into the daughter nuclei, and a micronucleus results. As a result of this, quantitation of micronucleus frequency may be used as a precise indicator of genetic damage.

A growing number of studies are showing similar results in both human and animal models. Giovannelli and coworkers (2000), using a modification of the single cell comet assay for DNA damage, showed a significant reduction in basal DNA oxidation in rats treated with a red wine complex phenolic compound/tannin powder blend. The rats had been treated at a dose equivalent to a 70 kg male drinking 500 mL wine daily, for ten days. Also supporting this work, a three-fold increase in the level of oxidative DNA damage in humans, seen following a high fat diet for 30 days was completely eliminated after supplementation of this diet with 240 mL red wine daily (Leighton *et al.*, 1999). The protective effect of red wine against DNA damage in these studies cannot be readily explained by the phenolic compound content of red wine because results for red wine were virtually identical to those for white wine even though white wine has a much lower concentration of phenolic compounds, mainly due to differences in production methods (Singleton, 1992).

Interestingly, the results from our pilot study were similar for both red and white wine, suggesting that the protection offered by wine consumption in terms of chromosome damage was not related to the increased phenolic compound content of red wine, but in fact to compounds, including certain non-flavonoid phenolic compounds equally present in both red and white wine, such as hydroxycinnamic acid and caffeic acid. Non-flavonoid phenolic compounds are structurally simpler than the flavonoids, and are generally stored

in the vacuoles of grape cells, thus being easily extracted on crushing. Indeed, the concentration of hydroxycinnamates in red and white wine is similar. It is interesting to note that caffeic acid accounts for the great majority of hydroxycinnamic acids in white wine, which represent approximately 75% by weight of the total phenolic compounds. Caffeic acid is mainly present as caftaric acid, that is, an ester with tartaric acid. Published data suggest that caffeic acid may be the most effective antioxidant amongst the hydroxycinnamates found in white wine (Nardini *et al.*, 1995). It could be that alcohol and glycerol from wine may also prevent oxidative damage by hydrogen peroxide, because it has been shown previously that such molecules are extremely effective in preventing DNA damage induced by ionising radiation (Worm *et al.*, 1993). Both hydrogen peroxide and ionising radiation mediate their DNA damaging effects via the hydroxyl radical, which is generated by the Fenton reaction within the nucleus in the former and by radiolysis of water in the latter (Imlay *et al.*, 1988; Wiseman and Halliwell, 1996).

A suitable tool for baseline or induced genetic damage measurement in either *in vitro* or *ex vivo* intervention studies is the cytokinesis-block micronucleus (CBMN) assay (Fenech *et al.*, 1993) ***(Figure III)***. In this sensitive and robust technique, micronuclei and nucleoplasmic bridges are scored in cells that have completed nuclear division—the cytokinesis block with cytochalasin-B allows these cells to be identified by their binucleate appearance. By including the novel assessment criteria of apoptosis and necrosis alongside the core assay protocol, a more comprehensive measure of genotoxic and cytotoxic damage can be obtained (Fenech *et al.*, 1999a).

3.3 Reduction in markers of cardiovascular disease

Subsequent research using *in vitro* models has demonstrated that the red wine phenolic compounds can inhibit oxidation of LDL, which is an important risk factor for atherosclerosis, and could explain, in part, the potential cardioprotective effect of wine (Frankel *et al.*, 1993). An *in vivo* intervention by Fuhrman and colleagues (1995) provided more direct evidence: daily consumption of red wine for two weeks with meals decreased the propensity of plasma lipids to undergo peroxidation *in vitro*, however, white wine consumption did not produce such a benefit and in fact appeared to increase lipid peroxidation. These results were interpreted to indicate that the high intake of red wine-derived phenolic compounds is required to prevent both endogenous and alcohol-induced lipid peroxidation. The intervention by Fuhrman and colleagues (1995), however, has several shortcomings, notably: (a) the experiment was not a cross-over so that differences between the white wine and red wine groups may have been accidental; (b) the study did not verify that there were significant differences in the concentration of phenolic compounds in LDL in the groups consuming red or white wine; (c) because diets were not appropriately controlled or recorded one cannot exclude the possibility that differences observed may have been due to diet (including tea drinking) between groups rather than the type of wine consumed; and (d) more importantly no reliable evidence was provided that wine phenols were actually absorbed during the intervention, or that more wine-derived phenolic compounds were absorbed after drinking red wine as compared to white wine. Furthermore, there is limited evidence that wine-derived phenolic compounds are actually absorbed into the blood and that wine consumption actually prevents lipid oxidation *in vivo*.

Following a submission for funding made to The Australian Wine Research Institute in 1993/94, collaborative studies between the AWRI's Health and Regulatory Information Manager and the Chemistry Group, and CSIRO Health Sciences and Nutrition (formerly

the Division of Human Nutrition) were undertaken. The broad objectives of that work were to evaluate the importance of wine-derived phenolic compounds to human health and

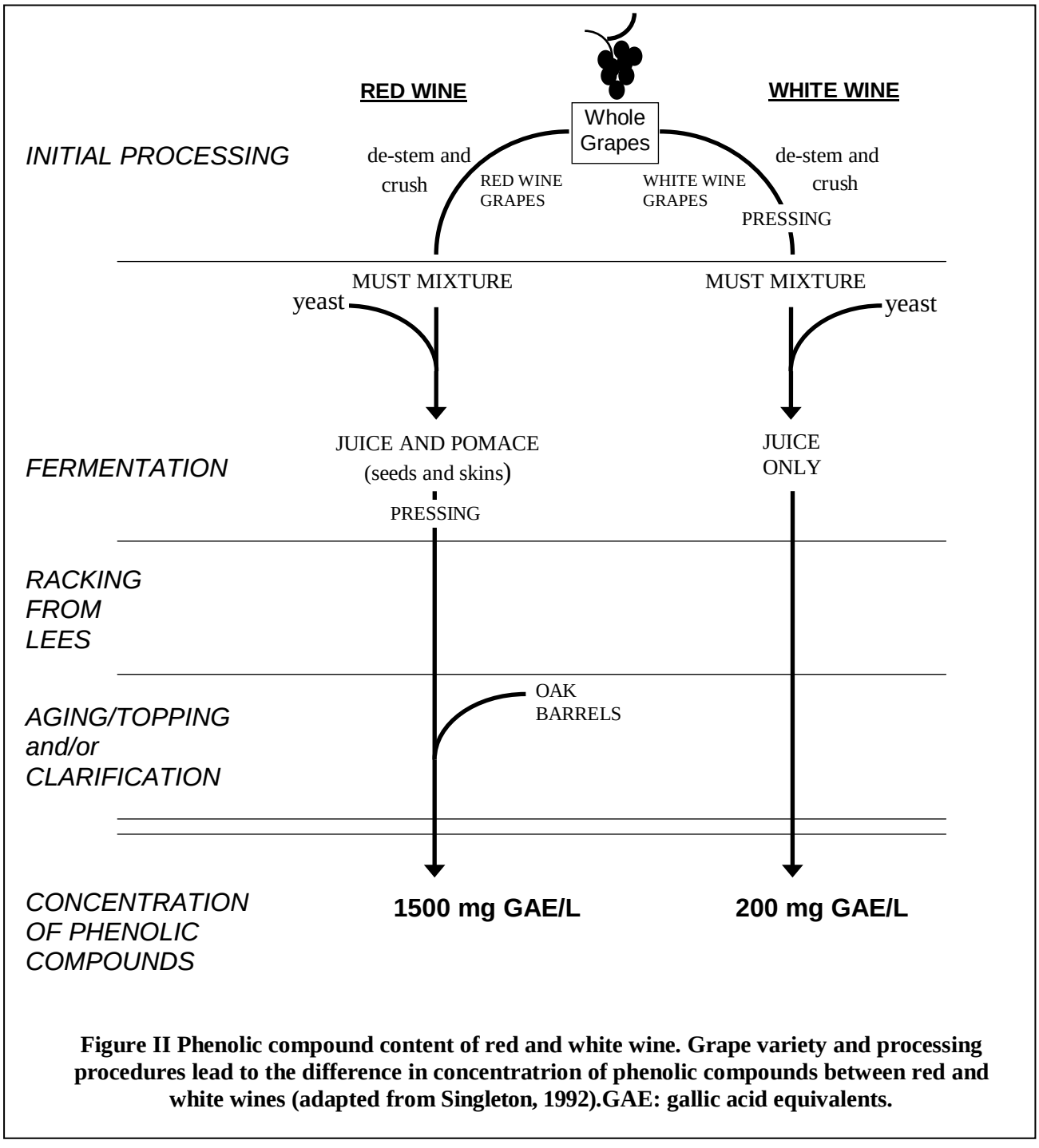


Figure II Phenolic compound content of red and white wine. Grape variety and processing procedures lead to the difference in concentratrion of phenolic compounds between red and white wines (adapted from Singleton, 1992).GAE: gallic acid equivalents.

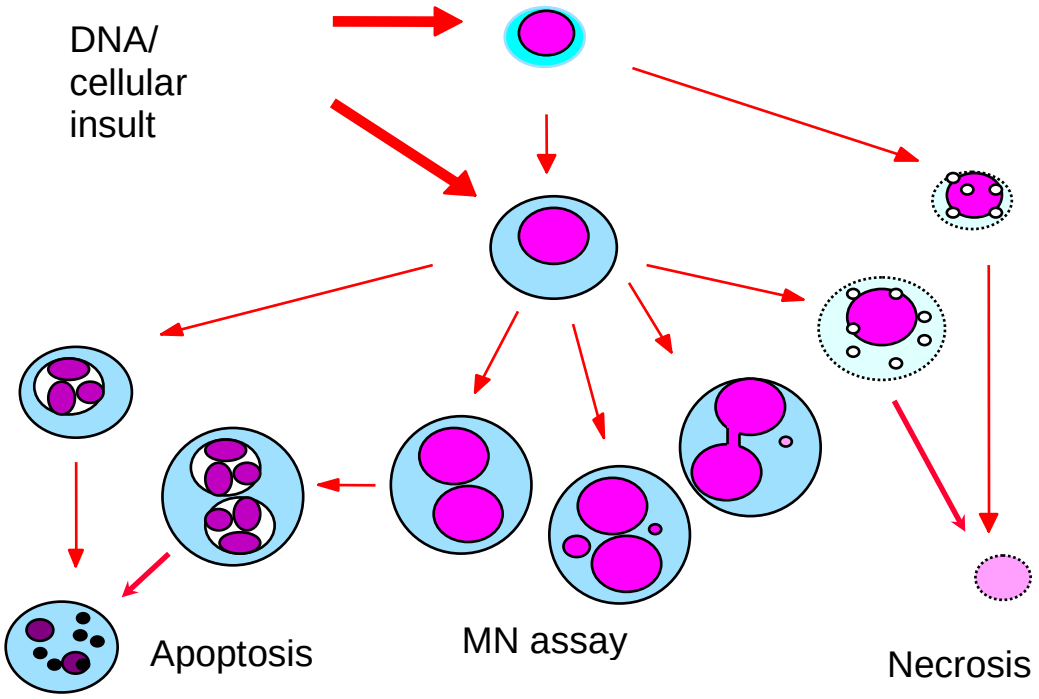


Figure III Cytogenetic endpoints of the micronucleus assay. Micronuclei (scored in the MN assay) are formed when DNA strand breaks, whole chromosome losses or defective kinetochore proteins prevent normal pairing of chromosomes at metaphase. Nucleoplasmic bridges are characteristic of dicentric chromosome formation and a marker for chromosomal rearrangements. Apoptotic cells are indicative of induced programmed cell death due to excessive genetic damage. Necrotic cells result from natural cell death due to excessive cellular structural damage.

nutrition. Initial *in vitro* experiments involved the addition of red or white wine to incubations of LDL in the presence of an oxidative agent. The results demonstrated that red wine-derived phenolic compounds can protect LDL from oxidation *in vitro;* red wine-derived phenolic compounds were approximately five-times more potent than those of white wine. The observation that both red and white wine stripped of phenolic compounds had no protective effect on LDL oxidation demonstrated that it was the phenolic component of the wine that conferred the protective effect.

Further preliminary research, both *in vitro* and *in vivo,* was funded by the Australian Wine Foundation in 1995/96 to develop protocols and analytical methodology. *In vitro* studies showed that red wine inhibited LDL oxidation in a dose dependent manner and that fractionated phenolic compounds from red wine were able to inhibit LDL oxidation, with the catechin fraction being the most effective (Kerry and Abbey 1997). Additionally, the non-phenolic fractions of both red and white wine protected DNA from oxidative free radicals (Fenech *et al*. 1997). In the *in vivo* cross-over study four male volunteers consumed three 100 mL glasses of wine per day for two days while on a low phenolic compound diet and after an overnight fast, consumed another 300 mL. The wines

administered were a red wine of known concentration of catechin (60 mg/l) and a white wine of known low concentration of catechin (11 mg/l); both wines were matched for concentration of ethanol, glucose/fructose, glycerol and ascorbic acid (Fenech *et al.* 1996) Although the preliminary *in vitro* research demonstrated that the wine-derived phenolic compounds may exert their protective effect on LDL by increasing the lag time before oxidation occurs in the presence of oxidative free radicals; that is, significantly less oxidation of LDL occurs, the initial *in vivo* research did not show an increase in lag time. The lack of effect may reflect the short duration of the study.

Fenech M., Abbey, M., Record, I., Williams, P.J., Waters, E. and Stockley, C. (1996) Collaborative study between CSIRO Division of Human Nutrition and The Australian Wine Research Institute. Progress report on the Effects of Wine Consumption on Plasma Antioxidant Activity.

3.4. Overall aim of the research project

There is now sufficient and significant epidemiological data in the literature to suggest that alcohol, when regularly consumed in moderation, has both cardio- and cancer-protective effects. There is also data to suggest that wine may offer additional or further protective effects than the other alcoholic beverages.

The investigation of both phenolic and non-phenolic compounds in wine is an area of substantial research activity. Whilst the antioxidant potential of some of these wine-derived compounds has been established clearly *in vitro* (Rice-Evans *et al.*, 1995), there is as yet, little evidence for their capacity to prevent disease processes *in vivo* (reviewed in Ferguson, 2001). If wine proves to be protective against the DNA damaging effect of ionising radiation it may be deduced: (a) that there may indeed be a valid role for wine in preventing genetic damage by hydroxyl radicals; and (b) that wine may have a useful function in reducing the health risks associated with exposure to diagnostic and therapeutic radiation (Roebuck, 1996).

The data from preliminary investigations has also indicated no measurable effect on the propensity of plasma lipids to undergo oxidation. This negative result for lipid oxidation can be explained either by insufficient absorption of the wine-derived phenolic compounds or inadequate transfer of the wine-derived phenolic compounds from the blood into the LDL.

Thus the approach of this project to understanding the *in vivo* effect of wine has been to investigate simultaneously its capacity to prevent lipid oxidation as well as damage to DNA, because both of these targets are important in the aetiology of degenerative disease. It is proposed that examination of the regular, moderate wine consumption for a four-week period, will demonstrate the bioavailabilty of wine components in human plasma, and also any beneficial effects on a number of markers of DNA damage (cancer) and cardiovascular disease.

4. Project Aims and Performance Targets

The aims of the project were as follows:

1. To test the hypothesis that the regular and moderate consumption of red wine elevates the plasma concentration of phenolic compounds, such as catechin, in humans.

2. To test the hypothesis that the regular and moderate consumption of red wine enhances in humans the antioxidant potential of plasma and thus:

    2.1 provides protection for DNA against oxidative free radical damage, such as *ex vivo* exposure to ionizing radiation and to hydrogen peroxide (cancer-protective) (phase 1); and

    2.2 provides protection for LDL against oxidative free radical damage *ex vivo* (cardioprotective) (phase 2).

Therefore, this research project comprised two phases as follows:

Phase I To determine a protective effect by wine of DNA against damage from oxidative free radicals in humans, which can initiate and progress cancer; and

Phase II To determine a protective effect by certain wine components of low density lipoprotein (LDL) against damage from oxidative free radicals in humans, which can initiate and progress cardiovascular disease, and the concomitant determination of the plasma concentration of wine-derived phenolic compounds, such as catechin.

Phase I was further divided into two separate studies

1. *in vitro* plasma spiking studies, to determine whether selected red wine components or groups of components protect against peroxide-induced or gamma radiation induced DNA damage; and

2. an *ex vivo* intervention to determine whether moderate consumption of red wine is able to protect DNA against radiation-induced DNA damage

The Gannt Chart milestones were as follows:

Task	1997				1998				1999			
Phase I *in vitro* study												
Phase I *in vivo* study												

Task	1998				1999				2000				2001			
Phase II intervention 2.1																
Phase II intervention 2.2																
Phase II intervention 2.3																

The outputs and performance targets for Phase II were as follows:

Outputs	Performance Target
1. Comparison of effects of red and white wine on vascular function using non-invasive techniques.	1. Completed by September 2000.
2. Comparison of effects of red wine and dealcoholised red wine using non-invasive Doppler techniques.	2. Completed by November 2000.
3. Comparison of effects of red wine and alcohol using non-invasive techniques.	3. Completed by April 2001.
4. Comparison of effects of red wine and white wine on biomarkers of vascular function in blood samples.	4. Completed by October 2001.
5. Comparison of effects of red wine and dealcoholised red wine on biomarkers of vascular function in blood samples.	5. Completed by December 2001.
6. Comparison of effects of red wine and alcohol on biomarkers of vascular function in blood samples.	6. Completed by May 2001.
7. Analysis and interpretation of results from items 1-6 to determine the relative importance of wine components in maintaining vascular function.	7. Completed by June 2001. Submit results for publication by July 2001.
8. Preparation of Final Report and manuscript.	8. Completed by July 2001.

5. Phase I—Potential benefits of wine in prevention of genetic damage induced by hydrogen peroxide and ionising radiation

5.1 Experimental design and protocol

5.1.1 Wine Components

The study examined the individual wine components ethanol, glycerol, tartaric acid (present in both red and white wine) as well as a mixture of catechin (flavanol) and caffeic acid (phenolic acid). All wine components examined were dissolved in Hank's Balanced Saline Solution as shown (Table I). The components were prepared at a concentration to achieve a final concentration *in vitro* equivalent to a 2.5% (1:40) and 10% (1:10) dilution of the level seen in white wine. This represents a heavy alcohol consumption. Control agents were the diluent Hanks Balanced Salt Solution (HBSS) and phenolic-stripped white wine.

	Component concentration		
		Final i*nvitro* concentration	
Wine component	White wine	*1:10 dilution*	*1:40 dilution*
Ethanol	13400 mg/l	13.4 mg/ml	3.35 mg/ml
Glycerol	680 mg/l	0.68 mg/ml	0.17 mg/ml
Tartaric acid	300 mg/l	0.3 mg/ml	$7.5x10^{-2}$ mg/ml
Catechin and caffeic acid mixture	1.1 mg/l	$1.1x10^{-3}$ mg/ml	$2.75x10^{-4}$ mg/ml
Mix*	-	All above components	All above components
PVPP stripped white wine**	-	10% v/v	2.5% v/v
Carrier (HBSS)	-	10% v/v	-

Table I Concentration of components used for in vitro plasma spiking experiments
***Mix sample contained all components listed above, at the concentrations listed**
****White wine stripped of phenolic compounds using polyvinyl poly-pyrolidone (PVPP)**

The white wine was sourced from Tucker Seabrook (WA) Pty Ltd and the red wine was sourced from the Orlando Wyndham Group. The transportation and bottling was undertaken by Vinpac International and the de-alcoholisation of the red wine was undertaken by Australian Vintage Ltd.

5.1.2 Volunteers

After approval by the CSIRO Health Sciences and Nutrition Human Experimentation Ethics Committee, and The University of Adelaide Human Research Ethics Committee, the study was advertised to students at both institutions. Volunteers were selected based upon their responses to a general health and alcohol consumption questionnaire. Those who supplemented their diet with antioxidant vitamins and/or multi-vitamins, and/or smoked, and/or drank in excess of moderate drinking guidelines were excluded from the study. Four healthy males aged 21–23 years were chosen for the *in vitro* studies, and six healthy males aged 21–26 years were selected for the *ex vivo* wine intervention studies. Both groups of volunteers gave informed consent, and were recruited to the study.

5.1.3 Study design

Phase I comprised two separate components:

a) *in vitro* plasma spiking studies; and
b) an *ex vivo* intervention.

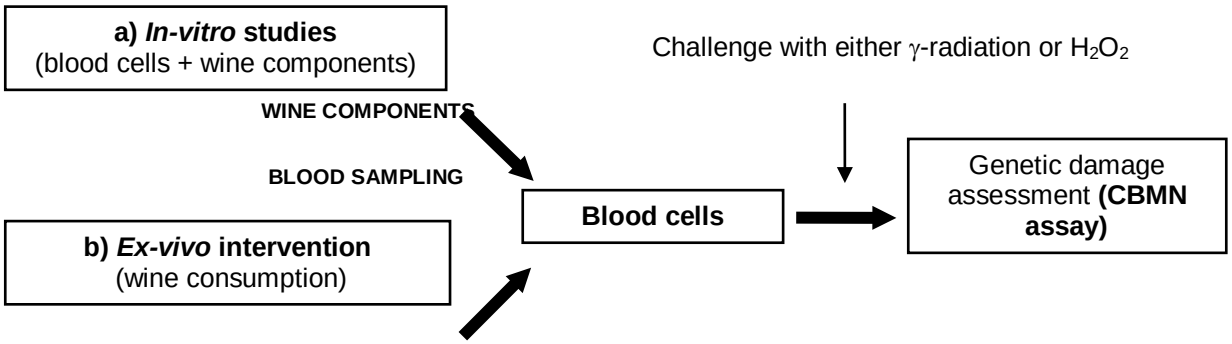
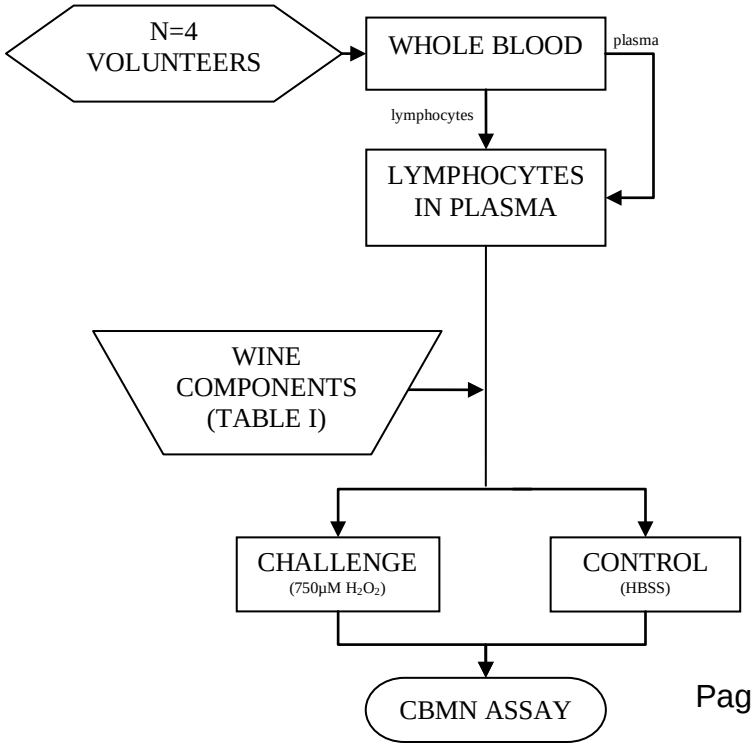


Figure IV Phase I experimental design

5.1.3.1 *In vitro* plasma spiking studies

To determine which compounds common to red and white wine are responsible for its antioxidant properties, a series of *in vitro* studies were performed. This work examined the effect of supplementing either plasma samples in isolated lymphocyte cultures (hydrogen peroxide experiment) or supplementing whole blood cultures (gamma radiation experiment) with known concentrations of wine components and control compounds. To challenge these *in vitro* systems, cultures were exposed to hydrogen peroxide (a known generator of reactive oxygen species; Figure V) or ionizing radiation (an energy source able to generate hydroxyl radicals from water and cause chromosome breakage; Figure VI). Cells were then assessed for genome damage using the cytokinesis-block micronucleus (CBMN) assay. By comparing challenged and control cultures, an assessment was made as to whether a given compound was able to prevent hydrogen peroxide or radiation-induced DNA damage. These types of studies have not been performed previously.

Figure V Experimental protocol for *in vitro* plasma 'spiking' experiments, hydrogen peroxide challenge



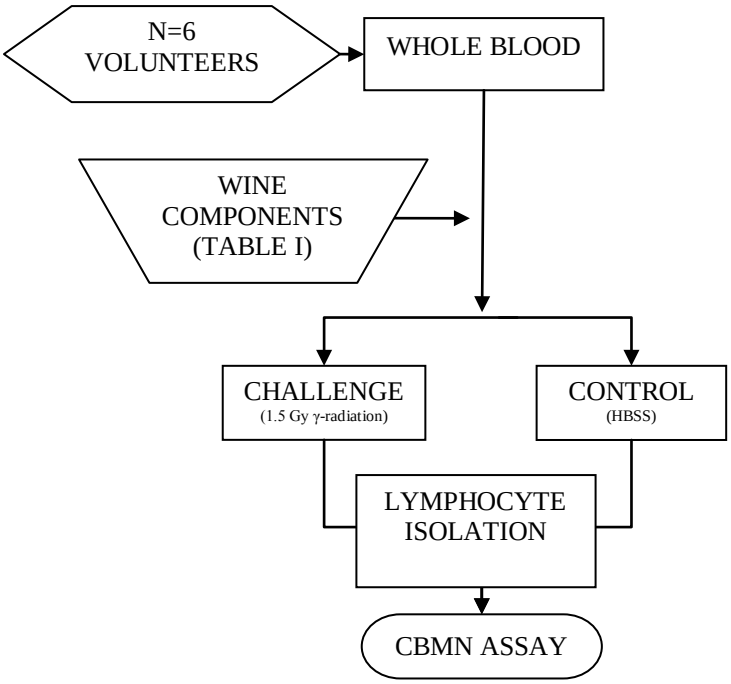


Figure VI Experimental protocol for in vitro plasma 'spiking experiments, gamma (γ)-radiation challenge.

5.1.3.2 *Ex-vivo* intervention—dietary cross-over study examining red wine consumption
In this pharmacokinetic-based study, the protection offered by red wine or other control drinks was assessed post-consumption in a crossover intervention (Figure VII). Whole blood was tested *ex vivo* for protection against DNA damage induced by ionizing radiation, that is, gamma rays.

Volunteers had a blood sample taken (25 mL) to assess baseline MN index. They were then asked to adhere to a special PPF diet (plant phenolic free diet) for 48 hours (Appendix A), prior to post-washout sample (25 mL) being taken. After consumption of either a moderate amount (300 mL) of red wine (RW), 11% alcohol solution (ALC), or de-alcoholised red wine (DE-ALC), samples (25 mL) were taken at 0.5, 1 and 2 hours post-consumption (Figure VII.) The same sampling schedule was used throughout the intervention. Volunteers were randomly assigned to red wine or alcohol solution in week 1. In the following week, all the volunteers consumed dealcoholised red wine before crossing over to the remaining red wine or alcohol solution. The analyses of the wines used in this study is detailed in Table IIIa.

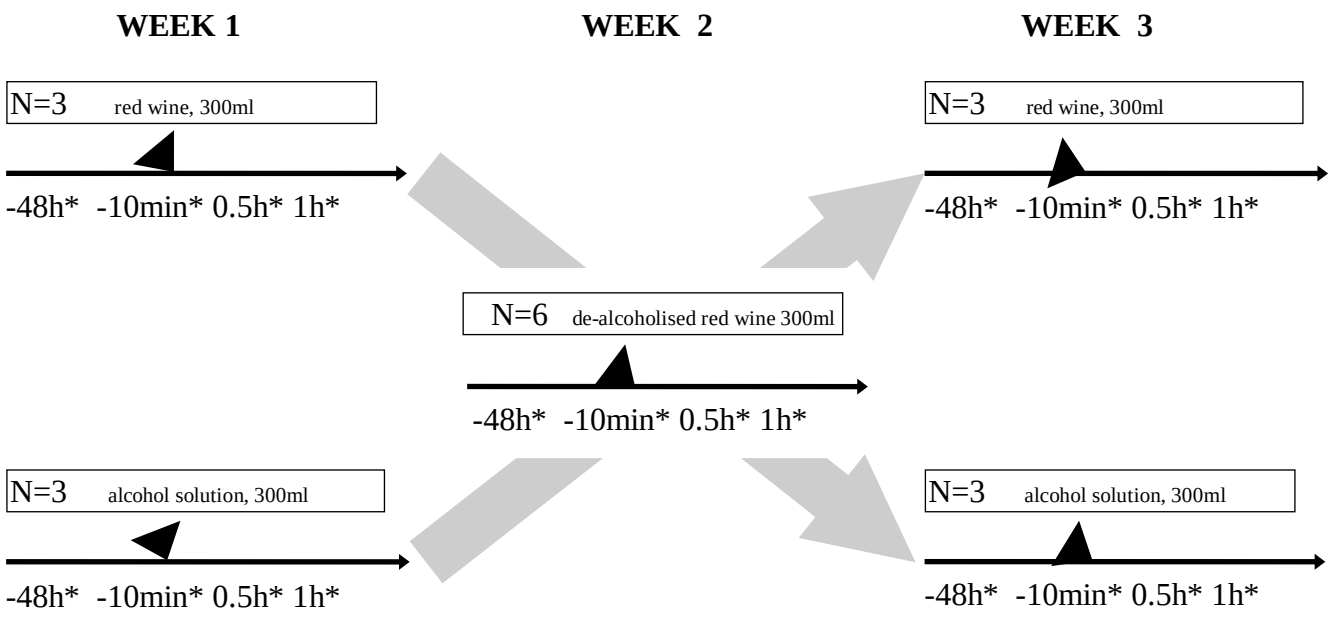


Figure VII Dietary intervention design. Intervention with double cross-over, six healthy males aged 20–30 years. Volunteers were randomly assigned to one of two groups on their first visit to the clinic (two groups, each N=3). A period of one week was allowed between the completion of each stage, and beginning of the next stage.

***At these time-points, 25ml of blood was collected in heparinised tubes**

Whole blood cytokinesis-block micronucleus assays were performed immediately to determine the radio-protective effect of wine consumption against ionising radiation, that is, gamma-rays.

5.2 Methods

For analytical methods for the whole blood cytokinesis-block micronucleus assays please refer to manuscripts attached in Appendix 10.1.

Table IIIa Component analysis of wines used
***ALC also contained potassium hydrogen tartrate at the same concentration as in the red wine**

Test	REF	UNITS	RW	DE-ALC	*ALC
Total anthocyanins - Red wine	GM05	mg/L	121	102	-
Total Phenolics - Red wine	GM05	a.u.	35	31	-
Total polyphenols		mg/L	1200	1100	-
Red wine colour – density	GM05	a.u.	5.2	5.1	-
Red wine colour – hue	GM05		0.87	0.84	-
Chemical age index #1	GM05		0.52	0.47	-
Chemical age index #2	GM05		0.2	0.22	-
Degree of ionisation of anthocyanins	GM05	%	14.88	19.5	-
Degree of ionisation of anthocyanins-abolishing effect of sulphur dioxide	GM05	%	28.39	39	-
Free sulphur dioxide, excluding anthocyanin bound sulphur dioxide	GM05	mg/L	3.49	3.84	-
Sulphur dioxide free	LM09	mg/L	1	1	-
Sulphur dioxide total	LM09	mg/L	16	7	-
ascorbic acid (HPLC)	GM08	mg/L	n.d.	n.d.	-
glucose + fructose	LM07	g/L	0.6	0.3	-
succinic acid (HPLC)	GM09	g/L	0.7	0.5	-
malic acid (HPLC)	GM09	g/L	n.d.	n.d.	-
lactic acid (HPLC)	GM09	g/L	0.8	0.7	-
acetic acid (HPLC)	GM09	g/L	n.d.	n.d.	-
Tartaric acid (HPLC)	GM09	g/L	n.d.	n.d.	-
citric acid	LM08	g/L	0.2	0.2	-
Alcohol (NIR)	LM13	% v/v	13.3	0.4	12

5.3 Results

5.3.1 *In vitro* plasma 'spiking' experiments

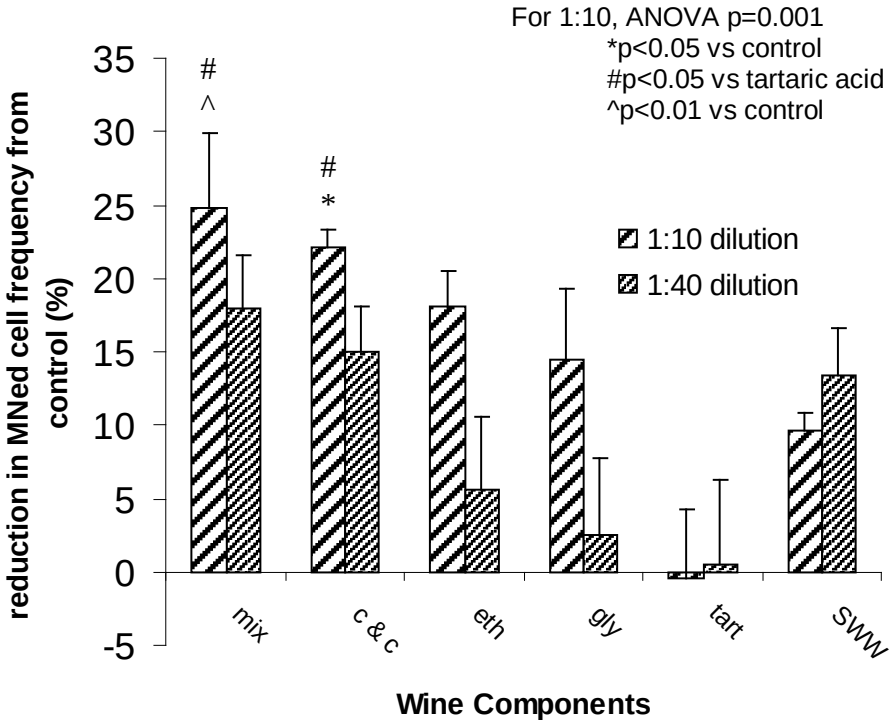


Figure 9 Reduction of hydrogen peroxide induced genetic damage on supplementation of plasma with wine components

Mix, all components; c&c, catechin and caffeic acid; eth, ethanol; gly, glycerol; tart, tartaric acid; SWW, phenolic-stripped white wine; MNed, micronucleated

All spiking components (with the exception of tartaric acid) appeared to reduce hydrogen peroxide induced micronucleated cell frequency at the 1:10 dilution in isolated lymphocyte cultures, the most effective being the mix of all components, and the catechin/caffeic acid mix (reductions of 25±5.1% and 22±1.1% respectively, $p<0.05$ relative to control, Figure 9). A similar, but less pronounced effect was noted on using the 1:40 dilution. No significant difference was seen in baseline micronucleated cell frequency ($p>0.05$).

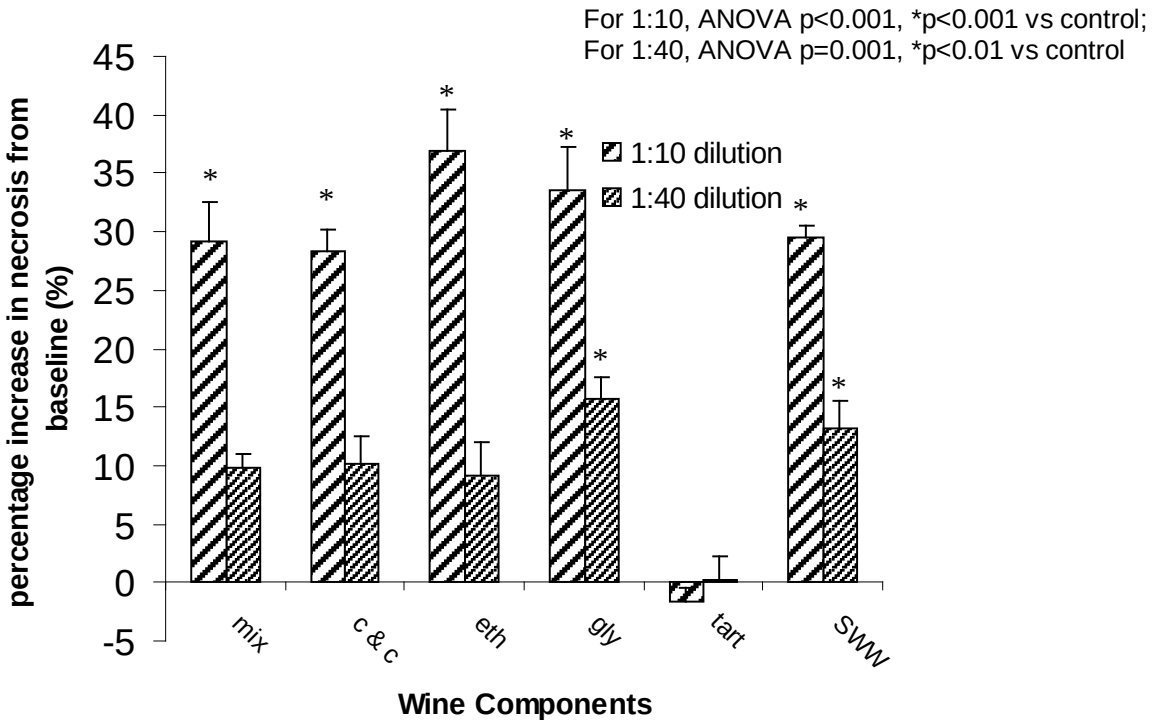


Figure 10 Increase of hydrogen peroxide induced cellular necrosis on supplementation of plasma with wine components.

Mix, all components; c&c, catechin and caffeic acid; eth, ethanol; gly, glycerol; tart, tartaric acid; SWW, polyphenol stripped white wine.

A significant increase in necrotic cell frequency was noted for all components at 1:10 dilutions ($p<0.001$), except tartaric acid. This increase in necrosis from baseline was up to 37±3.6% in the case of ethanol at the 1:10 dilution. At the lower concentration, less necrosis was observed in all cultures, and attained significance in those cultures spiked with either glycerol, or SWW ($p<0.05$) (Figure 10).

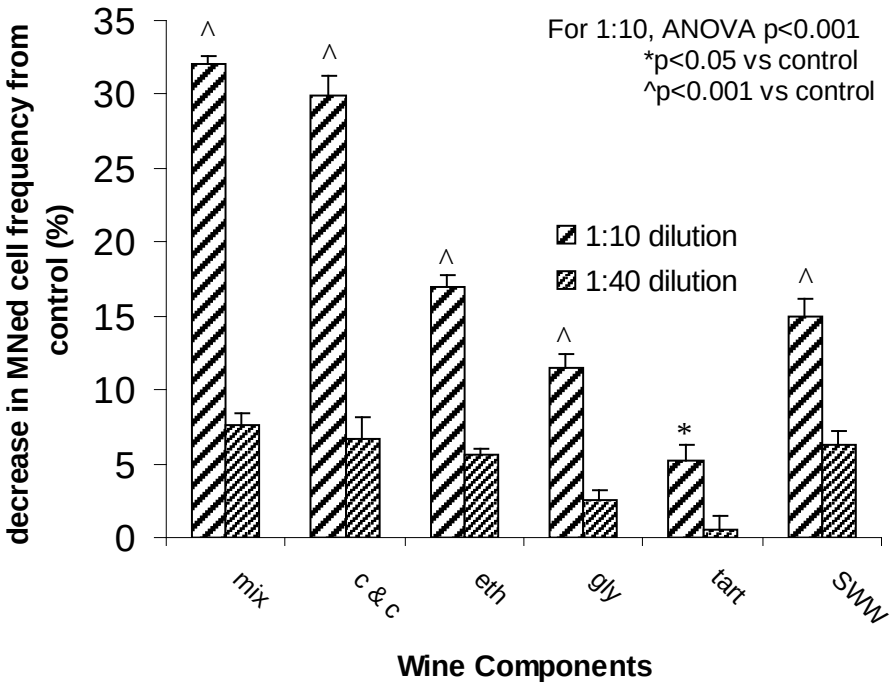


Figure 11 Reduction of gamma radiation induced genetic damage on supplementation of plasma with wine components

Mix, all components; c&c, catechin and caffeic acid; eth, ethanol; gly, glycerol; tart, tartaric acid; SWW, polyphenol stripped white wine

Whole blood cultures spiked with either a mix of all components, or catechin/caffeic acid at a 1:10 dilution, exhibited reduction in gamma-radiation induced micronucleated cell frequency by 32±0.5% and 30±1.3% respectively, both significant at $p<0.001$ vs control (Figure 11). These two spiking agents (mix and catechin/caffeic acid mix) were not significantly different in their protection ($p=0.636$). Weaker effects were observed for the 1:40 dilutions of the same compounds (8±0.8% and 7±1.5%). All other components, including phenolic stripped white wine (SWW) significantly reduced induced micronucleated cell frequency significantly from baseline ($p<0.001$), however to a lesser extent than the mix of all components or the catechin/caffeic acid solution.

5.3.2 *Ex-vivo* intervention—dietary cross-over examining red wine consumption

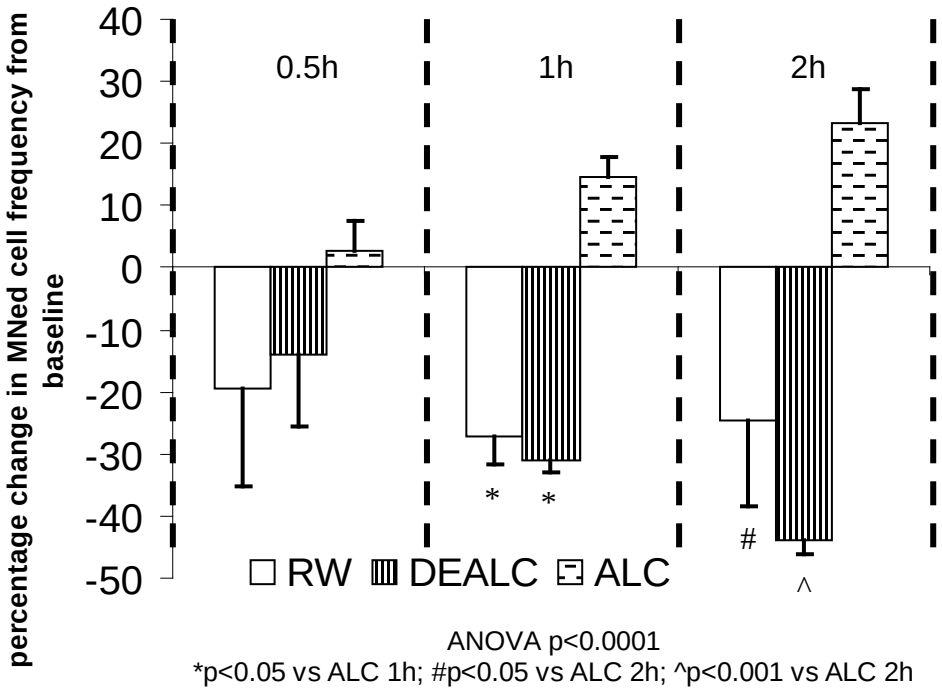


Figure 12 Change in micronucleated cell frequency over time compared to baseline following consumption of either red wine (RW), dealcoholised red wine (DEALC), or model wine solution (12% ethanol – ALC). Blood samples were taken 0.5 h, 1 h and 2 h post-consumption.

In the *ex vivo* intervention both red wine and dealcoholised red wine consumption significantly reduced gamma-radiation induced micronucleated cell frequency when compared to alcohol consumption. This reached significance for both drinks at 1 and 2 hours post-consumption ($p<0.05$). The most significant reduction (44±2.0%, $p<0.001$) was observed 2 hours after dealcoholised red wine consumption. Consumption of alcohol clearly increased induction of micronucleated cells in a time responsive manner post-consumption *(*Figure 12*)*.

5.4 Discussion

The epidemiological evidence linking light to moderate wine consumption with reduced cardiovascular disease risk is compelling and evidence is also accumulating for potential protective effects against cancer (Boffetta and Garfinkel, 1990; Renaud *et al*., 1998). Increased oxidative stress is a recognized risk factor for both cardiovascular disease and cancer (DeFlora *et al.*, 1997, Andreassi *et al*., 2000; Ferguson, 2001). A human intervention study suggested that moderate wine consumption reduces the concentration of 8-hydroxydeoxyguanosine (Leighton *et al*., 1999). A cross-sectional study of pre-menopausal, non-smoking women showed a significant inverse correlation between alcoholic drink consumption and oxidation of lymphocyte DNA (Bianchini *et al*., 2001). These results are also supported by experiments in rodents which showed that ingestion of wine solids reduces DNA oxidation in the colon (Giovanelli *et al*., 2000).

The main questions that needed to be resolved are essentially the following: (i) which of the major wine components are protective against oxidative stress at concentrations that are physiologically relevant; (ii) what is the relative contribution of the wine components that are protective against oxidative damage to DNA; and (iii) are the antioxidant effects of the various wine components additive or synergistic?

4.5.1 *In vitro* plasma spiking studies

These results provide a first comprehensive assessment of the relative antioxidant properties of tartaric acid, the phenolic compounds catechin and caffeic acid, ethanol and glycerol *in vitro*.

4.5.1.1 Hydrogen peroxide challenge

The results from our studies suggest that the wine components may have small but significant effects on the rate of baseline DNA damage, with ethanol tending to increase micronucleus formation when compared with the other wine components. The other wine components or ethanol in combination with the other wine components tended to reduce the rate of DNA damage, although the differences were not significant when compared with the Hanks Balanced Salt Solution (HBSS) control. The genotoxic effect of ethanol is not surprising given that there is ample evidence in the literature that ethanol can induce chromosome breakage and sister chromatid exchange, particularly in those with polymorphisms that alter the activity of aldehyde dehydrogenase (Morimoto *et al.*, 1994).

The results for the hydrogen peroxide challenge suggest that all wine components except tartaric acid significantly reduced the level of induced DNA damage, however, this decrease was at the expense of increased necrosis, with no effect on apoptosis.

While ethanol, glycerol and catechin and caffeic acid appeared equally effective in reducing DNA damage induced by hydrogen peroxide, the mixture of all wine components was not more effective than the individual contribution of ethanol, glycerol or catechin and caffeic acid. This suggests that the effect of the components in the mixture is not additive. These results seem to indicate that the protective effects of ethanol, glycerol and catechin and caffeic acid may be operating through a similar mechanism and that their protective effects are already at their maximum at the concentrations examined. The latter is also supported by the observation that there is virtually no difference between the extent of protection at the 2.5% dilution relative to the 10% dilution.

4.5.1.2 γ-Radiation challenge

The results for effects on γ-radiation-induced DNA damage show that all the components of wine examined, including tartaric acid, significantly reduced MNed cell frequency when present at the 10% dilution, while all but tartaric acid significantly protected against radiation-induced DNA damage at the 2.5% dilution, although to a lesser extent than that observed at the 10% dilution. Unlike the hydrogen peroxide challenge experiments, it is evident that the protective effect is not saturated at the 2.5% dilution. However, like the hydrogen peroxide challenge, the protective effect of the mixture was not greater than that of catechin and caffeic acid, and clearly did not exhibit an additive effect. An additive protective effect should have resulted in a reduction in MNed cell frequency of 64%, however, the observed reduction for the mixture was only 32%, which is similar to the effect of catechin and caffeic acid (30% reduction) alone.

Unlike the hydrogen peroxide challenge, catechin and caffeic acid were more effective than ethanol in reducing DNA damage induced by ionizing radiation. Therefore, it is apparent that wine and its components also protect against the genotoxic effect of ionizing radiation, however, it is still uncertain whether this protective effect is saturated at the 10% dilution. The lack of an additive effect suggests that the various wine components examined may be exerting their protective effect via a similar mechanism such as quenching of singlet oxygen, as has been reported recently for caffeic acid (Foley *et al.*, 1999), or hydroxyl radical scavenging, as has been reported for alcohols (Worm *et al.*, 1993). The observation that phenolic-stripped white wine was less protective than the mix also suggests that wine-derived phenolic compounds play an important role in the observed protective effects of the mixture. Our results support previous observations that ethanol and glycerol are protective against the cytotoxic effects of ionizing radiation (Worm *et al.*, 1993) and that plant-derived phenolic compounds prevent chromosome damage induction by ionizing radiation (Shimoi *et al.*, 1994; Parshad *et al.*, 1998; Virinda and Devi, 2001).

4.5.1.3 Conclusions

Taken together, the results of our *in vitro* plasma spiking studies support the hypothesis that the major components of white wine are protective against oxidative stress generated by hydrogen peroxide and ionizing radiation. Notable is the observation that ethanol was the only wine component increasing baseline MNed cell frequency and that this effect was neutralized in the mixture of all components. The results suggest that : (i) ethanol on its own is genotoxic compared with phenolic compounds; (ii) that under conditions of hydrogen peroxide stress ethanol and phenolic compounds appear equally protective; (iii) under conditions of ionizing radiation stress phenolic compounds appear to be more protective than ethanol and glycerol. Our results also suggest the hypotheses that dealcoholised wines may be equally protective as complete wine but wines stripped of phenolic compounds are likely to be less protective than complete wine and dealcoholised wines. These hypotheses need to be tested in properly controlled *in vivo* intervention studies.

4.5.2 *Ex-vivo* intervention

Controlled *in vivo* intervention studies were designed to determine whether ethanol, phenolic-stripped wine and whole wine differ significantly in their capacity to alter the antioxidant properties of blood *ex vivo* with respect to protection against hydrogen peroxide- and ionizing radiation-induced DNA damage.

4.5.2.1 Effect on baseline genome damage

There was no significant impact of moderate acute consumption of ethanol, red wine or dealcoholised red wine on the rate of baseline genome damage *ex vivo*. This is consistent with the results of the *in vitro* studies for red wine and dealcoholised red wine. In the *in vitro* studies, however, ethanol induced a significant increase in genome damage. The lack of a genotoxic effect of ethanol *ex vivo* can be explained by a lower concentration of ethanol *in vivo* due to detoxification by alcohol dehydrogenase and aldehyde dehydrogenase (Morimoto *et al.*, 1994; Ishikawa *et al.*, 2003).

The cross-correlation data indicated a significant negative correlation between total plasma catechin concentration with baseline MNed BNC frequency. This negative correlation is consistent with the potential role of catechin as an agent that can reduce oxidative stress within a cell and, therefore, reduce the risk of DNA strand break induction by reactive

oxygen species which has been shown to result in micronucleus formation (Umegaki and Fenech, 2000; Sugisawa *et al.*, 2004).

4.5.2.2 Effect on radiation-induced genome damage

Moderate consumption of dealcoholised red wine produced a significant reduction in radiation-induced chromosome damage and, relative to ethanol, both red wine and dealcoholised red wine provided protection against radiation-induced genome damage. The results do not support the hypothesis that ethanol may protect against reactive oxygen species (ROS)-induced genome damage.

The *in vitro* studies indicated that catechin, caffeic acid, glycerol and even ethanol (either on their own or in combination) could protect against radiation-induced micronuclei in human lymphocytes. The *in vitro* and *ex vivo* effects of ethanol are inconsistent because ethanol consumption was not protective *ex vivo*. The effects of ethanol on genome damage cannot be correctly predicted from *in vitro* studies because ethanol is metabolized rapidly *in vivo* to acetaldehyde and acetate, both of which are compounds that are not expected to protect against radiation-induced genome damage.

While the *in vitro* studies suggested that catechin and caffeic acid were the compounds with the strongest radiation-protective effect amongst the major components common to red and white wine, the results of the *ex vivo* study only showed a weak negative correlation between change in plasma catechin concentration and change in radiation-induced micronucleated cells ($R = -0.226$). Although the weak correlation could be explained by the non-specific nature of the assay used to measure plasma catechin it is probable that the protective effect of red wine and dealcoholised red wine cannot be explained solely by changes in total catechin concentration in the blood. Other compounds in red wine, such as anthocyanins, caffeic acid, quercetin, resveratrol and glycerol may have provided some additional quenching of ROS (Waterhouse 2002; Bianchini and Vainio, 2003; Dugo *et al.*, 2003). Clearly, it is necessary, in future studies, to include measurements of these and other components in plasma, and to take account of metabolites that may become inactive as antioxidants. For example, the studies of Caccetta *et al.* (2000) suggested that plasma concentration of caffeic acid and 4-*O*-methylgallic acid (the *O*-methylated metabolite of gallic acid) is maximized between 1 and 2 hours after red wine or dealcoholised red wine consumption but the same beverages also increase the concentration of plasma uric acid within this period. It is, therefore, possible that any antioxidant benefits of red wine and dealcoholised red wine consumption may be the result of multiple changes in plasma biochemistry. In addition, there is recent evidence that the wine-derived phenolic compound resveratrol, lowers the Michaelis constant of SIRT1 deacetylase for both the acetylated substrate and NAD (Howitz *et al.* 2003). This results in increased sirtuin deacetylase activity, and increased histone deacetylation which makes chromatin more compact and DNA less susceptible to DNA breaks induced by reactive oxygen species.

4.5.2 3. Effect on nuclear division index

Cross-correlation analysis indicated that baseline micronucleus frequency is positively correlated with NDI and could be explained by more micronuclei being expressed when the cell cycle checkpoint is more permissive. Plasma catechin concentration was negatively correlated with NDI especially in irradiated lymphocytes, which suggests that either catechin may inhibit the mitogenic response or that it may slow down the nuclear division cycle making the cells less permissive during cell cycle checkpoint processing. An analogous observation was made with ascorbic acid and dehydroascorbate that have been

shown to alter the redox state of Chinese hamster ovary cells and cause a transient arrest at the G2/M checkpoint possibly by delaying activation of cyclinB-cdc2 (Bijur *et al.*, 1999), however, there is as yet no direct evidence for catechin inducing these mechanisms.

4.5.2.4 Conclusions

In conclusion, the results of the *ex vivo* study provides preliminary evidence that the moderate consumption of de-alcoholised red wine protects lymphocytes against radiation-induced genome damage. This study has also determined that ethanol does not contribute to this protective effect and that the contribution of catechin is probably relatively small. More research is needed to explain all the contributing factors in red wine that protect against radiation-induced genome damage *in vivo*.

6. Phase II—The potential of regular moderate wine consumption to reduce markers of risk for cardiovascular disease

6.1 Experimental design and protocol

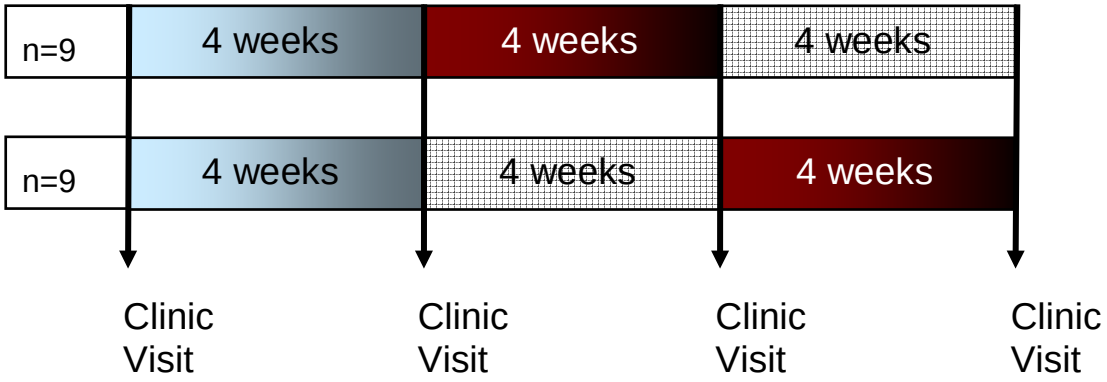
This study consisted of three different dietary "crossover" interventions, each of 12 weeks duration (Figure 8) as follows:

(a) Red wine was compared to white wine to determine whether the higher concentration of phenolic and non-phenolic components in red wine made a significant difference to the protective effect with regard to oxidative damage to lipids;

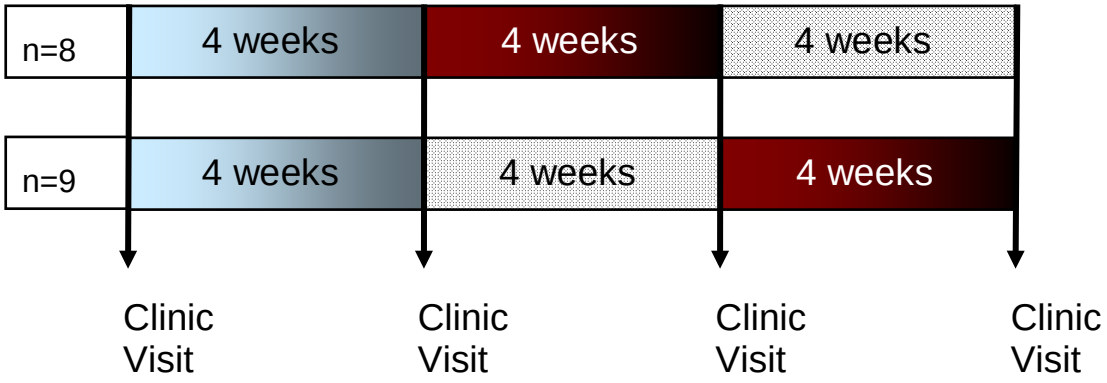
(b) De-alcoholised red wine was compared to red wine to determine the contribution of the alcoholic fraction to the observed effect; and

(c) An alcohol solution was compared to red wine to determine the relative contribution of the non-alcoholic phenolic fraction to protection and protective effects observed for red wine.

(a) Intervention: red wine vs white wine



(b) Intervention: red wine vs de-alcoholised red wine



(c) Intervention: red wine vs model wine (12% ethanol solution)

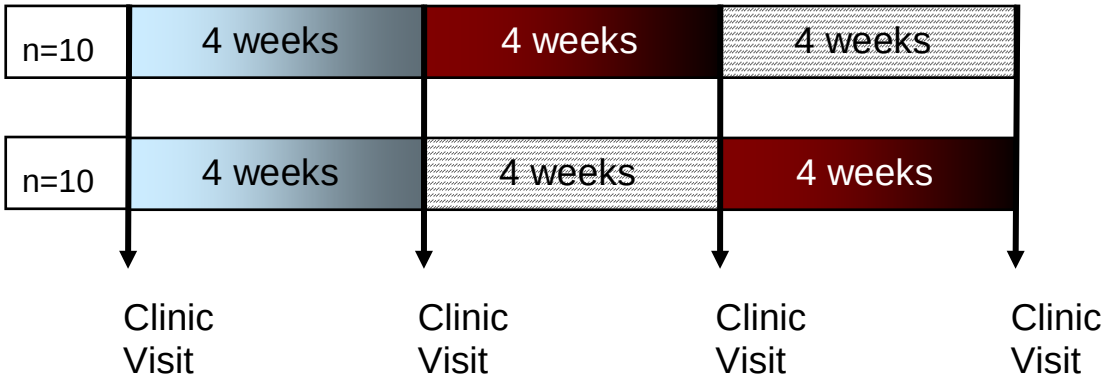
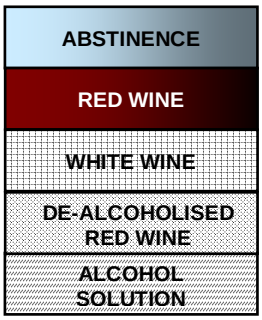


Figure 8 Phase II intervention design
Legend:



6.2 Volunteers

Healthy male volunteers from 40–60 years of age were required for the study. Volunteers were recruited by newspaper advertisement, or television news slot. Volunteers were screened using a confidential general health and alcohol consumption questionnaire, provided on initial contact. Those who: a) habitually supplemented their diet with anti-oxidant vitamins or multi-vitamins; and/or b) smoked cigarettes and/or c) had general health problems were excluded from the study. Binge drinkers and those who drank above the moderate drinking guidelines (2 to 3 units/standard drinks of alcohol per day) as determined from the questionnaire were also excluded from the study. The questionnaire also asked for background information about volunteers. If a volunteer was not accepted into the study, the personal questionnaire was destroyed. The age range and body mass index of the final cohort that completed the study (N=55 volunteers) are detailed below (Table 4) Four subjects withdrew due to work or family commitments, and one volunteer withdrew due to major illness (unconnected to the study). No significant differences were observed in age, height, mass and BMI between groups of the intervention ($P>0.05$).

	Intervention	N	Mean	±SEM
Mass (Kg)	a) RW vs WW	18	82.1	2.59
	b) RW vs DEALC	17	86.7	2.48
	c) RW vs ALC	20	82.8	2.54
	Total	55	83.8	1.47
Height (m)	a) RW vs WW	18	1.78	1.42
	b) RW vs DEALC	17	1.78	1.34
	c) RW vs ALC	20	1.78	1.21
	Total	55	1.78	0.75
BMI (Kgm^{-2})	a) RW vs WW	18	25.9	.816
	b) RW vs DEALC	17	27.5	.782
	c) RW vs ALC	20	25.9	.603
	Total	55	26.4	.425
Age (Years)	a) RW vs WW	18	50.6	1.16
	b) RW vs DEALC	17	51.4	1.42
	c) RW vs ALC	20	50.8	1.16
	Total	55	50.9	0.71

Table 4 Phase II cohort descriptive parameters

6.3 Low phenolic compound diet

To better assess the effects of polyphenols found in wine, for the period of the study volunteers were placed on to a self-selected low plant phenolic compound diet. Dietary compliance to this model was assessed by dietary checklist; volunteers were made aware of the high polyphenol foods to avoid or limit in their normal diet (as determined by resident Dietician (see Appendix 10.5), and any accidental deviation from the expected diet was recorded on the sheet provided for each block of the intervention.

On the first visit to the Clinic at CSIRO Health Sciences and Nutrition in Week 1, a blood sample was taken after fasting overnight. Volunteers were then asked to abstain from alcoholic drinks for a four-week period. After this time, blood was again collected after

fasting overnight, prior to the second phase of the study commencing. A beverage was provided for daily consumption (200mL) in weeks 5-8. A third blood sample was taken on a subsequent visit to the Clinic, prior to consuming beverage (200mL) daily for 4 weeks. A final blood sample was taken at the end of week 12.

6.4 Materials

The white wine was sourced from Tucker Seabrook (WA) Pty Ltd and the red wine was sourced from the Orlando Wyndham Group. The transportation and bottling was undertaken by Vinpac International and the de-alcoholisation of the red wine was undertaken by Tarac Technologies Pty Ltd. The model wine (alcohol solution) was prepared at CSIRO Health Sciences and Nutrition. Analyses of the wines used in Phase II are detailed in Table 5.

Table 5 Component analysis of wines used

Test	REF	UNITS	RW	DE-ALC	WHITE
Total anthocyanins - Red wine	GM05	mg/L	121	102	-
Total Phenolics - Red wine	GM05	a.u.	35	31	-
Total polyphenols		mg/L	1200	1100	345
Red wine colour – density	GM05	a.u.	5.2	5.1	-
Red wine colour – hue	GM05		0.87	0.84	-
Chemical age index #1	GM05		0.52	0.47	-
Chemical age index #2	GM05		0.2	0.22	-
Degree of ionisation of anthocyanins	GM05	%	14.88	19.5	-
Degree of ionisation of anthocyanins-abolishing effect of sulphur dioxide	GM05	%	28.39	39	-
Free sulphur dioxide, excluding anthocyanin bound sulphur dioxide	GM05	mg/L	3.49	3.84	-
sulphur dioxide free	LM09	mg/L	1	1	2
sulphur dioxide total	LM09	mg/L	16	7	61
ascorbic acid (HPLC)	GM08	mg/L	n.d.	n.d.	n.d.
glucose + fructose	LM07	g/L	0.6	0.3	0.8
succinic acid (HPLC)	GM09	g/L	0.7	0.5	n.d.
malic acid (HPLC)	GM09	g/L	n.d.	n.d.	1.2
lactic acid (HPLC)	GM09	g/L	0.8	0.7	n.d.
acetic acid (HPLC)	GM09	g/L	n.d.	n.d.	n.d.
tartaric acid (HPLC)	GM09	g/L	n.d.	n.d.	n.d.
citric acid	LM08	g/L	0.2	0.2	0.3
alcohol (NIR)	LM13	% v/v	13.3	0.4	13.7

6.5 Methods

At the beginning of the study and after each four-week intervention blood samples were taken from participants after an overnight fast. Because isoprostanes break down during storage antioxidants were added to the samples as follows: blood was collected into tubes containing Na_2EDTA (final concentration 1g/L) and reduced glutathione (final concentration 1mg/mL), plasma was separated by low speed centrifugation at 600 x g for 10 min at 4^oC and butylated hydroxytoluene (BHT) was immediately added to the plasma (final concentration 200µg/mL) and the plasma was stored at -80^oC. For other analyses blood was collected into tubes containing Na_2EDTA (final concentration 1g/L) and plasma was separated by low speed centrifugation at 600 x g for 10 min at 4^oC. An aliquot of fresh plasma was used immediately to isolate apoB-containing lipoproteins by affinity column chromatography for oxidation studies (Vinson and Hontz 1995) the remaining plasma was stored at -80^0C for further analysis.

6.5.1 Plasma catechins

Total plasma catechins were measured using a colorometric assay (Kivits *et al.*, 1997) with slight modifications. Catechins were purified from plasma samples by specific absorption to aluminium oxide (Ajax Chemicals, Australia). Methanol (3mL) was added to 1mL plasma. The precipitate was pelleted by centrifugation at 3000 rpm for 10 min at 4^oC and the supernatant was transferred to a fresh tube containing 100mg alumina and 0.6mL milliQ water. The tube was placed on a rotating disc at 4^oC for 15 min. The tube was then centrifuged for 3 min at 1000g. The supernatant was discarded and the residual alumina was suspended in 3 mL diethyl ether. The mix was transferred to a Pasteur pipette blocked with glass wool. The alumina was washed with a further 3mL diethyl ether. The alumina was dried under a stream of nitrogen. 0.5mL of 4-dimethylamino-cinnamaldehyde (DMACA) reagent (6mM DMACA dissolved in methanol/perchloric acid/milliQ water in the ration 8:1:1, v/v) was passed through the column to elute the catechin. The absorption of the solution was measured at a wavelength of 637nm 6 min post-DMACA reagent addition using a multicell spectrophotometer (Varian Cary 1E UV-Vis Spectrophotometer, Varian Aust Pty Ltd, Melbourne, Australia), The concentration of catechin was calibrated against a standard curve of catechin-spiked control plasma samples. Catechin standards were made by spiking plasma samples with known amounts of catechin-(+) (Sigma Chemical Co., St Louis, USA). A stock solution of 10mM catechin-(+) in absolute methanol was prepared and serially dilutions used to supplement plasma samples to provide final catechin-(+) concentrations in the range 0-5µmol/L.

6.5.2 Plasma lipids

Total plasma cholesterol and triglyceride were measured in duplicate on a Cobas-Bio automated analyser (Roche Diagnostica, Nutley, NJ) by standard enzymatic colorimetric techniques using test kits (F Hoffman –La Roche Diagnostica, Basel, Switzerland). HDL-cholesterol was determined on the Cobas-Bioanalyser after precipitation of apolipoprotein B-containing lipoproteins with polyethylene glycol 6000 (Warnick et al 1985). LDL-cholesterol was calculated using a modified Freidwald equation (DeLong et al 1986).

6.5.3 Plasma antioxidant capacity

Antioxidant capacity of plasma was measured as oxygen radical absorbance capacity (ORAC) (Cao and Prior, 1999) and ferric reducing ability of plasma (FRAP) (Benzie and Strain, 1996).

6.5.3.1 ORAC assay

Phosphate buffered saline (136mM NaCl, 10mM Na_2PO_4, 1.8mM KH_2PO_4, 2.7mM KCl, in milliQ water, pH 7.4) and stock PE (0.17g/L -1mg phycoerythrin, Sigma P1286, dissolved in 5.9ml PBS, stored in 300µl aliquots) were stored at 4^{O}C until use. 10ml tubes containing 868mg of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) were weighed out, and stored at room temperature.

A working solution of PE was made as necessary by diluting the 300µl aliquot of stock with 13.4ml PBS to a final concentration of 3.73mg/L. AAPH solution was also made just prior to use; the pre-weighed solid was washed into a 10ml volumetric flask with cold PBS, and made to final volume. This reagent was stored on ice until use. The same batch of each reagent was used for all experiments to minimise between batch variation. A Trolox standard stock solution was made by dissolving 5mg of Trolox in 200ml PBS. This stock was stored in 1ml aliquots at -80^{0}C, and diluted 1:4 v/v to prepare a working solution (20µM) when necessary.

Samples were measured on a COBAS FARA II fluorescent spectrophotometer operated at 37^{o}C. The reaction mode (Reaction mode 3, P-I-SRI-A) pipetted and transferred sample (20µl), phosphate buffer (5µl) and main reagent (PE) (365µl, 3.73mg/l) in parallel into the main reagent wells. The COBAS mixed and incubated the samples for 1 minute and recorded the initial fluorescence (excitation 540nm - monochromator, emission 565nm – Omega Optical band pass Filter XF3083).

Upon completion of initial fluorescence readings, a start reagent, 5µl of AAPH (320mM) plus 5µl of buffer is pipetted into the appropriate start reagent wells. Fluorescence readings were taken every 2mins for 70min. Phosphate buffer was used as a blank and Trolox (20µM) was used as a standard. A quality control plasma sample taken from a fasted volunteer was measured in each run. Results are expressed as Trolox equivalents

6.5.3.2 FRAP assay

Fresh working reagent was prepared by mixing 300mM acetate buffer (pH 3.6), 10mM TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40mM HCl, and 20mM Fe $Cl_3 \cdot 6H_2O$ in the ratio 10:1:1. Trolox was used as the standard. Samples were measured on a COBAS FARA at an absorbance of 593nm at 37^{o}C. Readings were taken at 15 second intervals for 4 min and the change in absorbance was used to calculate the FRAP value expressed as Trolox equivalents.

6.5.4 Lipoprotein oxidation

6.5.4.1 Isolation of apoB-containing lipoproteins

ApoB-containing lipoproteins (VLDL and LDL) were isolated from fresh plasma (Vinson and Hontz 1995). An Alltech extract-clean tube #210712 (Alltech Associates, Inc, IL) was fitted with a supplied polyethylene fritted disc. 2ml of heparin-agarose gel #H6508 **(**Sigma Chemical Co, MO) was added to the column. The gel was allowed to settle, and then another disc was inserted on top of the gel. The column was then washed with five volumes of 0.7% w/v saline (Buffer A) prior to sample addition. 250µl EDTA preserved plasma was added to the top of the column, and diluted with 250µl of Buffer A. The diluted sample was permitted to adsorb to the matrix for 5 minutes. Two volumes of buffer A was used to elute the HDL fraction from the column, which was discarded. 2.5ml of 2.9% (w/v) NaCl was then used to elute the VLDL/LDL fraction from the column. Total protein

concentration of the isolated LDL/VLDL fraction was determined using the Lowry protein assay (Lowry et al 1951).

6.5.4.2 Oxidation of VLDL/LDL

Copper-mediated oxidation of the apo-B-containing fraction of plasma (VLDL+LDL) was measured according to the method of Vinson and Hontz, (1995). LDL/VLDL (2mL) was added to a cuvette (final incubation concentration of 100µg protein/ml) and 25µl of 0.25mM $CuSO_4$ was added. The cuvettes were placed into a spectrophotometer (Varian Cary 1E UV-Vis Spectrophotometer, Varian Aust Pty Ltd, Melbourne, Australia). A peltier temperature control maintained temperature at 37^{O}C. Absorbance was measured (λ=234nm) at 120-second intervals for 180 minutes. The supplied kinetics software calculated lag time before oxidation commenced and maximum diene concentration.

6.5.6 Plasma F_2alpha isoprostane

10000cpm [^{3}H] $PGF_{2\alpha}$ was added as an internal standard to each 1mL plasma sample in glass culture tubes (12x75mm, Medos Company P/L, Adelaide, Australia). Each sample was hydrolysed by addition of 1mL 15% potassium hydroxide solution, followed by incubation in a shaking waterbath at 40^0C for 60min. 2mL chilled ethanol (containing 0.01% BHT) was added and the samples were vortexed and placed on ice for 5min. The samples were then centrifuged at 2000rpm at 4^0C for 10min. The supernatants were transferred to new culture tubes and the ethanol evaporated under a stream of nitrogen. The residues were acidified by addition of 2mL 1.0 M acetate buffer (pH 4.0). The samples were vortexed, chilled on ice for 10min, then centrifuged at 2000rpm at 4^0C for 10min. Isoprostane levels in the supernatants were determined by radioimmunoassay using a test kit (Cayman Chemical, MI, USA).

6.5.7 Blood pressure and arterial compliance

Blood pressures and arterial compliance were measured using pulse wave telemetry (McVeigh *et al.*, 1991) with a Hypertension Diagnostic CR-2000 Research Cardiovascular Profiling instrument (Hypertension Diagnostics Inc. Eagan, MN). The instrument uses two accessories to assess compliance: an oscillometric cuff to measure blood pressure and a tonometric probe. Volunteers were in a supine position for 10min prior to the commencement of the procedure and throughout the procedure. The tonometric probe was positioned on the skin surface of the right forearm, superficial to the location of strongest radial pulse. The oscillometric cuff was placed on the left upper arm. Details necessary for analysis were entered into the instrument (date of birth, weight, height and sex). Prior to recording measurements the probe was tensioned to achieve relative signal strength of greater than 23%, the minimum requirement for optimal waveform analysis. After a minimum of three passes of the waveform oscilloscope, measurements were begun. The instrument sampled at 200 Hz for 30 seconds. Measurements were taken in duplicate and downloaded to computer for analysis using Tera Term Pro software.

6.5.8 Prostaglandin measurements

6-keto-prostaglandin $F_{1\alpha}$, a stable metabolite of prostacyclin, was measured as a marker of plasma prostaglandin levels.

10000cpm [^{3}H]-6-keto-$PGF_{1\alpha}$ was added as an internal standard to each 500µL sample in glass culture tubes (12x75mm, Medos Company P/L, Adelaide, Australia). 2mL chilled methanol was added to each sample, and the samples were incubated at 4^0C for 5min.

Following this initial extraction, each sample was purified using solid phase extraction (C18 Sep-Pak® Plus, Waters Corporation, MA). Each cartridge was activated by washing with 5mL methanol, followed by 5mL Milli-Q® water. The sample was applied to the column and then washed with 5mL Milli-Q® water followed by 5mL hexane. 6-keto-$PGF_{1\alpha}$ was eluted using 5mL ethyl acetate (containing 1% methanol).

10% of the eluate was removed for scintillation counting of the labelled internal standard to determine recovery efficiency. The remaining sample was evaporated to dryness under nitrogen, and resuspended in 450µL EIA buffer. The concentration of 6-keto-$PGF_{1\alpha}$ in the samples was measured against a standard curve of pure 6-keto-$PGF_{1\alpha}$ using an EIA test kit (Cayman Chemical, MI).

6.5.9 Nitric oxide activity

Nitric oxide activity was measured as the ratio of nitrite to nitrate in plasma. Plasma samples were concentrated using a centrifugal filter device (Ultrafree®-0.5, Millipore, USA). The cartridge was pre-rinsed using 0.5mL Milli-Q® water, and 200µL EDTA preserved plasma was added to the column. The filter was centrifuged at 12,000g for 15min in a microtube centrifuge (Centrifuge 5414, Eppendorf). The concentrated sample was then assayed using a commercially available kit (Nitrate/Nitrite colorimetric Assay Kit Cat# 780001, Cayman Chemical, MI). Briefly, the nitrate in the sample was converted to nitrite using nitrate reductase. Addition of Greiss reagents (1, Sulfanilamide and 2, N-(1-Naphthyl)ethylenediamine) led to production of a deep purple coloured azo product. The absorbance at maximum wavelength of 540nm was measured in each sample against nitrite and nitrate reductase-treated nitrate standard curves in the range 1-35µm.

6.6 Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Data was analysed using linear regression or a general linear model of repeated measures (repeated measures ANOVA) with least significant difference (LSD) or Bonferonni tests of significance used where appropriate. A value of $p=0.05$ was the criterion of significance.

6.7 Results

6.7.1 Total plasma catechins concentration

Total plasma catechins are derived from consumption of catechins in the background low-catechin diet and the wines and controls used in the interventions. There were no significant differences in total plasma catechin concentrations after any of the interventions (Figure 9).

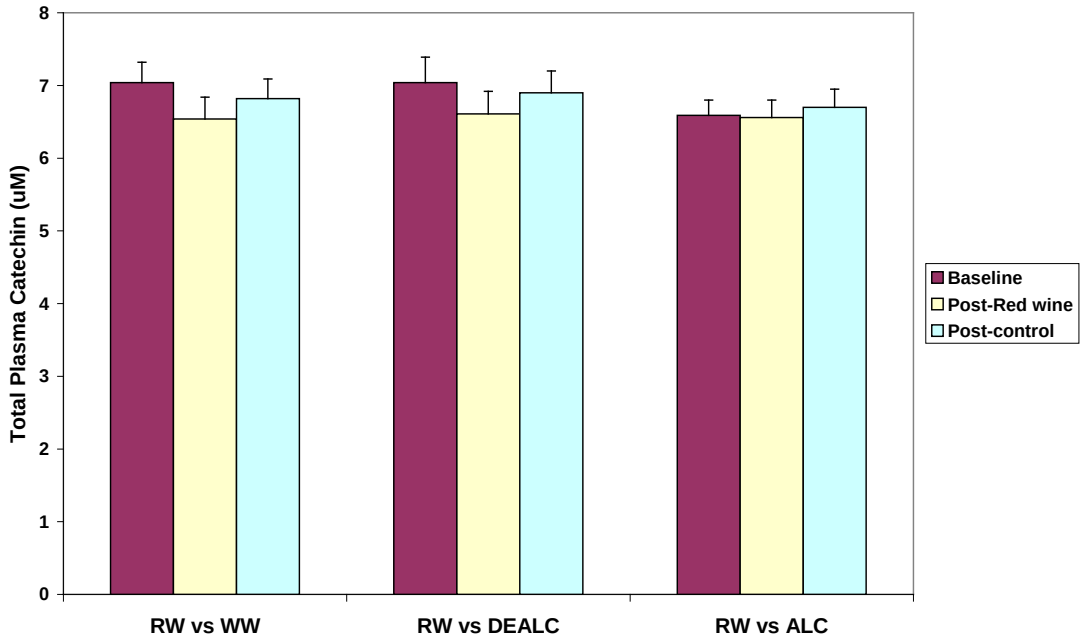


Figure 9 – Total Plasma catechin concentrations at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention. No significant differences.

6.7.2 Plasma lipid concentration

In the three experiments comparing red wine with control drinks (white wine, de-alcoholised red wine or alcohol), there were no significant differences in plasma LDL-cholesterol or plasma triglyceride concentrations between interventions (Figures 10 and 11).

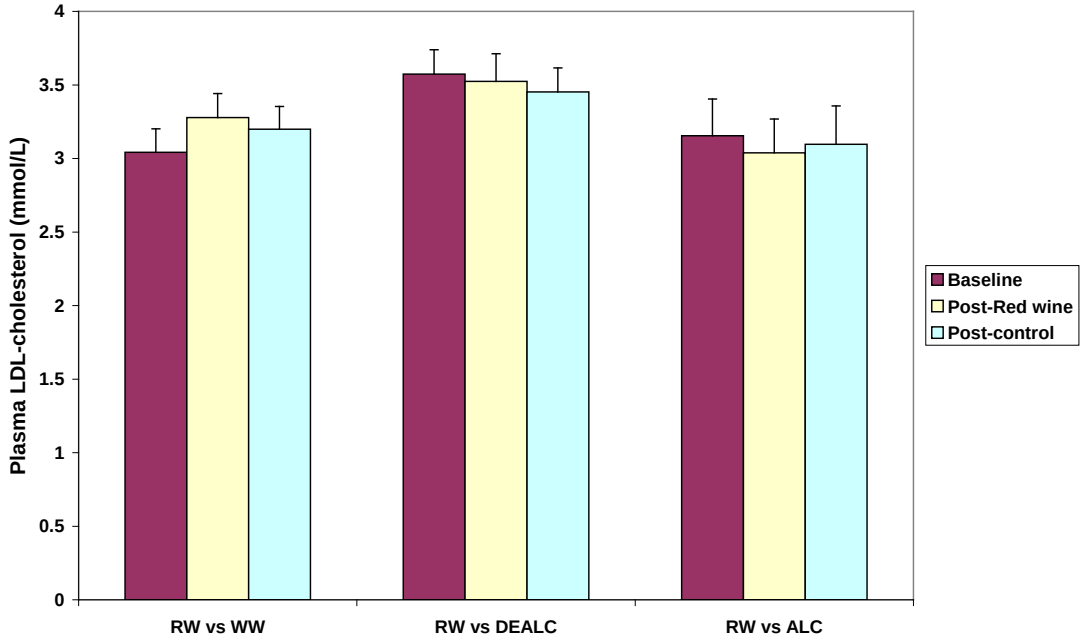


Figure 10 Plasma LDL-cholesterol concentrations at baseline and after 4-weeks intervention with red wine (RW) or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention. No significant differences were observed.

Plasma HDL-cholesterol was significantly higher than at baseline after intervention with red wine, white wine and alcohol ($p<0.02$) in the white wine and alcohol groups. The increase in HDL-cholesterol after red wine in the de-alcoholised red wine group did not reach significance (Figure 12). As a consequence of these changes in HDL-cholesterol the LDL:HDL ratio was significantly lower ($p<0.01$) in the post red wine group, compared to the alcohol group, after red wine consumption (Figure 13).

Since the subjects in all groups undertook the intervention with red wine these results were combined (n=55) and also indicated that red wine increased HDL-cholesterol significantly compared to baseline (1.02 ± 0.03 and 1.10 ± 0.03 mmol/L respectively, $p<0.001$), and the LDL:HDL ratio was decreased (3.25 ± 0.13 and 3.04 ± 0.11 respectively, $p<0.01$). Plasma LDL-cholesterol and triglyceride were not significantly different when looking at this combined group.

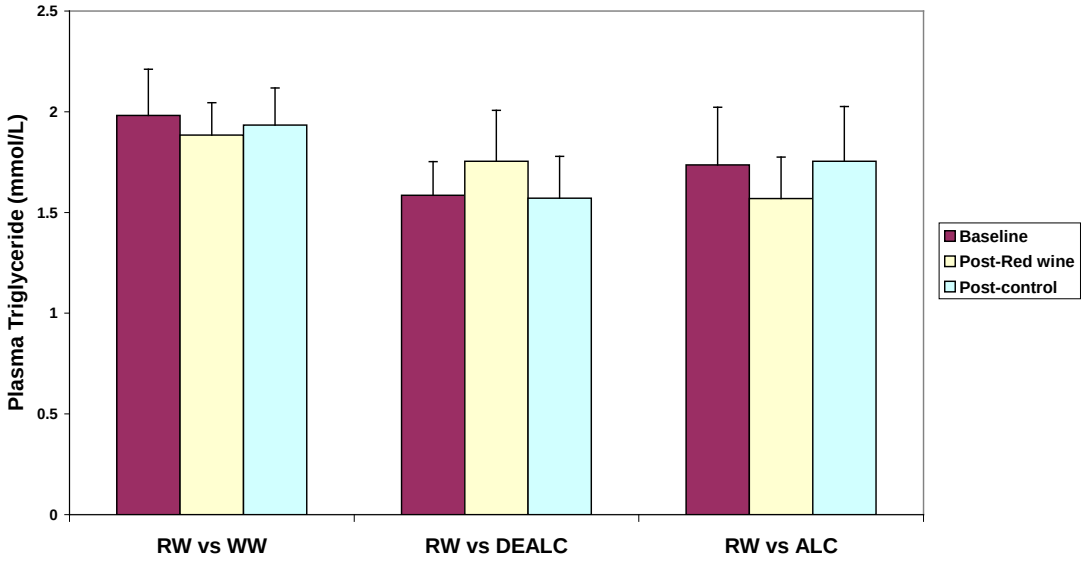


Figure 11- Plasma triglyceride concentrations at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention. No significant differences were observed.

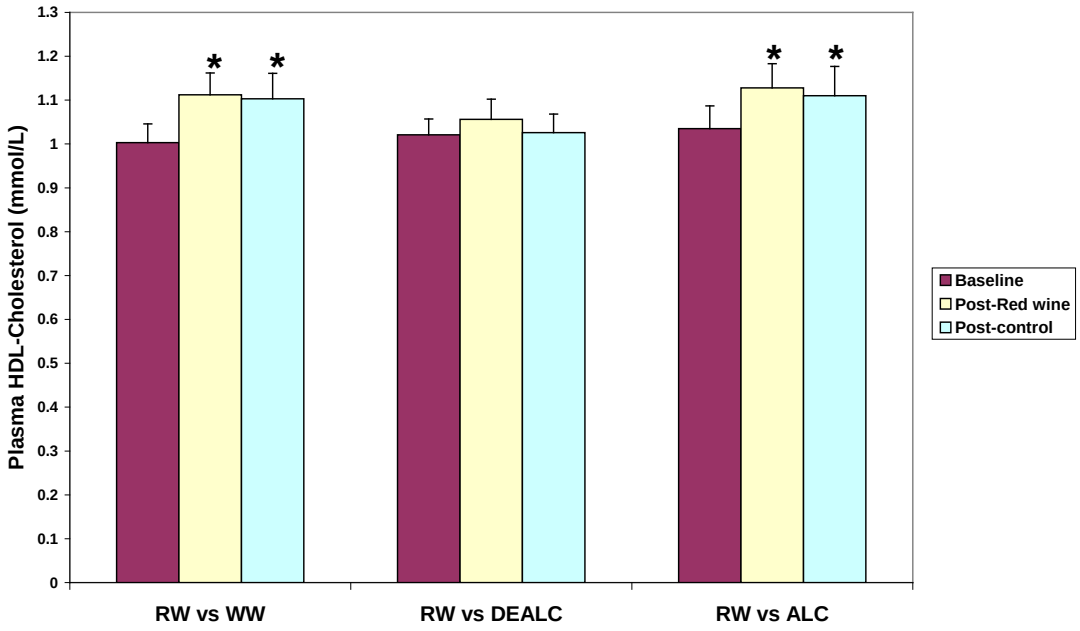


Figure 12 Plasma HDL-cholesterol concentrations at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention.
*** significantly different from baseline, p<0.02**

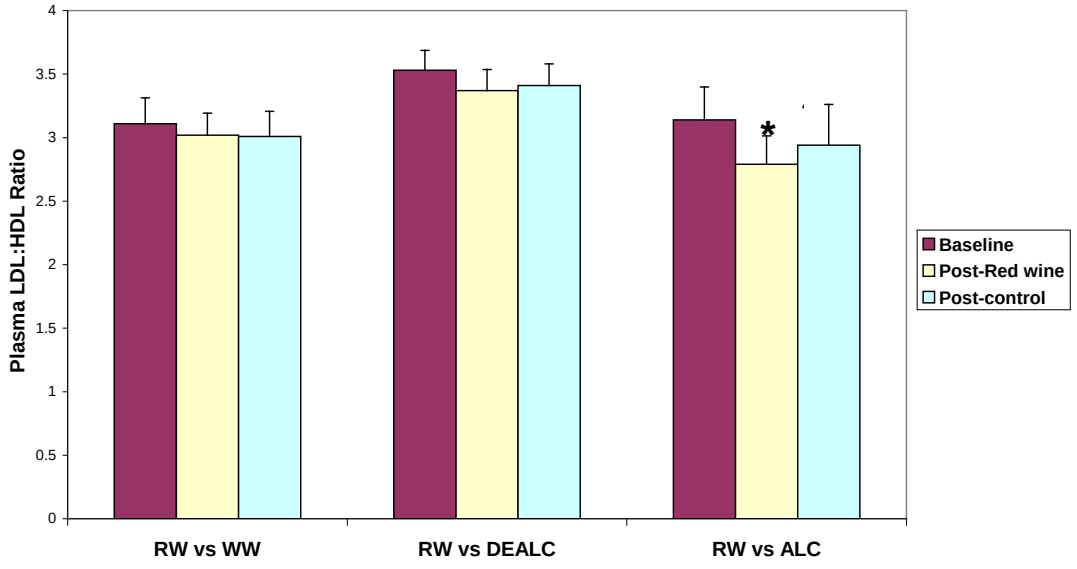


Figure 13. Plasma LDL:HDL ratio at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention.
*** significantly different from baseline, p<0.02**

6.7.3 Plasma antioxidant capacity

Antioxidant capacity is the ability of a substance or complex mixture to prevent oxidative damage. Two testing procedures were used to assess the ability of wine and alcohol to protect plasma lipids from oxidation, the ORAC (Oxygen Radical Absorbance Capacity) and FRAP (Ferric Reducing Ability of Plasma) assays. The ORAC assay assesses the antioxidant capacity of a sample to protect against free radical-mediated breakdown of the fluorescent substrate, phycoerythrin, whereas the FRAP assay measures the ability of a sample to act as a reductant, rather than an antioxidant. No change was seen in either index after wine or control drink consumption (Figures 14 and 15). Values for ORAC and FRAP between visits had high correlations as shown (Tables 6 and 7).

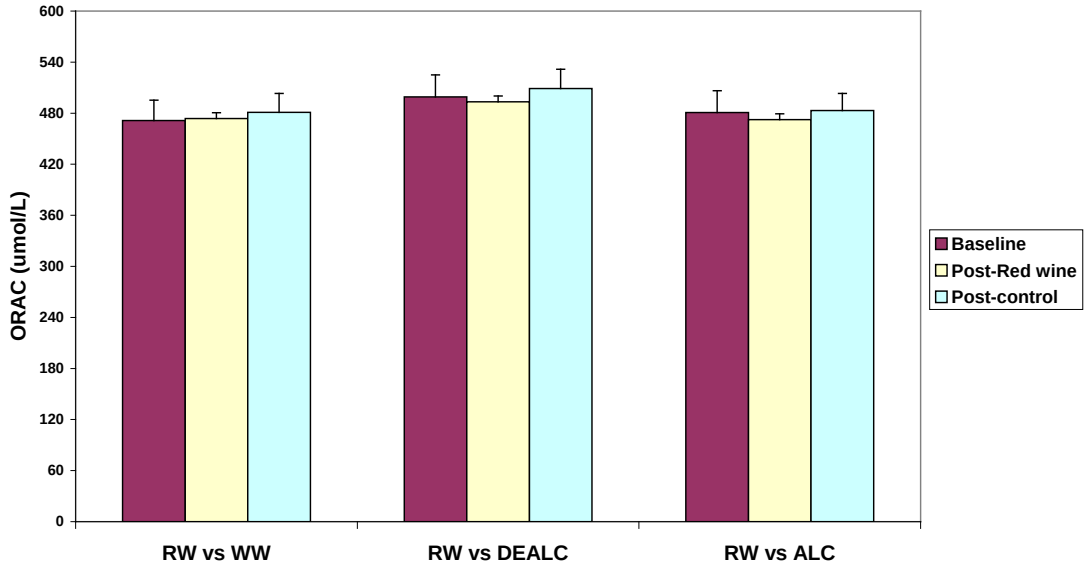


Figure 14. Oxygen Radical Absorbance Capacity (ORAC) at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention. No significant differences.

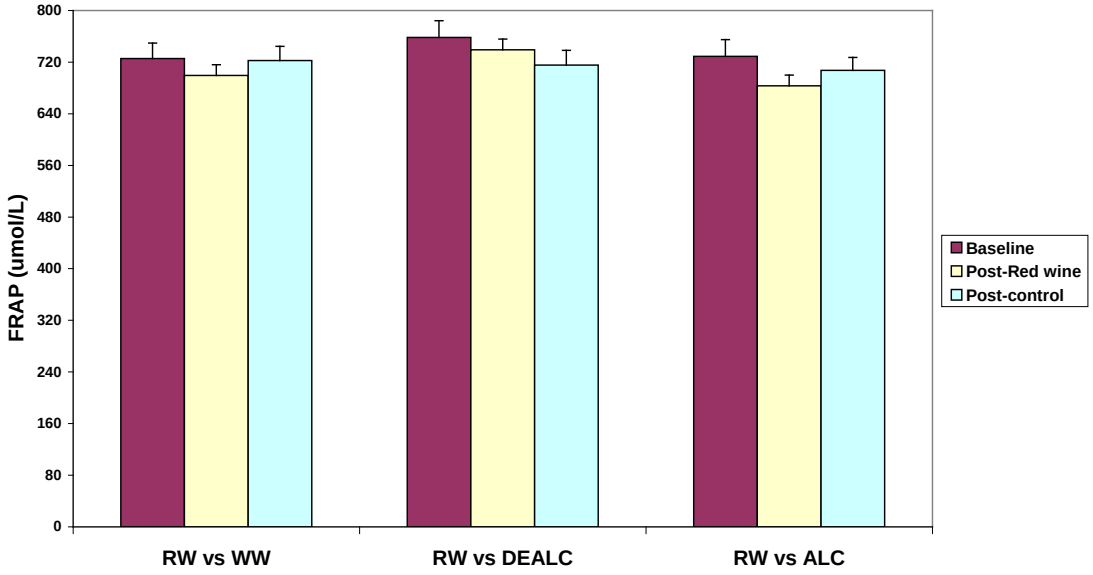


Figure 15. Ferric Reducing Ability of Plasma (FRAP) at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention. No significant differences

		Screening	*Baseline*	*Post-RW*	*Post-control*
Screening	Pearson Corr.		.761	.698	.790
	Sig		.000	.000	.000
Baseline	Pearson Corr.	.761		.799	.793
	Sig	.000		.000	.000
Post-RW	Pearson Corr.	.698	.799		.726
	Sig	.000	.000		.000
Post-control	Pearson Corr.	.790	.793	.726	
	Sig	.000	.000	.000	

Table 6 FRAP correlation values; all correlations significant at p<0.01

ORAC Correlations		*Screening*	*Baseline*	*Post-RW*	*Post-control*
Screening	Pearson Corr.		.762[a]	.571[b]	.582[b]
	Sig		.001	.021	.018
Baseline	Pearson Corr.	.762[a]		.652[a]	.690[a]
	Sig	.001		.006	.003
Post-RW	Pearson Corr.	.571[b]	.652[a]		.687[a]
	Sig	.021	.006		.003
Post-control	Pearson Corr.	.582[b]	.690[a]	.687[a]	
	Sig	.018	.003	.003	

Table 7 ORAC values; [a]indicates correlation is significant at p<0.01; [b]indicates significance at p<0.05.

6.7.4 Lipoprotein oxidation

The capacity of apoB –containing lipoproteins (VLDL/LDL), isolated from plasma after dietary intervention, to resist oxidation by a pro-oxidant *in vitro*, is a measure of the level of protective antioxidants present in the LDL particle. The lag time before the oxidation of LDL begins during the incubation with copper is a measure of this protection. There were no significant differences in lag time between samples after any of the interventions (Figure 16).

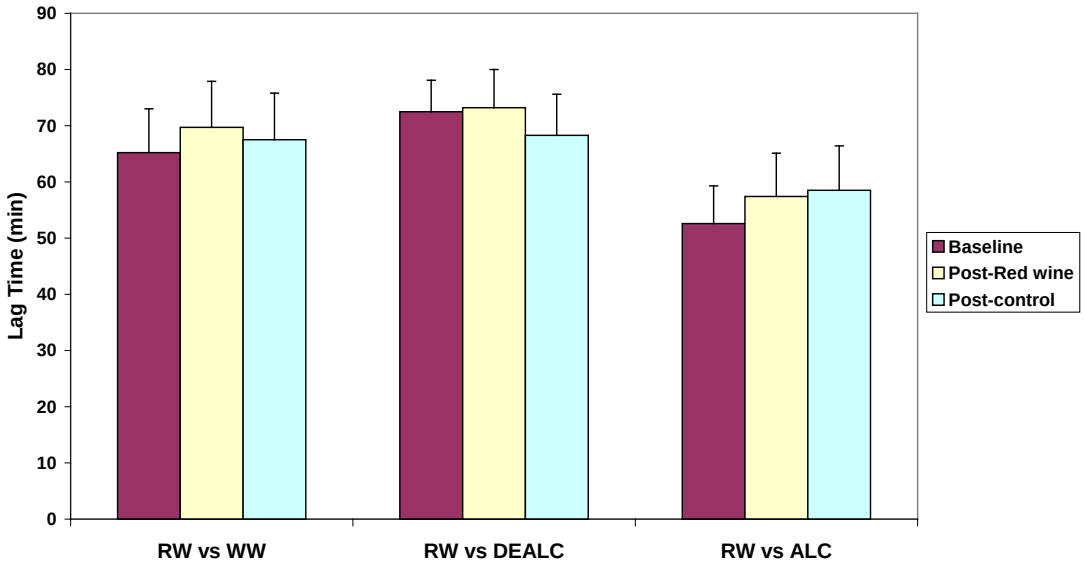


Figure 16 Lag time (min) before the onset of LDL-oxidation, induced by incubation in the presence of copper, at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention. No significant differences were observed.

6.7.5 Plasma F2alpha-isoprostane concentration

F2alpha-isoprostane is the end product of lipid peroxidation which occurs *in vivo* and therefore plasma levels give a direct measure of oxidation. There were no significant differences in plasma F2-isoprostane levels after any of the interventions (Figure 17).

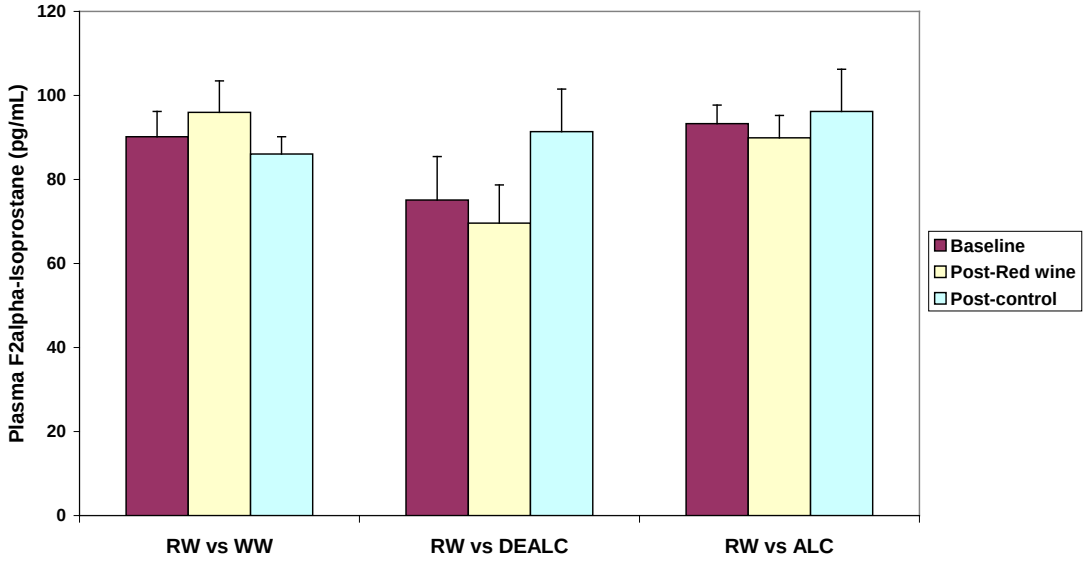


Figure 17 Plasma F2alpha-isoprostane concentrations at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention. No significant differences.

6.7.5.1 Correlation between plasma catechin concentration and F2alph-isoprostane

Total plasma catechin and F2alpha-isoprostane sequentially measured at each clinic visit. Visit 1 = screening (week 0), visit 2 = baseline (week 4), visit 3 = post first intervention, Visit 4 = post second intervention (week 12). When the type of intervention (RW, WW, DEALC, ALC) was ignored, and the concentrations of total plasma catechin and F2alpha-isoprostane were analysed in samples according to the sequence in which the volunteers attended the clinic, an inverse correlation appears to be seen between these two measures (Figure 18).

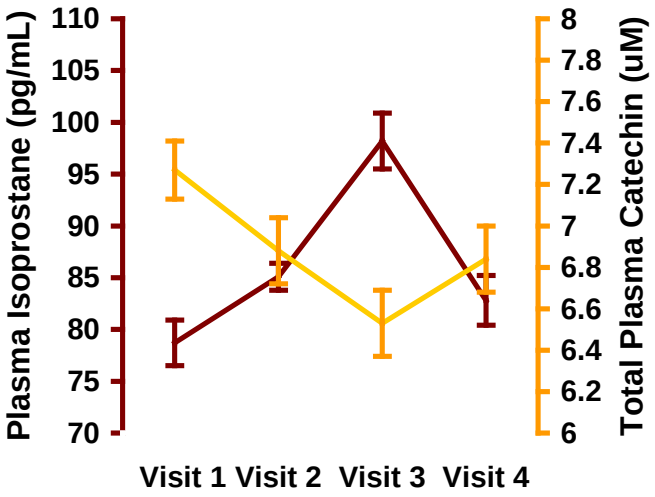


Figure 18 – Total plasma catechin and F2alpha-isoprostane sequentially measured at each clinic visit. Visit 1 = screening (week 0), visit 2 = baseline (week 4), visit 3 = post first intervention (week 8), visit 4 = post second intervention (week 12).

6.7.6 Arterial compliance

In terms of physiological change, arterial compliance has been suggested as a suitable pre-clinical marker for detection of cardiovascular disease states. Measures of arterial function were made using the HDI/PulseWave TM CR-2000 Research CardioVascular Profiling System. Such analysis was performed in parallel to blood sampling. No significant changes were seen in systolic, diastolic, mean arterial or pulse pressures throughout the course of the study when looking at individual intervention groups or when combining data and looking at the total number of subjects (Table 8).

			Pre-intervention			Baseline			Post-Red wine			Post-control wine		
	Intervention	N	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
SYS	RW vs WW	18	131.4	±	2.65	128.7	±	2.04	128.0	±	2.12	127.6	±	1.88
	RW vs DEALC	17	129.2	±	3.92	129.2	±	3.38	127.6	±	2.98	127.3	±	2.49
	RW vs ALC	20	128.8	±	3.72	127.7	±	3.43	130.4	±	3.53	129.9	±	3.35
	Total	55	129.8	±	1.98	128.5	±	1.73	128.7	±	1.70			
DIAS	RW vs WW	18	78.1	±	1.53	79.4	±	1.39	76.6	±	1.51	78.9	±	1.17
	RW vs DEALC	17	77.8	±	2.10	76.4	±	1.89	76.5	±	1.75	74.9	±	1.64
	RW vs ALC	20	76.9	±	2.43	76.3	±	2.61	78.6	±	2.45	78.6	±	2.13
	Total	55	77.6	±	1.19	77.3	±	1.20	77.3	±	1.14			
MAP	RW vs WW	18	98.1	±	2.32	79.1	±	2.02	95.3	±	1.77	96.9	±	1.70
	RW vs DEALC	17	97.7	±	2.76	95.3	±	2.82	94.6	±	2.25	93.3	±	2.40
	RW vs ALC	20	95.2	±	2.61	94.6	±	2.88	98.0	±	3.04	97.6	±	2.62
	Total	55	97.0	±	1.47	95.6	±	1.49	96.0	±	1.42			
PP	RW vs WW	18	53.4	±	2.28	49.3	±	1.47	51.4	±	1.52	48.7	±	1.62
	RW vs DEALC	17	51.3	±	2.19	52.9	±	2.22	51.1	±	1.71	52.4	±	1.33
	RW vs ALC	20	51.9	±	2.43	51.5	±	1.88	51.8	±	1.84	51.3	±	2.22
	Total	55	52.2	±	1.32	51.2	±	1.08	51.4	±	0.97			
PR	RW vs WW	18	59.1	±	1.89	58.0	±	1.87	58.4	±	1.34	58.8	±	1.46
	RW vs DEALC	17	58.7	±	2.46	57.7	±	2.54	55.8	±	1.75	56.1	±	1.91
	RW vs ALC	20	60.1	±	1.82	57.1	±	1.41	59.4	±	1.81	59.1	±	1.46
	Total	55	59.3	±	1.16	57.6	±	1.10	58.0	±	0.96			

Table 8 Blood pressure measures SYS: systolic blood pressure, DIAS: diastolic blood pressure, MAP: mean arterial pressure, PP: pulse pressure, PR: pulse rate.

No significant changes in small or large arterial elasticity, total vascular impedence, or systemic vascular resistance were noted from baseline after red wine, or control drinks, or when group data were combined (Table 9).

			Pre-intervention			Baseline			Post-Red wine			Post-control wine		
	Intervention	N	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
LAE	RW vs WW	18	14.46		0.79	17.38	±	0.79	17.21	±	0.70	17.71	±	0.89
	RW vs DEALC	17	17.51	±	1.34	16.80	±	0.84	16.71	±	0.73	16.67	±	0.64
	RW vs ALC	20	18.45	±	1.03	17.24	±	0.84	17.14	±	1.07	17.40	±	0.88
	Total	55	16.85	±	0.65	17.14	±	0.47	17.03	±	0.497			
SAE	RW vs WW	18	6.96	±	0.83	8.04	±	0.79	8.22	±	0.79	7.94	±	0.85
	RW vs DEALC	17	8.10	±	0.78	7.72	±	0.74	7.53	±	0.70	7.91	±	0.69
	RW vs ALC	20	8.31	±	0.68	8.45	±	0.65	7.72	±	0.70	8.18	±	0.55
	Total	55	7.80	±	0.44	8.09	±	0.41	7.83	±	0.42			
TVI	RW vs WW	18	152.4	±	10.35	127.7	±	7.59	126.5	±	6.42	124.3	±	7.18
	RW vs DEALC	17	132.4	±	8.35	132.5	±	5.82	140.0	±	8.85	136.7	±	5.43
	RW vs ALC	20	119.1	±	5.83	129.8	±	6.39	130.4	±	6.35	125.8	±	4.93
	Total	55	134.1	±	5.04	130.0	±	3.78	132.1	±	4.14			
SVR	RW vs WW	18	1497	±	52.2	1445	±	47.1	1388	±	41.2	1437	±	45.9
	RW vs DEALC	17	1412	±	49.0	1401	±	40.7	1363	±	49.0	1387	±	37.2
	RW vs ALC	20	1390	±	45.0	1396	±	42.9	1433	±	43.5	1444	±	38.6
	Total	55	1432	±	28.3	1413	±	25.0	1397	±	25.5			

Table 9 Arterial compliance measures—LAE: large artery elasticity, SAE: small artery elasticity, TVI: total vascular impedance, SVR: systemic vascular resistance.

6.7.7 Biochemical measures of vascular function

6.7.7.1 Prostaglandins (6-keto-PGF1a measurements)

Prostaglandins did not change significantly after any of the interventions, (Figure 19), supporting the lack of effect of interventions on blood pressure. There were significant correlations for 6-keto PGFalpha concentrations between visits (Table 10).

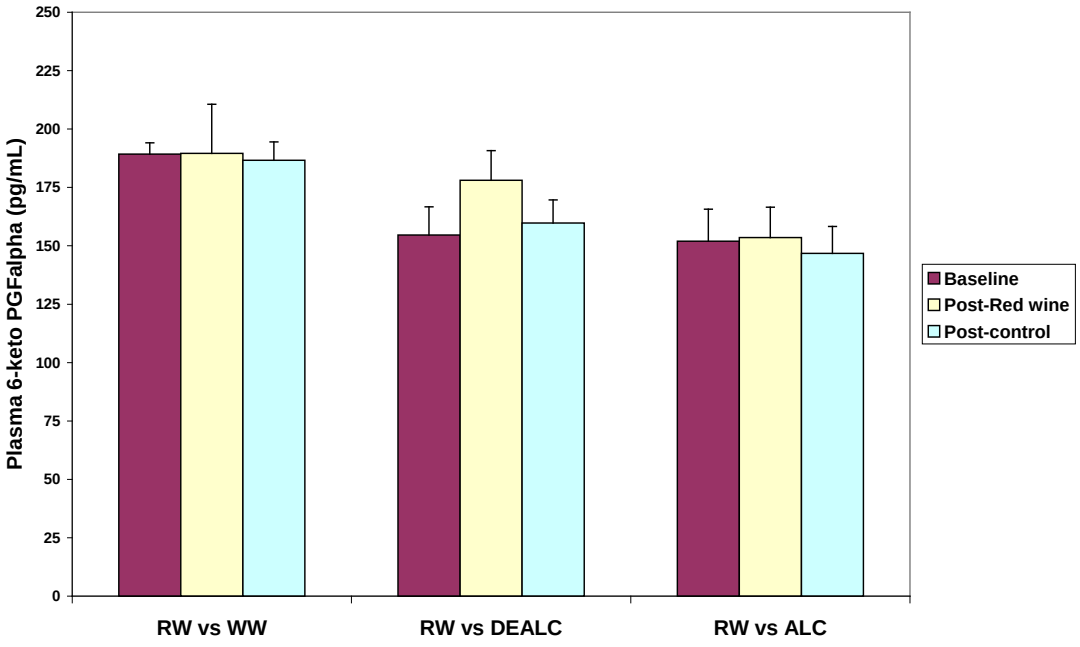


Figure 19 – Plasma 6-keto PGFalpha (pg/mL) at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention. No significant differences

PROSTAGLANDINS		*Screening*	*Baseline*	*Post-RW*	*Post-control*
Screening	Pearson Corr.		.299	.670[a]	.237
	Sig		.051	.000	.126
Baseline	Pearson Corr.	.299		.520[a]	.404[a]
	Sig	.051		.000	.007
Post-RW	Pearson Corr.	.670[a]	.520[a]		.381[b]
	Sig	.000	.000		.012
Post-control	Pearson Corr.	.237	.404[a]	.381[b]	
	Sig	.126	.007	.012	

Table 10 Correlation of prostaglandin values between visits; [a]indicates correlation is significant at p<0.01; [b]indicates significance at p<0.05.

6.7.7.2 Nitric oxide activity

Nitric oxide activity is measured in plasma as the ratio between nitrate and nitrite. There were no significant differences in nitric oxide activity between interventions (Figure 20), supporting the lack of effect of the interventions on blood pressure.

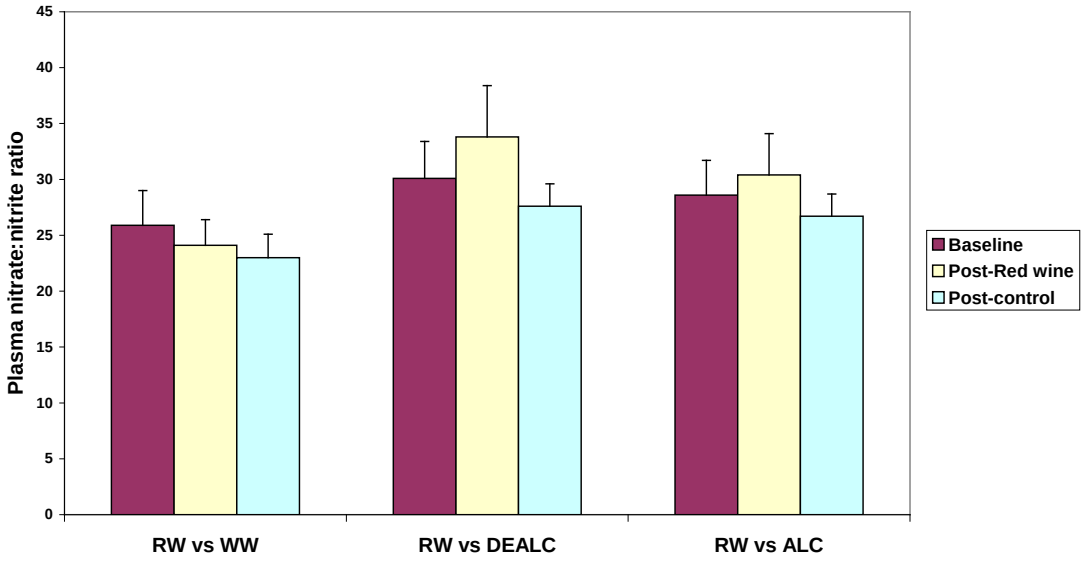


Figure 20 Nitric oxide activity (nitrate:nitrite ratio) at baseline and after 4-weeks intervention with red wine or control. Control wine is white wine (WW), de-alcoholised red wine (DEALC) or alcohol (ALC, 12% alcohol solution) dependent upon intervention. No significant differences were observed.

6.8 Discussion

In the human intervention study we examined markers of cardiovascular risk, including biomarkers of oxidative stress, after consumption of red wine, white wine, dealcoholised red wine and alcohol, against a background diet low in plant phenolic compounds.

Apart from an increase in the plasma concentration of HDL, which could be attributed to the alcohol component of the wine, there were no statistically significant differences in the other lipids, that is, total and LDL cholesterol and triglyceride. The increase in HDL cholesterol was after intervention with red or white wine or alcohol. This increase was not seen after the dealcoholised wine intervention indicating that the alcohol component and not the wine-derived phenolic compounds mediated the effect. The cumulative effect on LDL cholesterol and HDL cholesterol after the intervention with alcohol had a positive impact on the ratio of LDL:HDL, giving a protective profile.

No significant changes were observed in markers of oxidative stress such as LDL oxidation, FRAP, ORAC or nitrate/nitrite ratio following the consumption of red wine or the control beverages.

Because of the lack of effect on measures of oxidation, the assays to examine fatty acid composition and antioxidants in plasma were not undertaken. These assays would have

provided information in relation to possible modifiers of oxidation had the intervention been seen to have an effect.

From analysis of the data for sequential visits to the clinic irrespective of the type of intervention, it appeared that there was a trend towards a reduction in the plasma concentration of catechin as a result of the low plant phenolic compound background diet, that was not able to be countered by consumption of wine-derived phenolic compounds until the end of the study. For example, although no effect of moderate red and white wine consumption was seen on the plasma concentration of total catechin after any of the interventions, the plasma concentration appeared to decrease from baseline with the low plant phenolic compound diet, possibly countering or masking any change in the oxidative biomarkers. This decrease continued until the final third phase of the study when the plasma concentration of catechin began to increase and had an, albeit insignificant, effect on lipid oxidation. The plasma concentration of F2alpha isoprostane, which is an indicator of *in vivo* free radical-catalysed lipid peroxidation (Wang *et al* 1995), appeared however, to be inversely correlated with that of catechin, which implies that phenolic compounds may be protective against lipid oxidation *in vivo*, irrespective of their source. This potential correlation between the concentration of catechin and that of F2alpha isoprostane needs to be further investigated.

As the efficacy of the phenolic compounds is dose-dependent, this suggests that if the subjects had not been on a low plant phenolic compound diet, a beneficial effect of catechin on lipid oxidation and the other biomarkers may have been observed. This also needs to be confirmed by further investigation.

Using non-invasive measurements of vascular reactivity of the brachial artery it has been shown that wine (Leighton et al., 1999) or purple grape juice (Stein et al., 1999) consumption reverses the suppression of endothelial function by a high fat diet. A third study showed no clear effect of wine consumption (Djousee et al., 1999). In an early report of our work, using a tonometric technique for measurement of arterial compliance, a significant increase in small artery elasticity was observed (Greenrod and Abbey 2001). When the full complement of volunteers for this section of the intervention was included, however, the result lost significance. It would, therefore, be of interest to find out what type of red wines or grape juices are more likely to produce improved endothelial function or arterial compliance, which is a biomarker for cardiovascular disease risk.

The absence of changes in nitric oxide activity (measured as nitrate/nitrite ratio) may well be a related event. Nitric oxide production is known to influence flow-related vascular dilatation (Bevan et al., 1995), and since arterial elasticity is related to the degree of vessel relaxation, it could be conceived that no evident change in nitric oxide activity, results in no change in vascular compliance (Cohn, 1999). Related to this is the lack of change in the concentration of plasma prostaglandin the product of cyclooxygenase activity (quantified as 6-keto PGFalpha, the stable end product of prostacyclin) which could account for the lack of effect on blood pressure since prostaglandins are known to modulate blood pressure (Fujino, 2004).

These results do not account, however, for other factors that influence the dynamic characteristics of the peripheral vascular tree, and further work is necessary to determine the mechanism of the relationship between nitric oxide and prostaglandin production and vascular elasticity.

Overall, the results indicate that studies examining the effects of wine consumption on risk factors for cardiovascular disease should take into account the phenolic content of the background diet.

7. Outcomes and conclusions

7.1 Phase I - Potential benefits of wine in prevention of genetic damage induced by hydrogen peroxide and ionising radiation

It was observed that the phenolic compounds, such as catechin and caffeic acid, and the mixture including these components, significantly decreased baseline DNA damage and DNA damage caused by ionising radiation. It was observed that the ethanol component significantly increased base-line DNA damage, but the mixture that included both ethanol and the phenolic compounds, completely countered the DNA damaging effects of ethanol. These effects were observed for both the 2.5% and 10% concentration of the components, although the protective effect of the phenolic compounds was most significant for the 10% concentration. Ethanol, as well as the component mixtures, produced the strongest protective effects against DNA damage by hydrogen peroxide. The protective effect of the mixture did not account for the expected additive protective effects of the individual components, which suggests that the components may be exerting their effects through similar mechanisms, which are saturated at the concentration tested.

***In conclusion**:*

- *these observations suggest that the primary phenolic and ethanol components of wine can reduce the DNA damaging effects of two important oxidants, hydrogen peroxide and ionising radiation, in a physiologically relevant in vitro system.*

- *This has important clinical indications, such as in radiotherapy for the treatment of cancerous cells and the protection of normal tissue.*

The second study was an *ex vivo* study in which blood from six healthy male subjects was tested for its resistance to DNA damage induced by hydrogen peroxide or ionising radiation, following the consumption of 300 mL red wine, dealcoholised red wine or a model wine (12% alcohol solution). The subjects were placed on a plant phenolic compound free diet for 48 hours prior to each study day. The results of this study showed a clear protective effect of the dealcoholised wine, an aggravating or negative effect of alcohol and an intermediate but protective effect of whole wine. The most significant protective effects were observed at two hours post consumption. These results were important in verifying that it is the non-alcoholic fraction of wine that has DNA-protective properties in blood and body tissues.

Questions arising from these studies include:

- Why were results of ionising radiation challenge different to that from hydrogen peroxide for ethanol?

Limitations of these studies include:

- As only two concentrations of supplementary wine components were used in the initial *in vitro* study, further research is necessary to determine more accurately the dose response relationship between the wine components and protection against DNA damage;

- Further research is necessary to more accurately determine the time response relationship between the consumption of wine and protection against DNA damage; and

- The effects of caffeic acid and catechin were not tested individually on DNA damage.

7.2 Phase II - The effect of regular moderate wine consumption to reduce markers of risk for cardiovascular disease

No changes in blood pressure or arterial elasticity from baseline were observed following the consumption of red wine for four weeks compared to that following white wine, dealcoholised red wine or the 12% alcohol solution. In addition, there was no change in nitric oxide activity with any of the subject groups. This implies that physical changes such as arterial elasticity may take longer than the four-week red wine consumption period to occur.

There was, however, an increase in the plasma concentration of HDL from baseline in 74% of subjects following the consumption of red wine for four weeks compared to that following white wine, dealcoholised red wine or the 12% alcohol solution, and a concomitant improvement in the ratio of HDL to LDL. This observation confirms those of published studies, and support The Heart Foundation guidelines, which recommend that the plasma concentration of HDL should be increased to greater than 1.0 mM.

There was not, however, a change in the plasma concentration of LDL or that of triglycerides, or a change in the antioxidant capacity of the plasma. Again, this may reflect the short-term duration of the study and support the observations of van der Gaag et al. (2000) who observed no change in the antioxidant capacity of the plasma after a three-week period of red wine consumption on a controlled diet although small changes in specific plasma antioxidant compounds was observed, as supported by the study of Kerry and Abbey (1997).

Furthermore, there was no increase in the plasma concentration of the phenolic compound, catechin, but instead there appeared to be a continuous decrease over the 12-week study period from baseline, with an apparent concomitant increase in the plasma concentration of F_2-isoprostanes, primary plasma oxidation products. This implies that when on the low plant phenolic compound diet, the regular consumption of red wine in the short-term may be unable to improve vascular function or increase the antioxidant capacity of the plasma, or prevent the oxidation of lipids such as LDL. Therefore, the antioxidative and/or other cardioprotective effects of red wine may best be observed against a ‘normal’ or ‘higher’ background diet of plant phenolic compounds, such as a Mediterranean-style diet, and may be most observed against a background diet high in fat, when oxidative stress has been observed to increase (Leighton et al. 1999, Cuevas et al. 2000, Mezzano et al. 2001, Mansvelt et al. 2002).

In conclusion:

- *A regular and moderate consumption of red wine over a four-week period does not change endothelial and vascular function or prevent lipid oxidation when combined with a diet low in plant phenolic compounds.*

- *These results also add to the growing evidence that the cardioprotective effects of wine and the wine-derived phenolic compounds are not primarily related to plasma antioxidation, but may be primarily related to other effects, such as on haemostasis or inflammation.*

- *The protective effects of wine consumption, may therefore, be best observed against a combined diet of high fat, and high fruit and vegetable consumption.*

Questions arising from this study include:

- Are the same effects on plasma antioxidant capacity and other plasma lipids, and on endothelial function observed when the background diet is not low or is high in plant phenolic compounds or, conversely, is high fat?

- What dose of red wine, regularly consumed, would counteract the 'oxidative' and other negative nutrient effects of a diet is low in plant phenolic compounds;

- What is the minimal duration of a regular red wine consumption for antioxidant or other cardioprotective effects to be observed? and

- Is the amount of red wine consumed (200 mL) sufficient to observe a change in physiological and biochemical markers of cardiovascular disease?

Limitations of this study include:

- Background diet low in plant phenolic compounds; and

- Relatively short-term duration (four-weeks) of each treatment.

7.3 Overall conclusions and outcomes

The main conclusions that can be drawn from this research are as follows:

Concerning the potential cardioprotective effects of wine and wine-derived phenolic compounds:

- Wine-derived phenolic compounds are absorbed into, and are detectable in, human plasma following the consumption of wine, whether wine consumption is on one acute occasion or is regular for a short or longer period of time;

- The evidence in animal and human studies to date, consistently suggests the consumption of dealcoholised red wine can exert antioxidative effects on LDL and plasma in humans on a 'western' diet, when consumed regularly for a short or longer

period of time, and in a non-smoking population group; the results following consumption of red wine with its associated ethanol component have been mixed. This may be because of contrasting or countering anti-oxidative effects of wine-derived phenolic compounds with the established pro-oxidative effects of ethanol metabolism per se. Whether the presence of such antioxidant compounds in red wine offers a relative advantage for red wine compared to other alcoholic beverages in terms of the potential to cause less injury or even confer specific benefits (especially in terms of cardiovascular disease and cancer risk) requires considerably more basic, clinical and epidemiological research;

- Background diet, in particular of phenolic-rich fruit and vegetables, may interact with any antioxidative effects of the wine-derived phenolic compounds; and

- The primary cardioprotective effects of whole red and white wine consumption may be related to effects other than antioxidation.

Concerning the potential cancer-protective effects of wine and the wine-derived phenolic compounds:

- The acute consumption of dealcoholised and whole red wine may protect DNA from induced and spontaneous oxidative damage, but ethanol exacerbates DNA damage; and

- The effect of the whole red wine is observed to be intermediate to that of ethanol and dealcoholised red wine, which implies that the antioxidant wine-derived phenolic compounds may counter the peroxidative effects of the ethanol component in cells;

Specifically, the wine-derived phenolic compounds are bioavailable and exhibit antioxidative effects in plasma, LDL and cells as compared to ethanol, which exhibits peroxidative effects. The antioxidant effects of the wine-derived phenolic compounds, however, were observed to merely counteract the peroxidative effects of the ethanol component, when consumed together. Whether this is important for cardio- and cancer-protection remains to be answered.

8. Recommendations

Recommendations for further studies include:

- Investigation of the effects of wine consumption on other forms of cellular/DNA damage other than radiation or hydrogen peroxide;

- Confirmation of these results of this study in a study, which considers a background 'normal' diet versus a diet low in plant phenolic compounds for a longer treatment period; and

- A similar study but where the subjects have cardiovascular disease, such as increased blood pressure and a high lipid concentration with a skewed LDL:HDL ratio.

9. Appendices

9.1 Communications

9.1.1 Manuscripts

Greenrod, W. Abbey, M. (2001) Moderate wine consumption improves small arterial elasticity. Aust. Grapegrower Winemaker, 453: 52, 55–56.

Greenrod, W. and Fenech, M. (2003). The principal phenolic and alcoholic components of wine protect human lymphocytes against hydrogen peroxide- and ionizing radiation-induced DNA damage *in vitro*. Mutagenesis 18(2):119-26.

Greenrod, W., Stockley, C., Burcham, P., Abbey, M., Fenech, M. (2005) Moderate acute intake of de-alcoholised red wine, but not alcohol, is protective against radiation-induced DNA damage *ex vivo*—results of a comparative *in vivo* intervention study in younger men. Mutation Research 591(1-2):290-301.

9.1.2 Poster presentations

Greenrod, W.H., Stockley, C.S, Abbey, M. Background diet influences markers of risk for cardiovascular disease after moderate wine consumption. Second International Conference on Polyphenols and Health, Davis, CA, USA, 4─7 October 2005.

Greenrod, W.H., Fenech, M.F., Abbey, M., Stockley, C.S. Markers of risk for cancer and cardiovascular disease after moderate wine consumption. Office International de la Vigne et du Vin, Adelaide, South Australia, 11─17 October 2001.

Greenrod, W.H., Stockley, C.S., Abbey, M., Fenech, M.F. Genetic compounds from red wine are protective against the DNA damaging effect of ionising radiation *ex vivo*. 31st Annual meeting of the European Environmental Mutagen Society, Ghent, Belgium, 1─5 September 2001.

Greenrod, W.H., Abbey, A. Red wine but not white wine influences small artery elasticity. 17th International Congress of Nutrition, Vienna, Austria, 27─31 August 2001.

Greenrod, W.H., Fenech, M.F. The effect of moderate wine consumption on markers of genetic damage. 17th International Congress of Nutrition, Vienna, Austria, 27─31 August 2001.

Greenrod, W.H., Stockley, C.S., Abbey, M. Red wine but not white wine influences small artery elasticity. Australasian Atherosclerosis Society Meeting 2000, Adelaide, South Australia, 29 November─2 December 2000.

9.1.4 Media

4.1 Interviews
4.1.1 Print
Adelaidean —*Red wine study seeks reasons for healthy hearts*, Emily Collins (28/08/00)
The Advertiser—*Grape work if you can get it!*, Barry Hailstone (09/09/00)
The Australian—Diane Thorp
CSIRO National Awareness—Jason Major

4.1.2 Radio
5AA—Gerald Stone (16/09/00)
5UV—Radio for the third age segment (07/08/00)

4.1.2 Television
Channel 10 News—Melody Horall

4.2 External presentations
Rotary Clubs—Brownhill Creek (05/09/00), Edwardstown (03/10/00) and Unley (29/08/00)

9.1. 5 Abstracts

BACKGROUND DIET INFLUENCES MARKERS OF RISK FOR CARDIOVASCULAR DISEASE AFTER MODERATE WINE CONSUMPTION

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A Mediterranean-style diet, high in certain fruits, grains and vegetables, is observed to reduce the risk of cardiovascular disease. Traditionally wine is part of a Mediterranean-style diet and some epidemiological studies suggest that wine-derived phenolic compounds may supplement the cardioprotective effects of an already high plant-derived phenolic diet.

A randomized, controlled dietary crossover intervention was undertaken to investigate the effect of moderate red wine consumption coupled with a low plant-derived phenolic diet on biomarkers of cardiovascular risk. Healthy non-smoking males (n=17) aged 41–57 years were placed on a diet low in plant-derived phenolic compounds and followed a set drinking protocol of three 4-week periods: 1) alcohol-free washout; 2) 200 mL red wine per day; and 3) 200 mL white wine per day. At the beginning of the intervention and after each protocol period, a blood sample was collected.

The plasma concentration of catechin, a flavanoid phenolic compound, which was a biomarker for bioavailability, decreased from the beginning of the study, through baseline until post the first intervention when it increased during the second intervention. The concentration of F2alpha-isoprostane, a measure of lipid oxidation, was negatively correlated with plasma catechin throughout the intervention and suggests that the phenolic content of the wine was initially insufficient to counter the effect of the low phenolic background diet.

These results indicate that studies examining the effects of wine on risk factors for cardiovascular disease should take into account the phenolic content of the background diet.

MARKERS OF RISK FOR CANCER AND CARDIOVASCULAR DISEASE AFTER MODERATE WINE CONSUMPTION

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Free radical damage to vital body systems may contribute in the long term to the ailments of middle and old age including cancers, heart disease, diabetes, arthritis and Alzheimer's Disease. Polyphenolic compounds in important plant foods and beverages are emerging as preventive and therapeutic agents in radical-mediated disease. Their investigation is an area of substantial research activity. Whilst the antioxidant potential of these compounds has been established clearly *in vitro*, there is as yet, little evidence for their capacity to prevent disease processes *in vivo*. Epidemiological studies have indicated that moderate wine consumption may be beneficial in prevention of cancer and heart disease, and its protection may rely upon a number of components known to possess potent antioxidant activity. Whilst current measures indicate an increase in the antioxidant capacity of human plasma following wine consumption, and even a reduction in pre-cursor disease events, such as LDL oxidation or DNA damage, little is known of the possible mediators of these effects.

Currently, we are examining a number of biomarkers of oxidative damage relevant to vascular disease and cancer in volunteers (n=57, 40 to 60 years of age) consuming red wine and control drinks for one month. Markers of biochemical and cellular change include plasma lipid profiles, plasma antioxidant capacity (ORAC and FRAP assays), plasma antioxidant concentration, bioavailability of total catechins, copper-mediated LDL oxidation, plasma and urine isoprostane levels, and cytokinesis-blocked micronucleus assay.

In terms of physiological change, arterial compliance has been suggested as a suitable pre-clinical marker for detection of cardiovascular disease states. Measures of arterial function may be made by non-invasive tonometry and computer analysis of the diastolic decay through use of a modified Windkessel-type model (HDI/PulseWave TM CR-2000 Research CardioVascular Profiling System). Such analysis has been performed in parallel to blood sampling. No significant changes have been seen in systolic, diastolic, mean arterial or pulse pressures throughout the course of the protocol ($p > 0.1$). However, small artery elasticity has significantly increased from 7.77 ± 0.10 ml/mmHg x 100 at baseline to 9.12 ± 0.11 ml/mmHg x 100 after consuming red wine ($p < 0.02$). Further work is required to elucidate this action.

THE EFFECT OF MODERATE WINE CONSUMPTION ON MARKERS OF GENETIC DAMAGE

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A prospective study examining alcohol and mortality identified a reduced relative risk of 0.22 ($P<0.05$) for all cancers in people who drank less than 76 g of alcohol daily (the majority in the form of wine, 82%), when compared to non-drinkers (Renaud et al, 1992). Following the work of Fenech et al, 1997, we have tested the hypothesis that moderate consumption of red wine (RW) or RW components can protect DNA against the damaging effects of hydrogen peroxide or ionising radiation. Baseline and induced genetic damage was assessed by cytokinesis-blocked micronucleus assay. Plasma supplementation with wine components at expected *in-vivo* concentrations indicated a mix of phenolic compounds significantly reduced induction of micronuclei with hydrogen peroxide or gamma radiation insult from control (28.25 ± 2.25 to 22.25 ± 0.25, $P<0.05$ and 434.3 ± 1.74 to 303 ± 15.2, $P<0.001$, expressed as micronucleated (MNed) cells per 1000 binucleated cells, respectively), Since little is known of the bioavailability of RW components, a crossover intervention was used to assess the *in-vivo* effects of acute moderate consumption. Healthy non-smoking males (n=6) 23.3 ± 0.2 years of age adhered to a plant polyphenol free diet for 48 hours. Volunteers then drank 300 mL of RW, de-alcoholised RW (DE-ALC), or ethanol solution (ALC). Blood samples were taken prior to clearance and drinking and 30, 60 and 120 min after consumption. Radiation induced MNed cell frequency increased in a time dependent manner post-ALC consumption, but contrastingly decreased post-DE-ALC consumption ($P<O.OOI$). A difference of differences analysis of MNed cell frequency between treatments indicated that RW and DE-ALC were significantly different ($P<O.05$) from ALC at all times post-consumption. Moderate consumption of RW or specific RW components, therefore, leads to a reduction in induced micronuclei formation, a biomarker of genetic damage.

REDWINE, BUT NOT WHITE INFLUENCES SMALL ARTERY ELASTICITY

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In France, the levels of classical risk factors (tobacco smoking, diets high in fat leading to obesity, and a sedentary lifestyle) are particularly high in contrast to the low level of cardiovascular disease seen. “"The French Paradox" is a term used to describe this effect. Epidemiological studies have linked moderate alcohol consumption and particularly the red wine consumption in France with the low incidence of disease in populations consuming a Mediterranean diet.

To investigate the effect of moderate red wine consumption on biomarkers of cardiovascular disease risk, a randomised controlled dietary crossover intervention was undertaken. Healthy non-smoking males (n = 17) from 41-57 years of age (49.37 ± 0.12 years) were placed on a low polyphenol diet and followed a set drinking protocol of three 4-week periods, 1: alcohol free washout, 2: red wine 200 mL per day, 3: white wine 200 mL per day.

At the beginning of the study, and after each period of the protocol, a blood sample was taken. There was a highly significant increase in high density lipoprotein (HDL) concentration from baseline (1.00 ± 0.02 mmol/L) after consumption red (1.10 ± 0.03 mmol/L) and white (1.09 ± 0.03 mmol/L) wine ($p < 0.001$ and $p = 0.002$, respectively), with no significant increase in low density lipoprotein (LDL) concentration ($p > 0.1$). This accounts for the significant increase in total plasma cholesterol from baseline (4.94 ± 0.05 mmol/L) on consumption of red wine (5.20 ± 0.05 mmol/L, $p = 0.045$) and a similar, but insignificant trend for white wine (5.13 ± 0.05 mmol/L, $p > 0.05$). Total plasma triglycerides did not change significantly over the course of the study ($p > 0.05$).

Arterial compliance has been suggested as a suitable pre-clinical marker for detection of cardiovascular disease states. Measures of arterial function may be made by non-invasive ultrasonographic Doppler tonometry and computer analysis of diastolic decay through use of a modified Windkessel-type model. Such analysis was performed in parallel to blood sampling. No significant changes were seen in systolic, diastolic, mean arterial or pulse pressures throughout the course of the protocol ($p > 0.1$). However, small artery elasticity was seen to significantly increase from 7.77 ± 0.10 mL/mmHg x 100 at baseline to 9.12 ±: 0.11 mL/mmHg x 100 after consuming red wine. A significant difference was seen when comparing the effect mediated by either red or white wine ($p < 0.02$). Further work is required to elucidate this action.

In summary, we have shown that red and white wine consumption can beneficially effect the LDL:HDL ratio, and that red wine may improve small vessel compliance.

RED WINE, BUT NOT WHITE WINE INFLUENCES SMALL ARTERY ELASTICITY

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In France, the levels of classical risk factors (tobacco smoking, diets high in fat leading to obesity, and a sedentary lifestyle) are particularly high in contrast to the low level of cardiovascular disease seen. "The French Paradox" is a term used to describe this effect. Epidemiological studies have linked moderate alcohol consumption and particularly the red wine consumption in France with the low incidence of disease in populations consuming a Mediterranean diet.

To investigate the effect of moderate red wine consumption on biomarkers of cardiovascular (disease risk, a randomised controlled dietary crossover intervention was undertaken. Healthy non-smoking males (n = 17) from 41-57 years of age (49.37 ± 0.12 years) were placed on a low polyphenol diet and followed a set drinking protocol of three 4-week periods, 1: alcohol, free washout, 2: red wine 200 mL per day, 3: white wine 200 mL per day.

Arterial compliance has been suggested as a suitable pre-clinical marker for detection of cardiovascular disease states. Measures of arterial function may be made by non-invasive tonometry and computer analysis of the diastolic decay through use of a modified Windkessel-type model (HDI/PulseWave TM CR-2000 Research CardioVascular Profiling System). Such analysis was performed in parallel to blood sampling. No significant changes were seen in systolic, diastolic, mean arterial or pulse pressures throughout the course of the protocol ($p > 0.1$). However, small artery elasticity was seen to significantly increase from 7.77 ± 0.10 mL/mmHg x 100 at baseline to 9.12 ± 0.11 mL/mmHg x 100 after consuming red wine. A significant difference was seen when comparing the effect mediated by either red or white wine ($p < 0.02$). Further work is required to elucidate this action.

In summary, we have shown that moderate red wine consumption may improve small vessel compliance.

PHENOLIC COMPOUNDS FROM RED WINE ARE PROTECTIVE AGAINST THE DNA DAMAGING EFFECT OF IONISING RADIATION *EX VIVO*

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Using the cytokinesis-block micronucleus assay we: (a) investigated which compounds in red wine can prevent oxidative damage to DNA *in vitro* and (b) performed *in vivo* interventions with red wine (RW), dealcoholised red wine (DEALC) and alcohol (ALC) to distinguish the effects of alcohol from the other fractions of RW in prevention of oxidative damage to DNA ex *vivo*. Cells were either challenged with ionising gamma radiation or hydrogen peroxide, two different forms of oxidative stress. The relative contribution of ethanol, glycerol, tartaric acid, catechin + caffeic acid, a mixture of all of these and phenolic stripped RW at *in vivo* relevant concentrations on spontaneous and oxidative stress induced DNA damage was evaluated *in vitro*. The results from these studies have shown that: (a) only ethanol significantly increases spontaneous DNA damage, but this effect is eliminated when ethanol is included in a mixture of all the other wine components ($P<0.05$), (b) the strongest and only significant protective effect against hydrogen peroxide induced DNA damage was observed for the catechin + caffeic acid mixture ($P<0.05$) and (c) all compounds tested were significantly protective against ionising radiation-induced DNA damage in a dose dependent manner with the strongest protection being observed for the catechin + caffeic acid mixture and a mixture of all components ($P < 0.0001$). In the in vivo intervention studies with ex vivo challenge of whole blood showed that 1-2 h after consumption of 300 mL DEALC produced a significant protection against the DNA damaging effects of ionising radiation ($P = 0.0002$) but ALC significantly enhanced the damaging effects of ionising radiation ($P = 0.0002$) while RW produced an intermediate effect. The data from these studies are particularly interesting because they clearly demonstrate the potential beneficial effects of wine phenolics in counteracting the harmful effects of ionising radiation. Furthermore they demonstrate that the potential of alcohol to exacerbate the DNA damaging effects of oxidative stress is neutralised in the presence of the other RW components.

9.2 Intellectual property

N/A

9.3 References

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9.5 Miscellaneous

9.5.1. List of foods allowed/not allowed in the plant-phenolic compound-free (PPF) diet

Foods allowed:

meat (beef, lamb, pork), fish, chicken
cheese, eggs
white bread, white flour
ordinary (not wholemeal) pasta or noodles
skinned potatoes
butter, margarine, mayonnaise, oil, salt
milk, water, soda water, lemonade (clear, not homemade)

Foods not allowed:

all fruits
all vegetables, except skinned potatoes
vegetable juice
nuts, seeds
honey
fruit juice
wine, beer, alcoholic drinks
tea (black, green or herbal)
coffee

Taken from Fenech,, M., Stockley, C., Aitken, C. (1997). Moderate wine consumption protects against hydrogen peroxide-induced DNA damage. Mutagenesis 12 (4): 289-296.

9.5.2 Dietary guidelines for volunteers

Polyphenols are compounds present in plant foods such as fruit, vegetables, cereals and legumes and some beverages such as wine, cider, beer, tea and cocoa. For this research project, it is important that you maintain a low intake of phenolic compounds. These guidelines will help you to achieve a low phenolic compound diet.

AVOID
Legumes (baked beans, lentils, lima, soy and other beans, chickpeas)
Onions (except for very small amounts as a flavouring agent)
Orange juice
Blackcurrants
Blackcurrant juice
Dark grapes
Dark grape juice
Wine, Beer, and Spirits
Foods containing Millet, Barley or Sorghum
Seeds, including linseed

LIMIT
Fruit (fresh, tinned, stewed or juice) - 2 serves per day or less
Vegetables (fresh, tinned, frozen or juice) - 3 serves per day or less
Note: 1 serve = approximately ½ cup

Tea/Coffee/Cocoa or substitutes: MAXIMUM 1 cup (250ml) daily