Berryfruit Cognitive Study: Final Report

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February 2005

Report to: Blackcurrants New Zealand Ltd
Smith Kline Beecham plc t/a Glaxo Smith Kline Consumer Healthcare
Just the Berries Ltd
Broadacres Extracts and Marketing Ltd
Barkers Fruit Processors Ltd
Berryfruit Export NZ Ltd

HortResearch Client Report No. 16135
Contract No. C06X0407

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EXECUTIVE SUMMARY

Berryfruit Cognitive Study

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- There is increasing evidence that the consumption of fruit and vegetables promotes good health by providing protection against various degenerative diseases. The actual constituents responsible for these beneficial effects are not known and the mechanisms are not well understood. However, some evidence supports the view that the antioxidant properties of fruit and vegetable phytochemicals may be responsible.

- Oxidative damage to DNA, lipid and protein increases with age and is believed to contribute to the progression of some cancers and cardiovascular disease. Oxidative damage to both protein and lipid has been associated with age-related cognitive decline and consumption of dietary antioxidants may delay the onset of this decline. Previous studies with antioxidant-rich extracts of blackcurrant and Boysenberry showed that these berryfruit have the ability to reduce oxidative stress in *in vitro* cell experimental systems.

- The Berryfruit Cognitive Study was undertaken to determine if daily supplementation with either a blackcurrant or Boysenberry drink of an elderly population with below average memory abilities could improve measures of cognitive performance, oxidative stress and indicators of immune function and inflammation.

- The study design used was a fully blinded parallel intervention with a placebo control. Treatments were 1) Boysenberry; 2) blackcurrant; 3) synthetic drink (placebo). The study contained 51 participants and the intervention period was for 12 weeks with an extension period to 24 weeks for 30 participants.

- The Boysenberry drink was formulated as 100% single strength juice containing approximately 250 mg anthocyanin/serve. The blackcurrant drink was formulated as 20% single strength juice fortified with CurranteX20® blackcurrant powder containing approximately 500 mg anthocyanin. The placebo control was formulated from synthetic colours and flavours and had essentially zero antioxidant capacity and no anthocyanins.

- A total of six different measures of cognitive performance, six measures of oxidative stress, and six measures of immune function and inflammation were assessed at baseline, 6, 12, and 24 weeks after commencement of the intervention.

- No statistically significant differences were found between any of the treatments for any of the immune or cognitive parameters measured, indicating that neither of the treatments enhanced or degraded cognitive function, immune function and inflammation after 12, or 24 weeks of treatment compared with the placebo control.

- Of the six measures of oxidative stress, one parameter (plasma antioxidant capacity) increased for both the Boysenberry and blackcurrant treatments compared to the placebo-control. This increase was statistically significant. Plasma malondialdehyde (a marker of lipid peroxidation) decreased in both the Boysenberry and blackcurrant treatments but the

decrease was not statistically significant and can be regarded as a trend only. These changes in parameters of oxidative stress indicate that both the Boysenberry and blackcurrant function as antioxidants *in vivo* and therefore should have beneficial effects cardiovascular disease, some cancers, and age-related cognitive decline.

- Several measures of oxidative stress improved during the study (protein oxidation - carbonylation, and lipid peroxidation - malondialdehyde) with no statistical difference between the berryfruit and placebo treatments. The origin of this improvement in oxidative stress in the total study population is unknown but may have resulted from changes in lifestyle (e.g. diet) as study participants became more aware of nutrition and health.

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INTRODUCTION

There is increasing evidence that the consumption of fruit and vegetables promotes good health by providing protection against various degenerative diseases such as cardiovascular disease (CVD), some cancers and the onset of dementia (Steinmetz & Potter, 1996; Ness & Powles, 1997; Joshipura *et al.*, 2001; Youdim & Joseph, 2001; Knekt *et al.*, 2002). Fruit and vegetables contain a range of phytochemicals that may act individually or in concert to produce disease protective effects. Among the compounds thought to act in this way are carotenoids, polyphenolics, other antioxidants, vitamins, folate, calcium, selenium, potassium, iron and dietary fibre. The actual constituents responsible for these beneficial effects are not known and the mechanisms are not well understood. However, some evidence supports the view that the antioxidant properties of fruit and vegetable phytochemicals may be responsible for some of the disease protective effects, since oxidative damage to key cellular components is linked with cancer (Loft & Poulsen, 1996; Collins, 1999), cardiovascular disease (Halliwell & Chirico, 1993), dementia (Perry *et al.*, 2002; Pratico, 2002), and age-related decline (Bokov *et al.*, 2004; Junqueira *et al.*, 2004).

Reactive oxygen and nitrogen species are formed during normal cellular metabolism but when produced in amounts that exceed the capacity of the organism's endogenous antioxidant mechanisms, oxidative damage to essential cellular components such as DNA, lipid and protein occurs. Oxidatively damaged DNA is believed to contribute to the progression of cancers (Collins, 1999) whereas development and progress of atherosclerosis results from lipid (LDL) peroxidation (Halliwell & Chirico, 1993). Oxidative damage to both protein and lipid has been linked to the neuropathology associated with degenerating cognitive function both for normal ageing and degenerative disease (Joseph *et al.*, 1996; Levine, 2002; Sohal, 2002). Oxidative damage is likely to be a causal factor in age-related cognitive decline and consumption of dietary antioxidants may delay the onset of age-related cognitive decline. Indeed, clinical studies involving Alzheimer's patients showed that supplementation with vitamin E was able to delay institutionalization (Sano *et al.*, 1997), and in animal studies aged rats or dogs fed with dietary antioxidant showed improved cognitive function (Joseph *et al.*, 1998; Joseph *et al.*, 1999; Bickford *et al.*, 2000; Milgram *et al.*, 2005).

The pro-oxidant/antioxidant balance is important for the optimal functioning of the immune system and oxidative damage to immune cells is considered to be responsible for the age-related decline in immune function. A capable immune system is necessary for optimal health and wellbeing. Several dietary intervention studies in the elderly have shown that supplementation with anti-oxidants (vitamin E, carotenoids, Se) is able to enhance immune function. However, little is known about the effect of fruit-derived antioxidants on immune function in the elderly.

Previous studies with antioxidant-rich extracts of blackcurrant and Boysenberry showed that these berryfruit have the ability to reduce oxidative stress in *in vitro* cell experimental systems (McGhie *et al.*, 2003a; McGhie *et al.*, 2003b; Ghosh *et al.*, 2005). When these extracts were fed to aged rats some parameters of cognitive function and performance were moderately improved at statistically insignificant levels. However, the addition of blackcurrant extract to the diet significantly increased oxotremorine-enhanced dopamine release in rat striatum (Shukitt-Hale *et al.*, 2005). The addition of Boysenberry extract to the diet of young rats decreased some measures of oxidative damage but the effect was modulated by other constituents of the base diet (Barnett *et al.*, 2005). These experiments show that both

Boysenberry and blackcurrant polyphenolics behave as a biological antioxidant by reducing oxidative stress.

Since cognitive function appears to be related to oxidative damage, the Berryfruit Cognitive Study (BCS) was undertaken to determine if regular consumption of either a Boysenberry or blackcurrant drink would enhance cognitive function in an elderly population.

METHODS

STUDY POPULATION

The study population was composed of 52 elderly (≥65 years old), community-dwelling people. Volunteers were recruited from the Palmerston North area through advertisements in the local media, selection from existing participant databases maintained by IFNHH, Massey University and direct contact with retirement establishments. Volunteers had self-reported thinking problems described as 'not thinking as well as they used to'.

Study participants were selected from the volunteers. The following criteria were used to exclude potential participants:

- diagnosis of cancer (any form), vascular disease, diabetes mellitus, or mental illness;
- smokers;
- alcohol abuse;
- endocrine disease – myxidemia;
- conditions leading to amyloidosis;
- anaemia;
- malnutrition from malabsorption (B12, B6, folate, iron);
- neurological disorders – multiple scelorosis, Parkinson's Disease, Alzheimer's Disease;
- renal impairment;
- treatment with tranquillisers, antidepressants, anticonvulsants;
- impaired immune status – AIDS, multiple myeloma, chronic lymphatic leukemias;
- visual impairment – intact visual fields.

To be included in the study participants had to:

- have a BMI between 18 and 34 kg/m^2;
- be able and willing to provide written informed consent;
- have a reliable informant/carer;
- have acceptance by their regular General Practitioner that they were suitable for the study.

In addition each volunteer was assessed for age-related memory decline using the Rey Auditory Verbal Learning Test (RAVLT). During the RAVLT volunteers were presented with a 15-word list and asked to recall the list. Volunteers were included in the study if they scored below the mean for their age group on the total recall of words. Therefore the population selected for the study contained participants that had below average memory performance.

STUDY DESIGN

Study participants were randomised into three parallel arms: one arm was provided with placebo control drink, one arm received a Boysenberry drink, and the final arm received a blackcurrant drink (Table 1). All participants were blinded with respect to the treatment they received. The study contained two phases. In the first phase 52 participants were monitored for 12 weeks with assessments of the selected parameters at baseline, 6 weeks and 12 weeks. At the end of the first phase, 30 participants (10 for each treatment) continued supplements

for a further 12 weeks followed by a final assessment. Assessment included: cognitive function (CogState), plasma antioxidant capacity, plasma malondialdehyde, and plasma protein carbonyl, immune function, and inflammation. The cognitive assessments were performed at the INFF, Massey University under the supervision of a clinical psychologist. Non-fasting plasma and urine samples were provided the day following each cognitive assessment.

The ingredients used to prepare each drink are provided in Table 1. All three drinks were manufactured by Barkers Fruit Processors Ltd (Geraldine, NZ). Following formulation each drink was pasteurised and packed into 100 mL sachets. These were then frozen at -20°C until used. Each participant was instructed to consume 2 sachets (total 200 mL) of their allocated drink during the day while maintaining their normal diet.

TREATMENT DRINKS

Three treatment drinks were prepared according to the ingredients and specifications listed in Table 1. The treatments were:

1) **Placebo control:** synthetic berry fruit drink (200 mL) containing synthetic colours and flavours, granulated food grade sugar and citric acid to simulate a generic berryfruit drink.
2) **Blackcurrant drink:** freshly produced by reconstitution of blackcurrant juice concentrate (variety 'Ben Ard') commercially manufactured by the New Zealand Blackcurrant Co-Operative Ltd and diluted to simulate 20% pure single strength blackcurrant juice. The drink contained added granulated food grade sugar and a blackcurrant powdered concentrate (Currantex 20®, manufactured and marketed by Just the Berries Ltd). Currantex 20® was added to adjust the anthocyanin concentration of the drink to approximately 500 mg/200mL.
3) **Boysenberry drink:** freshly produced by reconstitution of a Berryfruit Export NZ Ltd juice concentrate and diluted to simulate a pure single-strength Boysenberry juice.

Prior to manufacture and packaging, test formulations were prepared and the palatability and acceptability determined by a panel of eight study participants. All the formulations were found to be acceptable as follows:

- **Blackcurrant:** Two formulations were prepared at 14 and 11.6 Brix. Both were well liked by the 8 participants and they were described as smooth drinks. A slight musty taste was apparent that probably was derived from the Currantex 20®. During preparation of these test drinks there were some problems in dissolving the Currantex 20® that were not present during the manufacture of the final test drinks.
- **Boysenberry:** Seven of the eight participants found this drink acceptable and were prepared to consume 200ml per day. A number expressed a preference for the Boysenberry drink although it has a 'tart' flavour compared with the other drinks.
- **Placebo:** No one identified this drink as a synthetic but many thought it was a little too sweet. The placebo is clear and has a much lighter colour than the other two drinks but was believed to be real fruit by all the participants.

Table 1: Ingredients and specifications of the drinks used as treatments and the placebo control.

Ingredients	Blackcurrant %w/w	Boysenberry %w/w	Placebo %w/w
Water	85.8	84.7	87.9
sugar	9.7		11.6
citric acid anhydrous			0.40
Currantex 20®	1.0		
Boysenberry Conc. N4003		15.3	
Blackcurrant Conc. N4037	3.55		
Flavour			0.50
Ponceau (124)			0.15
Colour (123+132)			0.07
Total	**100.0**	100.0	99.9
Target Specifications			
Boysenberry Juice (ssj[z]) %w/w		100.00	
Blackcurrant Juice (ssj[z]) %w/w	20.00		
°Brix (calculated)	12.00	9.98	11.60
Specific Gravity (g/mL)	1.048	1.040	1.048
Acidity %(eq citric acid anhydrous)	0.38		0.40

[z] – ssj=single strength juice

COGNITIVE FUNCTION

CogState Ltd (Melbourne, Australia) assessed cognitive function using a computer-based assessment procedure. The procedures are based on the manipulation of playing cards on the computer screen and have been validated for use in an aged population. In total six different cognitive performance parameters were measured: 1) simple reaction time; 2) choice reaction time; 3) one-back memory; 4) monitoring; 5) matching; and 6) learning.

Each study participant was assessed for all 6 parameters during a single session at baseline, 6 weeks, 12 weeks, and 24 weeks for the extension participants. The CogState programme was installed on a laptop computer and the assessment was administered by research nurses under the supervision of a registered clinical psychologist (Dr Patrick Dulin, Massey University). All data files were collated and sent electronically to CogState for processing.

Processing of the data consisted of two phases. Firstly, errors were identified and excluded from the dataset. These included anticipatory responses (response time, RT<100msec) and abnormally slow responses (RT>5000msec). Additionally, false positive and false negative responses were identified. The numbers of each error type were calculated and recorded for each test and the accuracy of performance was expressed as the total number of correct responses as a percentage of the number of trials.

Secondly, for each cognitive parameter the mean and standard deviation of the response times for correct trials were calculated. The process was repeated but with the distributions normalised using a logarithmic base 10 transformation before the mean and the standard deviation of the response time were calculated.

Three indices for each cognitive performance parameters were thus created.

1. The accuracy of performance expressed as a percentage;
2. The speed of performance expressed as the mean of the log response time initially as milliseconds;
3. The consistency of performance expressed as the standard deviation of the log response times.

Neuropsychological assessments were also conducted by Dr Patrick Dulin, School of Psychology, Massey University using the Repeatable Battery of the Assessment of Neuropsychological Status (RBANS). This procedure contained the following sub-tests: list learning, story memory, coding, list recall, story recall and figure recall.

OXIDATIVE STRESS

Biomarkers of Oxidative Damage

Oxidative stress occurs when exposure to oxidants overcomes the antioxidant defences, resulting in oxidative damage to biomolecules such as DNA, lipids, and proteins. In this study we selected oxidative damage to DNA - (urinary excretion of 8-oxo-deoxyguanosine, strand breaks – Comet assay), lipid (plasma malondialdehyde), and protein (carbonylated plasma protein) as measures of oxidative stress. In addition we also included measurements of plasma antioxidant capacity ($ORAC_{FL}$ total and non-protein) as indicators of antioxidant status. There are numerous methods for measuring oxidative damage *in vivo*, and following is a brief summary and justification for selection of the methods used in this study.

Oxidative damage to DNA occurs *in vivo* and produces lesions that may be mutagenic and lethal (Wallace, 2002). One of the most abundant lesions, 8-oxo-2'deoxyguanosine (8-oxodG), is widely used as a marker for oxidative damage to DNA and has been used to establish that a wide range of environmental and lifestyle factors are associated with increases in oxidative damage. 8-oxodG can be measured in cells and urine, but its measurement in urine provides an assessment of oxidative damage to DNA in the whole body. The exact origin of 8-oxodG in urine is not known, but nucleotide excision repair provides a plausible mechanism for its excretion in urine (Lunec *et al.*, 2002).

Lipids are readily damaged by free radicals result in the generation of a number of further products that damage other biomolecules. MDA has long been recognised as a marker for lipid oxidation (De Zwart *et al.*, 1999) and has been frequently measured by the thiobarbituric acid reactive substances (TBARS) method. Many compounds respond to the TBARS producing interference, although detection of thiobarbituric acid derivative of MDA by HPLC overcomes many of these problems. For this study we have employed a selected ion monitoring GC-MS method preceded by reaction of MDA with phenylhydrazine at room temperature. This method is highly selective and does not produce artefacts because of the mild derivatisation conditions (Fenaille *et al.*, 2001).

Proteins are readily damaged by free radicals resulting in the oxidation of amino acids forming protein carbonyls (Chevion *et al.*, 2000). The presence of protein carbonyls is one of the most widely used means of detecting oxidative damage to proteins and has been associated with ageing, diabetes and neurodegenerative disease (Chevion *et al.*, 2000; Levine, 2002). Protein carbonyls are measured by reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine followed by spectrophotometric, immunochemical, or radiometric techniques. For this study we detected protein carbonyls using a commercially available ELISA kit based on the method of Buss *et al*. (Buss *et al.*, 1997).

Total antioxidant status of plasma combines the antioxidant activity of all the components in plasma into a single value and can be determined by a number of methods. The antioxidant activity of the non-protein components is determined by removing the protein prior to the analysis. We selected the Oxygen Radical Absorption Capacity assay ($ORAC_{FL}$) (Ou *et al.*, 2001) to determine the total antioxidant activity of plasma.

The comet assay (also known as single cell gel electrophoresis) is a rapid, simple, visual and sensitive technique for measuring and analysing DNA breakage within single mammalian cells (Ostling & Johanson, 1984; Singh *et al.*, 1988). The generally adopted comet assay technique is that of Singh *et al*. 1988, in which the procedure of Ostling & Johanson is modified by performing the electrophoresis at high pH instead under neutral conditions. Since the publication by Singh and colleagues (Singh *et al.*, 1988) describing the alkaline comet assay, several laboratories worldwide have performed this technique in either its original or somewhat modified form (McKelvey-Martin *et al.*, 1993).

Protein Oxidation (protein carbonyls)

Plasma protein carbonyl content was measured by an enzyme-linked immunosorbent assay (ELISA) supplied by Zenith Technology (Dunedin, New Zealand). The assay was performed according to the instructions of the manufacturer.

Lipid Oxidation (plasma malondialdehyde)

Plasma MDA was measured by a GC-MS based method (Fenaille *et al.*, 2001). Briefly, MDA standards were prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane (Sigma Inc, Sacramento, CA) at 40^0C. Sample plasma (500 μL) was mixed with 350 μL of 10% trichloroacetic acid (TCA) to precipitate the protein and extracted with 0.8% butylated hydroxytoluene (BHT) in hexane. After discarding the upper hexane layer, MDA was derivatised with phenylhydrazine (PH) (Sigma Inc, Sacramento, CA) at room temperature. Derivatised MDA was extracted with hexane, internal standard (benzophenone) was added and the MDA concentration determined by selected ion monitoring GC-MS (Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer, Shimadzu Corporation, Kyoto, Japan; equipped with J & W Scientific DB™ – 5MS 30 m, 0.25 mm ID, 0.25 μm film thickness). MDA and the internal standard were recorded at mass to charge ratios (m/z) of 76.90, 104.85, 143.90 and 182.05, concentrations were calculated from a calibration curve (0-450 $ngmL^{-1}$) and presented as ng/mL of plasma.

Antioxidant capacity (ORAC)

Plasma $ORAC_{FL}$ was measured using a method similar to that described by Ou *et al*. (Ou *et al.*, 2001). Plasma samples were diluted 125-fold with 75 $mmolL^{-1}$ potassium phosphate buffer, pH 7.4. For non-protein $ORAC_{FL}$ (NP-$ORAC_{FL}$) analysis plasma proteins were precipitated with an equal volume of 0.3% perchloric acid, centrifuged at 12,000 g and the supernatant diluted 6.67-fold with 75 $mmolL^{-1}$ potassium phosphate buffer, pH 7.4. Standard curves were prepared using 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), a water-soluble analogue of vitamin E, at 50-200 $\mu molL^{-1}$. Each sample or standard was analysed in triplicate in a 96-well format. Plates were prepared by adding 10 μL of sample and 160 μL of fluorescein ($4.8x10^{-8}$ $molL^{-1}$) to each well. AAPH (25 μL, 0.1 $molL^{-1}$) was added to each well and the fluorescence measured every minute for 90 min. The relative fluorescence for each standard was calculated, to give the area under the curve (AUC), and a standard curve was plotted.

DNA Oxidation (Comet assay and 8-OHdG)

DNA damage in lymphocytes was measured by the alkaline comet assay according to Singh *et al*. (Singh *et al.*, 1988) with little modification. Lymphocytes were isolated by mixing 3 mL whole blood with 3 mL PBS, underlayering with Histopaque 1077 (3 mL) and centrifuging at 2000 rpm for 30 min at room temperature. Approximately 2 mL of the lymphocyte-containing 'buffy coat' was washed in PBS (8 mL) and the cell number adjusted to $2x10^6$/mL. The comet assay was performed by incubating washed lymphocytes in 1 mL PBS and embedding in agarose layers on microscope slides which were placed into a lysis solution to remove cellular proteins and leave the DNA as 'nucleotids'. The slides were then incubated in alkaline (pH >13) electrophoresis buffer to produce single stranded DNA and to express alkali-labile sites as single strand breaks. After alkali unwinding, the single stranded DNA in the agarose gels was electrophoresed under alkaline conditions (breaks in the DNA molecule disturb its supercoiling, allowing free DNA to migrate towards the anode, producing a 'comet'). Subsequently, microscope slides were neutralized and DNA visualized by fluorescence microscopy after staining with DNA-binding dye. Cells with damaged DNA display increased migration of DNA fragments from the nucleous. The length of the migration indicates the amount of DNA damage (Singh *et al.*, 1988). Quantification of DNA damage was performed using a visual scoring system (visual scoring is rapid, inexpensive and simple and at least as sensitive as computer-based image analysis (Kobayashi *et al.*, 1995). One hundred comets per sample were classified into one of 5 classes (0 = no tail; 4 = almost all DNA in tail) according to the relative intensity of fluorescence in the 'comet'-tail and the total score per sample was between 0 and 400 "arbitrary units". To measure lymphocyte DNA protective effects, washed lymphocytes were first incubated with PBS containing hydrogen peroxide (100 μM) for 5 min on ice, to induce DNA damage. DNA damage was then determined using the comet assay described.

8-OHdG was measured in urine using an ELIZA. 'New 8-OhdG Check' commercial kits were supplied by the Genox Corporation and the manufacturer was the Japan Institute for the Control of Aging, Fukuroi City, Japan. 8-Hydroxy-2'-deoxyguanosine (8OHdG) is a result of oxidation of bases in nuclear DNA. The oxidatively damaged bases are excised by DNA repair mechanisms and excreted in the urine. The concentration of the 8OHdG in urine is a result of both the level of oxidative stress and the rate at which the damaged bases are repair. The ELISA used is a competitive *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of 8OHdG in urine, plasma and serum.

IMMUNE FUNCTION AND INFLAMMATION

The immune system has numerous cells with different functions, which are controlled by a complex series of interactions. T-cells are involved in defence reactions against foreign organisms (e.g. viruses, parasites) and tissues (transplants, malignant cells). B-cells are responsible for immune responses that are mediated by immunogloblins, which are secreted in response to stimulation by antigens from bacteria, allergenic compounds and other substances. Cytokines are soluble molecules produced by many types of immune cells and are used to regulate lymphocyte differentiation and proliferation and help coordinate the activities of the various cells in immune and inflammatory responses. The following indices were selected as measures for immune function and inflammation.

IL-6 is associated with B cell differentiation, antibody synthesis, and blood cell synthesis (haematopoiesis). Serum levels of IL-6 are usually at the lower level of detection during

normal physiological states. A study of community-dwelling elderly people showed that a mean normal value would be around 1.7 pg/mL (Wilson *et al.*, 2003). Serum IL-6 increases with advancing age in various healthy populations and associated with various diseases. IL-6 is also in general associated with inflammatory diseases, post-menopausal osteoporosis, Alzheimer's disease, stroke and multiple sclerosis. As elevated IL-6 acts as a marker of subclinical cardiovascular disease and other diseases, it is hard to separate an isolated ageing effect from an effect of disease.

IL-10 and TGF-beta are regulatory/anti-inflammatory cytokines and may suppress the immune system. They exist mainly to dampen-down a burgeoning immune response to ensure that it doesn't become too aggressive (which could lead to inflammation). IL-10 specifically inhibits a broad spectrum of activated macrophage/ monocyte functions as well as CD8+ T cells and NK cells. IL-10 also inhibits secretion of IL-6, TNF and other cytokines from activated monocytes and macrophages. TGF-beta also plays an important role at the mucosal interfaces (e.g. the gut surface) to ensure that a person does not over-react to dietary proteins (i.e. it is involved in oral tolerance induction). IL-10 is more of a systemic-level immunoregulatory cytokine, and is particularly important in preventing the over-production of other inflammation- and fever- promoting cytokines (such as IL-1 and TNF-alpha). A study of 1726 community-dwelling elderly persons showed that a mean for this age group (77 years) for IL-10 is 9.7 pg/mL (Wilson *et al.*, 2003).

IL-8 is a neutrophil chemotactic cytokine, i.e. it promotes the migration of immune defence cells to sites of tissue inflammation. It is also one of the master players in the innate and acquired immune system. The relevance to the elderly is that this group often faces chronic, low- to medium-grade soft and hard tissue inflammatory reactions, so a reduction in the production of cytokines such as IL-8 might signify a reduced in vivo inflammatory capacity in these individuals.

IgA is one of 5 different classes of antibodies found in humans; it is most important in mucosal immune defence (e.g. protecting the gut and respiratory tract mucosal surfaces). IgA was measured as a proxy measure of mucosal immune defence. The relevance to the elderly is that they often have poor mucosal immune defence (especially in the airways, hence their increased susceptibility to upper respiratory tract infections). Normal values for IgA range between 0.8 and 4 g/L for adults and are dependent on age.

C-reactive protein (CRP), named for its capacity to precipitate the somatic polysaccharide of *Streptococcus pneumonia,* consists of 5 identical non-glycosylated 23-kD subunits that are synthesised mainly by hepatocytes, under control of IL-6. Elevated levels of CRP are one of the strongest predictors of progressive vascular disease and future cardiovascular events in healthy men and women. During the acute phase response to infection, inflammatory disease, surgery, trauma and cancer the levels of CRP in blood rise to extreme and return after resolution of the disease. Normal levels are below 1 mg/L. In patients with risk of atherosclerotic disease, CRP is elevated to around 10 mg/L and can remain at this level for months to years (Labarrere *et al*., 2004). Previous assays for CRP lacked sensitivity to measure below 8 mg/L, but with development of the newer high-sensitivity assays, levels can be detected in the 0.3 – 10 mg/L range.

Cytokine concentrations in serum were measured by enzyme-linked immunosorbent assay (ELISA). IL-6, IL-8, IL-10 and TNF-alpha DuoSet kits from R&D Systems (Minneapolis,

MN, USA) were used according to the protocol included with the kit. Essentially, a capture antibody was coated onto a 96-well plate (Nunc Maxisorb Immunoplate, cat no: 442404) and incubated at 4°C overnight. Next morning, excess antibody was washed away and non-specific binding sites were blocked for one hour. After a wash step, samples were added in triplicate and along with a 7-point standard and blank. After a two-hour incubation at room temperature and further washing, a biotinylated detection antibody was added for two hours. Again, after washing, streptavidin-conjugated horseradish peroxidase was added for twenty minutes and the amount of enzyme present was realised through the addition of the substrate TMB (Tetramethylbenzidine + hydrogen peroxide), and measured colormetrically. The amount of enzyme present is directly correlated to the amount of cytokine present in the serum sample, and was enumerated from the standard curve, present on each plate.

IgA and hs-CRP were analysed using immmunoturbidimetry techniques. Kits were purchased from Roche Diagnostics (NZ) Ltd and the assays were performed on Vitalab Flexor E and Cobas Fara II analysers, respectively. Samples were analysed in duplicate and the kits were used in accordance with the manufacturer's instructions.

RESULTS

Fifty-one of the original 52 participants completed the study. Generally the drink formulations were well tolerated and liked by the participants. A questionnaire administered to the participants at the end of the study showed that 5/12 participants described the Boysenberry drink as unpleasant and 15/16 described the blackcurrant drink as pleasant.

Ages ranged from 65-92 years with most participants in the 65-74 age groups. Approximately equal numbers of males and female were enrolled in the study (Table 2). The original intention was to recruit 60 participants, however this target was not achieved. This was mainly because of the high portion of prospective participants that failed the criteria for the RAVLT; that is their memory scores were average or better for their particular ages. After the initial advertisements and follow-ups there was a good response but this declined with time, presumably as the local pool of >65 year old people was exhausted. Because of time constraints to commence the study, it was decided to proceed with 52 participants.

Table 2. Number of participants completing the study according to treatment, gender and age.

	Year of birth	**1939-35**	**1934-30**	**1929-25**	**1924-20**	**1919-15**	**1914-10**	
	Age	**65-69**	**70-74**	**75-79**	**80-84**	**85-89**	**90-95**	***Sum***
Treatment	**Gender**							
Boysenberry	1	3	2	1	3	0	0	*9*
	2	6	1	0	1	0	0	*8*
	Sum	***9***	***3***	***1***	***4***	***0***	***0***	***17***
Blackcurrant	1	4	2	3	1	0	0	*10*
	2	2	2	2	0	0	1	*7*
	Sum	***6***	***4***	***5***	***1***	***0***	***1***	***17***
Control	1	3	5	0	1	0	0	*9*
	2	2	3	2	1	0	0	*8*
	Sum	***5***	***8***	***2***	***2***	***0***	***0***	***17***
All	1	10	9	4	5	0	0	*28*
	2	10	6	4	2	0	1	*23*
	Sum	***20***	***15***	***8***	***7***	***0***	***1***	***51***

The phytochemical components of the two treatment drinks were measured at the start of the study and then for each treatment drink at approximately 6 weeks, and again at the end of the study (12 weeks). The results are shown in Table 3. The phytochemical composition changed little during the study and no significant differences were detected.

Table 3: Phytochemical composition and antioxidant properties of the treatments +/- one standard deviation.

Component	Treatment	Baseline	6 Weeks	12 Weeks
Total anthocyanins (HPLC mg/serve)	Boysenberry[a]	247±8	241±28	235±25
	Blackcurrant[b]	742±20 (557)	741±15 (556)	754±57 (565)
	Control			
Total phenols (mg GAE/mL) (Folin C)	Boysenberry	4.5±0.2	3.9±0.4	3.8±0.4
	Blackcurrant	4.1±0.2	3.7±0.1	3.8±0.3
	Control			
Vitamin C (mg/serve)	Boysenberry	n.d.	n.d.	n.d.
	Blackcurrant	30±1.7	34±0.9	33±0.9
	Control			
Antioxidant Activity				
ORAC (mM TE)	Boysenberry	90±9.8	107±12	101±10
	Blackcurrant	88±8.1	121±16	96±24
	Control	n.a.	n.a.	n.a.
FRAP (mM TE)	Boysenberry	23.1±1.1	25.2±2.5	25.3±2.5
	Blackcurrant	23.7±2.6	20.9±1.2	24.3±2.1
	Control	n.a.	n.a.	n.a.

n.d. – not detected
n.a – not analysed
[a] – calculated as cyanidin-glucoside equiv.
[b].- calculated using relative response factors for blackcurrant anthocyanins. Cyanidin-glucoside equivalents in brackets.

The following tables present the results of the measures of cognitive performance (Tables 4,5,6), oxidative stress (Table 7) and immune function and inflammation (Table 8). Table 9 shows the results of the statistical analysis.

A full report for the assessment of cognitive function using the RBANS was provided by Dr Dulin and is attached as Appendix I. No statistical differences between treatment groups were detected for any of the RBANS sub-tests.

Table 4: Speed of performance (log transformed means of response time)

Parameter	Treatment	Baseline	6 Weeks	12 Weeks	Extension
Simple Reaction Time	Boysenberry	2.56	2.56	2.54	2.56
	Blackcurrant	2.51	2.54	2.52	2.52
	Control	2.56	2.54	2.52	2.51
Choice Reaction Time	Boysenberry	2.76	2.78	2.76	2.64
	Blackcurrant	2.76	2.75	2.72	2.59
	Control	2.75	2.73	2.74	2.64
One-back Memory	Boysenberry	2.99	3.00	2.94	2.72
	Blackcurrant	2.92	2.94	2.92	2.69
	Control	2.99	2.94	2.92	2.72
Monitoring	Boysenberry	2.69	2.70	2.66	2.94
	Blackcurrant	2.65	2.66	2.63	2.88
	Control	2.70	2.62	2.65	2.91
Matching	Boysenberry	3.32	3.33	3.30	3.32
	Blackcurrant	3.31	3.30	3.31	3.27
	Control	3.32	3.32	3.31	3.30
Learning	Boysenberry	3.23	3.25	3.22	3.23
	Blackcurrant	3.25	3.27	3.25	3.21
	Control	3.27	3.27	3.24	3.24

Table 5: Consistency of performance (standard deviations of the log transformed response times)

Parameter	Treatment	Baseline	6 Weeks	12 Weeks	Extension
Simple Reaction Time	Boysenberry	0.108	0.120	0.121	0.131
	Blackcurrant	0.113	0.109	0.085	0.096
	Control	0.117	0.116	0.092	0.099
Choice Reaction Time	Boysenberry	0.113	0.108	0.114	0.190
	Blackcurrant	0.116	0.110	0.097	0.195
	Control	0.086	0.099	0.085	0.175
One-back Memory	Boysenberry	0.175	0.171	0.157	0.089
	Blackcurrant	0.162	0.158	0.159	0.091
	Control	0.155	0.142	0.146	0.107
Monitoring	Boysenberry	0.205	0.220	0.198	0.144
	Blackcurrant	0.211	0.206	0.200	0.146
	Control	0.244	0.197	0.187	0.139
Matching	Boysenberry	0.107	0.118	0.097	0.100
	Blackcurrant	0.095	0.103	0.100	0.097
	Control	0.098	0.098	0.093	0.110
Learning	Boysenberry	0.146	0.191	0.153	0.160
	Blackcurrant	0.182	0.172	0.176	0.140
	Control	0.161	0.152	0.139	0.141

Table 6: Accuracy of performance (transformed percentage correct)

Parameter	Treatment	Baseline	6 Weeks	12 Weeks	Extension
Simple Reaction Time	Boysenberry	1.56	1.52	1.57	1.55
	Blackcurrant	1.51	1.55	1.53	1.55
	Control	1.54	1.54	1.56	1.57
Choice Reaction Time	Boysenberry	1.45	1.41	1.43	1.57
	Blackcurrant	1.33	1.37	1.42	1.57
	Control	1.36	1.37	1.45	1.57
One-back Memory	Boysenberry	1.24	1.25	1.25	1.40
	Blackcurrant	1.27	1.28	1.26	1.51
	Control	1.24	1.33	1.34	1.41
Monitoring	Boysenberry	1.54	1.51	1.54	1.26
	Blackcurrant	1.54	1.57	1.55	1.29
	Control	1.55	1.57	1.57	1.30
Matching	Boysenberry	1.05	0.96	1.10	1.15
	Blackcurrant	1.11	1.15	1.15	1.10
	Control	1.07	1.04	1.19	1.20
Learning	Boysenberry	0.72	0.65	0.75	0.69
	Blackcurrant	0.74	0.78	0.75	0.79
	Control	0.72	0.72	0.70	0.73

Table 7: Means (*and standard deviations*) of the parameters of oxidative stress

Parameter	Treatment	Baseline	6 Weeks	12 Weeks	Extension
Oxidised protein (carbonylation)	Boysenberry	0.17 *(0.08)*	0.18 *(0.26)*	0.10 *(0.03)*	0.05
	Blackcurrant	0.20 *(0.13)*	0.17 (0.07)	0.13 *(0.07)*	0.05
	Control	0.18 *(0.13)*	0.13 *(0.06)*	0.11 *(0.05)*	0.05
Oxidized lipid (malondialdehyde)	Boysenberry	17.1 *(8.0)*	11.5 *(7.9)*	9.6 *(8.4)*	3.8*(2.0)*
	Blackcurrant	17.3 *(8.5)*	11.9 *(7.2)*	7.0 *(4.4)*	3.2*(1.5)*
	Control	23.5 *(17.2)*	14.6 *(9.4)*	10.1 *(7.6)*	5.9*(4.7)*
Oxidised DNA (8-OH dG excretion)	Boysenberry	6.9 *(9.2)*	10.2 *(14.3)*	6.7 *(8.4)*	
	Blackcurrant	4.3 *(2.0)*	11.9 *(14.3)*	3.1 *(1.2)*	
	Control	6.7 *(5.2)*	10.3 *(10.0)*	4.1 *(2.3)*	
Plasma ORAC	Boysenberry	13.1 *(1.6)*	14.3 *(2.4)*	14.8 *(1.5)*	14.1 *(2.8)*
	Blackcurrant	13.1 *(2.0)*	13.5 *(2.6)*	15.0 *(2.1)*	14.4 *(3.5)*
	Control	13.4 *(1.5)*	13.7 *(2.3)*	15.3 *(2.2)*	11.3 *(1.9)*
Plasma NP-ORAC	Boysenberry	1.54 *(0.73)*	1.35 *(0.26)*	1.58 *(0.30)*	1.5 *(0.2)*
	Blackcurrant	1.29 *(0.18)*	1.22 *(0.20)*	1.44 *(0.18)*	1.3 *(0.2)*
	Control	1.44 *(0.31)*	1.30 *(0.31)*	1.56 *(0.30)*	1.4 *(0.3)*
DNA fragmentation (Comet Assay)	Boysenberry	32 *(24)*	51 *(62)*	*29* (40)	
	Blackcurrant	25 *(18)*	82 *(104)*	*31* (33)	
	Control	43 *(27)*	37 *(56)*	*18* (15)	
DNA protection (H_2O_2 Comet Assay)	Boysenberry	249 *(60)*	269 *(29)*	*253* (34)	
	Blackcurrant	266 *(64)*	263 *(32)*	*231* (48)	
	Control	235 *(39)*	252 *(44)*	*246* (43)	

Table 8: Means of the parameters of immune function and status of inflammation

Parameter	Treatment	Baseline	6 Weeks	12 Weeks
Cytokine IL-6 (pg/L)	Boysenberry	9.2	9.0	9.4
	Blackcurrant	25.7	34.1	25.7
	Control	27.5	27.9	38.3
Cytokine IL-8 (pg/L)	Boysenberry	16.0	19.1	17.2
	Blackcurrant	22.7	23.1	22.2
	Control	17.6	15.9	18.8
Cytokine IL-10 (pg/L)	Boysenberry	210.3	183.4	114.9
	Blackcurrant	194.6	226.3	213.8
	Control	104.0	105.6	98.2
TNF-alpha (pg/L)	Boysenberry	25.8	25.0	36.2
	Blackcurrant	133.5	94.1	88.8
	Control	67.1	68.7	76.8
IgA (g/L)	Boysenberry	2.86	2.86	2.88
	Blackcurrant	2.66	2.63	2.58
	Control	2.81	2.78	2.65
C-Reactive Protein (mg/L)	Boysenberry	3.08	3.41	2.39
	Blackcurrant	3.53	1.78	1.82
	Control	3.61	2.74	2.57

Table 9: Summary of F-probabilities. Probabilities significant at the 5% level (i.e. P<0.05) are in bold type face. For some analyses the residual plots indicated that the assumptions of the analysis were violated, even after transformation, and F-probabilities are given for these. [Ext] indicates that the F-probability calculations include the 24 week (extension) assessment.

Parameter	Treatment Effect[A]	Time Effect[B]	Treatment/time Effect[C]
Simple Reaction Time			
Speed	0.499 [Ext]	0.296 [Ext]	0.250 [Ext]
Consistency	0.130	0.676	0.595
Accuracy	0.582 [Ext]	0.809 [Ext]	0.861 [Ext]
Choice Reaction Time			
Speed	0.422 [Ext]	**<0.001** [Ext]	0.263 [Ext]
Consistency	0.120	0.339	0.371
Accuracy	0.573 [Ext]	**<0.001** [Ext]	0.323 [Ext]
One-back Memory			
Speed	0.577 [Ext]	**<0.001** [Ext]	0.220 [Ext]
Consistency	0.393	0.638	0.761
Accuracy	0.551 [Ext]	0.020 [Ext]	0.929 [Ext]
Monitoring			
Speed	0.238 [Ext]	**<0.001** [Ext]	0.776 [Ext]
Consistency	0.897	0.064	0.147
Accuracy	0.293 [Ext]	**<0.001** [Ext]	0.994 [Ext]
Matching			
Speed	0.707 [Ext]	0.277 [Ext]	0.506 [Ext]
Consistency	0.271	0.806	0.668
Accuracy	0.821 [Ext]	0.367 [Ext]	0.121 [Ext]
Learning			
Speed	0.760 [Ext]	0.668 [Ext]	0.539 [Ext]
Consistency	0.053	0.170	0.381
Accuracy	0.182 [Ext]	0.839 [Ext]	0.849 [Ext]
Protein Oxidation *(Protein carbonyls)*	0.071 [Ext]	**<0.001** [Ext]	0.778 [Ext]
DNA Damage			
DNA breaks	0.994	**0.006**	0.228
DNA protection	0.454	0.375	0.078
Lipid Peroxidation *(malondialdehyde)*	0.258 [Ext]	**<0.001** [Ext]	0.434 [Ext]
Antioxidant Capacity			
Total ORAC	0.842 [Ext]	0.410 [Ext]	**0.032** [Ext]
Non-protein ORAC	0.053 [Ext]	0.158 [Ext]	0.807 [Ext]
Cytokine IL-8	0.538	0.148	0.791
IgA	0.713	0.279	0.413
C-Reactive Protein	0.569	0.142	0.616

[A] Significance of differences between treatments for all data points
[B] Significance of differences during the time course of study
[C] Significance of differences between treatments during the time course of the study.

To explore the data for oxidative stress further scatter plots were constructed against time for each participant for each treatment. These are shown in Appendix II. These graphs allow a visualisation of the data.

The concentration of plasma protein carbonyls appears to decrease in all treatments including the control. There is a striking reduction in the variation in the population for each treatment particularly for the extension sample time. The concentration of plasma MDA, the marker for lipid peroxidation, also decreases for all treatments, however there appears to be a greater reduction for the two berryfruit treatments compared with the control but these differences were not significant. In the case of 8OHdG, the marker for DNA damage, there are several very high values, however it appears that a number of participants on the Boysenberry treatment increase excretion of 8OHdG. This is similar to observations in some of our previous studies in which rats were treated with a Boysenberry extract. For plasma ORAC there appears to be gradual increase in plasma oxidative capacity for both blackcurrant and boysenberry treatments compared with the control. These differences were statistically significant and indicate that both the blackcurrant and Boysenberry drinks have *in vivo* antioxidant activity.

At the completion of the study each of the participants were asked to complete a questionnaire (Appendix II) to determine if other side effects were generated by the treatments that were not measured directly during the study. The questionnaire also provided study participants with the opportunity to comment on aspects of the treatment drinks such as taste and palatability and general aspects of health. The results from the questionnaire are summarised in Table 10. Generally all treatments were well tolerated by the study participants during the 12 or 24 week study period and no significant side effects of the treatments were observed. However, some negative comments were made for the Boysenberry treatment, which might be due to the greater acidity of the 100% juice formulation compared with the blackcurrant and placebo formulations.

Compliance of the participants was assessed by diary. Each participant was required to enter the time of the daily consumption. Thirty participants indicated 100% compliance, 2 participants 99%, 2 participants 97%, one participant 91%, one participant withdrew due to ill health, and 16 diaries were not returned. For the non-returned diaries six were from the Boysenberry group, three from blackcurrant, and 7 from the placebo group. Apart from this there was no direct measure of compliance. This level of compliance is considered good for the type of study that was undertaken.

Table 10: Results from the questionnaire (Appendix II) completed by participants at the conclusion of the study.

	Boysenberry		**Blackcurrant**		**Placebo**	
Returned	12/17		16/17		10/17	
Comments: flavour	2 pleasant, 2 mediocre, 5 unpleasant		15 pleasant		1 false, 9 enjoyable	
	Yes	***No***	***Yes***	***No***	***Yes***	***No***
Q1 after taste	4	8	3	11	2	8
Q2 ease to drink	8	4	15	1	10	0
Q3 long term	6	6	15	1	9	1
Q4 side effects	1	11	1	15	0	10
Q5 nausea	3	9	1	15	0	10
Q6 belch	0	12	0	16	1	9
Q7 bororygmia	0	12	0	16	0	10
Q8 indigestion	3	9	1	15	0	10
Q9 colic	0	12	0	16	0	10
Q10energy	1	11	2	10	3	7
Q11 hair/nails	2	10	3	13	2	8
Q12 mood	0	12	1	14	1	9
Q13weight	0	12	1	15	0	10
Q14medical	1	11	1	15	0	10
Q15 appetite	1	11	1	15	0	10
Q16 memory	4	8	5	11	2	8
General Comments:						
Positive	less leg cramping, wife says memory better, cuts &scratches healed		good for general health, sight improved		numbers and words	
Negative	dark stools, too sour, staining, unpleasant taste reduced appetite		stained dentures, dark stools, stained bench		sachets leaked - bottles are better, juice stained	

DISCUSSION

This study attempted to determine if daily supplementation with either a blackcurrant or Boysenberry drink by an elderly population with below average memory abilities could improve measures of cognitive performance, the status of oxidative damage and indicators of immune and inflammation. To our knowledge this is the first study in which dietary antioxidants derived from berryfruit have been assessed for ability to enhance cognitive function in a human population. Despite previous evidence of cognitive enhancement in animals, in this study we observed no statistically significant differences in cognitive performance between the placebo control and either of the blackcurrant or Boysenberry treatments for any measures during the 24-week study period. A total of six different cognitive performance indices (simple reaction time, choice reaction time, one-back memory, monitoring, matching; and learning) were assessed for speed of reaction, consistency and accuracy of performance. We also detected no differences in measures of immune function (cytokines, IgA) and inflammation status (hs-CRP) during over the study period. In contrast changes in oxidative damage and protection indicated a reduction in oxidative stress. For some measures of oxidative stress these changes were found for all treatments. However, plasma ORAC showed a statistically significant increase and plasma MDA showed a decrease (not-significant) indicating a reduction of oxidative stress for the Boysenberry and blackcurrant drinks.

Cognitive performance declines as a normal part of ageing but further substantive declines occur in dementia diseases. One theory to explain both the performance declines in normal ageing and dementia disease is that increasing oxidative stress generates oxidative damage to lipid and protein components of the brain. In this study we specifically selected a study population with aged (≥65 yrs) individuals with slightly impaired memory performance but clinically free of symptoms of dementia. The aim of the study was to determine whether supplementation with either Boysenberry or blackcurrant antioxidant drink would reverse the cognitive impairment in these individuals by alleviating oxidative stress through the consumption of antioxidant rich food. Furthermore, since antioxidant vitamins affect immune function (Beharka *et al.*, 1994; Bergman *et al.*, 2004) which in turn can have an effect on cognitive performance, we measured several parameters of immune function and inflammation.

The antioxidant capacities of dietary antioxidants are often determined by *in vitro* measurements, such as the oxygen radical absorbance capacity (ORAC) (Cao *et al.*, 1996; Wang *et al.*, 1996; Wu *et al.*, 2004) and ferric-reducing antioxidant power (FRAP) assays (Gil *et al.*, 2002; Ou *et al.*, 2002). However, although these measures are valid indicators of the antioxidant potential of a dietary substance (Frankel & Meyer, 2000), *in vitro* assays of this type do not provide evidence that a specific substance acts as an *in vivo* antioxidant when consumed. *In vivo* measurement of antioxidant capacity requires confirmation that oxidative stress is actually reduced and such studies are much less common. In this study we investigated 6 different measures of oxidative stress and damage (plasma MDA, protein carbonyls, ORAC, non-protein ORAC, lymphocyte DNA strand breaks (comet assay), and induced DNA protection to treatment with H_2O_2). We found only one statistically significant effect of treatment on oxidative stress with an increase in plasma antioxidant capacity and a trend to lower plasma MDA concentrations (Table 7 & Table 9). However there was also a general trend towards decreased oxidative stress over the time course of the study as indicated by the placebo results. The reason for this trend over time is not known but the changes for each measure reflect an improved antioxidant status. For example, the concentrations of

oxidised protein and lipid both significantly decreased ($P<0.001$ for both). In the case of DNA, the number of strand breaks appeared to decrease significantly. Thus, over the course of the study there was a reduction in oxidative stress. This may have been due to seasonal variation in oxidative stress due to factors such as diet, but such effects have not been reported. Previous studies of this type have used a randomized, cross-over design rather than the parallel and such effects would not have been observed. This is a recognised weakness of the cross-over study design. The placebo-controlled parallel design as used in this study is considered the best design for detecting real changes and placebo effects are frequently encountered. It is also possible that enrolment in the study may have changed awareness and encouraged a change in dietary, or other lifestyle patterns (e.g. exercise), which had a greater effect than the effect induced by the treatments.

A number of other studies have reported reductions of *in vivo* oxidative stress following the consumption of dietary antioxidants. For example a highly enriched anthocyanin extract of *Abies koreana* improved plasma antioxidant capacity in vitamin-E deficient rats and reduced the concentration of hydroperoxides and 8-oxodG in the liver (Ramirez-Tortosa *et al.*, 2001). When oxidative stress was induced in rats by treatment with paraquat, anthocyanins isolated from eggplant and red cabbage were able to provide some protection and reduced the oxidative damage as measured by several indices (Kimura *et al.*, 1999; Igarashi *et al.*, 2000). Furthermore anthocyanin-containing berry fruit incorporated into juice mixtures increased both antioxidant status (plasma TEAC) and reduced oxidative damage (plasma MDA) in human studies (Netzel *et al.*, 2002; Bub *et al.*, 2003). In contrast, a study in which human subjects consumed 750, 1000 or 1500 mL of a blackcurrant/apple juice combination found no change in plasma antioxidant status, a decrease in plasma MDA, and increase in plasma protein oxidation. (Young *et al.*, 1999). A recent intervention study in which an elderly population was given a phenolic-rich dessert composed of grape, cherry, blackberry, blackcurrant and raspberry for two weeks, found no changes in antioxidant status (Ramirez-Tortosa *et al.*, 2004).

In comparison to oxidative stress there appeared to be no effect on any of the markers of immune function or inflammation. Previous *in vitro* studies have shown that flavonoids, the primary antioxidants in the study treatments, can affect functioning of immune cells (Middleton *et al.*, 2000), and potentially could modulate immune function. The immune system is a highly complex and finely regulated system that is essential to health. Effects of flavonoids have been observed of the functions of T-cells, B-cells NK cells macrophages and neutrophils (Middleton *et al.*, 2000). In studies with humans it has been found that two different fruit juice formulations modulated immune function (Bub *et al.*, 2003) and vitamin E had a positive effect on IL-2 production and serum immunoglobulin concentrations (Meydani, 2000). In contrast, a study involving polyphenolics in red wine for a period of two weeks had no effect on the immune system of healthy men (Watzl *et al.*, 2004). In this study, even though there was an effect on oxidative stress, no effect was observed for two measures of immune system functioning (IL-8, IgA).

Previous studies with animals have shown that dietary antioxidants and extracts of fruit have improved measures of cognitive and motor performance. Aged Beagle dogs given a diet fortified with a cocktail of antioxidants showed improved learning accuracy after both one and two years (Milgram *et al.*, 2005). In a further study with α-tocopherol, phenyl-αtert-butylnitrone and ascorbic acid, no effect was seen in avoidance memory but the antioxidant treatments improved water maze learning (Socci *et al.*, 1995). In aged rats, improvements were observed in spatial and motor learning and brain functioning (Joseph *et al.*, 1999; Bickford *et al.*, 2000). In contrast, long-term supplementation with α-tocopherol for two

years did not improve cognitive functioning in moderately demented Alzheimer's disease patients (Sano *et al.*, 1997).

In summary, this study in which an elderly population was given either a blackcurrant, Boysenberry, placebo drink did not show any statistically significant effects (between treatments) for the six measures of cognitive performance. Neither were any changes in immune function observed. However, several of the measures of antioxidant status showed a reduction in oxidative stress during the 12 (or 24-week) period of the study. While some measures of oxidative stress showed no difference compared with the control, other measures did. This indicates that the blackcurrant and Boysenberry drink did function *in vivo* as antioxidants, and do have potential to reduce oxidative stress and associated disease conditions in the human population.

FUTURE RESEARCH

Previous cell and animal-based research has indicated that berry fruit have the potential to reverse deficits in cognitive function and therefore to positively influence age-related neuro-degeneration. This study was designed to rigorously test this hypothesis in an aged, human population with slight memory impairment using blackcurrant and Boysenberry formulations. While some positive effects were observed for the parameters of oxidative stress, we observed no effects on the measures of cognitive performance. Thus this study is evidence that supplementation with blackcurrant or Boysenberry for 6 months has no effect in reversing deficits in cognitive performance in the elderly. An alternative view would be that blackcurrant and Boysenberry may improve cognition in the elderly but this was no shown due to limitations in the design of the study.

The Berryfruit Cognitive Study was developed using the best science and knowledge available from previous research as outlined in the Introduction. The study design is described as : parallel; double blind; and placebo controlled. For a clinical intervention study this design is considered the best available. The study design was evaluated and approved by both the Massey University Human Ethics Committee (PN 04/30) and the Manawatu Human Ethics Committee (04/05/014). The ethics approval process included a review of the study proposal by Dr Garg, Associate Professor of Medicinal Chemistry, The University of Newcastle.

Although the study design was the best available, limitations of the study may have prevented effects from being observed. These include; variability of response in the selected population, the selected population being recalcitrant to the treatments, insufficient statistical power, and insufficient treatment times. These are discussed separately below.

- **Population selection:** Various criteria were used in an attempt to restrict variation in the selected population, these included; excluding participants with a range of diagnosed diseases and conditions, and those consuming supplements. In addition, only participants assessed with below average memory were admitted into the study. Despite these attempts the population had relatively high variance in the measures of cognitive performance and no statistically significant effects of the treatments were observed. Furthermore it may be possible that the population selected was recalcitrant to the treatments provided. The underlying assumption in the selection of elderly people with slight memory impairment was that the deficit(s) causing the impairment

would be reversed by an antioxidant treatment such as berry fruit. This maybe an incorrect hypothesis.

- **Number of participants:** This study appears to be the first of this type in which the effects of berryfruit anthocyanins on cognitive function was determined in a human population and it was not possible to power the current study based on previous research. Consequently the numbers of participants included in the study were determined by comparison with human studies that investigated the reduction of parameters of oxidative stress. During the development of the study this was accepted as a rational way to proceed. However, greater numbers will always provide greater power for statistically determining differences and a larger number of participants may have enabled differences to be detected between treatments and placebo in the current study.
- **Treatment Time:** As already stated, the underlying hypothesis was that treatment with blackcurrant or Boysenberry could reverse the deficits responsible for the reduction in memory of the study participants. The treatment time of 3 months was considered suitable as it was appropriate for the resources available and based on treatment times used for previous rat studies by Joseph et al. A further extension of 3 months was undertaken. However, in retrospect if a reversal of memory deficits can be achieved, a longer treatment period may be required.

Any future study to investigate the effects of blackcurrant and Boysenberry on cognitive function should take the following into account:

1. It may be more appropriate to test the hypothesis that berry fruit **prevents** cognitive decline with aging rather than **reverses** declines that are already evident. This would require a much greater treatment time. Suggest that we implement a 5-10 year prospective study to investigate the health benefits of berryfruit consumption.
2. Consider investigation of short-term (acute) effects of berry fruit on cognitive function rather than long-term effects.
3. Consider the effect in a different population, for example 40-60 year old people.
4. Attempt to further reduce variability in the population and increase study numbers. For example, selecting a population known to suffer from oxidative stress and elevated risk factors for CVD such as smokers, diabetics, and the obese.

ACKNOWLEDGEMENTS

HortResearch acknowledges the contribution of the staff of the Institute for Food, Nutrition, and Human Health, Massey University, Palmerston North to the study. Christine Booth, Mirian Hendriks, and Hilary McKinnon ran the operational aspects of the study. Linley Fray carried out the immune assays and Dr Phil Pearce carried out the assays for hs-CRP and IgA. Dr Patrick Dulin supervised the psychological assessments. The Massey University activities of the study were coordinated by A.Prof. Marlena Kruger.

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APPENDIX I

Neuropsychological Test Results of the

Berryfruit Cognitive Study

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Neuropsychological Test Results of the Berryfruit Cognitive Study

This report addresses the neuropsychological findings of the Cognitive Berryfruit Study, whose aim is to ascertain the physiological and cognitive effects of ingesting Blackcurrant and Boysenberry juice among mildly memory impaired older adults. As a consultant to this study, my involvement revolved around designing and ethically implementing neuropsychological tests that would measure memory and other cognitive functions among the participants. Following is a discussion of the procedures and results of this study.

Procedure:

A double-blind, placebo controlled design with three groups (Blackcurrant, Boysenberry and Placebo) was utilised to test the cognitive effects of berryfruit juice supplementation within a sample of older adults with mild memory impairment. Participants were recruited from the Manawatu area through advertisements in news papers. Inclusion criteria included being over 65 years old and having mild memory impairment. Our screening procedure for mild memory impairment involved administering the Rey Auditory Verbal Learning Test, (RAVLT) to all potential participants and including them if their scores fell at or below the mean. The RAVLT is a widely used test of memory functioning and has adequate normative data for use with older adults. Two post graduate students in the School of Psychology, with specific training in psychological assessment administered and scored the psychological instruments in this study. The initial aim of the study was to obtain 60 participants in total, 20 in each group. Due to time constraints and difficulty attracting enough participants, the final number included in the study was 52. It was noted that approximately 50% of the participants scores fell at or below the mean on the RAVLT, which is to be expected. Two potential participants were screened out of the study due to apparent psychological difficulties (depression and grief) that precluded their participation. Both of these individuals were referred to their physician and encouraged to seek further mental health treatment. It was also

noted that none of the volunteers for the study performed exceptionally poorly on the RAVLT, which indicated that it was unlikely that any of the participants were experiencing a dementing illness.

Participants were then randomly assigned to the three experimental conditions and were given juice to drink daily for 12 weeks. Assessment occurred at baseline, 6 weeks and 12 weeks. Participants were administered the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) at the three time periods. This test was chosen due to it having multiple forms for longitudinal assessment and it's solid psychometric properties for use with an older adult sample. It should be noted that this is perhaps the first study to examine the cognitive effects of berryfruit juice supplementation in a human sample. Therefore, no previous studies were available to guide the choice of specific aspects of cognition to examine. It was deemed appropriate to examine a broad array of cognitive abilities that are reflected in 6 sub-tests of the RBANS; List Learning, Story Memory, Coding, List Recall, Story Recall and Figure Recall. The List Learning sub-test measures immediate verbal memory by asking subjects to recall a list of words four times, each time after having the list read to them again. The List Recall sub test assesses long term verbal memory by asking the participant to recall the list of words after a 15 minute time delay. The Story Memory and Story Recall also measure verbal memory by asking the participant to attend to a story that the examiner reads aloud. The participant's ability to remember the story immediately and after a 15 minute time delay constitutes their scores on the Story Memory and Story Recall sub-tests, respectively. The Coding sub test assesses psychomotor speed and concentration abilities as it is a timed test that asks participants to match numbers with symbols and code them as quickly as possible. The Figure Recall sub test is a measure of visual memory. It asks the participant to draw a complex figure that was presented to them 15 minutes previously.

The same two postgraduate students in the School of Psychology administered the RBANS. I provided instruction to them regarding administering and scoring the RBANS as

well as bi-monthly supervision during the study. The study appeared to have proceeded quite smoothly and no ethical or practical problems were evident. One participant withdrew from the study due to acute health-related issues, which lowered the total number of participants that completed the study to 51. There were 18 participants in the Boysenberry group, 16 participants in the Blackcurrant group, and 17 participants in the Control group.

Results:

Data from this study were analysed with the Statistical Package for the Social Sciences (SPSS) 10.1. Tables 1 and 2 provide a visual display of the means and standard deviations of each sub test across groups and time. On all sub tests, a higher score reflects a better performance on the test. Of particular interest in these tables is the last row for each sub-test, which describes the means of the different groups at 12 weeks. A visual examination of each of the six, 12 week, between subjects rows indicates that the means do not differ greatly from one another. In fact, in 4 out of 6 sub-tests, the placebo group is higher than or equal to the Boysenberry and Blackcurrant groups. The Figure Recall and Story Memory sub tests are the two tests in which the Boysenberry and Blackcurrant groups are higher than the Placebo group, but the differences appear small in light of the standard deviations for both sub tests.

A repeated measures ANOVA was then performed with time as the within subjects factor and group (Blackcurrant, Boysenberry and Placebo) as the between subjects factor for all sub tests of the RBANS. Results of these analyses indicated a similar pattern of results for all sub tests. The Time factors were significant, but the Time X Group and the between subjects factor of Group were not significant. Results from the Story Memory indicated that the Time factor was significant, $F(2,45) = 7.2$, $p<.05$, the Time X Group interaction was not significant, $F(4,92) = .65$, n.s., and the between subjects factor was not significant, $F(2, 46) = .05$, n.s. The List Learning sub test results indicated that the Time factor was significant, $F(2,45) = 7.3$, $p<.05$, the Time X Group interaction was not significant, $F(4,92) = .55$, n.s., and the between subjects factor was not significant, $F(2,46) = .17$, n.s. The Coding sub-test

results were that the Time factor was significant, $F(2,45) = 8.6$, $p<.05$, the Time X Group interaction was not significant, $F(4,92) = 1.01$, n.s., and the between subjects factor was not significant, $F(2,46) = .26$, n.s. Results from the List Recall were again similar. The Time factor was significant, $F(2,45) = 8.1$, $p<.05$, the Time X Group interaction was not significant, $F(4,92) = .60$, n.s., and the between subjects factor was not significant, $F(2,46) = .67$, n.s. The Story Recall evidenced a similar pattern. The Time factor was significant, $F(2,45) = 22.9$, $p<.05$, the Time X Group interaction was not significant, $F(4,92) = .10$, n.s., and the between subjects factor was not significant, $F(2,46) = .67$, n.s. The Figure Recall results indicated that the Time factor was significant , $F(2,45) = 10.0$, $p<.05$, the Time X Group interaction was not significant, $F(4,92) = .82$, n.s., and the between subjects factor was not significant, $F(2,46) = .17$, n.s.

Discussion

This study sought to understand the effects of berryfruit supplementation on cognitive functioning within a sample of older adults with mild memory impairment. The results do not indicate that 12 weeks of berryfruit supplementation enhances cognitive functioning among this sample of older adults. Across all sub tests, there was a noted improvement in performance over time, but the interaction of time and group and the effect of being in the different groups were not significant. It is to be expected that the participants all improved on the tests over time due to practice effects. Even thought this study controlled for the influence of practice effects as much as possible by using alternate forms of the same tests, the effect of knowing what to expect and being able to relax on subsequent administrations of the test likely resulted in better performance over time. The real test of whether or not the berryfruit juices had an effect on cognition lies in the interaction of time and group and the between subjects group factor, which were all not significant. It is noteworthy that the F values were very small and not at all close to being significant in all interactional and between subjects analyses. In fact, it is difficult to discriminate any pattern of effects among the analyses. It

appears that the berryfruit juice supplementation was not effective in enhancing cognitive functioning among this older adult sample with mild memory impairment.

It is possible that the cognition enhancing effects of berryfruit juice supplementation needs more time in order to become evident. Or perhaps a different method of ingestion is necessary. These are both possible avenues for future research.

Table 1: Means and standard deviations of study groups of sub-scales of RBANS

	Boysenberry	Blackcurrant	Placebo
LIST LEARNING Baseline	Mean = 22.79 S.D. = 5.5	Mean =23.6 S.D. = 5.2	Mean =23.1 S.D. =4.1
6 weeks	Mean = 25.1 S.D. = 6.9	Mean =25.4 S.D. =4.5	Mean =24.6 S.D. =3.7
12 weeks	Mean = 23.6 S.D. = 6.5	Mean =25.5 S.D. =4.5	Mean =25.7 S.D. =3.9
STORY MEMORY Baseline	Mean = 12.8 S.D. = 4.7	Mean =12.8 S.D. = 3.9	Mean =13.6 S.D. =4.8
6 weeks	Mean =14.7 S.D. =4.6	Mean =15.3 S.D. =4.1	Mean =16.0 S.D. =2.5
12 weeks	Mean =14.8 S.D. =4.4	Mean =14.2 S.D. =5.9	Mean=13.8 S.D.=4.3
CODING Baseline	Mean = 34.6 S.D. = 9.4	Mean =36.0 S.D. = 10.8	Mean =38.2 S.D. =8.8
6 weeks	Mean=38.4 S.D.=7.2	Mean=35.8 S.D.=6.9	Mean=38.1 S.D.=8.1

12 weeks	Mean=40.2 S.D.=8.7	Mean=38.6 S.D.=9.6	Mean=40.5 S.D.=9.3

Table 2: Means and standard deviations of study groups of sub-scales of RBANS

	Boysenberry	Blackcurrant	Placebo
LIST RECALL Baseline	Mean = 3.7 S.D. = 2.1	Mean = 2.9 S.D. = 2.1	Mean = 3.5 S.D. = 1.3
6 weeks	Mean = 4.1 S.D. = 2.9	Mean = 3.7 S.D. = 2.3	Mean = 3.9 S.D. = 2.3
12 weeks	Mean = 4.4 S.D. = 2.5	Mean = 4.3 S.D. = 1.7	Mean = 4.4 S.D. = 1.7
STORY RECALL Baseline	Mean = 5.7 S.D. = 2.8	Mean = 5.3 S.D. = 3.0	Mean = 6.8 S.D. =2.7
6 weeks	Mean = 8.2 S.D. = 2.9	Mean = 8.1 S.D. = 2.7	Mean = 8.8 S.D. = 2.3
12 weeks	Mean = 7.3 S.D. = 3.3	Mean = 7.1 S.D. = 3.3	Mean= 8.1 S.D.= 1.9
FIGURE RECALL Baseline	Mean = 8.1 S.D. = 3.2	Mean = 9.1 S.D. = 3.2	Mean = 9.2 S.D. = 3.3
6 weeks	Mean= 9.7 S.D.= 3.6	Mean= 10.6 S.D.= 4.9	Mean= 10.1 S.D.= 4.1

12 weeks	Mean= 11.7 S.D.= 4.7	Mean= 11.4 S.D.= 3.5	Mean= 10.8 S.D.= 3.3

APPENDIX II

Berries for Memory – Health Questionnaire

Trial ID:

Trial Juice:

A Taste and after taste

How did you find the flavour of the juice?

Was there an after taste?

B. Ease of drinking

Did you find it easy to drink the juice each day? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the No box above, please provide a written description, if possible.

C. Long term drinking

Do you think you would find it easy to drink the juice each day? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the No box above, please provide a written description, if possible.

D. Side-effects

Question 1.

Did you experience heartburn after drinking the juice? (heartburn is a burning sensation in the stomach, rising to the upper chest and sometimes as far as the throat; it can be accompanied by an acidic taste in the mouth). Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please provide a written description, if possible

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Question 2.

Did you experience any feelings of nausea, and/or vomiting? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state how you experienced these symptoms in the boxes below, if possible.

Mild symptoms < ----------> *Severe symptoms*		
Slight queasy feeling	General nausea	Strong feeling of wanting to (or actually did) vomit

Question 4.

Wind – did you experience any noticeable changes in your belching (or feelings of wanting to belch)? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state how you experienced these symptoms, and also provide a written description, if possible.

Question 5.

Did you experience any borborygmia? (this is unusual rumbling or gurgling sounds in your stomach, and normally occurs shortly after a meal or drink). Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state how you experienced these symptoms, and also provide a written description, if possible.

Question 6.

Did you suffer from indigestion? (indigestion can be defined as a prolonged, steady ache in your stomach following a meal, somewhat like the dull ache of a toothache). Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state how you experienced these symptoms, and also provide a written description, if possible.

Question 7.

Did you suffer from colic at any point? (colic can be defined as a painful, piching sensation in your stomach that comes and goes in waves). Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please how you experienced these symptoms, and also provide a written description, if possible.

E. General well-being

Question 1.

Did you feel that you had any changes in your energy levels (i.e. that you felt noticeably any more or less energetic at any stage)? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state how you experienced these symptoms, and also provide a written description, if possible.

Question 2.

Did you feel that you had any changes in the condition of your hair or nails. Please tick the appropriate box.

NO ☐

YES ☐

Question 3.

Did you notice any marked changes in your mood? (e.g. feelings of happiness, melancholy, optimism or pessimism)? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state how you experienced these symptoms, and also provide a written description, if possible.

Question 4.

Did your weight change noticeably (either up or down)? Please tick appropriate box.

NO ☐

YES ☐

Question 5.

Did you notice any marked changes in any pre-existing medical conditions that you might have (e.g. your hay fever/asthma, if you have this condition)? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state how you experienced these symptoms, and also provide a written description, if possible.

Question 6.

Did you notice any marked changes in your appetite at any point? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state how you experienced these symptoms, and also provide a written description, if possible.

Question 7.

Do you think the juice had an effect on your memory? Please tick the appropriate box.

NO ☐

YES ☐

Can you provide a written description, if possible.

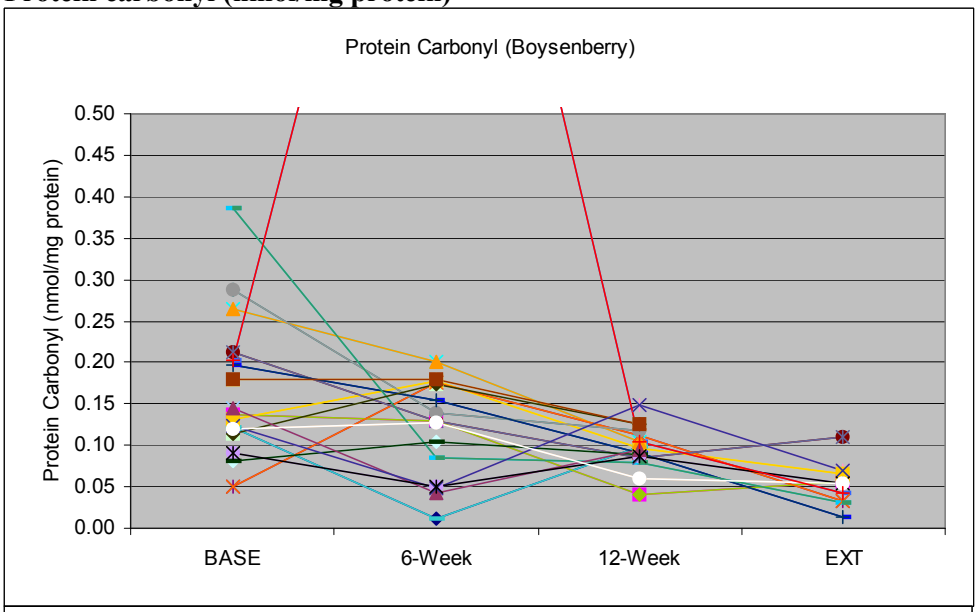
Do you have any other comments?

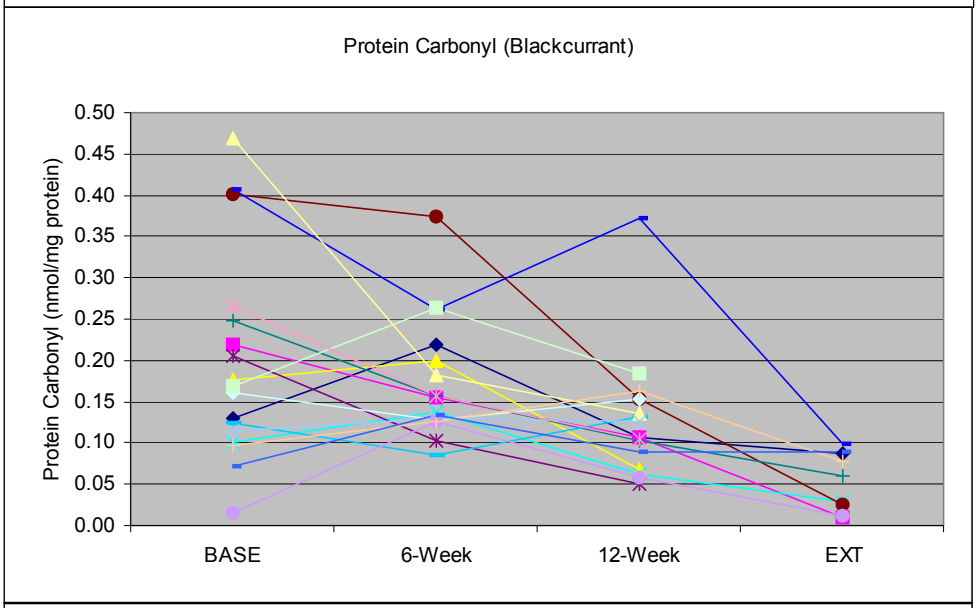
Thank-you for completing this questionnaire

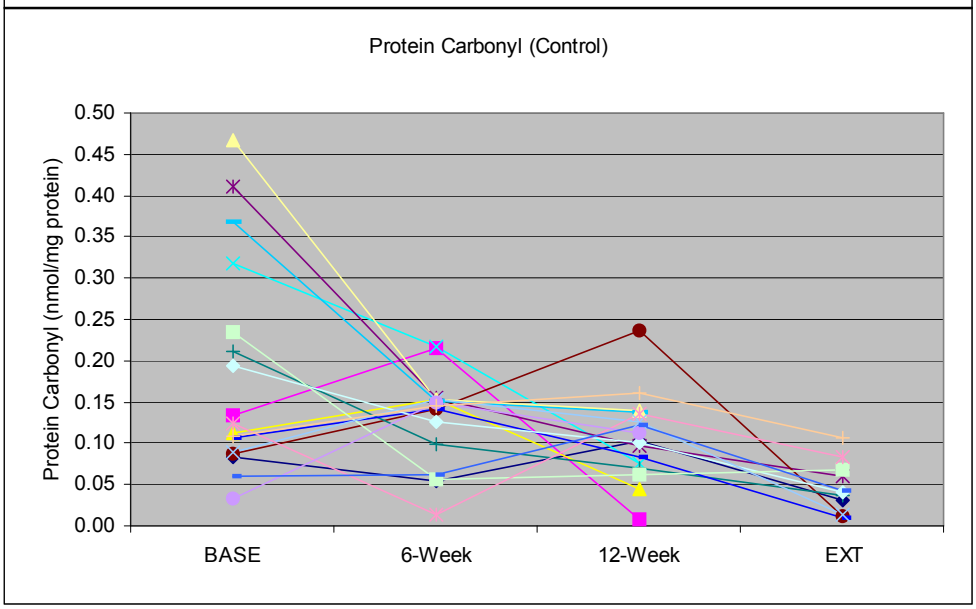
APPENDIX III

Plots of measures of oxidative stress for participants by treatment during the time course of the study.

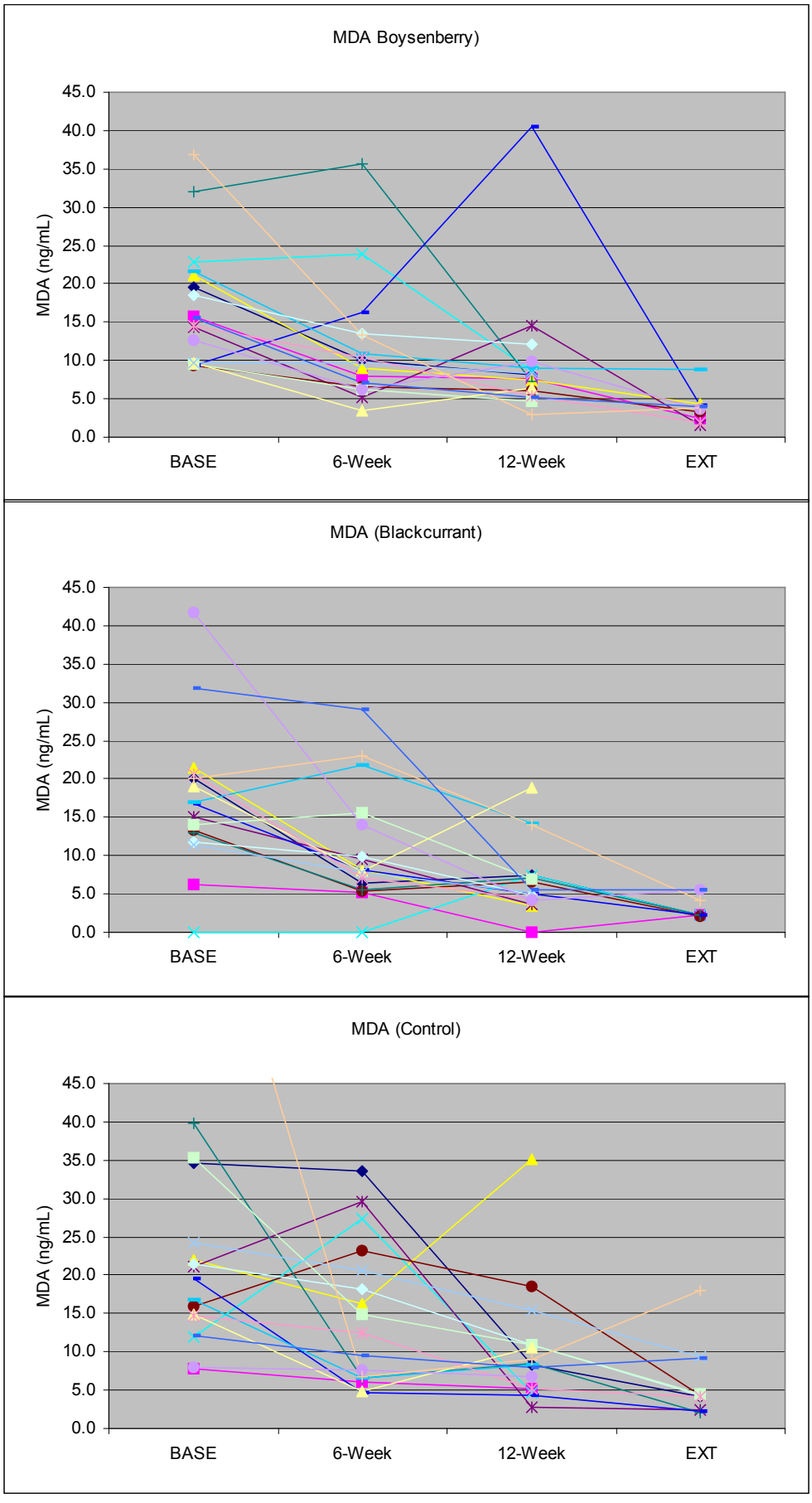
Protein carbonyl (nmol/mg protein)



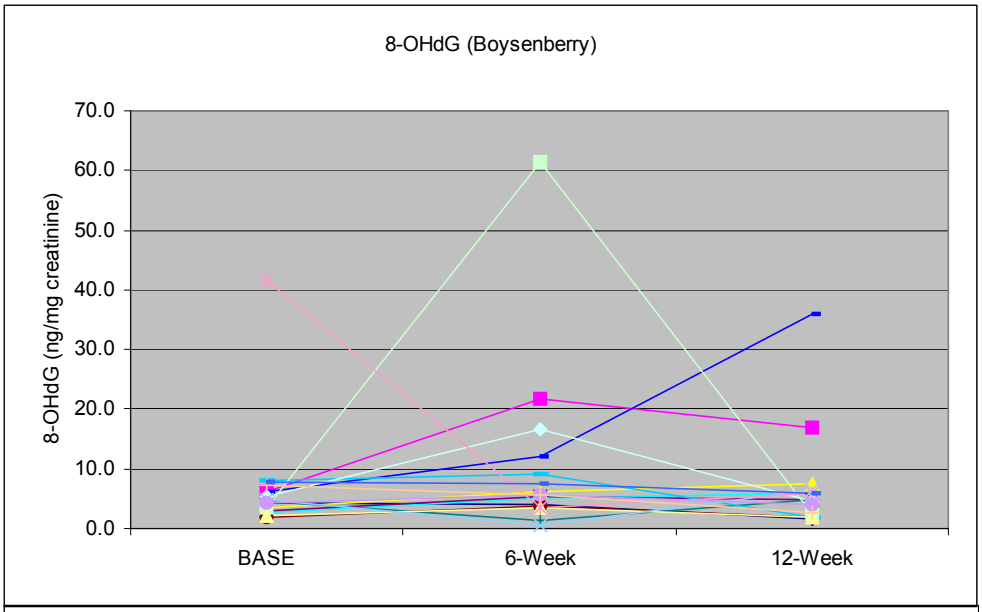


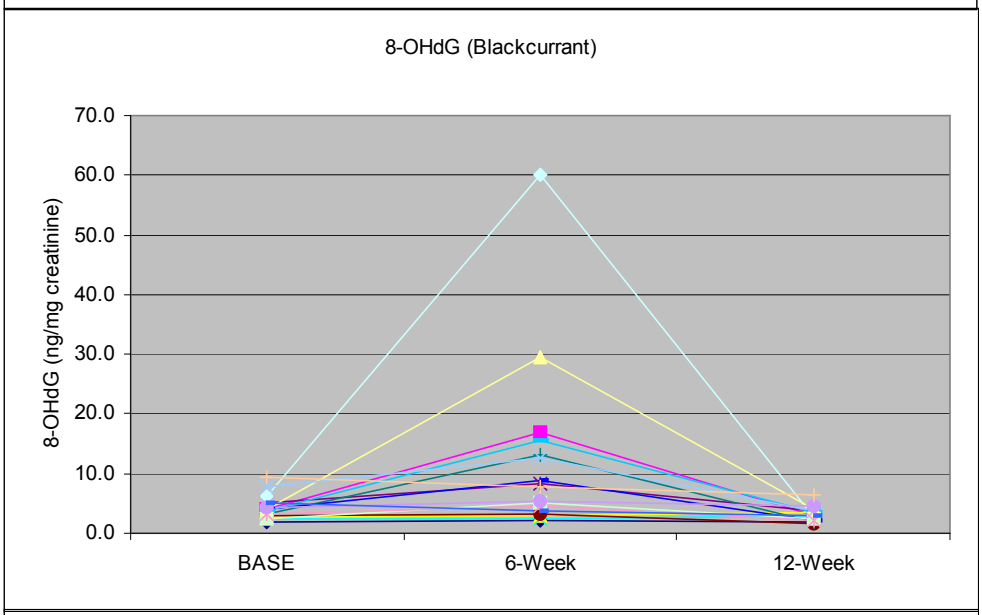


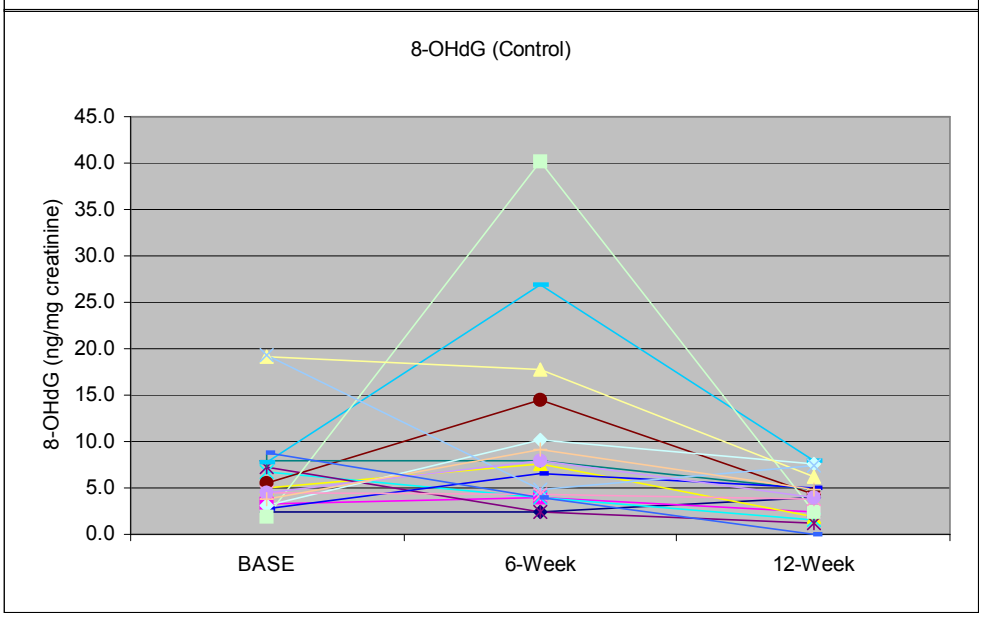
MDA (ng/mL plasma)



8-OHdG (ng/mg creatinine)







ORAC (mmol TE/L)