ASSOCIATION BETWEEN ANTIOXIDANT STATUS AND MnSOD ALA-9VAL POLYMORPHISM IN TRAINED MALE ATHLETES (RUGBY PLAYERS) AND SEDENTARY MALE STUDENTS CONTROLLED FOR ANTIOXIDANT INTAKE

by

Maria Seele

Thesis presented for the partial fulfilment of the requirements for the degree of Master of Physiological Sciences at the University of Stellenbosch

Supervisor: Dr M Senekal

Co-supervisor: Dr NP Steyn

March 2007

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

…….. ……..

Signature Date

SUMMARY

The human body has developed an integrated antioxidant defence system to protect against free radical damage. Acute exercise may result in the increased generation of free radicals, including reactive oxygen species, and this may overwhelm antioxidant defence systems resulting in oxidative stress. However, it has been shown that individuals who undergo regular exercise training may have improved antioxidant capacity when compared to sedentary controls. Results from research regarding the association between antioxidant capacity and exercise training are however not conclusive and further investigation is required. Therefore, the aim of this study was to investigate the association between the total plasma antioxidant status and selected plasma indicators of antioxidant status and the MnSOD Ala-9Val (-28C→T) polymorphism in trained male athletes (rugby players) and sedentary male students while controlling for dietary intake of the major antioxidants using a validated dietary assessment method.

In order to address the potential confounding effect of dietary antioxidant intake on antioxidant status in the main study, a FFQ that measures vitamin C, vitamin E, carotenoid and flavonoid intake was developed. The reproducibility was assessed by the repeat administration of the FFQ (n = 38), while the validity was assessed using a 28-day close-ended dietary record and repeated plasma vitamin C values (n = 18). Several statistical tests were conducted to compare the values obtained from the FFQ with values obtained from the various reference methods. While results from Bland-Altman plots suggested that the reproducibility and validity of FFQ was not completely satisfactory, similar mean values, moderate to strong correlation coefficients, and a high percentage of individuals classified correctly according to quartiles of intake indicated satisfactory reproducibility and validity of the FFQ in assessing antioxidant intake. Furthermore, moderate to strong validity coefficients obtained from the method of triads also indicated satisfactory validity for the FFQ.

The main study involved a cross-sectional study that compared plasma vitamin C and carotenoid levels as well as total plasma antioxidant status in trained rugby players (n = 76) and sedentary male subjects (n = 39) with different MnSOD genotypes, while controlling for dietary antioxidant intake. Rugby players had significantly higher plasma vitamin C and carotenoid levels compared to sedentary students, which indicated more satisfactory plasma antioxidant status. This was also reflected in the tendency for total plasma

antioxidant status (ORAC assay) to be higher in rugby players than sedentary students. MnSOD genotype did not influence plasma vitamin C and carotenoid levels or plasma total antioxidant status, with or without control for dietary antioxidant intake. Dietary vitamin C, vitamin E, carotenoid an flavonoid intake (from foods + supplements) was similar for rugby players and sedentary students and was adequate for both groups. Thus the association between antioxidant status and MnSOD genotype in rugby players and sedentary students seemed not to be influenced by dietary antioxidant intake. In conclusion therefore, rugby players undergoing regular exercise training had a more satisfactory antioxidant status compared to sedentary students. Based on this conclusion, the widespread use of antioxidant supplements by athletes is questioned.

OPSOMMING

Die menslike liggaam beskik oor ‘n geintegreerde antioksidantmeganisme om dit teen vryradikaalskade te beskerm. Akute oefening kan bydra tot ‘n verhoogde produksie van vry radikale, insluitend reaktiewe suurstofspesies, wat kan veroorsaak dat die antioksidantbeskermingsmeganisme oorlaai word, wat dan kan aanleiding gee tot die ontstaan van oksidatiewe stress. Dit is aangetoon dat persone wat gereeld oefening doen verbeterde antioksidantkapasiteit toon in vergelyking met persone wat geen oefening doen nie. Die resultate van navorsingstudies wat die verband tussen antioksidantkapasiteit en oefening ondersoek is egter teenstrydig en verdere navorsing op hierdie gebied is essensieël om uitsluitsel te kry oor kontensieuse vraagstukke. Die doel van hierdie studie was dus om ondersoek in te stel na die verband tussen plasma antioksidant status, die MnSOD Ala-9Val (-28C T) polimorfisme en geselekteerde plasma antioksidantmerkers in geoefende manlike atlete (rugby spelers) en ‘n onaktiewe manlike kontrolegroep terwyl gekontroleer word vir die dieetinname van die vernaamste antioksidante.

Om vir die potensiële invloed van dieetantioksidantinname op die antioksidantstatus van proefpersone in die hoofstudie te kontroleer, is ‘n voedsel frekwensievraelys wat vitamien C-, vitamien E-, karotenoïed- en flavinoïedinname meet, ontwikkel. Die herhaalbaarheid (betroubaarheid) van die vraelys is getoets deur herhaalde voltooiing daarvan deur ‘n toetsgroep (n=38), terwyl die geldighied getoets is deur gebruik te maak van ‘n 28-dag geslote dieetrekord en herhaalde plasma vitamien C bepalings as verwysingswaardes (n=18). Verskeie statistiese toetse is uitgevoer om die frekwensievraelys waardes met die verskillende verwysingswaardes te vergelyk. Alhoewel die Bland-Altman grafieke nie dui op bevredigende herhaalbaarheid en geldigheid van die voedselfrekwensie vraelys nie, dui gelyke gemiddelde waardes, matig tot sterk en betekenisvolle korrelasiekoeffisiënte en ‘n hoë persentasie individue korrek geklassifiseer volgens kwartiele van inname, wel op bevredigende herhaalbaarheid en geldigheid. Matige tot sterk geldigheidskoeffisiënte is ook verkry met die toepassing van “The method of Triads”, wat verdere steun bied vir bevredigende geldigheid.

In die hoofstudie is plasma vitamien C, karotenoïedvlakke en totale plasma antioksidantstatus in manlike rugby spelers (n=76) vergelyk met dié van onaktiewe manlike kontroles (n=39). Vergelykings tussen MnSOD genotipes binne die aktiwiteitsgroepe is ook getref. Al genoemde analises is gekontroleer vir dieet-

antioksidantinname. Resultate dui daarop dat die plasma vitamien C en karotenoïedvlakke van rugby spelers betekenisvol hoër was as dié van die kontrolegroep, wat dui op ‘n meer bevredigende antioksidantstatus. Hierdie resultaat is ook weerspieël in die feit dat totale plasma antioksidantstatus (ORAC) in die rugby spelers oog geneig was om hoër te wees as dié van die kontrole groep. Dit het ook geblyk dat MnSOD genotipe nie ‘n effek gehad het op plasma vitamien C-, karotenoïed- of totale antioksidantstatus nie, met of sonder kontrole vir dieet antioksidantinname. Die dieet vitamien C-, vitamien E-, karotenoïed- en flavinoïedinname (vanaf voedsel en supplemente) was dieselfde vir rugby spelers en kontrole en was toereikend vir beide groepe. Dit blyk dus dat dat die verband tussen antioksidantstatus en MnSOD genotipe in die twee groepe nie beinvloed is deur antioksidantinname nie. Ten slotte kan die gevolgtrekking gemaak word dat manlike rugby spelers ‘n meer bevredigende antioksidant status het as onaktiwe manlike kontroles. Op grond van hierdie gevolgtrekking word die algemene gebruik van antioksidant supplemente deur atlete bevraagteken.

To my parents

ACKNOWLEDGEMENTS

My gratitude and appreciation goes to the following people:

- My family, friends and fellow students for their support
- My supervisor, Dr M Senekal, and co-supervisor, Dr NP Steyn, for their input, advice and guidance
- Dr H Nel for assistance with statistical analyses
- Dr J van Rooyen for liaising with the Maties Rugby Club
- House Committees of the university residences for assistance with recruiting study participants
- Staff of the Maties Rugby Club, especially Alec and Danville Brown, for assistance with recruiting study participants
- Dr P Viviers and staff of Student Health Services for assistance with blood drawing
- Abrie Eksteen for assistance with blood drawing
- Dr T Nell for assistance with blood drawing, laboratory analyses and anthropometric measurements
- All the rugby players and students who volunteered to take part in the study

TABLE OF CONTENTS

LIST OF TABLES **PAGE**

CHAPTER 2

CHAPTER 3

LIST OF FIGURES **PAGE**

CHAPTER 1

CHAPTER 2

CHAPTER 3

ADDENDA **PAGE**

CHAPTER 1

INTRODUCTION

INTRODUCTION

1. Introduction and problem identification

The benefits of regular moderate exercise have been well-documented and include reduced risk of obesity, cardiovascular disease, cancer, osteoporosis and diabetes among others (Astrand, 1992; Durstine & Haskell, 1994; Rippe & Hess, 1998; Powers & Lennon, 1999). However, at another level of exercising, where the focus is not necessarily on health, but on competitive performance, exercise may result in the increased generation of free radicals and reactive oxygen species (ROS) (Davies *et al*., 1982; Ashton *et al*., 1998). These reactive species can attack and cause oxidative damage to a wide variety of biological molecules including proteins, lipids and DNA (Halliwell & Gutteridge, 1999). To protect against free radical attack and subsequent oxidative damage, antioxidant defence systems have developed (Halliwell & Gutteridge, 1999; Benzie, 2000). A disturbance in this pro-oxidant-antioxidant balance in favour of the former, leading to potential damage has been defined as oxidative stress (Sies, 1991). Several, but not all, studies have reported an increase in markers of oxidative stress in response to exercise (Dillard *et al*., 1978; Niess *et al*., 1996; Alessio *et al*., 2000; Lee *et al*., 2002).

However, while acute exercise may result in increased ROS production and oxidative stress, there is increasing evidence that exercise training may enhance the antioxidant defence system (Powers & Lennon, 1999). Studies have shown that the antioxidant system is able to adapt to the increased ROS exposure by upregulating antioxidant enzyme activities and possibly increasing non-enzymatic antioxidant levels (Oberley *et al*., 1987; Ji, 1998; Powers & Sen, 2000). This adaptation of the antioxidant system would thus enable the body to cope with the exercise-induced ROS production and minimise associated oxidative damage (Vollaard *et al*., 2005). Animal studies have generally shown that the activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX) in skeletal muscle improve with regular exercise training (for summary see (Ji, 1998; Powers & Lennon, 1999). Evidence regarding the training-induced adaptation of antioxidant defence systems in humans is based on training intervention studies and cross-sectional studies in which trained individuals are compared to sedentary ones. However, the effect of training on antioxidant enzyme adaptation in humans is not clear. Furthermore, findings from human studies investigating exercise training-induced changes in total antioxidant status and concentrations of individual antioxidants such as

vitamin E and vitamin C are not consistent (Robertson *et al*., 1991; Rokitzki *et al*., 1994; Brites *et al*., 1999; Evelson *et al*., 2002; Cazzola *et al*., 2003).

A variety of factors may contribute to the discrepancies in results, including the markers of oxidative stress and analytical methodology used, the study population characteristics, and the diversity of exercise training protocols. In addition, according to Sen and Goldfarb (2000): “Depending on nutritional habits and genetic disposition susceptibility to oxidative stress may vary from person to person”.

Although there are various analytical methods that can be used to assess oxidative stress, each is associated with difficulties and some of the reported inconsistencies may be attributed to this. Free radicals have a short lifetime, making their direct detection extremely difficult and this approach is thus rarely used (Han *et al*., 2000). ROS attack of proteins, lipids, and DNA results in the formation of unique oxidatively modified biomolecules, which can be used as biomarkers of oxidative stress in *in vivo* studies (Han *et al*., 2000). An increase in these molecules provides strong evidence of oxidative stress in biological systems (Han *et al*., 2000). The determination of antioxidant enzymatic activity or level and the measurement of both individual plasma markers of antioxidant status and total antioxidant status have also been used to indirectly assess oxidative stress. A decrease in these indicators does not necessarily indicate oxidative stress, but does point to a compromised antioxidant defence due to increased production of ROS (Packer, 1997). However, the measurement of markers of oxidative stress is also a difficult task due to the lack of specific and sensitive assays (Jenkins, 2000; Han *et al*., 2000).

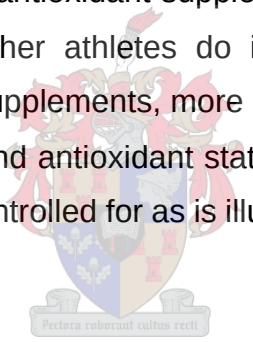
In human studies variations in the characteristics of the study population may contribute to inconsistent findings. Differences in factors such as gender, age, genotype, the type of exercise and the levels and years of training could account for conflicting results regarding exercise-induced ROS production and the subsequent antioxidant system adaptation (Jackson, 2000; Jenkins, 2000). Human studies investigating antioxidant capacity in relation to exercise training have done so employing a wide variety of exercise training protocols and sports, including endurance sports (Robertson *et al*., 1991; Ohno *et al*., 1992), soccer (Brites *et al*., 1999; Cazzola *et al*., 2003), athletics (Watson *et al*., 2005), rugby (Evelson *et al*., 2002), and others (Rokitzki *et al*., 1994). The different types of sports have different energy requirements, oxygen consumption and mechanical stresses on

tissue and which may potentially influence free radical generation and the subsequent antioxidant response (Jackson, 2000).

Genetic variation is an aspect that has, to our knowledge, not been investigated in the context of oxidative stress and antioxidant adaptation associated with exercise training. Human genetic variation is quite common and is largely in the form of single nucleotide polymorphisms (SNP's) (Forsberg *et al*., 2001). In the context of oxidative stress and antioxidant enzymes, many potentially significant genetic variants have been identified and are reviewed in Forsberg (Forsberg *et al*., 2001). Oxidative-stress related genetic variation can be found in for example CuZnSOD, MnSOD, glutathione peroxidase, catalase, glutathione synthase, glutathione reductase and other enzymes (in Forsberg *et al*. (2001). Variations in MnSOD genotype have been investigated in relation to cancer (Ambrosone *et al*., 1999; Mitrunen *et al*., 2001; Woodson *et al*., 2003). The effect of these polymorphic genes on oxidative stress susceptibility and subsequent antioxidant status in general and more specifically in athletes is not clear and requires further investigation in order to determine whether certain individuals may be at an increased risk of oxidative stress.

A further potential confounding factor that has been alluded to is the dietary habits of individuals, specifically the dietary intake of antioxidants. The major dietary antioxidants include vitamin C, vitamin E, carotenoids and flavonoids (Powers & Sen, 2000). Studies have shown that variations in circulating levels of dietary antioxidants generally reflect dietary antioxidant intakes (Block *et al*., 2001; Record *et al*., 2001; Anlasik *et al*., 2005). In addition, some studies have reported that increases in dietary antioxidant intake improves general indicators of plasma antioxidant capacity (Cao *et al*., 1998; Lesgards *et al*., 2002). The improvement in antioxidant capacity and antioxidant levels associated with training may thus be as a result of dietary habits and not necessarily a training-induced adaptation. Therefore, it is necessary to control for the dietary intake of antioxidants when assessing training-induced antioxidant adaptation to eliminate this possibility. Many studies have failed to adequately control/determine dietary antioxidant intake in the exercise training context and this may have been a confounding factor in the findings reported. In order to control for dietary antioxidant intake, dietary intake must be assessed very meticulously. This however is a challenging task due to the fact that available assessment methods, e.g. recall, food frequency questionnaire (FFQ), and records are all associated with specific challenges (Thompson & Byers, 1994; Willett, 1998).

The finding that exercise may increase ROS production, which may result in reduced antioxidant capacity and oxidative damage, has led to the general perception that athletes have increased antioxidant requirements and in order to meet these increased needs should consume antioxidant supplements. This belief is evident from the availability of specialized antioxidant supplement formulations for athletes and the high prevalence of supplement use among athletes. Recent studies have reported prevalence of supplement use among university athletes of above 80% (Froiland *et al*., 2004; Kristiansen *et al*., 2005). The type of dietary supplement used varies, but antioxidant vitamin containing supplements are among the most common types of supplement used (Krumbach *et al*., 1999; Schroder *et al*., 2002; Froiland *et al*., 2004). However, as is evident from the above background information there seems to be no clear evidence at this point in time that athletes actually do have an increased antioxidant requirement and a need for supplements. On the contrary, there is evidence pointing to the fact that athletes may actually adapt to the increased oxidative damage by upregulating antioxidant defence systems and therefore do not need antioxidant supplements, especially if dietary intake is adequate. In order to clarify whether athletes do indeed have a greater antioxidant requirement and need antioxidant supplements, more research is needed that investigates the link between exercise training and antioxidant status in the body as well as genotype, while dietary antioxidant intake is controlled for as is illustrated in Figure 1.



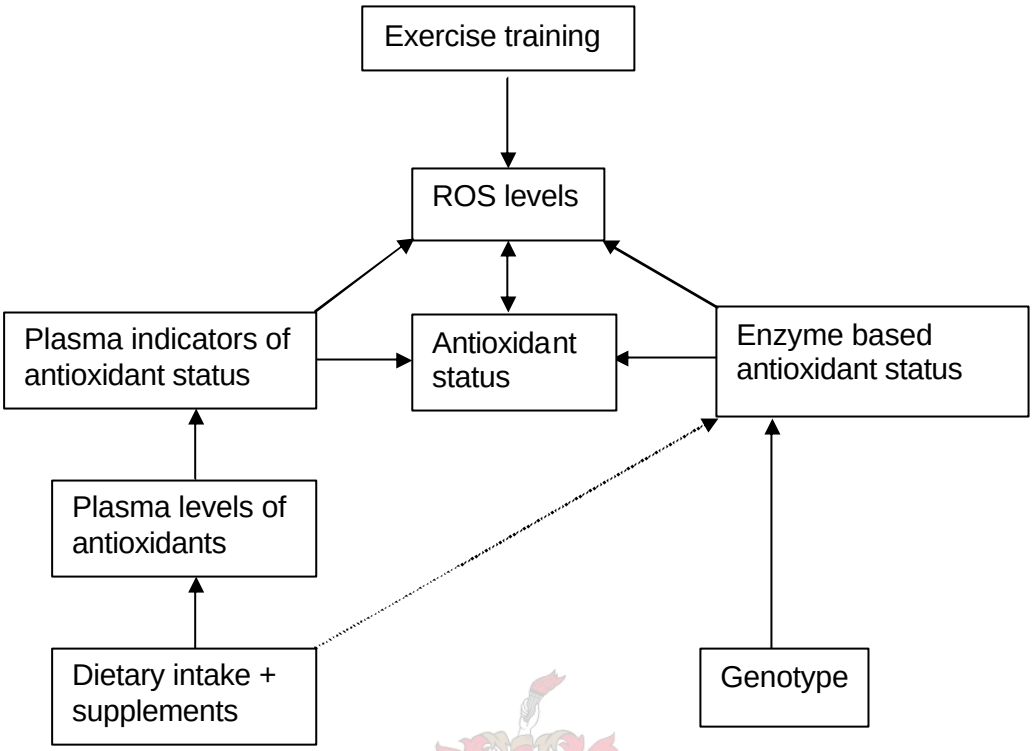


Figure 1: Conceptual framework of the possible interaction between the exercise training, antioxidant status, dietary antioxidant intake and genotype.
ROS = reactive oxygen species.

2. Aim and objectives

The aim of this study was to investigate the association between plasma antioxidant status (total plasma antioxidant status as well selected plasma indicators of antioxidant status) and the MnSOD Ala-9Val (-28C→T) polymorphism in trained male athletes (rugby players) and sedentary male students while controlling for dietary intake of the major antioxidants.

In order to achieve these aims the following objectives were formulated:

Objective 1: To develop and assess the reproducibility and validity of a quantified FFQ that measures the dietary intake of vitamin C, vitamin E, carotenoids and flavonoids

Objective 2: To determine and compare the total intake, including dietary and supplement intake, of the major antioxidants, namely vitamin C, vitamin E , carotenoids and flavonoids of subjects

Objective 3: To assess the plasma total antioxidant status and plasma vitamin C and carotenoid concentrations in subjects

Objective 4: To screen DNA samples of subjects for the MnSOD Ala-9Val polymorphism and determine the association between any specific MnSOD genotype (Ala/Ala = CC, Ala/Val = CT, Val/Val = TT), physical training group (rugby players and sedentary students), and :

- total plasma antioxidant status
- and plasma vitamin C and carotenoid concentration,

while controlling for dietary antioxidant intake

.

3. Outline of the thesis

Chapter 2 of this thesis is an overview of the literature regarding oxidative stress and antioxidant status and the effects of dietary antioxidant intake, exercise and exercise training. The development and validation of a FFQ that measures antioxidant intake is presented in the first article in Chapter 3. The second article is an exploratory study investigating the association between plasma antioxidant status, MnSOD genotype and physical exercise training while controlling for dietary antioxidant intake, and is presented in Chapter 4. A general discussion of the two articles and general conclusions and recommendations are presented in Chapter 5.

The articles in this thesis are referenced according to *The British Journal of Nutrition*.

4. References

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

Ambrosone CB, Freudenheim JL, Thompson PA, *et al* (1999) Manganese superoxide dismutase (MnSOD) genetic polymorphisms, dietary antioxidants, and risk of breast cancer. *Cancer Res* **59,** 602-606

Anlasik T, Sies H, Griffiths HR, Mecocci P, Stahl W & Polidori MC (2005) Dietary habits are major determinants of the plasma antioxidant status in healthy elderly subjects. *Br J Nutr* **94,** 639-642

Ashton T, Rowlands CC, Jones E, Young IS, Jackson SK, Davies B & Peters JR (1998) Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. *Eur J Appl Physiol Occup Physiol* **77,** 498-502

Astrand PO (1992) Physical activity and fitness. *Am J Clin Nutr* **55,** 1231S-1236S

Benzie IF (2000) Evolution of antioxidant defence mechanisms. *Eur J Nutr* **39,** 53-61

Block G, Norkus E, Hudes M, Mandel S & Helzlsouer K (2001) Which plasma antioxidants are most related to fruit and vegetable consumption? *Am J Epidemiol* **154,** 1113-1118

Brites FD, Evelson PA, Christiansen MG, Nicol MF, Basilico MJ, Wikinski RW & Llesuy SF (1999) Soccer players under regular training show oxidative stress but an improved plasma antioxidant status. *Clin Sci (Lond)* **96,** 381-385

Cao G, Booth SL, Sadowski JA & Prior RL (1998) Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *Am J Clin Nutr* **68,** 1081-1087

Cazzola R, Russo-Volpe S, Cervato G & Cestaro B (2003) Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. *Eur J Clin Invest* **33,** 924-930

Davies KJ, Quintanilha AT, Brooks GA & Packer L (1982) Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun* **107,** 1198-1205

Dillard CJ, Litov RE, Savin WM, Dumelin EE & Tappel AL (1978) Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. *J Appl Physiol* **45,** 927-932

Durstine JL & Haskell WL (1994) Effects of exercise training on plasma lipids and lipoproteins. *Exerc Sport Sci Rev* **22,** 477-521

Evelson P, Gambino G, Travacio M, Jaita G, Verona J, Maroncelli C, Wikinski R, Llesuy S & Brites F (2002) Higher antioxidant defences in plasma and low density lipoproteins from rugby players. *Eur J Clin Invest* **32,** 818-825

Forsberg L, de Faire U & Morgenstern R (2001) Oxidative stress, human genetic variation, and disease. *Arch Biochem Biophys* **389,** 84-93

Froiland K, Koszewski W, Hingst J & Kopecky L (2004) Nutritional supplement use among college athletes and their sources of information. *Int J Sport Nutr Exerc Metab* **14,** 104-120

Halliwell B & Gutteridge JMC (1999) *Free Radicals in Biology and Medicine*, 3rd ed. New York: Oxford University Press.

Han D, Loukianoff S & McLaughlin L (2000) Oxidative stress indices: analytical aspects and significance. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 433-483 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Jackson MJ (2000) Exercise and oxygen radical production by muscle. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 57-86 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Jenkins RR (2000) Exercise and oxidative stress methodology: a critique. *Am J Clin Nutr* **72,** 670S-674S

Ji LL (1998) Antioxidant enzyme response to exercise and training in the skeletal muscle. In *Oxidative Stress in Skeletal Muscle*, pp. 103-125 [Reznick AZ, Packer L, Sen CK, Holloszy JO and Jackson MJ, editors]. Basel: Birkhäuser Verlag.

Kristiansen M, Levy-Milne R, Barr S & Flint A (2005) Dietary supplement use by varsity athletes at a Canadian university. *Int J Sport Nutr Exerc Metab* **15,** 195-210

Krumbach CJ, Ellis DR & Driskell JA (1999) A report of vitamin and mineral supplement use among university athletes in a division I institution. *Int J Sport Nutr* **9,** 416-425

Lee J, Goldfarb AH, Rescino MH, Hegde S, Patrick S & Apperson K (2002) Eccentric exercise effect on blood oxidative-stress markers and delayed onset of muscle soreness. *Med Sci Sports Exerc* **34,** 443-448

Lesgards JF, Durand P, Lassarre M, Stocker P, Lesgards G, Lanteaume A, Prost M & Lehucher-Michel MP (2002) Assessment of lifestyle effects on the overall antioxidant capacity of healthy subjects. *Environ Health Perspect* **110,** 479-486

Mitrunen K, Sillanpaa P, Kataja V, Eskelinen M, Kosma VM, Benhamou S, Uusitupa M & Hirvonen A (2001) Association between manganese superoxide dismutase (MnSOD) gene polymorphism and breast cancer risk. *Carcinogenesis* **22,** 827-829

Niess AM, Hartmann A, Grunert-Fuchs M, Poch B & Speit G (1996) DNA damage after exhaustive treadmill running in trained and untrained men. *Int J Sports Med* **17,** 397-403

Oberley LW, St Clair DK, Autor AP & Oberley TD (1987) Increase in manganese superoxide dismutase activity in the mouse heart after X-irradiation. *Arch Biochem Biophys* **254,** 69-80

Ohno H, Yamashita H, Ookawara T, Saitoh D, Wakabayashi K & Taniguchi N (1992) Training effects on concentrations of immunoreactive superoxide dismutase iso-enzymes in human plasma. *Acta Physiol Scand* **146,** 291-292

Packer L (1997) Oxidants, antioxidant nutrients and the athlete. *J Sports Sci* **15,** 353-363

Powers SK & Lennon SL (1999) Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle. *Proc Nutr Soc* **58,** 1025-1033

Powers SK & Sen CK (2000) Physiological antioxidants and exercise training. In *Handbook of oxdidants and antioxidants in exercise*, pp. 221-242 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Record IR, Dreosti IE & McInerney JK (2001) Changes in plasma antioxidant status following consumption of diets high or low in fruit and vegetables or following dietary supplementation with an antioxidant mixture. *Br J Nutr* **85,** 459-464

Rippe JM & Hess S (1998) The role of physical activity in the prevention and management of obesity. *J Am Diet Assoc* **98,** S31-S38

Robertson JD, Maughan RJ, Duthie GG & Morrice PC (1991) Increased blood antioxidant systems of runners in response to training load. *Clin Sci (Lond)* **80,** 611-618

Rokitzki L, Hinkel S, Klemp C, Cufi D & Keul J (1994) Dietary, serum and urine ascorbic acid status in male athletes. *Int J Sports Med* **15,** 435-440

Schroder H, Navarro E, Mora J, Seco J, Torregrosa JM & Tramullas A (2002) The type, amount, frequency and timing of dietary supplement use by elite players in the First Spanish Basketball League. *J Sports Sci* **20,** 353-358

Sen CK & Goldfarb AH (2000) Antioxidants and physical exercise. In *Handbook of oxidants and antioxidants in exercise*, pp. 297-320 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Sies H (1991) *Oxidative Stress II. Oxidants and Antioxidants*. London: Academic Press.

Thompson FE & Byers T (1994) Dietary assessment resource manual. *J Nutr* **124,** 2245S-2317S

Vollaard NB, Shearman JP & Cooper CE (2005) Exercise-induced oxidative stress:myths, realities and physiological relevance. *Sports Med* **35,** 1045-1062

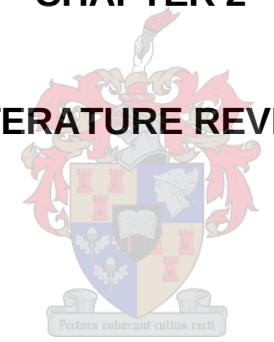
Watson TA, MacDonald-Wicks LK & Garg ML (2005) Oxidative stress and antioxidants in athletes undertaking regular exercise training. *Int J Sport Nutr Exerc Metab* **15,** 131-146

Willett WC (1998) *Nutritional Epidemiology*, 2nd ed. New York: Oxford University Press.

Woodson K, Tangrea JA, Lehman TA, Modali R, Taylor KM, Snyder K, Taylor PR, Virtamo J & Albanes D (2003) Manganese superoxide dismutase (MnSOD) polymorphism, alpha-tocopherol supplementation and prostate cancer risk in the alpha-tocopherol, beta-carotene cancer prevention study (Finland). *Cancer Causes Control* **14,** 513-518

CHAPTER 2

LITERATURE REVIEW



LITERATURE REVIEW

The aim of this chapter is to provide a summary of the literature relating to oxidative stress and antioxidant status in relation to dietary antioxidant intake, exercise and exercise training. This literature review will begin with a brief description of reactive oxygen species and oxidative stress and the assessment thereof and will follow with an overview of antioxidant defence systems. The potential pro-oxidant activity of antioxidants as well as genetics relating to antioxidant defence will be discussed briefly. Thereafter, the influences of dietary antioxidant intake and exercise on oxidative stress and antioxidant status will be discussed. The literature regarding training-induced adaptation of the antioxidant systems will be reviewed in the final section.

1. Reactive molecules in biological systems

1.1. Overview

Free radicals are defined as any species that is capable of independent existence and that contains one or more unpaired electrons (Halliwell & Gutteridge, 1999). Free radicals are conventionally symbolised by a radical dot "•". When a free radical reacts with a molecule that is a non-radical, the molecule becomes a new radical, and this can result in a radical chain reaction as further reactions with non-radicals take place (Halliwell, 1998). As most biological molecules are non-radicals, the generation of reactive radicals in vivo will usually set off a chain of radical reactions. Typically a radical reaction involves three steps: initiation (the formation of free radicals), propagation (the formation of subsequent radicals) and termination (radicals combine with other radicals or are scavenged resulting in a stable form) (Jenkins, 1988). Free radicals can interact with and damage a variety of substrates in the human body including lipids, proteins, DNA and carbohydrates, as was first suggested by Harman in 1956 (Harman, 1956).

Although most of the biologically important free radicals and reactive species are derived from or are associated with molecular oxygen, they are not limited to oxygen species. Reactive oxygen species (ROS) is a collective term that includes oxygen radicals and certain non-radicals that are oxidising agents and/or easily converted into radicals (Halliwell, 1998). Included in this group are superoxide, hydrogen peroxide, hydroxyl, hypochlorous acid, ozone and peroxynitrite (Halliwell & Gutteridge, 1999). Other terms used to describe this group include the term oxygen-derived species, based on the fact that some molecules, for example hydrogen peroxide, are not particularly reactive

(Halliwell, 1998). Another term that has been used is ‘oxidants’, but its use is less popular due to the fact that hydrogen peroxide and superoxide can act as both oxidising and reducing agents in different systems in aqueous solution (Halliwell & Gutteridge, 1999). For the focus of this review reactive oxygen species (ROS) will be used, which includes the oxygen radicals and other oxygen derived non-radical species.

1.2. Reactive oxygen species

1.2.1. ROS chemistry

The production of highly reactive oxygen-containing molecular species in biological systems is a normal consequence of a variety of essential biochemical reactions (Spitzer, 1995). Superoxide ($O_2^{\bullet-}$) is produced by the addition of a single electron to oxygen (Halliwell & Gutteridge, 1999) (see Equation 1). As a result of a spontaneous dismutation reaction, which is catalysed by superoxide dismutase, superoxide will form hydrogen peroxide (H_2O_2) (Halliwell & Gutteridge, 1999) (see Equation 2). Although hydrogen peroxide is less reactive than other oxygen-derived reactive species, it is a biologically important oxidant due to its ability to diffuse considerable distances from its site of production and react with reduced metal ions in the Haber-Weiss reaction (referred to as the Fenton reaction when it is iron catalyzed (see Equation 3) forming the highly reactive and damaging hydroxyl radical ($OH^{\bullet}$) (Halliwell & Gutteridge, 1999). Thus the incomplete reduction of oxygen may result in the formation of superoxide radical, hydrogen peroxide, and hydroxyl radical.

$O_2 + e^- \rightleftharpoons O_2^{\bullet-}$ Equation 1

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

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

1.2.2. Physiological effects of ROS

The production of controlled amounts of ROS may be physiologically useful in various biological processes, including cell signalling and gene expression (Suzuki *et al*., 1997; Allen & Tresini, 2000; Jackson *et al*., 2002). However, the production of these highly reactive molecules may also be harmful as they are able to attack and damage a wide variety of biological molecules including lipids, proteins and DNA. These processes have been associated with various pathophysiological conditions such as the process of ageing and chronic degenerative diseases (Halliwell & Gutteridge, 1999; Beckman & Ames, 2000). There are a variety of complex reactions that can take place when free radicals attack

molecules. The species and site of the oxidants produced, the target molecule, the availability of transition metals and the action of enzymes are factors that determine the fate of radical species and the cellular response (Thomas, 1999; Finkel & Holbrook, 2000).

1.2.2.1. Lipid peroxidation

When free radicals attack lipids, such as fatty acid side chains in membranes and lipoproteins, a self-propagating chain of chemical reactions can be initiated, known as lipid peroxidation (Alessio, 2000). ROS that can initiate and propagate lipid peroxidation include superoxide radical, hydroxyl radical, perhydroxyl radical and the conjugated peroxyl radical (Alessio, 2000). The first step in lipid peroxidation reactions is the formation of a lipid radical. This lipid radical can combine with molecular oxygen to form lipid hydroperoxides, which decompose to form alkoxyl and peroxyl radicals, that can react further and thus propagate oxidative damage (Yu, 1994). Lipid peroxidation may lead to a changed or damaged lipid molecular structure (Alessio, 2000). In the case of membranes, lipid peroxidation may result in altered membrane fluidity and permeability and ultimately impaired membrane function (Tyler, 1975; Chia *et al*., 1983; Yu *et al*., 1992). In addition to the production of harmful radicals, lipid peroxidation is also a source of products such as hydrocarbon gases (e.g. ethane and pentane) and aldehydes (e.g. MDA) that are produced from the decomposition of lipid hydroperoxides (Esterbauer *et al*., 1987). Aldehydes can in turn be harmful due to their carcinogenic, mutagenic and protein cross-linking properties (Basu & Marnett, 1984; Halliwell & Gutteridge, 1999).

1.2.2.2. Protein oxidation

Proteins are a prime target of free radical attack due to their abundance as cell constituents, their complex structure and the numerous oxidizable functional groups of amino acids (Tirosh & Reznick, 2000). Protein oxidation can result in modifications to the secondary and tertiary protein structure, increased susceptibility to proteolytic degradation and can influence essential cell-regulatory processes, causing amongst others receptor modification, intracellular ionic homeostasis disturbance and altered signal transduction ultimately resulting in impaired biological activity (Davies, 1986; Stadtman, 1990; Sen, 2001). Free radical attack may also result in the conversion of amino acid residues to reactive carbonyl derivatives (Levine *et al*., 1990). The accumulation of these derivatives has been linked to a variety of pathophysiological conditions including ageing, Alzheimer's disease, rheumatoid arthritis, atherosclerosis, muscular dystrophy and diabetes (Tirosh & Reznick, 2000).

1.2.2.3. DNA oxidation

DNA damage caused by ROS includes base lesions, single- and double strand breaks, base/nucleotide modifications and DNA-protein crosslinks (Birnboim, 1982; Breen & Murphy, 1995). If these are not repaired or misrepaired, it may lead to gene and/or chromosome mutations, which may then alter gene/protein activity and initiate carcinogenesis (Hartmann & Niess, 2000). Oxidised DNA is abundant in human tissues and besides its role in cancer development, oxidative DNA damage, especially mitochondrial DNA, has been associated with age-related degenerative diseases such as Alzheimer disease, Parkinson disease and aging heart failure (Hartmann & Niess, 2000).

1.2.3. Neutralization of ROS

A variety of processes have evolved in order to eliminate ROS. These include specific channelling of ROS into harmless products by enzymatic diversion or neutralisation, scavenging ROS through the sacrificial interaction with ROS by replaceable or recyclable substrates and quenching of ROS by the absorption of electrons and/or energy (Gutteridge, 1994; Benzie, 2000; Benzie, 2003). These processes involve the action of antioxidants systems, including enzymatic and non-enzymatic antioxidants (Halliwell, 1998). The antioxidant systems are discussed in Section 2 of this literature review.

1.3. Oxidative stress

In order to minimize the risk of damage caused by oxidants, a fine balance must be kept between ROS production and antioxidants. This balance is referred to as antioxidant status (Papas, 1996), and an imbalance in this system is referred to as oxidative stress. Sies (1991) defined oxidative stress as a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage. This imbalance can be as a result of depletion or weakening of the antioxidant defence system or as a result of an excess production of ROS (Halliwell & Gutteridge, 1999). A depressed antioxidant system can be a result of depletion of the endogenous antioxidant system, caused by, for example, mutations affecting the antioxidant enzymes such as MnSOD, CuZnSOD and glutathione peroxidase, and/or depletion of the dietary antioxidants caused by, for example, malnutrition (Halliwell & Gutteridge, 1999). A variety of factors can cause excess ROS production, including increased oxygen exposure, environmental toxins, and excessive activation of “natural” free radical-producing systems, caused by, for example, exercise (Halliwell & Gutteridge, 1999).

1.4. Assessment of oxidative stress in biological systems

Oxidative stress is generally characterised by one or more of the following parameters: an increase in the formation of ROS; a decrease in the levels of low molecular weight, water and/or lipid soluble antioxidants; and an increase in oxidative damage to proteins, lipids and DNA (Han *et al*., 2000). These can be used as indicators in the measurement of oxidative stress in an individual.

1.4.1. Direct assessment of oxidative stress: ROS levels

Currently the only method that can directly detect free radical species is electron paramagnetic resonance (EPR or also known as electron spin resonance, ESR), which is a spectrophotometric technique that relies on the detection of unpaired electrons (Halliwell & Gutteridge, 1999). EPR can be used to quantify free radicals and can also identify the free radical species generated. ROS, particularly free radicals, are highly reactive and have very short lifetime, thus their detection using EPR in biological samples is very difficult (Han *et al*., 2000). Exogenously added traps and probes have been used to overcome this problem (Halliwell & Gutteridge, 1999). These probes or traps react with free radicals to form a relatively stable radical with a relatively long lifetime that can readily be detected and quantitated by EPR as a measure of ROS (Halliwell & Gutteridge, 1999). Many of the traps and probes are toxic and their use in *in vivo* measurements is therefore limited (Halliwell & Gutteridge, 1999). Also, the addition of these molecules to biological systems may disrupt the system being measured (Han *et al*., 2000). Despite these limitations, EPR together with spin traps remains the most useful method of ROS detection and measurement in biological systems (Han *et al*., 2000).

1.4.2. Indirect assessment: Measurement of oxidatively modified biomolecules

Mild oxidative stress can usually be tolerated by cells and often results in the increase in the synthesis of antioxidant defence systems to help protect the cells (Halliwell & Gutteridge, 1999). However, severe oxidative stress may cause lipid peroxidation, protein modification and degradation and DNA damage, which can lead to alterations in membranes and organelle structure and function causing cell and tissue damage and ultimately can even lead to cell death (Reznick *et al*., 1998). Oxidative damage of lipids, proteins and DNA has been shown to result in a wide range of unique break-down products in *in vitro* studies, and these can be used as biomarkers of oxidative stress in in vivo studies (Han *et al*., 2000). An increase in these molecules provides strong evidence of oxidative stress in biological systems (Han *et al*., 2000).

1.4.2.1. Lipid peroxidation by-poducts

Lipid peroxidation by-products that are measured include malondialdehyde (MDA) and other aldehydes, thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LH), and 4-hydroxyalkenals (4-HNE) (Alessio, 2000). Increases in these by-products are directly linked to increased lipid peroxidation rates (Alessio, 2000). Many human studies make use of the TBARS assay, which is thought to reflect the production of MDA, one of the secondary products formed during the oxidation of polyunsaturated fatty acids (Gutteridge & Quinlan, 1983; McCall & Frei, 1999; Alessio, 2000). Although its use has been criticised due to its lack of specificity, it is still commonly because it is inexpensive and easy to perform (Halliwell & Gutteridge, 1999; Han *et al*., 2000). Other assessments have included the measurement of levels of isoprostanes, isoleukotrienes, ethane and pentane (Han *et al*., 2000). Currently, F_2-isoprostanes, which are isomers of prostaglandin F_2 and are produced by peroxidation of arachidonic acid, are being suggested as a reliable index of *in vivo* free radical generation and lipid oxidative damage (Morrow *et al*., 1990; McCall & Frei, 1999).

1.4.2.2. Protein oxidation by-products

ROS induced protein oxidation results in the formation of carbonyls from amino acid residues, which can be used as markers of oxidative damage (Han *et al*., 2000). Similar to the TBARS assay, this assay is also widely used despite its lack of specificity and reproducibility (Cao & Cutler, 1995; Han *et al*., 2000). Other indicators of protein oxidation that have been used include protein thiol/disulfide redox status, oxidized amino acids, nitration of protein-bound tyrosine residues and protein peroxides/hydroxides (Han *et al*., 2000).

1.4.2.3. DNA oxidation by-products

DNA oxidation has been assessed by measuring DNA strand breaks and more commonly products of DNA base oxidation, such as thymidine, glycol and 8-hydroxydeoxyguanosine (Han *et al*., 2000). The measurement of urinary 8-hydroxydeoxyguanosine (8-OHdG) represents a potentially useful measure of whole-body DNA base oxidation in humans and animals, although controversy does exist with regards to its accuracy as an indicator of oxidative stress (Collins *et al*., 1996; Helbock *et al*., 1998; Jackson, 1999). Different methods that have been used to measure 8-hydroxydeoxyguanosine formed by free radicals induced damage have however resulted in a wide discrepancy of values, with

artefacts generated during extraction and derivatization being responsible for most of the discrepancies in results (Ravanat *et al*., 1995; Collins *et al*., 1997).

1.4.3. Indirect assessment: Measurement of antioxidant levels

The third parameter that can be used to indicate oxidative stress is the decrease in levels of antioxidants. Vitamin C, vitamin E and glutathione are commonly used as biomarkers to assess oxidative stress as these tend to decrease during oxidative stress (Han *et al*., 2000). In addition to assays of individual antioxidant levels, assays that measure the total antioxidant status or antioxidant capacity of biological fluids have been developed to assess oxidative damage (Han *et al*., 2000). In these assays, a free radical species is generated by a variety of chemical methods and is subsequently monitored. Biological samples (tissue or blood) or different compounds are then added and the ability of the added compounds to resist oxidative damage or quench the radicals is used to assess its antioxidant capacity. Many different assays are available, the common ones include the ORAC (oxygen radical absorbance capacity) assay, TAC (total antioxidant capacity) assay, the TRAP (total peroxyl radical trapping antioxidant capacity of plasma) assay, the FRAP (ferric-reducing ability of plasma) assay and the TEAC (TROLOX –equivalent antioxidant capacity) assay. It has however been shown that several of the commonly used assays do not correlate well when compared to each other (Cao & Prior, 1998). In addition, assays developed to measure total antioxidant capacity are not always as sensitive as assays used to measure individual antioxidants (Han *et al*., 2000). Although the total antioxidant assay does not indicate which antioxidants are specifically being measured, its strength lies in its ability to provide a quantitative value for the general antioxidant status of biological systems without having to measure each individual antioxidant separately (Han *et al*., 2000). It must be noted however, that the use of individual or total antioxidant levels as biomarkers offers only an indirect measure of oxidative stress that shows that the antioxidant system is working (Halliwell, 1998). Antioxidant depletion does not prove oxidative damage but only points to a compromised antioxidant system (Packer, 1997).

1.4.4. Oxidative stress assessment methods: Conclusion

Due to the complex nature of oxidative stress and the many sources and target molecules of ROS as well as the limitations of various assays that were mentioned above, the measurement of oxidative stress in biological systems is difficult. As such, no marker or group of markers has been established as a standard and there is no single marker that can be used to accurately measure oxidative stress in an organism (Prior *et al*., 2000).

There is no best assay to assess oxidative stress and generally a combination of parameters that characterise oxidative stress should be used in order to provide an accurate picture of oxidative stress (Prior & Cao, 1999; Han *et al*., 2000).

2. Antioxidant defence systems

2.1. Overview of antioxidants

To protect against free radical attack and subsequent oxidative damage, antioxidant defence systems have evolved in aerobic organisms (Benzie, 2000). As mentioned in Section 1.2.3, these systems include both enzymatic and non-enzymatic antioxidants that work as a complex unit to minimize the generation and counter-act the potential oxidative damaging effects of ROS (Benzie, 2000). An antioxidant is defined as "any substance that when present at low concentrations, compared to those of the oxidisable substrate, significantly delays, or inhibits, oxidation of that substrate" (Halliwell & Gutteridge, 1999). In general terms an antioxidant is therefore anything which can prevent or inhibit oxidation of a susceptible substrate (Benzie, 2003).

Within the cell antioxidants protect against oxidative damage at different levels including preventing radical formation, intercepting formed radicals, repairing damage caused by radicals, eliminating damaged molecules and preventing mutations occurring by non-repair-recognition of excessively damaged molecules (Gutteridge, 1994).

The intra- and extra cellular location of the enzymatic and non-enzymatic antioxidant defences are illustrated in Figure 1.

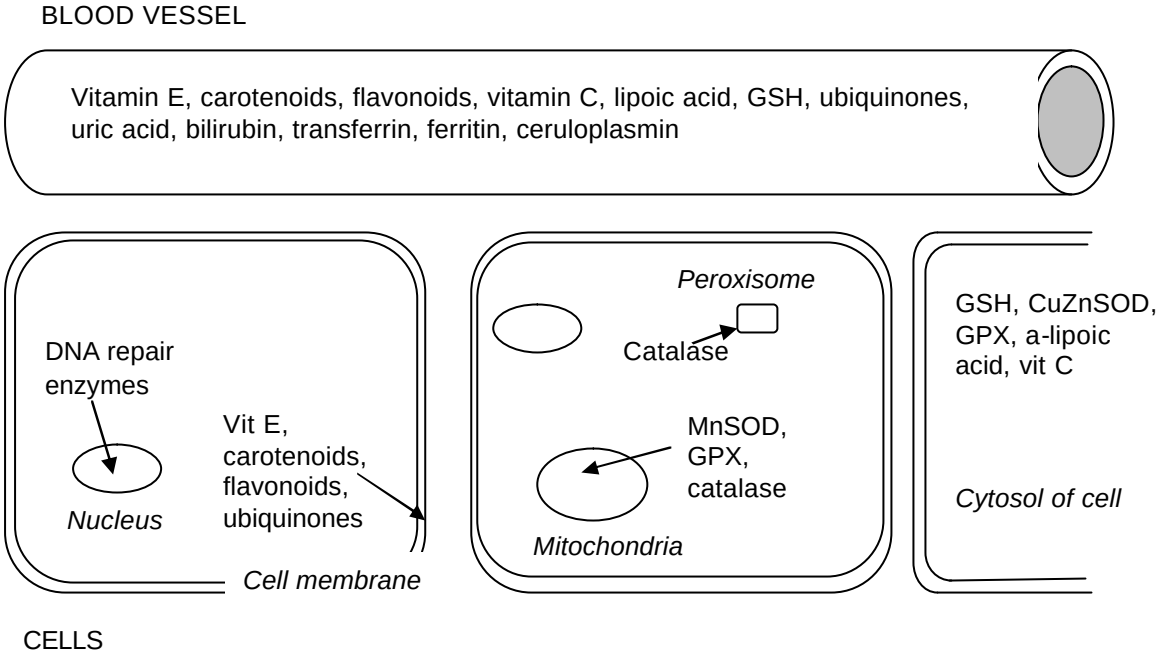


Figure 1: A schematic representation of the locations of the various major enzymatic and non-enzymatic antioxidants.
Vit C = vitamin C, Vit E = vitamin E, GSH = glutathione, MnSOD = manganese superoxide dismutase, CuZnSOD = copper zinc superoxide dismutase, GPX = glutathione peroxidase.
Adapated from Powers and Lennon (1999); Powers and Sen (2000).

2.2. Major enzymatic antioxidants

Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase, catalase, thioredoxin and glutaredoxin. Table 1 summarises the location, properties and antioxidant action of these enzymatic systems in the human body.

Table 1: Cellular location, properties and antioxidant mechanism of the major enzymatic antioxidant enzymes

Enzymatic antioxidant	Cellular location	Properties	Target ROS and antioxidant action
Superoxide dismutase (SOD)	Both in cell cytoplasm (copper-zinc enzyme) and mitochondria (manganese enzyme)	Two isozymes – copper-zinc (CuZn SOD) and manganese (MnSOD) Highest levels found in liver, spleen, kidney and adrenal gland	Catalyzes dismutation of superoxide anion
Catalase	Widely distributed in cell, high concentrations in peroxisomes and mitochondria	Heme protein Greatest activity in liver and erythrocytes	Catalyzes decomposition of hydrogen peroxide
Glutathione peroxidase (GPX)	Cell cytosol, mitochondria and plasma membrane	Selenium dependent Activity dependent on constant availability of reduced glutathione Highest concentration in liver	Catalyzes reduction of hydrogen peroxide or organic hydroperoxides to H2O and alcohol respectively
Thioredoxin (Trx)	Widely distributed in mammalian cells, especially in endoplasmic reticulum	Found in both prokaryotes and eukaryotes	Repairs oxidised sulfhydryl proteins. Removes hydrogen peroxide and radicals.
Glutaredoxin	Widely distributed in mammalian cells	Thiodisulfide oxidoreductase enzyme	Involved in the protection and repair of protein and non-protein thiols under oxidative stress

Sources: Halliwell and Gutteridge (1999); Powers and Sen (2000); Young and Woodside (2001)

Trace minerals such as selenium, copper, iron, manganese and zinc play an indirect but important role in contributing to the effectiveness of specific antioxidant enzymes by acting as co-factors in the enzymes mentioned in Table 1 (Powers *et al*., 2004). Selenium's role as an antioxidant resides in its involvement in the active site of the seleno-enzyme glutathione peroxidase (GSH-PX) (Flohe *et al*., 1973; Rotruck *et al*., 1973). Glutathione peroxidase is one of the major free-radical scavenging enzymes in the antioxidant defence systems and a deficiency in selenium results in depleted GSH-PX thus altering antioxidant defence (Ji *et al*., 1988).

Copper and zinc contribute to antioxidant protection as co-factors for the antioxidant enzyme CuZn superoxide dismutase (CuZnSOD) and a deficiency would result in decreased levels of enzyme activity (Powers *et al*., 2004). Similarly, manganese plays an important role as co-factor in the key antioxidant enzyme manganese superoxide dismutase (MnSOD) in the mitochondria (Halliwell & Gutteridge, 1999). Iron is an essential co-factor in the antioxidant enzyme catalase (Halliwell & Gutteridge, 1999). Deficiencies in the above nutrients could therefore contribute to an impaired antioxidant activity (Powers *et al*., 2004).

2.3. Major non-enzymatic antioxidants

The major non-enzymatic antioxidants include the dietary antioxidants vitamin E, vitamin C, glutathione, carotenoids, flavonoids, α-lipoic acid, and ubiquinones and the non-dietary antioxidants uric acid, and bilirubin (Powers & Sen, 2000; Powers *et al*., 2004). The dietary antioxidants are discussed in greater detail in Section 3. Table 2 summarises the location, properties and antioxidant action of the major non-enzymatic antioxidants in the human body.

Table 2: Cellular location, properties, and antioxidant function of the major non-enzymatic antioxidants

Antioxidant	Cellular location	Properties	Target ROS and antioxidant action
Vitamin C	Located in cytosol Concentration in plasma: 25-80µM	Exists in 2 forms: ascorbic acid and oxidised dehydroascorbic acid form At physiological pH exists as ascorbate anion Water soluble	Directly scavenge wide variety of aqueous-phase ROS Regenerates vitamin E from its oxidised product Can exert pro-oxidant effects at high levels in the presence of transition metals Pro-oxidant activity
Vitamin E	Cell membranes Concentration in plasma: 15-40µM	Most widely distributed antioxidant in nature. Primary chain breaking antioxidant in cell membranes. Lipid-soluble phenolic compound. Occurs in at least eight structural isomers of tocopherols and tocotrienols. ?-tocopherol most potent antioxidant.	Converts superoxide, hydroxyl and lipid peroxyl radicals to less reactive forms. Breaks lipid peroxidation chain reactions by reacting with lipid peroxyl and alkoxyl radicals. Pro-oxidant activity
Carotenoids	Membranes of tissues Concentration in plasma: <1µM	Lipid soluble Pro-oxidant activity Most important is ß-carotene	Scavenge several ROS including singlet oxygen, superoxide radicals and peroxyl radicals. Pro-oxidant activity
Flavonoids and other plant phenols	Throughout cell	Major component of phytochemicals. Amphipathic antioxidants	Scavenge radicals in lipid and aqueous environments Inhibit metal ion-mediated radical formation Inhibit formation of lipid peroxyl radical species Pro-oxidant activity

Table 2 (continued)

Antioxidant	Cellular location	Properties	Target ROS and antioxidant action
Ubiquinone	Relative high levels in heart, liver and kidney. Intracellularly, about 50% in mitochondria, rest in nucleus, endoplasmic reticulum and cytosol. Concentration in plasma 0.4-1.0 µmol/l	Lipid soluble quinone derivatives. Reduced form is efficient antioxidant. Predominant form in humans is ubiquinone-10 (coenzyme Q)	Prevent lipid peroxidation by reacting with oxygen radicals and singlet oxygen. Function in vitamin E recycling.
Glutathione	Located in cytosol and mitochondria	Tripeptide Most abundant non-protein thiol in mammalian cells. Highest levels in lens of eye and liver. Found in both the reduced (GSH) or oxidised (GSSG) state Liver is primary site of GSH synthesis	Interacts with variety of radicals, including hydroxyl and carbon radicals. Removes hydrogen and organic peroxides. Important role in vitamin E and C recycling. Pro-oxidant activity
a-Lipoic acid	Located in both lipid and aqueous phase of cell	Endogenous thiol Unbound form may be effective as an antioxidant.	Reduced form is potent antioxidant against all forms of ROS and can assist in vitamin C recycling.
Uric acid	Intracellular and extracellular antioxidant	By-product of purine metabolism in humans and higher apes.	Scavenges hydroxyl radicals. Preserves plasma ascorbate.
Bilirubin	Intracellular and extracellular antioxidant.	By-product of heme-metabolism. Partially soluble in water. Bound to albumin in human plasma.	Can protect albumin-bound fatty acids from lipid peroxidation.

Sources: Yu (1994); Halliwell (1998); Halliwell and Gutteridge (1999); Powers and Lennon (1999); Powers and Sen (2000)

2.4. Transition metal binding proteins in antioxidant systems

As mentioned in Section 2.2, iron and copper are co-factors for antioxidant enzymes. However, these transition metals also play a key role in the production of hydroxyl radicals via Haber-Weiss reactions in vivo (Stohs & Bagchi, 1995). These reactions only take place in the presence of free metal ions. Once absorbed, metals such as copper are rapidly transported to enzymes requiring them, and only a small amount is stored in the body (Halliwell & Gutteridge, 1999). Thus, in healthy humans, extra cellular fluids have essentially no transition metal ions that can catalyse free radical reactions. However, with regards to iron, extracellular unbound iron may be increased in some cases, such as in iron-overload diseases or where iron intake is very high (as can occur through supplementation) and this free iron is then available to catalyse free radical reactions (Halliwell & Gutteridge, 1999). Transferrin, ferritin, lactoferrin and caeruloplasmin are transition metal binding proteins that sequester free iron and copper in a form that is not available to drive the formation of the hydroxyl radical, thereby playing a crucial role in the antioxidant defence system (Halliwell & Gutteridge, 1999; Young & Woodside, 2001).

2.5. Antioxidant repair systems

As the human antioxidant system is not 100% effective against free radical attack, some damage of lipids, proteins and DNA occurs, which must be dealt with by repair processes (Halliwell, 1998). Such repair processes can therefore be regarded as part of antioxidant defence systems (Halliwell, 1998). Proteins that are damaged, including damage due to oxidative processes, are recognised and degraded by cellular proteases, especially the proteasome (Stadtman, 1992; Berlett & Stadtman, 1997; Halliwell & Gutteridge, 1999). This prevents the build up of altered and damaged proteins in the cell (Halliwell & Gutteridge, 1999). Oxidised lipids can be repaired or removed by various enzymes including phospholipases and glutathione dependent enzyme systems (Pacifici & Davies, 1991). Phopsholipases cleave lipid peroxides from membranes, thus allowing them to be converted to alcohol by glutathione peroxidase (Halliwell & Gutteridge, 1999). Cells are equipped with various enzymes that are able to recognise DNA abnormalities. Enzymes such as endonucleases and glycosylases are able to remove these abnormalities by excision, resynthesis and rejoining of the DNA strands (Halliwell & Gutteridge, 1999). Even though cells are equipped with these repair systems, some oxidative damage may still occur and this has been linked to the process of ageing and chronic degenerative diseases (Halliwell & Gutteridge, 1999; Beckman & Ames, 2000).

2.6. Interaction between antioxidant systems

Antioxidants function as a network to minimise oxidative damage and interactions between vitamin C, vitamin E and glutathione, are evident (Powers & Sen, 2000). For example, the importance of vitamin C as an antioxidant is not only based on its ability to directly scavenge radicals, but also on its ability to recycle vitamin E (Packer *et al*., 1979). When vitamin E reacts with a radical species, a vitamin E radical is formed that can be reduced back to its non-radical form by the interaction with vitamin C. In the process, vitamin C is oxidised to a relatively stable radical form, semidehydroascorbic acid. Semidehydroascorbic acid is reduced back to non-radical vitamin C by NADH semiascorbyl reductase or by cellular thiols such as glutathione and dihydrolipoic acid (Packer *et al*., 1979; Sevanian *et al*., 1985). The reaction of vitamin C and glutathione ensures that the antioxidant functions of vitamin C and E are preserved until there is a significant oxidation (Jones & deLong, 2000). An interaction between selenium and vitamin E has also been observed as selenium can prevent or reduce the severity of several symptoms of vitamin E deficiency in animals (Chow, 2000). Selenium forms an integral part of the glutathione peroxidase enzymes and it complements the antioxidant function of vitamin E via the role of these selenoenzymes in reducing lipid peroxidation (Chow, 2000). Other, as yet non-detected, interactions may also be present.

2.7. Effectiveness of antioxidant systems

The effectiveness and relative importance of antioxidants as protective agents against oxidative damage depends on a variety of factors, including the type of reactive species that is generated, how it is generated, where it is generated and what target of damage is being measured (Halliwell & Gutteridge, 1999). This is illustrated by the following examples: In human blood plasma exposed to oxidising conditions that generate peroxyl radicals, superoxide anion, hydrogen peroxide, hypochlorite, and chloramines, it was found that ascorbate was the most effective antioxidant in preventing lipid peroxidation (Frei *et al*., 1990). In contrast, ascorbate was not effective in decreasing protein oxidation in plasma exposed to gas-phase cigarette smoke (Reznick *et al*., 1992). The transition metal binding proteins, transferrin and ceruloplasmin, are found to be the most important protective agents in inhibiting iron ion-dependent lipid peroxidation in human blood (Gutteridge & Quinlan, 1983). However, lipid peroxidation in cell membranes is most effectively inhibited by the chain-breaking antioxidant vitamin E, probably due to its close association with the polyunsaturated components of the membrane (Thomas, 1999).

2.8. Pro-oxidant activity of antioxidants

What is often disregarded by the promoters of antioxidant supplements, is that some antioxidants including vitamin C, vitamin E, carotenoids, GSH and flavonoids have shown pro-oxidant behaviour *in vitro* (Halliwell & Gutteridge, 1999). The presence of these antioxidants may therefore increase oxidative stress in certain circumstances. The above-mentioned antioxidants are reducing agents (can donate electrons) and are able to reduce Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+} respectively, thus generating transition metal ions that can stimulate free radical generation (Halliwell & Gutteridge, 1999). In equations 4 and 5 below, vitamin C is used as an example to illustrate the pro-oxidant activity of antioxidants (Halliwell & Gutteridge, 1999). Whether these reactions occur *in vivo* and result in oxidative damage is however still unclear; the main point of contention being the actual availability of catalytic metal ions (Carr & Frei, 1999).

Fe(III) + ascorbate ? Fe^{2+} + ascorbate$^{\cdot}$ Equation 4

$Fe^{2+} + H_2O_2$? Fe (III) $OH^{\bullet}$ + OH^{-} Equation 5

2.9. Genetics and antioxidant systems

Variations in genes encoding antioxidant enzymes may also influence their effectiveness in antioxidant systems. Human genetic variation is quite common and is largely in the form of single nucleotide polymorphisms (SNP's), which involve the change of a nucleotide (Forsberg *et al*., 2001). Antioxidant enzyme genes for which polymorphisms have been identified include superoxide dismutase (SOD) (copper.zinc SOD and manganese SOD), glutathione peroxidase, catalase, glutathione synthase, glutathione reductase and are reviewed in Forsberg *et al*., (2001).

One polymorphic enzyme that has been researched in relation to breast and prostate cancer is MnSOD (Ambrosone *et al*., 1999; Mitrunen *et al*., 2001; Woodson *et al*., 2003). The antioxidant enzyme MnSOD is a key enzyme in the antioxidant defence system and provides the first line of defence against ROS produced in the mitochondria. MnSOD is encoded by a single gene, containing 5 exons, located on chromosome 6q25 (Church *et al*., 1992; Wan *et al*., 1994). A single nucleotide polymorphism located at position 16 in the mitochondrial targeting sequence involving an Alanine (GCT) - Valine (GTT) amino acid change has been described (Rosenblum *et al*., 1996)[1]. This polymorphism has been

[1] Note alternative numbering for the MnSOD polymorphism: DNA change: C to T change at base 47 (47C→T); Protein change: Alanine to Valine amino acid change at codon 16 (Ala16Val)

suggested to alter the conformation of the leader signal and thereby affect the import of MnSOD into the mitochondria (Shimoda-Matsubayashi *et al*., 1996). Rosenblum *et al*., (1996) suggested that the MnSOD Ala allele is associated with decreased protection against superoxide radicals in the mitochondria and thus increased oxidative stress, as the transport of the enzyme into the mitochondria, where it is biologically available, seems to be affected. Inefficient targeting of MnSOD could leave the mitochondria without their full defence against superoxide radicals, which could lead to protein oxidation and mitochondrial DNA mutations (Rosenblum *et al*., 1996). Allele frequencies of about 0.5 have been reported for the two alleles in the Caucasian population (Ambrosone *et al*., 1999; Van Landeghem *et al*., 1999). The effect of MnSOD genotype on oxidative stress susceptibility and the influence of it on antioxidant system adaptation in response to exercise training is not clear and requires investigation.

3. Dietary antioxidant intake and antioxidant capacity

3.1. Introductory perspectives

Dietary antioxidants have been defined by the Food and Nutrition Board of the National Institute of Medicine USA as: 'a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species, or both on normal physiological function in humans'. Dietary antioxidants can be classified as those that are essential to health [vitamins A (carotenoids), C and E] and those that are not essential to health but may be beneficial to health (Decker & Clarkson, 2000). These non-essential dietary antioxidants consist of a wide variety of compounds, including flavonoids and other phenolic compounds, ubiquinone, α-lipoic acid and glutathione, which inhibit oxidative reactions by a variety of mechanisms (see Table 2) (Decker & Clarkson, 2000). The sources, bioavailability, and dietary reference intakes of vitamin C, vitamin E, carotenoids and flavonoids are summarised in Table 3.

Table 3: Sources, dietary reference intakes and bioavailability of vitamin C, vitamin E, carotenoids and flavonoids.

Antioxidant	Sources	DRI (mg/day)*	Bioavailability
Vitamin C	Green vegetables, citrus fruits, tomatoes, berries, potatoes	RDA • Males 19-50y: 90 • Females 19-50y: 75 UL • 2000	Dose dependent: 100% at doses < 100mg decreasing to <15% at doses >10g
Vitamin E	Plant oils, green leafy vegetables, nuts, seeds	RDA • Males and females 19-50y: 15 UL • 1000	10-95%, saturable hepatic uptake
Carotenoids	Orange/ yellow/ red fruit and vegetables, green leafy vegetables	NE	Unclear, dose and form dependent (for ß-carotene, 5-50% is absorbed)
Flavonoids	Berries, apples, onions, tea, red wine, citrus fruits, grapes, cherries	NE	Poorly absorbed, depends on form and dose

* DRI obtained from Food and Nutrition Board (2000)
DRI = dietary reference intakes; RDA = recommended dietary allowance; NE = DRI not established
Sources: Decker and Clarkson (2000); Benzie (2003)

3.2. Dietary antioxidant intake, plasma antioxidant levels and plasma antioxidant capacity

As fruit and vegetables represent the main sources of dietary antioxidants (Table 3), researchers have focussed on assessing the effect of fruit and vegetable intake on antioxidant capacity to reflect the effect of dietary antioxidants on markers of antioxidant capacity.

Plasma levels of antioxidants are generally influenced by the dietary antioxidant intake. For example, Block *et al*. (2001) examined the correlation between fruit and vegetable intake measured with an abbreviated food frequency questionnaire and several plasma antioxidants. Both plasma vitamin C and carotenoids were highly significantly associated with frequency of fruit and vegetable consumption. After adjustment for age and energy intake, the correlation between fruit and vegetable intake and vitamin C was 0.64; while

lipid adjusted total carotenoids was 0.44. In a study by Dragsted *et al*. (2004), a diet devoid in fruit and vegetables resulted in significantly lower plasma ascorbic acid and serum ß-carotene levels. Others have also reported significant increases in plasma levels of antioxidants in response to high antioxidant diets (Record *et al*., 2001; Nelson *et al*., 2003; Anlasik *et al*., 2005).

It is important to note that the first level of the assessment of the association between antioxidant intake and antioxidant capacity is the correlation between intake and plasma levels. However, a positive link between these two parameters does not necessarily reflect a positive link between intake and antioxidant capacity, thus the actual functional outcome of the presence of the compound in question. Therefore, an assessment of the actual antioxidant capacity and the link with dietary antioxidant intake should be included to serve as conclusive evidence that increases/decreases of intake and plasma levels can actually be used to predict antioxidant capacity.

The effect of dietary antioxidant intake on total antioxidant capacity and markers of oxidative damage is not clear (see Section 1.4 for detail on indicators of oxidative damage and antioxidant capacity). Studies that support an association between antioxidant intake and antioxidant capacity include the following: Lesgards *et al*. (2002) conducted an assessment of lifestyle effects (including fruit and vegetable intake) on overall antioxidant capacity (assessed using a test based on free radical induced blood hemolysis) in 184 healthy subjects. Subjects with an intake of less than one fruit per day and less than two vegetables per week showed impaired antioxidant capacity compared to those who ate more. Anlasik *et al*. (2005) reported that subjects with high fruit and vegetable diets (≥4 portions/day) had higher plasma antioxidant levels and lower plasma malondialdehyde levels (a biomarker of lipid peroxidation) than subjects with low fruit and vegetable diets (0-1 portion/day). Cao *et al*. (1998) also found that baseline fasting plasma total antioxidant capacity (measured using the ORAC assay) was significantly correlated with estimated daily intake of total antioxidants from fruit and vegetables. In this study subjects had an estimated average intake of five servings of fruit and vegetables per day, and when intake was increased to ten servings per day, the plasma antioxidant capacity increased significantly.

In contrast, Record *et al*. (2001) reported that an increase in fruit and vegetable intake did not improve plasma antioxidant capacity (assay not specified), even though plasma levels

of vitamin C, a- and ß-carotene and lutein+zeaxanthin were increased. In a well-designed 25-day, randomized partly-blinded intervention trial, Dragsted *et al*. (2004) investigated the effects of fruit and vegetable intake and antioxidant supplementation on markers of oxidative stress and antioxidant defence. The markers that were used included plasma vitamin C, plasma carotene and plasma tocopherol, markers of lipid and protein oxidation, antioxidant enzyme activities and total antioxidant capacity (assessed using the TEAC and FRAP assay). In their comparison of a basic diet without fruit and vegetables (placebo group), a fruit and vegetable diet (fruveg group) and a basic diet plus an antioxidant combination supplement (supplement group), they found that plasma vitamin C and ß-carotene were significantly decreased in the placebo group. However, total plasma antioxidant capacity was not significantly affected by any intervention. Erythrocyte antioxidant enzyme activities were not different between treatments, with the exception of GPx, which was significantly higher in the fruveg group compared to the placebo and supplement groups. Biomarkers of plasma lipid peroxidation did not change significantly, whereas plasma protein carbonyl formation (a marker of protein oxidation) was significantly increased in the fruveg and supplement groups compared to placebo group, which the researchers ascribe to a possible pro-oxidant effect of some antioxidant. Therefore, they concluded that within the 25-day study period there was no positive effect of fruit and vegetable intake on plasma antioxidant capacity. Similarly, Nelson *et al*. (2003) reported that a five week intervention with a high carotenoid diet or an antioxidant combination supplement did not change markers of oxidative damage or total antioxidant capacity (assessed using the ORAC assay). Thompson *et al*. (1999) reported a decrease in markers of DNA damage in response to an increase in fruit and vegetable intake. However, markers of lipid peroxidation did not change.

As can be seen from the findings of the studies mentioned, the effect of dietary antioxidants on antioxidant capacity is not conclusive and more research is needed in healthy individuals to determine the effect of antioxidant-rich diets on antioxidant capacity and oxidative stress. A variety of factors may influence the effectiveness of dietary antioxidants in improving antioxidant capacity or decreasing markers of oxidative stress, including the study population, baseline antioxidant levels, and the intervention period studied (Trevisan *et al*., 2001; Galan *et al*., 2005). In addition, the issues of suitability and validity of biomarkers of oxidative damage may contribute to discrepancies in results. Better in vivo markers of oxidative stress need to be developed and used in order to

improve our knowledge of the effect of antioxidants on oxidative stress (McCall & Frei, 1999).

3.3. Antioxidant supplementation and indicators of oxidative stress and antioxidant status

While the possibility cannot be ruled out that some as yet unidentified compound(s) found in fresh products is responsible for the beneficial effect of fruit and vegetable intake, it is generally believed that the healthful effects of fruit and vegetables is a result of the antioxidant vitamins, other compounds with antioxidant activity or most likely from a concerted action of a combination of different antioxidants present in these foods (Cao *et al*., 1998). The latter notion is supported by studies that have shown that the protection against chronic degenerative diseases is much stronger and consistent for foods rich in antioxidants than for single compounds. For example, epidemiological research conducted more than 20 years ago attempted to elucidate the link between fruit and vegetable intake and the decreased risk of degenerative diseases. At that point in time data and statistical analysis pointed to the fact that the benefits were linked to ß-carotene (Peto *et al*., 1981). This resulted in large ß-carotene supplementation trials, including the Alpha-Tocopherol/Beta-Carotene Trial (ATBC) (The ATBC study group, 1994) and the beta Carotene and Retinol Efficiency Trial (CARET) (Omenn *et al*., 1996). The ATBC trial tested ß-carotene and/or vitamin E in 29 133 male smokers in Finland and the CARET tested the combination of ß-carotene and vitamin A compared to a placebo in 18 314 men and women who were at high risk for lung cancer and heart disease in the US. Results from these intervention trials however showed an unexpected increase in lung cancer incidence and mortality with ß-carotene supplementation resulting in termination of the studies. A possible explanation that was given for these unexpected adverse findings was the pro-oxidant activity of ß-carotene resulting in carcinogenic effects (Omenn *et al*., 1996).

Despite this clear indication that supplementation of single biologically active compounds does not necessarily result in the hypothesised health benefits, the belief that antioxidant supplementation could result in a range of health benefits via protection against oxidative stress is still strong. This is also reflected in ongoing research in this regard. However, results from studies investigating the effects of antioxidant supplementation on antioxidant capacity and markers of oxidative stress are still equivocal. The use of dietary antioxidant supplements generally seems to increase plasma levels of antioxidants (Papas, 1996; Block *et al*., 2001; Record *et al*., 2001; Jacob *et al*., 2003; Nelson *et al*., 2003). With

regards to antioxidant capacity, Lesgards *et al*. (2002), found that subjects who reported regular vitamin use had significantly higher blood antioxidant capacity than the non-consumers. No further detail as to the type of supplements that were used was given, other than that they were mainly in the form of vitamins C, E, B and magnesium.

McCall and Frei (1999) conducted a comprehensive review to examine the scientific evidence relating to the ability of antioxidant vitamin supplements to reduce in vivo oxidative damage based on the measurement of biomarkers of oxidative damage. The researchers reported that from the data that was reviewed, it appeared that vitamin E supplementation and perhaps vitamin C could reduce oxidative damage to lipids, whereas supplementation with ß-carotene appeared to have little effect on lipid peroxidation. They therefore concluded that current evidence was insufficient to conclude that antioxidant vitamin supplementation actually reduces oxidative damage in humans.

3.4. Assessment of dietary intake of antioxidants

A challenge in research that is aimed at assessing the link between dietary antioxidant intake and antioxidant capacity is the valid and reliable assessment of dietary intake (Willett, 1998). Dietary intake measurement is based on food intake and the energy and nutrient content of these foods. Therefore, dietary intake assessments are only an indicator of, and not a direct measure of the amount of nutrients that are available for metabolism (Willett, 1998).

3.4.1. Appropriate methodology

Various approaches to measure dietary nutrient intake have been developed, including food frequency questionnaires (FFQ), diet recalls and diet records. Each method is characterised by specific strengths and limitations. The choice of assessment tool therefore depends on several factors including amongst others, the aims of the study (e.g. nutrients, other food constituents, foods, food groups and dietary patterns), the nutrient(s) being assessed, and the subject population (Dwyer, 1999).

Antioxidant nutrient intake demonstrates considerable within- and between-person variability over time. Therefore, in order to accurately estimate intake, the dietary intake assessment tool should capture usual or habitual intake of the antioxidants in question. In addition, antioxidant nutrient intake is contributed to by relatively few food sources (mainly fruit and vegetables). For these reasons, a food frequency questionnaire (FFQ) that

specifically measures these nutrients is recommended as it is most likely to provide the most accurate estimates of usual intake (Willett, 1998).

3.4.2. Development of a FFQ

The FFQ was developed as an alternative to diet records and food recalls to assess long-term dietary intake in large population studies (Horwath, 1990; Willett, 1998). Food frequency questionnaires can be developed to assess the complete dietary intake of individuals or can be designed to only measure intake of selected foods or nutrients. The basic FFQ, known as a qualitative FFQ, consists of a food list and a frequency response section and subjects are required to report their usual frequency of consumption of each food from the food list for a specific time period. Quantitative FFQs contain additional questions regarding usual portion sizes for each food item. Information obtained from the FFQ can then be used to estimate daily nutrient intake by multiplying the food frequency, the portion size and the nutrient content for each food item (Willett, 1998).

Advantages of the FFQ include its ability to assess usual intake, its relatively low respondent burden, and its cost- and time-effectiveness (Horwath, 1990; Thompson & Byers, 1994). The major limitation of the FFQ is that it is not able to measure many details of dietary intake and that the quantification of intake is not as accurate as with dietary records (Thompson & Byers, 1994). Incomplete food lists as well as errors in frequency and portion size estimation may contribute to inaccuracies (Thompson & Byers, 1994). A further source of possible source of error, that is not limited to FFQs, is the nutrient database that is used to calculate nutrient intake (Willett, 1998). An underlying assumption for these calculations is that the nutrient content of a specific food is relatively constant (Willett, 1998). This is not completely correct as foods vary in nutrient content based on various factors including, its size, growing and harvesting conditions, processing, cooking, storage (Willett, 1998). This may contribute to inaccuracies in nutrient calculations. With regards to antioxidants, for some nutrients such as flavonoids, complete food composition databases are not available, which restricts dietary antioxidant intake estimations. Improvements in dietary assessment methods are essential in order to provide conclusive evidence regarding the association between antioxidant intake and antioxidant capacity.

As with any research instrument the reproducibility and validity of a FFQ needs to be determined for application in a particular study.

3.4.3. Reproducibilty of a FFQ

Reproducibility refers to how consistent the measurements obtained from the questionnaire are when the questionnaire is administered to the same person at different times (Willett, 1998). The reproducibilty of a FFQ can be assessed using various statistical tests. It must be noted though, that no single test provides a definite answer on either the reproducibility or validity of a FFQ (Willett, 1998).

While it is useful to compare means and standard deviations for the two methods to give an indication of the average tendency of individuals to over- or underestimate dietary intake, it is more important to give information regarding the associations between the intakes measured by the respective methods (Kaaks *et al*., 1994; Willett, 1998). In the past, various forms of correlation coefficients, including the Pearson's, Spearman rank and intraclass have been used to describe the associations between intakes estimated from the two administrations (Willett, 1998).

However, the appropriateness of the correlation coefficient as a measure of the agreement between two methods, or between administrations, has been questioned (Bland & Altman, 1986). According to Bland and Altman (1986), a high correlation between two measurements, or administrations, does not necessarily imply agreement. They motivate this statement as follows: 1) The correlation coefficient measures the strength of a relation between two variables, not the agreement between them. A correlation is perfect if the data points lie along any straight line, whereas for a perfect agreement, the points lie along the line of equality; 2) a change in scale of measurement does not affect the correlation but affects the agreement; 3) the correlation depends on the range of the true value in the sample, with the correlation being greater for a wide range; 4) the test of significance may show that the two methods are similar, but two methods that are designed to measure the same quantity must be related; and 5) data which seem to be in poor agreement can produce quite high correlations. To address these limitations, these researchers have developed an alternative statistical technique to assess agreement. This method uses the mean and the difference between measurements obtained from either two administrations of an instrument or from two instruments that are being compared. The mean and standard deviation of the differences is used to obtain limits of agreement (LOA), which define the boundaries within which 95% of differences are expected to fall (Bland & Altman, 1986). Bland-Altman plots can be used to show the relationship between the size of the difference and the mean intakes of the two FFQ administrations. If a relationship is present,

this is most often manifested as an increasing between-method difference as the size of the measurement increases and is referred to as proportional bias (Bland & Altman, 1995). This will result in LOA being wider apart than necessary thereby indicating poorer agreement (Bland & Altman, 1986). The Spearman rank correlation coefficient between the mean intakes and the difference in intakes will be significant if proportional bias is present (Bland & Altman, 1995). In these cases, log transformation of the raw data is recommended to remove the relationship, and the analysis can then be applied to the log-transformed data (Bland & Altman, 1986). The LOA will be narrower but because they are on a log scale, it is not possible to relate them back to the original measurement scale, thus limiting the interpretation of the data. The interpretation of the Bland-Altman and the judgement of agreement is based on the following: The width of the LOA, the closeness of the mean difference to 0, the scatter of data and the presence of proportional bias (Bakker *et al*., 2003). Generally, differences that are within the LOA (d±2SD) are in agreement, provided that these differences are not clinically relevant (Bland & Altman, 1986). However, the width of the LOA (i.e. the clinical relevance of the differences) and the scatter around the mean difference line must be judged by eye. Therefore, the decision about whether the methods are in agreement is a subjective one based on the mentioned criteria and does not rely on statistical tests (Bland & Altman, 1999; Bakker *et al*., 2003).

Other statistical tests that have been used to compare measurements obtained from separate administrations include contingency tables (cross-classification) and regression coefficients, both of which have their own problems (Bland & Altman, 1995; Willett, 1998). With regards to the cross-classification, the agreement between administrations is assessed by grouping individuals into quartiles for each of the nutrient intake distributions. The percentage of individuals classified into the same and adjacent quartiles of intake on each administration gives an indication of the agreement between the administrations (Willett, 1998).

To summarize: Reproducibility of a FFQ will be indicated by similar means and standard deviations, correlation coefficients that are significant and moderate to strong, Bland-Altman plots with narrow limits of agreement, mean differences situated around the 0, data points that are scattered around the line of the mean difference, and no proportional bias, and lastly by the ability to classify a high percentage of individuals correctly into quartiles of intake obtained from the two administrations (Bland & Altman, 1986; Willett, 1998).

3.4.4. Validity of a FFQ

Validity refers to how well a dietary assessment method actually measures the aspect of diet that it was designed to measure (Willett, 1998). For the assessment of the validity of a new dietary assessment method (test method), the outcome should be compared to the outcome generated by a method that reflects the true intake of the study population, i.e. a golden standard. However, as no golden standard for determining dietary intake is available, comparisons in validity studies are made with another dietary assessment method that is judged to be superior (reference method) (Willett, 1998). It must, however, be borne in mind that neither method (test or reference) will provide an accurate reflection of the truth and each is associated with different types of measurement error. It is therefore crucial that the errors of both methods are as independent as possible to avoid spuriously high estimates of validity (Horwath, 1990). For FFQ validations, dietary records represent the best comparison method as the two methods have independent sources of error, namely the food record is not restricted by a food list, does not depend on memory and has the ability to accurately estimate frequency of intake and portion size (Horwath, 1990).

For the assessment of agreement between the test and reference methods, the same statistical tests described above for reproducibility testing can be applied in validation studies to compare the measurements obtained from the test method (e.g. a FFQ) with the reference method (e.g. a dietary record). As mentioned previously for reproducibility assessment, no single statistical test can provide an answer regarding the validity of the FFQ. It is therefore recommended that several statistical tests be used to assess validity. The more the various tests point towards satisfactory agreement, the more certain one can be that the validity is satisfactory.

Due to the unavailability of a golden standard in the form of a dietary assessment method, biochemical indicators (e.g. nitrogen, sodium, vitamins, fatty acids) and/or estimates of energy expenditure (e.g. doubly labelled water (DLW), and direct/indirect calorimetry/formulae) have been investigated as independent standards for validation studies (Willett, 1998). The major advantage of using these indicators is that the sources of measurement error are different from those of questionnaire measurements (Kaaks, 1997; Willett, 1998). These indicators are however influenced by a variety of factors that are unrelated to intake, which result in the weakening of correlations between indicator

and questionnaire measurements (Willett, 1998). In addition, suitable indicators are not available for all nutrients, thus limiting their use (Willett, 1998).

In most cases where biochemical indicators or energy expenditure estimates have been used in dietary assessment validation studies, the information is presented as a correlation coefficient between the indicator and the dietary assessment method (Willett, 1998). More recently, structural equation modelling (SEM) has been used to assess the validity of dietary assessment tools. One of these models that have been developed is referred to as 'the method of triads', which makes triangular comparisons between the test questionnaire, reference method (e.g. dietary record) and a biochemical indicator/ energy expenditure estimate (Ocke & Kaaks, 1997). This approach can be used to estimate a validity coefficient (VC), which is the correlation between observed intake and an individual's unknown true intake, for each of the three methods. The higher the VC for a particular method, the more accurately that method estimates true intake. This technique assumes that positive linear correlations between estimates obtained from the dietary assessment methods and true intake exist and that random measurement errors in the methods are mutually independent (Ocke & Kaaks, 1997; Kaaks, 1997).

3.4.5. Dietary assessment of antioxidant intake: Conclusion

As mentioned, there are several challenges that researchers are faced with regarding the assessment of dietary intake. These need to be addressed to obtain conclusive evidence regarding the influence of dietary antioxidant intake on oxidative stress and antioxidant capacity. Such information can then be used to formulate appropriate guidelines and recommendations regarding the intake of antioxidants.

Data regarding reproducibility and validity should be analysed using several statistical tests as "no single method for relating a surrogate measure to a measure of truth conveys all the available information" (Willett, 1998). Rankin and Stokes (1998) also recommend the use of more than one statistical test for evaluating agreement as the judgement regarding the agreement between two methods is a rather subjective one.

4. Exercise and ROS generation

4.1. Overview

Regular physical exercise has been associated with a variety of health benefits including the reduced risk of obesity, coronary artery disease, myocardial infarction stroke and osteoporosis, a reduction in blood pressure, improved glucose tolerance, maintenance of ideal body weight and improved lipid profile (Astrand, 1992; Durstine & Haskell, 1994; Rippe & Hess, 1998; Powers & Lennon, 1999). However, it has been shown that exercise may also result in the increased production of ROS, which may lead to disturbances in muscle redox balance, which may in turn contribute to oxidative injury and muscle fatigue (Powers *et al*., 2004). There are several proposed mechanisms of exercise-induced increases in ROS production, which are briefly discussed in Section 4.2.

4.2. Exercise-induced production of free radicals and ROS

There are a number of potential sources of ROS production during exercise, including primary sources such as mitochondrial activity, cellular enzyme systems, and prostanoid metabolism (Jackson, 2000). A number of other mechanisms have been identified that are potentially responsible for increases in ROS production secondary to damage induced by other processes (Pattwell & Jackson, 2004). These secondary sources include autoxidation of catecholamines, generation of radicals by phagocytic white cells, and disruption of iron-containing proteins (Jackson, 2000). The contributions and significance of these various mechanisms is however not clear and conclusive evidence for the occurrence of some of the proposed mechanisms in an exercise related context is still lacking.

4.2.1. Mitochondrial production of free radicals and ROS (primary source)

During ATP production in the mitochondria, 95-98% of molecular oxygen is reduced to water via electron reduction catalyzed by cytochrome oxidase (Jackson, 2000). However, 2-5% of the oxygen consumed may undergo one electron reduction leading to the formation of the superoxide radical (Jackson, 2000). This in turn can form other harmful ROS such as hydrogen peroxide, which subsequently can leak out of the mitochondria and enter the extracellular fluid where they can generate hydroxyl radicals (Jackson, 2000). During maximal aerobic exercise whole body oxygen consumption increases and in working muscles this may increase by 100-fold compared to resting levels (Keul *et al*., 1972). Therefore, there will be an increase in oxygen flow through the mitochondria and

with it a potential increase in electron flux through the mitochondrial electron transport chain. Theoretically this would then lead to a proportional increase in free radical and ROS production (Jackson, 1998; Powers *et al*., 2004). However, conclusive evidence for this theory is still lacking at this point in time and its role in exercise-induced ROS generation is therefore not clear (Ji, 1999; Vollaard *et al*., 2005).

4.2.2. Xanthine oxidase (primary source)

Another possible mechanism of increased free radical generation during exercise is via the xanthine oxidase pathway during ischemia-reperfusion (McCord, 1985; Jackson, 2000). During ischemia, ATP is broken down to AMP via the adenylate kinase reaction, and is further broken down to hypoxanthine, which is a substrate for xanthine oxidase. Xanthine oxidase is converted from its reduced form xanthine dehydrogenase via activation of a calcium-dependent protease. Using molecular oxygen as an electron acceptor, xanthine oxidase converts hypoxanthine to xanthine (and eventually uric acid) and the superoxide radical (McCord, 1985). Therefore, strenuous exercise performed at or above VO_2max, that leads to muscular ischemia (especially anaerobic or eccentric exercise), and a failure of calcium homeostasis (necessary for activation of the calcium-dependent protease) may result in increased ROS production via the xanthine oxidase pathway, provided that the substrate hypoxanthine is produced in substantial amounts by the exercising muscle (Jackson, 2000). This situation is most likely to occur in very high-intensity, short-duration exercise, such as sprinting, which produces the cellular environment that is necessary for the activation of this pathway (Ji, 1999; Jackson, 2000).

4.2.3. Phagocytic white cells (secondary source)

Exercise that results in skeletal muscle fibre damage can elicit a response that resembles the acute phase immune response to infection, which involves the activation and infiltration of blood-borne neutrophils that are attracted to the area of tissue damage by chemotactic factors (Ji, 1999; Cannon & Blumberg, 2000). As part of the phagocytic process, neutrophils produce substantial amounts of ROS in the NAD(P)H oxidase catalysed oxidative burst reaction (Jackson, 2000; Cannon & Blumberg, 2000). These ROS are essential for the regeneration of tissue by removing damaged proteins and preventing bacterial and viral infection (Jackson, 2000). The production of ROS is however non-specific and can result in damage to surrounding tissues (Jackson, 2000). The ROS produced by this mechanism is therefore not an exercise-induced ROS production, but is a

result of exercise-related damage that causes a secondary increase in ROS in skeletal muscles.

4.2.4. Iron-containing protein disruption (secondary source)

A possible further mechanism for the secondary production of ROS after cellular damage is the disruption of iron-containing proteins. Certain types of exercise (especially eccentric contractions) may cause cellular damage, including erythrocyte destruction and skeletal muscle fibre damage, which may result in the release of amongst other things iron or iron-containing proteins into the circulation (Jackson, 2000). This free iron is then available for free radical production reactions via Fenton chemistry (Haber-Weiss reaction) resulting in further ROS generation.

4.2.5. Other potential primary and secondary sources

Other potential sources of increased free radical production in response to exercise that have been proposed include the following: metal ion-catalyzed oxidation of catecholamines, such as epinephrine, to free radical products (Singal *et al*., 1982); and the metabolism of the arachidonic acid precursor, prostaglandin, which forms free radical intermediates (Jackson, 2000). Evidence for the actual presence of these mechanisms is however lacking and their role in the generation of free radicals during exercise is thus not clear (Jackson, 2000).

5. Effect of exercise on ROS production and oxidative stress

5.1. Overview

While the sources and mechanisms of increased free radical production during exercise remain unclear, the occurrence of exercise-induced oxidative stress is generally accepted. As mentioned in Section 1.4.1, EPR (electron paramagnetic resonance) is the only method that can directly measure free radical production, but due to the difficulties involved it has not been used to a great extent in exercise studies. The majority of studies investigating the effect of exercise on free radical production and oxidative stress have made use of biomarkers of oxidative damage as an indirect assessment of oxidative stress.

5.2. Exercise and free radical production

Davies *et al*. (1982) were the first group to demonstrate that free radical generation was increased in skeletal muscle of rats exercising to exhaustion. Using ESR (electron spin resonance), they reported two- to three-fold increases in free radicals in muscle and liver

tissue following exercise. This technique has also been used by Jackson *et al*. (1985) to show an increase in free radicals in mammalian skeletal muscle following exercise, although they interpreted the increase as a result of a secondary consequence of alternative damaging processes. More recently, Ashton *et al*. (1998) used ESR together with spin trapping to measure free radical species in blood in response to exercise in humans. In their study, using 19 healthy untrained males, exhaustive incremental cycling resulted in an approximately threefold increase in free radical production.

5.3. Effect of exercise on markers of oxidative stress

5.3.1. Oxidatively modified biomolecules

Dillard *et al*. (1978) were the first to report an exercise-induced increase in lipid peroxidation products in humans. Since then a large number of studies have investigated the effect of various types of exercise on a range of lipid peroxidation by-products. Results from these studies are however conflicting. A commonly measured bio-marker of oxidative damage to lipids is MDA, which is normally assessed using the TBARS assay. Several studies have reported increases in MDA after sprint exercise (Marzatico *et al*., 1997), after a 30 min treadmill test at 60% and 90% VO2max (Kanter *et al*., 1993) and after submaximal resistance exercise (Ramel *et al*., 2004). Other by-products of lipid peroxidation that have been shown to increase with exercise include F2-isoprostanes (Child *et al*., 1999; Mastaloudis *et al*., 2001) and conjugated dienes (Marzatico *et al*., 1997). Another marker of lipid peroxidation, namely lipid hydroperoxides, was found to increase after exercise (Alessio *et al*., 2000). In the latter study lipid hydroperoxides were increased whereas MDA showed no change. In contrast, Ashton *et al*. (1998) reported an increase in both lipid hydroperoxides and MDA following exhaustive incremental cycling exercise. Other studies have however reported no change or even decreases in lipid peroxidation by-products as a result of exercise (Duthie *et al*., 1990; Maxwell *et al*., 1993; Rokitzki *et al*., 1994b; Marzatico *et al*., 1997; Quindry *et al*., 2003).

Even though the TBARS assay is widely used, its use in human studies has been criticised due to its non-specificity (Han *et al*., 2000). This may account for some of the discrepancies in the results of exercise induced oxidative stress studies. Therefore, at this stage the evidence for the effect of exercise on lipid peroxidation is inconclusive, but it can be said that some forms of exercise may result in increased lipid peroxidation.

Protein oxidation by-products have also been used to assess exercise induced oxidative stress. Protein carbonyls have been reported to increase after exhaustive aerobic exercise (Alessio *et al*., 2000) and after eccentric exercise in humans (Lee *et al*., 2002). However, the use of protein carbonyls as marker is controversial, and the methods have been criticised as being non-specific and having a lack of reproducibility (Han *et al*., 2000). On the other hand, oxidised amino acids appear to be one of the best methods to assess oxidative stress in biological systems as they are specific markers of oxidative damage (Han *et al*., 2000). However, due to technical difficulties not many studies have measured oxidised amino acids in response to exercise. Orhan *et al*. (2004) used this method and reported increases in urinary excretion of o,ó-dityrosine (a tyrosine oxidation product) following 60 minutes of exercise at 70% VO2max on a cycle ergometer. Similarly, Radak *et al*. (2003) reported increases in urinary and serum nitrotyrosine as well as protein carbonyls during a four day super marathon. These findings suggest that protein oxidation may increase in response to exercise.

Results from studies investigating exercise and DNA oxidative damage are conflicting. Several studies have reported significant increases in DNA strand breaks of leukocyte DNA after exercise of various intensities (Sen *et al*., 1994; Hartmann *et al*., 1995; Niess *et al*., 1996). Measuring oxidised nucleosides or DNA bases excreted in urine is a widely used indicator for oxidative DNA damage, but results from exercise studies report conflicting results. Elevated levels of 8-OHdG were reported after a marathon run (Alessio, 1993) as well as after a training camp (Okamura *et al*., 1997) and after 60 minutes of cycle exercise (Orhan *et al*., 2004). In contrast others have found no significant increase in markers of DNA oxidation after exercise (Viguie *et al*., 1993; Hartmann *et al*., 1998).

Due to the inaccuracy, lack of specificity, or reliability of some of the assays used to measure oxidatively modified biomolecules, it is generally agreed that results from such studies have to be interpreted with caution. Nevertheless, evidence does point to the possibility that some types of exercise may increase free radical production and may result in oxidative damage as indicated by increases in by-products of oxidative processes.

5.3.2. The effect of exercise on antioxidant enzymes

Changes in antioxidant enzymes have also been used to investigate exercise-induced oxidative stress. The antioxidant enzymes that are most commonly measured include

SOD (total SOD, MnSOD and CuZnSOD), catalase, glutathione peroxidase (GPx), with the majority of studies investigating enzyme activity changes.

Marzatico *et al*. (1997) measured SOD, glutathione peroxidase and catalase activity in erythrocytes of highly trained marathon runners after a half marathon and highly trained sprint athletes after a sprint training session. SOD and GPx activity was significantly elevated immediately after exercise in the sprinters, whereas catalase activity was unchanged. SOD and GPx activity returned to baseline values six hours after the sprint exercise. In the marathon runners, only SOD was significantly elevated after the endurance exercise, and returned to baseline values six hours after the marathon. GPx activity remained unchanged and catalase activity was elevated only at 24 and 48 hours after the marathon. In contrast, Tauler *et al*. (1999) reported an increase in erythrocyte catalase activity while SOD activity did not change after a duathlon in endurance athletes. Catalase activity returned to baseline values within an hour after exercise. The authors ascribe the difference in catalase activity to haemolysis that occurred during the exercise, which may change the response pattern of antioxidant enzymes in erythrocytes. Duthie *et al*. (1990) and Rokitzki *et al*. (1994b) reported no change in erythrocyte catalase and GPx after a marathon in trained athletes, while Minami *et al*. (1981) reported that light exercise increased plasma SOD activity. The variable results of these studies indicate that the effect of acute exercise on antioxidant enzyme activity in humans is not clear.

5.3.3. The effect of exercise on non-enzymatic antioxidant compounds and total antioxidant capacity

Levels of non-enzymatic antioxidants have also been used to investigate the effect of exercise on antioxidant capacity. The most commonly measured antioxidants in exercise studies include vitamin E, vitamin C, and glutathione.

Glutathione is found in both the reduced form (GSH) and the oxidised form (GSSG) and the ratio of GSH-GSSG decreases under oxidative conditions (Urso & Clarkson, 2003). Increased levels of GSSG following exercise have been reported by several researchers including (Viguie *et al*., 1993; Tessier *et al*., 1995; Dufaux *et al*., 1997). However, some studies have reported no changes in blood GSH and/or GSSG levels in response to exercise (Camus *et al*., 1994; Lee *et al*., 2002).

Reported changes in circulating vitamin C following exercise are not consistent. Gleeson *et al*. (1987) reported increased plasma and lymphocyte vitamin C immediately after a 21km run, with decreased levels below baseline at 24h post exercise. Mastaloudis *et al*. (2001) reported a similar pattern with plasma vitamin C increasing immediately after a 50km ultramarathon and a decrease to below pre-race levels 24h after the race. Transient increases in plasma vitamin C were reported by Duthie *et al*. (1990) with plasma vitamin C increasing five minutes after a half marathon, and returning to normal 24 hours later. Others have also reported elevated plasma vitamin C after various forms of acute exercise (Viguie *et al*., 1993; Rokitzki *et al*., 1994b; Aguilo *et al*., 2003; Groussard *et al*., 2003). Although it is unclear what causes the exercise-induced increases in plasma vitamin C, possible explanations that have been put forward include an efflux of vitamin C from the adrenal gland in response to cortisol and/or the release of vitamin C from other tissue sites such as erythrocytes or leukocytes (Peake, 2003). In contrast to the studies reporting increased vitamin C levels following exercise, Camus *et al*. (1994) reported that plasma vitamin C was decreased immediately after downhill running with levels returning to baseline 20 min later, while uphill walking did not affect plasma vitamin C. Similarly, Quindry *et al*. (2003) reported decreased plasma vitamin C levels immediately after maximal exercise with a return to baseline levels at one hour and two hours post exercise, whereas submaximal exercise had no effect. Plasma vitamin C levels did not change after submaximal resistance exercise (Ramel *et al*., 2004), immediately after or four days after a marathon run (Liu *et al*., 1999), or after downhill treadmill running (Meydani *et al*., 1993).

Similar to the results of the effect of exercise on vitamin C, vitamin E response to exercise is also inconsistent. While some reported increases in plasma vitamin E in response to exercise (Camus *et al*., 1990; Aguilo *et al*., 2003), others have reported no changes in plasma vitamin E following exercise (Duthie *et al*., 1990; Meydani *et al*., 1993; Viguie *et al*., 1993; Liu *et al*., 1999). Groussard *et al*. (2003) even reported a decrease in plasma vitamin E immediately after an acute exercise test, but values returned to basal levels 20 minutes after exercise. Packer *et al*. (1989) proposed an interorgan transport of vitamin E as a possible reason for exercise-induced changes in vitamin E levels. This may involve vitamin E being exported from the liver and adipose tissue and imported by the muscles and heart. Studies that have shown increases in plasma vitamin E levels following exercise lend support to this theory. However, in view of the discrepant results, the effect of exercise on vitamin E levels remains unclear. In a recent study, Mastaloudis *et al*. (2001) compared the rate of vitamin E disappearance using a deuterium labelled vitamin E

supplement during a 50 km ultramarathon with a sedentary trial. They found an increased rate of vitamin E turnover during endurance exercise compared to the sedentary period providing evidence that exercise results in an increase in vitamin E disappearance. As lipid peroxidation was also increased in response to exercise, the researchers suggested that the increase in oxidative stress resulted in an increase in vitamin E oxidation.

Other antioxidants that have been measured in response to exercise include ß-carotene and uric acid. ß-carotene concentrations have been reported to decrease after exercise by some researchers (Aguilo *et al*., 2003; Groussard *et al*., 2003). In contrast, Ramel *et al*. (2004) and Liu *et al*. (1999) reported no change in plasma ß-carotene levels after submaximal resistance exercise and a marathon run respectively. Exercise appears to transiently increase plasma uric acid (Duthie *et al*., 1990; Liu *et al*., 1999; Mastaloudis *et al*., 2001; Groussard *et al*., 2003). Quindry *et al*. (2003) reported that plasma uric acid decreased immediately after maximal exercise with an increase to above pre exercise values at one and two hours after exercise, whereas submaximal exercise did not change plasma uric acid. The increase in plasma uric acid may be due to enhanced purine oxidation in the muscle (Hellsten *et al*., 1997).

Changes in total antioxidant capacity have also been used to assess oxidative stress in biological fluids following exercise. Various assays that measure total antioxidant capacity have been used in exercise studies including the oxygen radical absorbance capacity (ORAC) assay, the total antioxidant capacity (TAC) assay and the total peroxyl radical trapping antioxidant capacity of plasma (TRAP) assay. Alessio *et al*. (2000) reported that aerobic exercise and to a lesser extent isometric exercise, resulted in improved antioxidant capacity, as measured by the ORAC assay, immediately after the exercise, with values remaining elevated (not significantly) an hour after the exercise in untrained subjects. In contrast, Alessio *et al*. (1997) reported unchanged ORAC levels after 30 min of submaximal exercise. In another study, Child *et al*. (1999) investigated TAC in response to eccentric exercise. While serum TAC did not change in response to exercise, muscle TAC was elevated on day four and seven after eccentric exercise in untrained but active subjects. Serum TAC was, however, elevated after a simulated half marathon in trained subjects (Child *et al*., 1998). Ashton *et al*. (1998) reported that serum TAC did not change significantly following exhaustive aerobic exercise despite increases in free radicals (measured by ESR) and plasma lipid peroxidation markers. Using the TRAP assay, Liu *et al*. (1999) reported higher values both immediately and four days after a marathon

compared to baseline values in trained subjects, indicating an improved antioxidant capacity.

These results thus indicate that acute exercise may result in changes in antioxidant levels and may result in changes in antioxidant status. However, the direction of change (increases or decreases in response to exercise) is not consistent as can be seen from the variable results reported in the above-mentioned studies.

5.4. Antioxidant supplementation and exercise induced oxidative stress

The finding that exercise may increase oxidative stress, has led to a number of studies investigating the effects of antioxidant supplementation on oxidative stress markers. In theory, antioxidant supplementation should protect against the negative consequences of exercise-induced generation of ROS (Viitala & Newhouse, 2004). However, human studies investigating the effects of antioxidant supplementation on exercise-induced markers of oxidative stress are not conclusive.

The most common antioxidant supplements tested include vitamin E, vitamin C and antioxidant combination supplements. In a critical review of studies investigating the effect of vitamin E supplementation on exercise-induced markers of lipid peroxidation in humans, Viitala and Newhouse (2004) concluded that vitamin E supplementation alone does not appear to decrease markers of lipid peroxidation following exercise. In addition, McAnulty *et al*. (2005) reported a pro-oxidant effect of prolonged large dose vitamin E supplementation in highly trained athletes during exhaustive exercise. Results of vitamin C supplementation and exercise-induced oxidative stress in humans are inconsistent, with some reporting a positive effect (Ashton *et al*., 1999; Close *et al*., 2006) and others reporting no effect on markers of oxidative damage after exercise (Maxwell *et al*., 1993; Alessio *et al*., 1997).

Due to the synergistic nature of antioxidant functioning, several studies have investigated the effect of antioxidant combination supplements on oxidative stress markers following exercise. Antioxidant combination supplements that have been investigated typically contain two or more of the following antioxidants: vitamin C, vitamin E, ß-carotene, selenium and glutathione. While some studies report increased tissue antioxidant levels after supplementation and an attenuation of the oxidative stress response to exercise (Kanter *et al*., 1993; Goldfarb *et al*., 2005; Bloomer *et al*., 2006; Tauler *et al*., 2006), others

reported that antioxidant supplementation did not change markers of oxidative stress (Rokitzki *et al*., 1994b; Bryant *et al*., 2003). Differences in antioxidant combinations, duration of supplementation and dosages amongst other factors make comparison between studies difficult and as such no clear conclusion can be drawn as to the effect of combination supplements on exercise-induced oxidative stress.

5.5. Exercise-induced oxidative stress: Conclusion

Due to the lack of consistent results, no definitive conclusion can be drawn at this stage as to the effect of exercise on oxidative stress and antioxidant capacity. However, it does seem that some, but not all exercise may cause an increase in the generation of ROS and may result in oxidative stress as indicated by an increase in biomarkers of oxidative damage. Thus, more well-designed studies are needed in order to determine the effect of exercise on oxidative stress and the possible role of antioxidant supplementation in preventing exercise-induced oxidative stress.

Various factors could contribute to some of the discrepancies found in the results of studies investigating exercise-induced oxidative stress. These include the following: A lack of reliable and valid biomarkers and methodological difficulties of assays may account for some differences in results (Jenkins, 2000). Furthermore exercise may result in plasma volume changes, which may consequently modify plasma molecule concentrations measured, including antioxidant concentrations (Meydani *et al*., 1993). The lack of controlling for plasma volume changes may have been the reason for significant changes reported in some studies (Camus *et al*., 1990; Ashton *et al*., 1998). The timing of the biomarker measured (i.e. the sampling of the biomarker in relation to the exercise test) is also critical and inconsistent measuring times may account for some discrepancies (Marzatico *et al*., 1997; Viitala & Newhouse, 2004). The type, duration and intensity of exercise used may play a role in the exercise-induced production of ROS and consequential oxidative stress (Marzatico *et al*., 1997; Jackson, 2000; Aguilo *et al*., 2003; Quindry *et al*., 2003). In addition, factors relating to the subjects used, including age, nutritional status, gender, genotype and training status could potentially influence the findings of different studies (Sen, 2001; Ginsburg *et al*., 2001; Aguilo *et al*., 2003; Vollaard *et al*., 2005). The influence of some of these factors has not been thoroughly investigated and their contribution to the discrepancies in results is still unclear. Furthermore, Balog *et al*. (2006) report seasonal variations in lipid peroxidation markers (TBARS) and antioxidant enzyme activities in both trained and untrained men and women.

Based on the inconsistent findings of antioxidant supplementation studies and exercise-induced oxidative stress, there is to date not enough conclusive evidence to recommend antioxidant supplements to athletes or persons who exercise regularly (Sharpe, 1999; Clarkson & Thompson, 2000; ADA, 2000; Powers *et al*., 2004; Williams *et al*., 2006).

6. Training and antioxidant system adaptation

Cellular levels of oxygen use are correlated with the rates of free radical production and tissues with the highest rates of resting oxygen consumption show the greatest antioxidant enzyme activity (Powers & Lennon, 1999). Similarly, highly oxidative muscles show greater antioxidant capacity than muscles with lower oxidative capacity (Powers & Lennon, 1999). Thus, antioxidant defence capacity seems to match the rate of radical production. Furthermore, it has been shown that antioxidant defence systems are capable of adapting to chronic exposure to oxidants (Oberley *et al*., 1987).Therefore, since exercise may increase the production of ROS, repeated exercise exposure during chronic training can be expected to upregulate the antioxidant systems in order to protect against potential oxidative damage (Ji, 1998).

Findings from animal studies provide convincing evidence that endurance exercise training results in increased SOD and GPX activity in skeletal muscle (for comprehensive summary in this regard see Ji (1998); Powers and Lennon (1999); Powers and Sen (2000) and Suzuki *et al*. (2000). High intensity exercise training is generally superior to low-intensity training in the upregulation of both SOD and GPX activity in skeletal muscle (Powers *et al*., 1994). In addition, exercise-induced upregulation of these enzymes may be fibre type specific with highly oxidative muscles being most responsive (Powers *et al*., 1994). Differences in training protocols and muscle fibre recruitment may explain why some have reported that endurance training does not promote an increase in SOD and GPX activity in skeletal muscles (Powers & Sen, 2000). In contrast to SOD and GPX adaptability, there is little evidence to suggest that exercise training results in increased CAT activity in skeletal muscle. While some have shown catalase activity to increase in response to training, most studies report either no change in catalase activity or even a decrease in activity (summarised in Ji (1998); Powers and Lennon (1999) and Powers and Sen (2000).

Results from human training intervention studies are not consistent. Elosua *et al*. (2003) found that sixteen weeks of aerobic training significantly increased blood GPx and plasma glutathione reductase activity, while erythrocyte SOD did not change significantly. Markers of lipid peroxidation were also improved after training. In addition, the researchers investigated the effect of training on the markers of lipid peroxidation during an acute bout of exercise, and reported that the response pattern to exercise did not change significantly with training. Similarly, Miyazaki *et al*. (2001) reported that twelve weeks of endurance training resulted in an improvement in erythrocyte GPx activities as well as SOD activities, whereas CAT activity did not change after training. In their study, however, training attenuated the effect of acute exercise on lipid peroxidation and neutrophil superoxide anion production. Protein oxidation was not affected by either training or acute exercise in their study. Tiidus *et al*. (1996) examined the effects of eight weeks of aerobic exercise training on skeletal muscle antioxidant activity in seven males and six females. Training did not affect skeletal muscle SOD, Cat and GPx activity. Muscle vitamin E, GSH, GSSG, ratio GSH/GSSG, and total glutathione were also similar before and after exercise training. The results therefore suggest that the exercise training intensity, duration, and frequency were not sufficient oxidative stressors to induce muscle antioxidant status adaptation. Similarly, Tonkonogi *et al*. (2000) found that a 6 week endurance training period did not improve skeletal muscle SOD or GPX activity and muscle glutathione status.

Bergholm *et al*. (1999) measured circulating antioxidants in nine semi-fit males before and after three months of running training. Plasma levels of uric acid, vitamin E, ß-carotene, retinol and serum sulfhydryl groups were significantly lower after the training period, whereas plasma vitamin C was significantly higher and total antioxidant capacity (measured by the TRAP assay) did not change. Although subjects were on a weight-maintaining diet during the training period and did not report antioxidant supplement use, no information regarding dietary antioxidant intake was provided. Therefore the possibility that dietary antioxidant intake could have influenced the results cannot be excluded.

Whether regular exercise training has a protective effect on oxidative stress in response to an acute bout of exercise is also not clear. Niess *et al*. (1996) showed that exercise-induced DNA damage was reduced in trained subjects compared to untrained subjects. In addition trained subjects also had lower plasma MDA levels at rest and 15minutes after exercise compared to the untrained group. In contrast, Ortenblad *et al*. (1997) did not find any significant differences in plasma or muscle MDA levels after exercise between trained

and untrained subjects. However, resting muscle total SOD, GPX, and MnSOD activity was higher in trained subjects than untrained. Blood antioxidant enzymes were similar in both groups. The two studies involved different exercise tests and differently trained subjects, which may contribute to the discrepancy in the results.

Other researchers have conducted cross-sectional studies that compare antioxidant capacity and/or levels of biomarkers of oxidative stress between trained and sedentary individuals to investigate whether antioxidant systems are upregulated with training (see Table 4). From the table it is evident that this type of research has not provided conclusive evidence regarding the effect of training on oxidative stress markers. Some have found that trained individuals have improved antioxidant capacity as shown by decreased markers of oxidative damage and/or improved antioxidant levels when compared to sedentary individuals. In contrast, others studies have reported similar or no differences in oxidative stress markers or antioxidant levels between trained individuals and sedentary controls.

A variety of factors may play a role in the adaptation of the antioxidant system in response to training including the type, duration and intensity of exercise training (Powers *et al*., 1994; Tiidus *et al*., 1996). Dietary antioxidant intake, which is often poorly controlled for, influences plasma antioxidant levels and may therefore also influence antioxidant capacity and oxidative stress. Therefore, the differences reported between studies investigating antioxidant adaptation in response to training, may be in part due to differences in dietary antioxidant intake and also due to mobilisation of plasma antioxidants.

Table 4: Summary of cross-sectional studies comparing markers of oxidative stress between athletes and sedentary controls.

Reference	Subjects	Dietary intake control and results	Markers of oxidative damage	Antioxidant levels
Robertson *et al*., 1991	6 high-training runners, 6 low-training runners and 6 sedentary, all males	7-day weighed food record, no results of intake given, vitamin supplement users excluded	TBARS, plasma conjugated dienes,	Plasma vitamin C, vitamin E, albumin, caeruloplasmin, similar, erythrocyte vitamin E, selenium, glutathione, higher in runners, leokocyte vitamin C higher in high-training runners than low-training, plasma uric acid lower in high-training runners than sedentary controls. SOD activity similar, erythrocyte GSHPx activity, CAT activity higher in runners
Rokitzki *et al*., 1994a	44 male athletes, various sports, 16 sedentary controls	7-day weighed food record, no significant difference in vitamin C intake, no supplements used during study period		Plasma and urinary vitamin C similar
Balakrishnan & Anuradha, 1998	26 male sport science students, 27 sedentary students	Determined food intake but no results reported (similar food habits), supplement users excluded	Trained group higher plasma TBARS, higher conjugated dienes	Similar blood vitamin E, lower blood vitamin C and GSH and ceruloplasmin in athletes
Brites *et al*., 1999	30 soccer players, 12 sedentary males	Dietary food intake with FFQ, similar foods, supplement users excluded		Plasma total antioxidant capacity, plasma vitamin C, uric acid, vitamin E higher in athletes, bilirubin similar. Plasma SOD higher in athletes
Evelson *et al*., 2002	15 rugby players, 15 sedentary controls	Dietary food intakes similar using FFQ, supplement users excluded	TBARS similar	TRAP, plasma vitamin C, vitamin E higher in sportsmen plasma uric acid, bilirubin, similar. SOD activity higher in sportsmen

Table 4 (continued)

Reference	Subjects	Dietary intake control and results	Oxidative stress markers	Antioxidant status
Ohno *et al*., 1992	19 long-distance cross country skiers, 12 sedentary males	No dietary control		Plasma MnSOD levels higher in athletes, similar CuZnSOD levels
Cazzola *et al*., 2003	20 soccer players, 20 sedentary controls, male	3-day weighed food record, higher vitamin C intake by soccer players, supplement users excluded	Lipoperoxides lower in athletes	Plasma albumin, bilirubin similar, plasma uric acid, vitamin C, vitamin E, higher in athletes. Plasma GPX activity similar, plasma SOD activity higher in athletes
Rousseau *et al*., 2004	115 athletes, 16 sedentary controls, male and female	Antioxidant containing therapeutic agents excluded, 7-day food record, similar intakes	Plasma TBARS similar	(Plasma antioxidants not compared between sedentary and active, only between male and female)
Watson *et al*., 2005	20 track and field athletes, 20 sedentary controls, male and female	4 day weighed food records (vitamin E intake not measured), dietary intake similar, supplement users excluded	F2-isoprostanes similar	Uric acid similar in both groups, plasma vitamin E and ß-carotene higher in athletes, total antioxidant capacity lower in male athletes than male controls. SOD and GPx similar in both groups

7. Conclusion

Reactive oxygen species are produced as part of normal metabolism and their generation may be increased under certain circumstances such as exercise. While the human body has developed an integrated antioxidant system to remove these potentially damaging molecules, studies have shown that exercise-induced oxidative damage may occur. This has been indicated by increases in markers of oxidative protein, lipid and DNA damage and decreases in levels of antioxidants and/or decreased antioxidant capacity. On the other hand, exercise training has been shown to upregulate antioxidant defences, suggesting that the antioxidant system is able to adapt to exercise-induced ROS production and so reduce the risk of oxidative stress. The effect of dietary antioxidant intake on antioxidant capacity and oxidative stress is not clear and the role thereof in the training-induced adaptation of the antioxidant system requires further investigation. However, results from studies investigating oxidative stress and antioxidant capacity in response to training are not consistent and further research is needed.

Susceptibility to oxidative stress may vary considerably from person to person and may be influenced by genetic disposition, which may account for some of the discrepancies reported. However, the effect of genetic variation on antioxidant capacity in an exercise-training context has not yet been investigated and it is therefore not clear whether certain individuals may be predisposed to oxidative stress based on a specific genotype. Thus the effect of inter-individual genetic variation and dietary antioxidant intake on antioxidant capacity in trained adaptation needs to be explored further.

As mentioned in Section 5.4, methodological problems relating to exercise-induced oxidative stress measurement may explain some of the discrepancies found in studies investigating training-induced changes in antioxidant capacity. The lack of appropriate assays may influence some of the findings and this must be taken into account when interpreting the results. Furthermore, factors relating to the type and intensity of exercise training and the subjects, including the dietary habits, could also play a role in the conflicting results and need to be investigated further. Errors relating to dietary antioxidant intake assessments may contribute to contrasting findings. Several methods of dietary assessment are available and while some researchers have made use of these to measure antioxidant intake, others have failed to adequately control for dietary intake. In addition, different dietary assessment methods have different strengths and limitations, thus making dietary assessment a challenging task.

8. References

ADA (2000) Position of the American Dietetic Association, Dietitians of Canada, and the American College of Sports Medicine: Nutrition and athletic performance. *J Am Diet Assoc* **100,** 1543-1556

Aguilo A, Tauler P, Pilar GM, Villa G, Cordova A, Tur JA & Pons A (2003) Effect of exercise intensity and training on antioxidants and cholesterol profile in cyclists. *J Nutr Biochem* **14,** 319-325

Alessio HM (1993) Exercise-induced oxidative stress. *Med Sci Sports Exerc* **25,** 218-224

Alessio HM (2000) Lipid peroxidation in healthy and diseased models: influence of different types of exercise. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 115-127 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Alessio HM, Goldfarb AH & Cao G (1997) Exercise-induced oxidative stress before and after vitamin C supplementation. *Int J Sport Nutr* **7,** 1-9

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

Allen RG & Tresini M (2000) Oxidative stress and gene regulation. *Free Radic Biol Med* **28,** 463-499

Ambrosone CB, Freudenheim JL, Thompson PA, *et al* (1999) Manganese superoxide dismutase (MnSOD) genetic polymorphisms, dietary antioxidants, and risk of breast cancer. *Cancer Res* **59,** 602-606

Anlasik T, Sies H, Griffiths HR, Mecocci P, Stahl W & Polidori MC (2005) Dietary habits are major determinants of the plasma antioxidant status in healthy elderly subjects. *Br J Nutr* **94,** 639-642

Ashton T, Rowlands CC, Jones E, Young IS, Jackson SK, Davies B & Peters JR (1998) Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. *Eur J Appl Physiol Occup Physiol* **77,** 498-502

Ashton T, Young IS, Peters JR, Jones E, Jackson SK, Davies B & Rowlands CC (1999) Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study. *J Appl Physiol* **87,** 2032-2036

Astrand PO (1992) Physical activity and fitness. *Am J Clin Nutr* **55,** 1231S-1236S

Bakker I, Twisk JW, van Mechelen W, Mensink GB & Kemper HC (2003) Computerization of a dietary history interview in a running cohort; evaluation within the Amsterdam Growth and Health Longitudinal Study. *Eur J Clin Nutr* **57,** 394-404

Balakrishnan SD & Anuradha CV (1998) Exercise, depletion of antioxidants and antioxidant manipulation. *Cell Biochem Funct* **16,** 269-275

Balog T, Sobocanec S, Sverko V, Krolo I, Rocic B, Marotti M & Marotti T (2006) The influence of season on oxidant-antioxidant status in trained and sedentary subjects. *Life Sci* **78,** 1441-1447

Basu AK & Marnett LJ (1984) Molecular requirements for the mutagenicity of malondialdehyde and related acroleins. *Cancer Res* **44,** 2848-2854

Beckman KB & Ames BN (2000) Oxidants and aging. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 755-796 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Benzie IF (2000) Evolution of antioxidant defence mechanisms. *Eur J Nutr* **39,** 53-61

Benzie IF (2003) Evolution of dietary antioxidants. *Comp Biochem Physiol A Mol Integr Physiol* **136,** 113-126

Bergholm R, Makimattila S, Valkonen M, Liu ML, Lahdenpera S, Taskinen MR, Sovijarvi A, Malmberg P & Yki-Jarvinen H (1999) Intense physical training decreases circulating antioxidants and endothelium-dependent vasodilatation in vivo. *Atherosclerosis* **145,** 341-349

Berlett BS & Stadtman ER (1997) Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* **272,** 20313-20316

Birnboim HC (1982) DNA strand breakage in human leukocytes exposed to a tumor promoter, phorbol myristate acetate. *Science* **215,** 1247-1249

Bland JM & Altman DG (1986) Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **1,** 307-310

Bland JM & Altman DG (1995) Comparing two methods of clinical measurement: a personal history. *Int J Epidemiol* **24 Suppl 1,** S7-14

Bland JM & Altman DG (1999) Measuring agreement in method comparison studies. *Stat Methods Med Res* **8,** 135-160

Block G, Norkus E, Hudes M, Mandel S & Helzlsouer K (2001) Which plasma antioxidants are most related to fruit and vegetable consumption? *Am J Epidemiol* **154,** 1113-1118

Bloomer RJ, Goldfarb AH & McKenzie MJ (2006) Oxidative stress response to aerobic exercise: comparison of antioxidant supplements. *Med Sci Sports Exerc* **38,** 1098-1105

Breen AP & Murphy JA (1995) Reactions of oxyl radicals with DNA. *Free Radic Biol Med* **18,** 1033-1077

Brites FD, Evelson PA, Christiansen MG, Nicol MF, Basilico MJ, Wikinski RW & Llesuy SF (1999) Soccer players under regular training show oxidative stress but an improved plasma antioxidant status. *Clin Sci (Lond)* **96,** 381-385

Bryant RJ, Ryder J, Martino P, Kim J & Craig BW (2003) Effects of vitamin E and C supplementation either alone or in combination on exercise-induced lipid peroxidation in trained cyclists. *J Strength Cond Res* **17,** 792-800

Camus G, Felekidis A, Pincemail J, Deby-Dupont G, Deby C, Juchmes-Ferir A, Lejeune R & Lamy M (1994) Blood levels of reduced/oxidized glutathione and plasma concentration of ascorbic acid during eccentric and concentric exercises of similar energy cost. *Arch Int Physiol Biochim Biophys* **102,** 67-70

Camus G, Pincemail J, Roesgen A, Dreezen E, Sluse FE & Deby C (1990) Tocopherol mobilization during dynamic exercise after beta-adrenergic blockade. *Arch Int Physiol Biochim* **98,** 121-126

Cannon JG & Blumberg JB (2000) Acute phase immune responses in exercise. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 177-193 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Cao G, Booth SL, Sadowski JA & Prior RL (1998) Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *Am J Clin Nutr* **68,** 1081-1087

Cao G & Cutler RG (1995) Protein oxidation and aging. I. Difficulties in measuring reactive protein carbonyls in tissues using 2,4-dinitrophenylhydrazine. *Arch Biochem Biophys* **320,** 106-114

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

Carr A & Frei B (1999) Does vitamin C act as a pro-oxidant under physiological conditions? *FASEB J* **13,** 1007-1024

Cazzola R, Russo-Volpe S, Cervato G & Cestaro B (2003) Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. *Eur J Clin Invest* **33,** 924-930

Chia LS, Thompson JE & Moscarello MA (1983) Disorder in human myelin induced by superoxide radical: an in vitro investigation. *Biochem Biophys Res Commun* **117,** 141-146

Child R, Brown S, Day S, Donnelly A, Roper H & Saxton J (1999) Changes in indices of antioxidant status, lipid peroxidation and inflammation in human skeletal muscle after eccentric muscle actions. *Clin Sci (Lond)* **96,** 105-115

Child RB, Wilkinson DM, Fallowfield JL & Donnelly AE (1998) Elevated serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half-marathon run. *Med Sci Sports Exerc* **30,** 1603-1607

Chow CK (2000) Vitamin E. In *Biochemical and Physiological Aspects of Human Nutrition*, pp. 584-598 [Stipanuk MH, editors]. Philadelphia: W.B. Saunders Company.

Church SL, Grant JW, Meese EU & Trent JM (1992) Sublocalization of the gene encoding manganese superoxide dismutase (MnSOD/SOD2) to 6q25 by fluorescence in situ hybridization and somatic cell hybrid mapping. *Genomics* **14,** 823-825

Clarkson PM & Thompson HS (2000) Antioxidants: what role do they play in physical activity and health? *Am J Clin Nutr* **72,** 637S-646S

Close GL, Ashton T, Cable T, Doran D, Holloway C, McArdle F & MacLaren DP (2006) Ascorbic acid supplementation does not attenuate post-exercise muscle soreness following muscle-damaging exercise but may delay the recovery process. *Br J Nutr* **95,** 976-981

Collins A, Cadet J, Epe B & Gedik C (1997) Problems in the measurement of 8-oxoguanine in human DNA. Report of a workshop, DNA oxidation, held in Aberdeen, UK, 19-21 January, 1997. *Carcinogenesis* **18,** 1833-1836

Collins AR, Dusinska M, Gedik CM & Stetina R (1996) Oxidative damage to DNA: do we have a reliable biomarker? *Environ Health Perspect* **104 Suppl 3,** 465-469

Davies KJ (1986) Intracellular proteolytic systems may function as secondary antioxidant defenses: an hypothesis. *J Free Radic Biol Med* **2,** 155-173

Davies KJ, Quintanilha AT, Brooks GA & Packer L (1982) Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun* **107,** 1198-1205

Decker EA & Clarkson PM (2000) Dietary sources and bioavailbality of essential and non-essential antioxidants. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 323-358 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Dillard CJ, Litov RE, Savin WM, Dumelin EE & Tappel AL (1978) Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. *J Appl Physiol* **45,** 927-932

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

Dufaux B, Heine O, Kothe A, Prinz U & Rost R (1997) Blood glutathione status following distance running. *Int J Sports Med* **18,** 89-93

Durstine JL & Haskell WL (1994) Effects of exercise training on plasma lipids and lipoproteins. *Exerc Sport Sci Rev* **22,** 477-521

Duthie GG, Robertson JD, Maughan RJ & Morrice PC (1990) Blood antioxidant status and erythrocyte lipid peroxidation following distance running. *Arch Biochem Biophys* **282,** 78-83

Dwyer J (1999) Dietary assessment. In *Modern Nutrition in Health and Disease*, pp. 937-959 [Shils ME, Olson JA, Shike M and Ross AC, editors]. Philadelphia: Lippincott Williams & Wilkins.

Elosua R, Molina L, Fito M, Arquer A, Sanchez-Quesada JL, Covas MI, Ordonez-Llanos J & Marrugat J (2003) Response of oxidative stress biomarkers to a 16-week aerobic physical activity program, and to acute physical activity, in healthy young men and women. *Atherosclerosis* **167,** 327-334

Esterbauer H, Jurgens G, Quehenberger O & Koller E (1987) Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J Lipid Res* **28,** 495-509

Evelson P, Gambino G, Travacio M, Jaita G, Verona J, Maroncelli C, Wikinski R, Llesuy S & Brites F (2002) Higher antioxidant defences in plasma and low density lipoproteins from rugby players. *Eur J Clin Invest* **32,** 818-825

Finkel T & Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408,** 239-247

Flohe L, Gunzler WA & Schock HH (1973) Glutathione peroxidase: a selenoenzyme. *FEBS Lett* **32,** 132-134

Food and Nutrition Board IoM (2000) *Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids.*, Washington, DC: National Academic Press.

Forsberg L, de Faire U & Morgenstern R (2001) Oxidative stress, human genetic variation, and disease. *Arch Biochem Biophys* **389,** 84-93

Frei B, Stocker R, England L & Ames BN (1990) Ascorbate: the most effective antioxidant in human blood plasma. *Adv Exp Med Biol* **264,** 155-163

Galan P, Briancon S, Favier A, *et al* (2005) Antioxidant status and risk of cancer in the SU.VI.MAX study: is the effect of supplementation dependent on baseline levels? *Br J Nutr* **94,** 125-132

Ginsburg GS, O'Toole M, Rimm E, Douglas PS & Rifai N (2001) Gender differences in exercise-induced changes in sex hormone levels and lipid peroxidation in athletes participating in the Hawaii Ironman triathlon. Ginsburg-gender and exercise-induced lipid peroxidation. *Clin Chim Acta* **305,** 131-139

Gleeson M, Robertson JD & Maughan RJ (1987) Influence of exercise on ascorbic acid status in man. *Clin Sci (Lond)* **73,** 501-505

Goldfarb AH, Bloomer RJ & McKenzie MJ (2005) Combined antioxidant treatment effects on blood oxidative stress after eccentric exercise. *Med Sci Sports Exerc* **37,** 234-239

Groussard C, Machefer G, Rannou F, Faure H, Zouhal H, Sergent O, Chevanne M, Cillard J & Gratas-Delamarche A (2003) Physical fitness and plasma non-enzymatic antioxidant status at rest and after a wingate test. *Can J Appl Physiol* **28,** 79-92

Gutteridge JM (1994) Biological origin of free radicals, and mechanisms of antioxidant protection. *Chem Biol Interact* **91,** 133-140

Gutteridge JM & Quinlan GJ (1983) Malondialdehyde formation from lipid peroxides in the thiobarbituric acid test: the role of lipid radicals, iron salts, and metal chelators. *J Appl Biochem* **5,** 293-299

Halliwell B (1998) Free radicals and oxidative damage in biology and medicine: An introduction. In *Oxidative stress in skeletal muscle*, pp. 1-27 [Reznick AZ, Packer L, Sen CK, Holloszy JO and Jackson MJ, editors]. Basel: Birkhäuser Verlag.

Halliwell B & Gutteridge JMC (1999) *Free Radicals in Biology and Medicine*, 3rd ed. New York: Oxford University Press.

Han D, Loukianoff S & McLaughlin L (2000) Oxidative stress indices: analytical aspects and significance. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 433-483 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Harman D (1956) Aging: a theory based on free radical and radiation chemistry. *J Gerontol* **11,** 298-300

Hartmann A & Niess AM (2000) Oxidative DNA damage in exercise. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 195-217 [Sen CK, Packer L and Hanninen O, editors]. Amsterdam: Elsevier.

Hartmann A, Niess AM, Grunert-Fuchs M, Poch B & Speit G (1995) Vitamin E prevents exercise-induced DNA damage. *Mutat Res* **346,** 195-202

Hartmann A, Pfuhler S, Dennog C, Germadnik D, Pilger A & Speit G (1998) Exercise-induced DNA effects in human leukocytes are not accompanied by increased formation of 8-hydroxy-2'-deoxyguanosine or induction of micronuclei. *Free Radic Biol Med* **24,** 245-251

Helbock HJ, Beckman KB, Shigenaga MK, Walter PB, Woodall AA, Yeo HC & Ames BN (1998) DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. *Proc Natl Acad Sci U S A* **95,** 288-293

Hellsten Y, Tullson PC, Richter EA & Bangsbo J (1997) Oxidation of urate in human skeletal muscle during exercise. *Free Radic Biol Med* **22,** 169-174

Horwath C (1990) Food frequency questionnaires: a review. *Australian Journal of Nutrition and Dietetics* **47,** 71-76

Jackson MJ (1998) Free radical mechanisms in exercise-related muscle damage. In *Oxidative Stress in Skeletal Muscle*, pp. 75-86 [Reznick AZ, Packer L, Sen CK, Holloszy JO and Jackson MJ, editors]. Basel: Birkhäuser Verlag.

Jackson MJ (1999) An overview of methods for assessment of free radical activity in biology. *Proc Nutr Soc* **58,** 1001-1006

Jackson MJ (2000) Exercise and oxygen radical production by muscle. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 57-86 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Jackson MJ, Edwards RH & Symons MC (1985) Electron spin resonance studies of intact mammalian skeletal muscle. *Biochim Biophys Acta* **847,** 185-190

Jackson MJ, Papa S, Bolanos J, *et al* (2002) Antioxidants, reactive oxygen and nitrogen species, gene induction and mitochondrial function. *Mol Aspects Med* **23,** 209-285

Jacob RA, Aiello GM, Stephensen CB, Blumberg JB, Milbury PE, Wallock LM & Ames BN (2003) Moderate antioxidant supplementation has no effect on biomarkers of oxidant damage in healthy men with low fruit and vegetable intakes. *J Nutr* **133,** 740-743

Jenkins RR (1988) Free radical chemistry. Relationship to exercise. *Sports Med* **5,** 156-170

Jenkins RR (2000) Exercise and oxidative stress methodology: a critique. *Am J Clin Nutr* **72,** 670S-674S

Ji LL (1998) Antioxidant enzyme response to exercise and training in the skeletal muscle. In *Oxidative Stress in Skeletal Muscle*, pp. 103-125 [Reznick AZ, Packer L, Sen CK, Holloszy JO and Jackson MJ, editors]. Basel: Birkhäuser Verlag.

Ji LL (1999) Antioxidants and oxidative stress in exercise. *Proc Soc Exp Biol Med* **222,** 283-292

Ji LL, Stratman FW & Lardy HA (1988) Antioxidant enzyme systems in rat liver and skeletal muscle. Influences of selenium deficiency, chronic training, and acute exercise. *Arch Biochem Biophys* **263,** 150-160

Jones DP & deLong MJ (2000) Detoxification and protective functions of nutrients. In *Biochemical and Physiological Aspects of Human Nutrition*, pp. 901-916 [Stipanuk MH, editors]. Philadelphia: W.B. Saunders Company.

Kaaks R, Riboli E, Esteve J, van Kappel AL & van Staveren WA (1994) Estimating the accuracy of dietary questionnaire assessments: validation in terms of structural equation models. *Stat Med* **13,** 127-142

Kaaks RJ (1997) Biochemical markers as additional measurements in studies of the accuracy of dietary questionnaire measurements: conceptual issues. *Am J Clin Nutr* **65,** 1232S-1239S

Kanter MM, Nolte LA & Holloszy JO (1993) Effects of an antioxidant vitamin mixture on lipid peroxidation at rest and postexercise. *J Appl Physiol* **74,** 965-969

Keul J, Doll E & Koppler D (1972) *Energy metabolism and human muscle*, Basel: S. Karger.

Lee J, Goldfarb AH, Rescino MH, Hegde S, Patrick S & Apperson K (2002) Eccentric exercise effect on blood oxidative-stress markers and delayed onset of muscle soreness. *Med Sci Sports Exerc* **34,** 443-448

Lesgards JF, Durand P, Lassarre M, Stocker P, Lesgards G, Lanteaume A, Prost M & Lehucher-Michel MP (2002) Assessment of lifestyle effects on the overall antioxidant capacity of healthy subjects. *Environ Health Perspect* **110,** 479-486

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S & Stadtman ER (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* **186,** 464-478

Liu ML, Bergholm R, Makimattila S, Lahdenpera S, Valkonen M, Hilden H, Yki-Jarvinen H & Taskinen MR (1999) A marathon run increases the susceptibility of LDL to oxidation in vitro and modifies plasma antioxidants. *Am J Physiol* **276,** E1083-E1091

Marzatico F, Pansarasa O, Bertorelli L, Somenzini L & Della VG (1997) Blood free radical antioxidant enzymes and lipid peroxides following long-distance and lactacidemic performances in highly trained aerobic and sprint athletes. *J Sports Med Phys Fitness* **37,** 235-239

Mastaloudis A, Leonard SW & Traber MG (2001) Oxidative stress in athletes during extreme endurance exercise. *Free Radic Biol Med* **31,** 911-922

Maxwell SR, Jakeman P, Thomason H, Leguen C & Thorpe GH (1993) Changes in plasma antioxidant status during eccentric exercise and the effect of vitamin supplementation. *Free Radic Res Commun* **19,** 191-202

McAnulty SR, McAnulty LS, Nieman DC, Morrow JD, Shooter LA, Holmes S, Heward C & Henson DA (2005) Effect of alpha-tocopherol supplementation on plasma homocysteine and oxidative stress in highly trained athletes before and after exhaustive exercise. *J Nutr Biochem* **16,** 530-537

McCall MR & Frei B (1999) Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic Biol Med* **26,** 1034-1053

McCord JM (1985) Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* **312,** 159-163

Meydani M, Evans WJ, Handelman G, *et al* (1993) Protective effect of vitamin E on exercise-induced oxidative damage in young and older adults. *Am J Physiol* **264,** R992-R998

Minami M, Mori K & Nagatsu T (1981) The effect of light exercise on the plasma superoxide dismutase activity and on the plasma noradrenaline concentration. *Ind Health* **19,** 133-138

Mitrunen K, Sillanpaa P, Kataja V, Eskelinen M, Kosma VM, Benhamou S, Uusitupa M & Hirvonen A (2001) Association between manganese superoxide dismutase (MnSOD) gene polymorphism and breast cancer risk. *Carcinogenesis* **22,** 827-829

Miyazaki H, Oh-ishi S, Ookawara T, Kizaki T, Toshinai K, Ha S, Haga S, Ji LL & Ohno H (2001) Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. *Eur J Appl Physiol* **84,** 1-6

Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF & Roberts LJ (1990) A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc Natl Acad Sci U S A* **87,** 9383-9387

Nelson JL, Bernstein PS, Schmidt MC, Von Tress MS & Askew EW (2003) Dietary modification and moderate antioxidant supplementation differentially affect serum carotenoids, antioxidant levels and markers of oxidative stress in older humans. *J Nutr* **133,** 3117-3123

Niess AM, Hartmann A, Grunert-Fuchs M, Poch B & Speit G (1996) DNA damage after exhaustive treadmill running in trained and untrained men. *Int J Sports Med* **17,** 397-403

Oberley LW, St Clair DK, Autor AP & Oberley TD (1987) Increase in manganese superoxide dismutase activity in the mouse heart after X-irradiation. *Arch Biochem Biophys* **254,** 69-80

Ocke MC & Kaaks RJ (1997) Biochemical markers as additional measurements in dietary validity studies: application of the method of triads with examples from the

European Prospective Investigation into Cancer and Nutrition. *Am J Clin Nutr* **65,** 1240S-1245S

Ohno H, Yamashita H, Ookawara T, Saitoh D, Wakabayashi K & Taniguchi N (1992) Training effects on concentrations of immunoreactive superoxide dismutase iso-enzymes in human plasma. *Acta Physiol Scand* **146,** 291-292

Okamura K, Doi T, Hamada K, Sakurai M, Yoshioka Y, Mitsuzono R, Migita T, Sumida S & Sugawa-Katayama Y (1997) Effect of repeated exercise on urinary 8-hydroxy-deoxyguanosine excretion in humans. *Free Radic Res* **26,** 507-514

Omenn GS, Goodman GE, Thornquist MD, *et al* (1996) Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med* **334,** 1150-1155

Orhan H, van Holland B, Krab B, Moeken J, Vermeulen NP, Hollander P & Meerman JH (2004) Evaluation of a multi-parameter biomarker set for oxidative damage in man: increased urinary excretion of lipid, protein and DNA oxidation products after one hour of exercise. *Free Radic Res* **38,** 1269-1279

Ortenblad N, Madsen K & Djurhuus MS (1997) Antioxidant status and lipid peroxidation after short-term maximal exercise in trained and untrained humans. *Am J Physiol* **272,** R1258-R1263

Pacifici RE & Davies KJ (1991) Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited. *Gerontology* **37,** 166-180

Packer JE, Slater TF & Willson RL (1979) Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* **278,** 737-738

Packer L (1997) Oxidants, antioxidant nutrients and the athlete. *J Sports Sci* **15,** 353-363

Packer L, Almada AL, Rothfuss LM & Wilson DS (1989) Modulation of tissue vitamin E levels by physical exercise. *Ann N Y Acad Sci* **570,** 311-321

Papas AM (1996) Determinants of antioxidant status in humans. *Lipids* **31 Suppl,** S77-S82

Pattwell DM & Jackson MJ (2004) Contraction-induced oxidants as mediators of adaptation and damage in skeletal muscle. *Exerc Sport Sci Rev* **32,** 14-18

Peake JM (2003) Vitamin C: effects of exercise and requirements with training. *Int J Sport Nutr Exerc Metab* **13,** 125-151

Peto R, Doll R, Buckley JD & Sporn MB (1981) Can dietary beta-carotene materially reduce human cancer rates? *Nature* **290,** 201-208

Powers SK, Criswell D, Lawler J, Ji LL, Martin D, Herb RA & Dudley G (1994) Influence of exercise and fiber type on antioxidant enzyme activity in rat skeletal muscle. *Am J Physiol* **266,** R375-R380

Powers SK, Deruisseau KC, Quindry J & Hamilton KL (2004) Dietary antioxidants and exercise. *J Sports Sci* **22,** 81-94

Powers SK & Lennon SL (1999) Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle. *Proc Nutr Soc* **58,** 1025-1033

Powers SK & Sen CK (2000) Physiological antioxidants and exercise training. In *Handbook of oxdidants and antioxidants in exercise*, pp. 221-242 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Prior RL & Cao G (1999) In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radic Biol Med* **27,** 1173-1181

Prior RL, Cao G, Prior RL & Cao G (2000) Analysis of botanicals and dietary supplements for antioxidant capacity: a review. *J AOAC Int* **83,** 950-956

Quindry JC, Stone WL, King J & Broeder CE (2003) The effects of acute exercise on neutrophils and plasma oxidative stress. *Med Sci Sports Exerc* **35,** 1139-1145

Radak Z, Ogonovszky H, Dubecz J, Pavlik G, Sasvari M, Pucsok J, Berkes I, Csont T & Ferdinandy P (2003) Super-marathon race increases serum and urinary nitrotyrosine and carbonyl levels. *Eur J Clin Invest* **33,** 726-730

Ramel A, Wagner KH & Elmadfa I (2004) Plasma antioxidants and lipid oxidation after submaximal resistance exercise in men. *Eur J Nutr* **43,** 2-6

Rankin G & Stokes M (1998) Reliability of assessment tools in rehabilitation: an illustration of appropriate statistical analyses. *Clin Rehabil* **12,** 187-199

Ravanat JL, Turesky RJ, Gremaud E, Trudel LJ & Stadler RH (1995) Determination of 8-oxoguanine in DNA by gas chromatography--mass spectrometry and HPLC--electrochemical detection: overestimation of the background level of the oxidized base by the gas chromatography--mass spectrometry assay. *Chem Res Toxicol* **8,** 1039-1045

Record IR, Dreosti IE & McInerney JK (2001) Changes in plasma antioxidant status following consumption of diets high or low in fruit and vegetables or following dietary supplementation with an antioxidant mixture. *Br J Nutr* **85,** 459-464

Reznick AZ, Cross CE, Hu ML, Suzuki YJ, Khwaja S, Safadi A, Motchnik PA, Packer L & Halliwell B (1992) Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. *Biochem J* **286 (Pt 2),** 607-611

Reznick AZ, Packer L & Sen CK (1998) Strategies to assess oxidative stress. In *Oxidative Stress in Skeletal Muscle*, pp. 43-58 [Reznick AZ, Packer L, Sen CK, Holloszy JO and Jackson MJ, editors]. Basel: Birkhäuser Verlag.

Rippe JM & Hess S (1998) The role of physical activity in the prevention and management of obesity. *J Am Diet Assoc* **98,** S31-S38

Robertson JD, Maughan RJ, Duthie GG & Morrice PC (1991) Increased blood antioxidant systems of runners in response to training load. *Clin Sci (Lond)* **80,** 611-618

Rokitzki L, Hinkel S, Klemp C, Cufi D & Keul J (1994a) Dietary, serum and urine ascorbic acid status in male athletes. *Int J Sports Med* **15,** 435-440

Rokitzki L, Logemann E, Sagredos AN, Murphy M, Wetzel-Roth W & Keul J (1994b) Lipid peroxidation and antioxidative vitamins under extreme endurance stress. *Acta Physiol Scand* **151,** 149-158

Rosenblum JS, Gilula NB & Lerner RA (1996) On signal sequence polymorphisms and diseases of distribution. *Proc Natl Acad Sci U S A* **93,** 4471-4473

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG & Hoekstra WG (1973) Selenium: biochemical role as a component of glutathione peroxidase. *Science* **179,** 588-590

Rousseau AS, Hininger I, Palazzetti S, Faure H, Roussel AM & Margaritis I (2004) Antioxidant vitamin status in high exposure to oxidative stress in competitive athletes. *Br J Nutr* **92,** 461-468

Sen CK (2001) Antioxidants in exercise nutrition. *Sports Med* **31,** 891-908

Sen CK, Rankinen T, Vaisanen S & Rauramaa R (1994) Oxidative stress after human exercise: effect of N-acetylcysteine supplementation. *J Appl Physiol* **76,** 2570-2577

Sevanian A, Davies KJ & Hochstein P (1985) Conservation of vitamin C by uric acid in blood. *J Free Radic Biol Med* **1,** 117-124

Sharpe P (1999) Oxidative stress and exercise: need for antioxidant supplementation? *Br J Sports Med* **33,** 298-299

Shimoda-Matsubayashi S, Matsumine H, Kobayashi T, Nakagawa-Hattori Y, Shimizu Y & Mizuno Y (1996) Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. A predictive evidence for conformational change to influence mitochondrial transport and a study of allelic association in Parkinson's disease. *Biochem Biophys Res Commun* **226,** 561-565

Sies H (1991) *Oxidative Stress II. Oxidants and Antioxidants*. London: Academic Press.

Singal PK, Kapur N, Dhillon KS, Beamish RE & Dhalla NS (1982) Role of free radicals in catecholamine-induced cardiomyopathy. *Can J Physiol Pharmacol* **60,** 1390-1397

Spitzer JA (1995) Active oxygen intermediates--beneficial or deleterious? An introduction. *Proc Soc Exp Biol Med* **209,** 102-103

Stadtman ER (1990) Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic Biol Med* **9,** 315-325

Stadtman ER (1992) Protein oxidation and aging. *Science* **257,** 1220-1224

Stohs SJ & Bagchi D (1995) Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med* **18,** 321-336

Suzuki K, Ohno H, Oh-ishi S, Kizaki T, Ookawara T, Fujii J, Radak Z & Taniguchi N (2000) Superoxide dismutases in exercise and disease. In *Handbook of oxidants and*

antioxidants in exercise, pp. 243-296 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Suzuki YJ, Forman HJ & Sevanian A (1997) Oxidants as stimulators of signal transduction. *Free Radic Biol Med* **22,** 269-285

Tauler P, Aguilo A, Gimeno I, Fuentespina E, Tur JA & Pons A (2006) Response of blood cell antioxidant enzyme defences to antioxidant diet supplementation and to intense exercise. *Eur J Nutr* **45,** 187-195

Tauler P, Gimeno I, Aguilo A, Guix MP & Pons A (1999) Regulation of erythrocyte antioxidant enzyme activities in athletes during competition and short-term recovery. *Pflugers Arch* **438,** 782-787

Tessier F, Margaritis I, Richard MJ, Moynot C & Marconnet P (1995) Selenium and training effects on the glutathione system and aerobic performance. *Med Sci Sports Exerc* **27,** 390-396

The Alpha-Tocopherol BCCPSG (1994) The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med* **330,** 1029-1035

Thomas JA (1999) Oxidative stress and oxidant defense. In *Modern Nutrition in Health and Disease*, pp. 751-760 [Shils ME, Olson JA, Shike M and Ross AC, editors]. Philadelphia: Lippincott Williams & Wilkins.

Thompson FE & Byers T (1994) Dietary assessment resource manual. *J Nutr* **124,** 2245S-2317S

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

Tiidus PM, Pushkarenko J & Houston ME (1996) Lack of antioxidant adaptation to short-term aerobic training in human muscle. *Am J Physiol* **271,** R832-R836

Tirosh O & Reznick AZ (2000) Chemical bases and biological relevance of protein oxidation. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 89-114 [Sen CK, Packer L and Hanninen O, editors]. Amsterdam: Elsevier.

Tonkonogi M, Walsh B, Svensson M & Sahlin K (2000) Mitochondrial function and antioxidative defence in human muscle: effects of endurance training and oxidative stress. *J Physiol* **528 Pt 2,** 379-388

Trevisan M, Browne R, Ram M, Muti P, Freudenheim J, Carosella AM & Armstrong D (2001) Correlates of markers of oxidative status in the general population. *Am J Epidemiol* **154,** 348-356

Tyler DD (1975) Role of superoxide radicals in the lipid peroxidation of intracellular membranes. *FEBS Lett* **51,** 180-183

Urso ML & Clarkson PM (2003) Oxidative stress, exercise, and antioxidant supplementation. *Toxicology* **189,** 41-54

Van Landeghem GF, Tabatabaie P, Kucinskas V, Saha N & Beckman G (1999) Ethnic variation in the mitochondrial targeting sequence polymorphism of MnSOD. *Hum Hered* **49,** 190-193

Viguie CA, Frei B, Shigenaga MK, Ames BN, Packer L & Brooks GA (1993) Antioxidant status and indexes of oxidative stress during consecutive days of exercise. *J Appl Physiol* **75,** 566-572

Viitala P & Newhouse IJ (2004) Vitamin E supplementation, exercise and lipid peroxidation in human participants. *Eur J Appl Physiol* **93,** 108-115

Vollaard NB, Shearman JP & Cooper CE (2005) Exercise-induced oxidative stress:myths, realities and physiological relevance. *Sports Med* **35,** 1045-1062

Wan XS, Devalaraja MN & St Clair DK (1994) Molecular structure and organization of the human manganese superoxide dismutase gene. *DNA Cell Biol* **13,** 1127-1136

Watson TA, MacDonald-Wicks LK & Garg ML (2005) Oxidative stress and antioxidants in athletes undertaking regular exercise training. *Int J Sport Nutr Exerc Metab* **15,** 131-146

Willett WC (1998) *Nutritional Epidemiology*, 2nd ed. New York: Oxford University Press.

Williams SL, Strobel NA, Lexis LA & Coombes JS (2006) Antioxidant requirements of endurance athletes: implications for health. *Nutr Rev* **64,** 93-108

Woodson K, Tangrea JA, Lehman TA, Modali R, Taylor KM, Snyder K, Taylor PR, Virtamo J & Albanes D (2003) Manganese superoxide dismutase (MnSOD) polymorphism, alpha-tocopherol supplementation and prostate cancer risk in the alpha-tocopherol, beta-carotene cancer prevention study (Finland). *Cancer Causes Control* **14,** 513-518

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

Yu BP (1994) Cellular defenses against damage from reactive oxygen species. *Physiol Rev* **74,** 139-162

Yu BP, Suescun EA & Yang SY (1992) Effect of age-related lipid peroxidation on membrane fluidity and phospholipase A2: modulation by dietary restriction. *Mech Ageing Dev* **65,** 17-33

CHAPTER 3

DEVELOPMENT AND VALIDATION OF A QUANTIFIED FOOD FREQUENCY QUESTIONNAIRE TO ASSESS DIETARY ANTIOXIDANT INTAKE

Introduction

Antioxidant compounds that are consumed as part of our diet are an important part of the body's antioxidant defence system that protects against damage caused by free radicals. Such dietary antioxidants include vitamin C, vitamin E, carotenoids (or ß-carotene) and flavonoids and are found primarily in fruit and vegetables but also in plant oils, nuts, tea, red wine, legumes and spices (Willett, 1998b). Diets rich in fruit and vegetables are associated with positive health benefits, which are often attributed to the antioxidant nutrient intake (Cao *et al*., 1998; Benzie, 2003; Prior, 2003).

Antioxidant nutrient intake demonstrates considerable within- and between-person variability and therefore in order to accurately estimate intake, the dietary intake assessment method should capture usual or habitual intake. Assessment of the habitual intake of antioxidants poses an additional challenge, namely that many of the relatively few major sources may not be consumed regularly. Therefore, a method that captures intake over as many as possible days, possibly covering seasonal variation, must be considered. For these reasons, a food frequency questionnaire (FFQ) that specifically measures antioxidant intake (vitamin C, vitamin E, carotenoid and flavonoid) is recommended as it is most likely to provide the most accurate estimates of usual intake (Willett, 1998b).

The FFQ consists of a food list and a frequency response section and subjects are required to report their usual frequency of consumption of each food from the food list for a specific time period. The food list can be tailored to suit the specific aims of a particular study (Zulkifli & Yu, 1992). Quantitative FFQs contain additional questions regarding usual portion sizes for each food item. Information obtained from the FFQ can then be used to estimate daily nutrient intake by multiplying the food frequency, the portion size and the nutrient content for each food item (Willett, 1998b).

As with any research instrument, the reproducibility and validity of a FFQ needs to be determined for application in a particular study. Reproducibility refers to the degree of consistency of measurements obtained from the measurement instrument from more than one administration to the same persons at different times (Willett, 1998b). Validity refers to the degree to which the questionnaire actually measures the aspect of diet that it was intended to measure (Willett, 1998b). For effective validity assessment, the FFQ should ideally be compared to a 'gold standard'. However, as there is no perfect standard for

determining dietary intake, comparisons in validity studies are made with another dietary method that is judged to be superior (Horwath, 1990; Willett, 1998b). As neither method is perfect and each is associated with different types of measurement error, it is crucial that the errors of both methods are as independent as possible to avoid spuriously high estimates of validity (Horwath, 1990; Willett, 1998b). For FFQ validations, dietary records represent the best comparison method as the two methods have independent sources of error, namely the food record is not restricted by a food list, does not depend on memory and has the ability to accurately estimate frequency of intake and portion size (Horwath, 1990). Biochemical indicators of dietary intake and energy expenditure estimations represent attractive independent standards for validation studies (Willett, 1998b). The major advantage of using such indicators is that the sources of measurement error are different from those of questionnaire measurements (Kaaks, 1997; Willett, 1998b). Biochemical indicators and energy expenditure estimations are however influenced by a variety of factors that are unrelated to intake, which result in the weakening of correlations between indicator and questionnaire measurements (Willett, 1998b).

Various approaches can be used to assess the level of agreement between the test method (e.g. a FFQ) and the reference method (e.g. a dietary record) for reproducibility and validity testing. However, it must be borne in mind that no single test provides definitive answers on either the reproducibility or validity and that the judgement thereof is based largely on the subjective interpretation of the statistical tests (Rankin & Stokes, 1998). It is therefore recommended that more than one statistical test be used to assess the reproducibility and validation of a FFQ (Rankin & Stokes, 1998; Willett, 1998b). The more of the tests that point towards satisfactory agreement, the more certain one can be that the reproducibility and validity is satisfactory.

For the assessment of reproducibility, the following statistical tests are applicable: comparison of means and standard deviations for the two administrations; correlation coefficients, including the Pearson's, Spearman rank and intraclass, which describe the associations between intakes estimated from the two administrations; Bland-Altman plots and the assessment of the agreement between the classification of individuals according to quartile distributions of intake obtained from the two administrations (Bland & Altman, 1986; Willett, 1998b). For the assessment of the validity of the FFQ the same statistical methods can be used to compare the FFQ with a reference dietary assessment method (or a biomarker or an energy expenditure estimation).

Furthermore, structural equation modelling (SEM), e.g. the method of triads, can be applied to assess the validity of the test questionnaire in relation to the unknown true intake if data from two dietary assessment methods and a biomarker are available (Kaaks *et al*., 1994; Kaaks, 1997).

It is clear that the effective development and validation of FFQ involves a number of important steps from the development of the food list to the choice of statistical tests to be used in the assessment of the reproducibility and validity. Bearing this in mind, the aims of this study were to firstly develop a quantified FFQ to measure vitamin C, vitamin E, carotenoid and flavonoid intake in a young adult male population, and secondly, to assess the reproducibility and validity of the developed FFQ.

Materials and methods

Study design

The study consisted of two parts. In the first part, a quantified food frequency questionnaire was developed to measure dietary intake of vitamin C, vitamin E, carotenoids and flavonoids during the previous month in male athletes and sedentary male controls. The second part of the study involved the assessment of the reproducibility and validity of the FFQ. The study design is depicted in Figure 1.

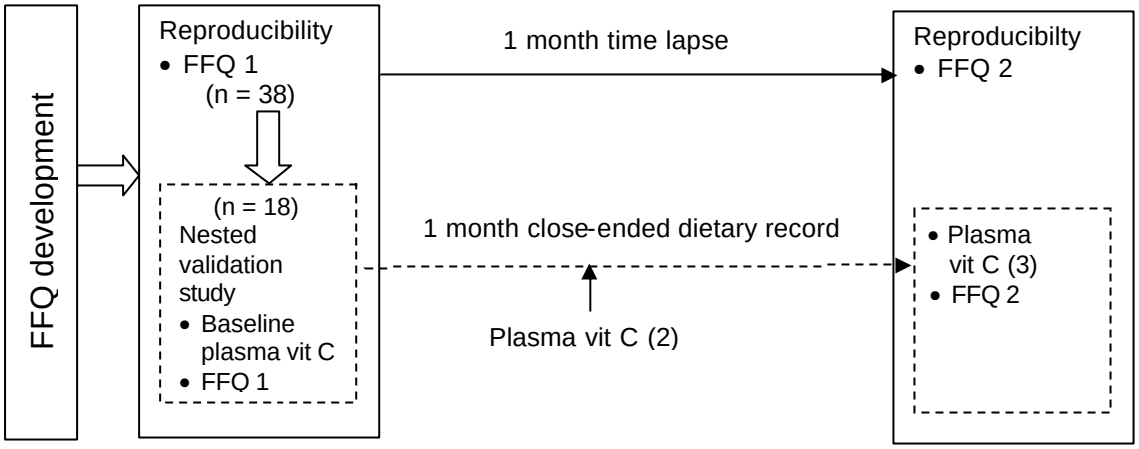


Figure 1: Schematic diagram of study design
FFQ = food frequency questionnaire; vit C = vitamin C

Food frequency questionnaire development

The development of the FFQ involved generating a food list, identifying frequency categories, developing a portion size estimation section and tools as well guidelines for the administration of the final questionnaire to subjects using the picture-sort method (Kumanyika *et al*., 1996).

Food list

The foods to be included in a food list depend on the objectives of the study, for example, measuring intakes of specific foods or nutrients or conducting a comprehensive dietary assessment (Willett, 1998b). In either circumstance, it is important to carefully select the most informative food items for the food list, as lists that are too short may omit important foods resulting in underestimation of intakes, whereas an excessively long food lists can lead to fatigue and boredom, which can impair concentration and accuracy (Willett, 1998b). As the aim of this study was to assess dietary antioxidant intake, only foods that were important contributors to vitamin C, vitamin E, carotenoid and flavonoid intake were included in the food list. According to Willett (1998b), three criteria relating to food items need to be assessed when it is considered for inclusion in a particular food list, namely: 1) the food item has to be a good source of the specific nutrient; 2) the food item has to be used reasonably often by an appreciable number of individuals, and 3) the use of the food item must vary between persons.

To meet the first criterion, foods that were good sources of the nutrients in question were identified from the literature as well as from nutrient composition tables (Langenhoven *et al*., 1991a; Shils *et al*., 1999; Decker & Clarkson, 2000; Pietta, 2000; Stipanuk, 2000). To meet the second criterion information regarding population frequencies of intake of specific food items was obtained from a study that determined usual food consumption of the South African population by analysing secondary data of dietary surveys undertaken in South Africa (Steyn *et al*., 2003). In addition, dietary intake data from 7-day food records, which was gathered during a study conducted with a similar group of rugby players (Personal communication, 2005, Dr. J. van Rooyen, Department of Physiological Sciences, University of Stellenbosch) was analysed and commonly eaten foods that contained the nutrients under investigation were identified and added to the FFQ list. As no information could be found regarding the variation in the use of the food items between persons, the third criterion was not considered in the development of the food list in this study. All the food items identified in the above described steps were integrated in order to finalise the food list. Both single-food items and mixed dishes, such as salads or vegetable soups, were included. The food items were divided into three categories, namely fruit and fruit juices, vegetables and other foods.

Recall period, frequency categories and portion size estimation

The recall period for a FFQ is usually six months to a year, but can be made shorter for a specific study (Willett, 1998b). In the present study the recall period for the FFQ was set at one month. This time period was chosen based on the rugby season and the university timetable, to ensure a homogenous time period (e.g. no holidays). This ensured that the dietary intake would be fairly consistent and well-controlled, in order to be able to link nutrient intake with blood levels.

An open-ended frequency response was used according to which subjects were required to report their intake in terms of frequency per day, week or month. In order to quantify dietary antioxidant intake, subjects were required to provide information regarding usual portion sizes of the foods consumed during the preceding month. Usual portion sizes could be described in terms of 1) small, medium or large compared to diagrams of single food items; 2) common household measurements; 3) an amorphous flour model of half a cup with portion size being half the size, the same, one-and-a-half times the size or twice the size. See addendum 1 for the final questionnaire.

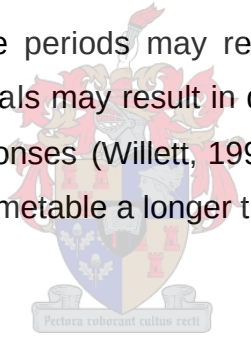
FFQ administration

For the FFQ administration the picture-sort approach was used, which was first developed by Kumanyika *et al*. (1996) and Kumanyika *et al*. (1997) for use in the Cardiovascular Health study. The picture-sort method is an adaptation of a written FFQ that involves respondents having to sort colour picture cards of foods into piles representing frequency of use. The food cards that were used for this study were part of the Food Photo Manual developed by Senekal and Steyn (2004).

FFQ reproducibility and validation

FFQ reproducibility

For the purposes of this study, reproducibility was defined as the consistency of the FFQ measurements in repeated administrations under similar conditions. To assess the reproducibility of our FFQ, subjects were required to complete the FFQ twice approximately one month apart, and nutrient intake derived from the two administrations were compared. When deciding on a time interval for repeat FFQ administrations, it must be borne in mind that shorter time periods may result in subjects remembering their answers, whereas longer time intervals may result in decreased reproducibility due to true changes in diet or variations in responses (Willett, 1998b). Due to time constraints related to the rugby season and university timetable a longer time interval could not be used.



FFQ validity

For the purpose of this study, validity was defined as the ability of the FFQ to accurately estimate dietary antioxidant intake. Validity testing in this study was based on the following: two FFQ administrations one month apart, a 28-day dietary record and three plasma vitamin C samples. The validity was assessed by: 1) Comparing the means ± SD of the reported frequencies of intake of specific food items derived from the FFQs to the means ± SD of the frequencies of intake of the same food item derived from the 28-day dietary record; 2) Comparing the frequencies of intake of specific food items derived from the FFQs to the frequencies of intake of the same food item derived from the 28-day dietary record; 3) Comparing the vitamin C and carotenoid intake derived from the FFQs (two repetitions: FFQ 1 and FFQ 2, and the mean of the two FFQ: FFQmean) with values obtained from a 28-day close-ended dietary record; and 4) comparing plasma vitamin C levels to vitamin C intake derived from the FFQs and the 28-day dietary record. The vitamin C intake derived from FFQs, 28-day dietary record as well as plasma vitamin C

levels were also included in a structural equation model (SEM) to assess the relationship thereof with the true unknown intake.

The comparison of frequency of intake derived from the FFQs and the 28-day dietary record is essential in the validity testing of a FFQ, as Thompson and Byers (1994) and Willett (1998b) mention that frequency estimation of intake is a greater contributor than estimated portion size to variance in intakes for most foods and therefore has a greater influence on FFQ validity.

Vitamin E and flavonoid intake were not considered for the validation assessments as inclusion of the additional food items in the close-ended dietary record would have resulted in a too long list of items, which would increase respondent burden and thus decrease compliance.

Study sample

Subjects in a validation study should ideally be a random sample of the study population for which the FFQ is being used (Willett, 1998b). Although this is often not practical, the sample should at least be representative of the main study cohort. According to Willett (1998b) and Kaaks *et al*. (1994) a similar reproducibility estimation of a FFQ can be achieved by using a larger sample size with fewer days of recording intake versus an increased number of days using a smaller sample size. As the recruitment of a large sample was not possible for the purposes of this study, it was decided to combine a smaller sample with 28 recording days. For the purposes of reproducibility and validity testing of the present FFQ, subjects therefore included a sub-sample of volunteers who were participating in the main study (n = 115) that investigated the association of plasma antioxidant status and genotype in trained rugby players and sedentary male students controlled for dietary antioxidant intake. Thirty-eight subjects participated in the reproducibility study and a subgroup of these (n = 18) volunteered for the validation study. Subjects were healthy, non-smoking males between the ages of 18 and 25 years. Both supplement users and non-users were included.

Dietary assessment procedures and methods

FFQ administration

For the administration of the FFQ, the mentioned picture-sort method was used. After the procedure was thoroughly explained, subjects were asked to sort the food cards into three

separate piles based on the frequency of consumption over the previous month, namely never in the last month, sometimes in the last month and frequently during the past month. The cards corresponding to foods eaten never in the past month were placed aside and no further information regarding those foods was collected. Food items included in the FFQ that were not represented by a photograph, such as vitamin C containing sweets, were asked about separately. Subjects were then required to give detail regarding the exact frequency of consumption (per day / per week / per month) and the usual portion size of the food items that were consumed sometimes or frequently. Visual aids were used to ensure accurate portion size estimation. Life size line drawings depicting small, medium or large servings of fruit and some vegetable items were used (Senekal & Steyn, 2004) as well as a three-dimensional amorphous flour model of half a cup. For the latter, subjects could indicate portion size as half the size, the same size, one-and-a-half times the size or twice the size of the standard portion size. In addition, life size line drawings of different sizes of cups, glasses, bowls and spoons were used (Senekal & Steyn, 2004).

28-day close-ended dietary record

The close-ended dietary resembles FFQs as it also consists of a list of food items that the subject needs to indicate whether it had been consumed (Thompson & Byers, 1994). However, in contrast to the FFQ the subject does not rely on memory to complete this checklist as it is completed either concurrent with actual intake or at the end of a day for that day's intake (Thompson & Byers, 1994).

The dietary record checklist for this study consisted of a booklet with a page for each of the four weeks. Each page contained the same list of food items and response space for each of the seven days. The checklist included a list of food items judged to be frequently consumed sources of vitamin C and carotenoids that were also part of the FFQ. These food items included fruit and fruit juices, vegetables, and vitamin C containing sweets (see Addendum 2).

The purpose of the booklet as well as the recording procedure was thoroughly explained to the subjects. Subjects were also instructed to maintain their usual eating habits and keep the food diary with them at all times, completing it concurrent with actual intake. Therefore, every time a subject consumed a food that was in the food list, a mark had to be made in the block corresponding to the food item and the specific day. If the same food item was

eaten again during that day, another mark had to be made in that particular block. Subjects were not required to indicate portion sizes in the checklist.

The recording started immediately after the first administration and continued for 28 days. The second administration of the FFQ took place after the completion of the dietary record

Dietary supplement intake

As part of the first FFQ administration subjects were asked whether they had taken a dietary supplement during the previous month. Those subjects who had used dietary supplements were then requested to specify the type of supplement(s) they had used, the dosage, frequency and duration of use. For reproducibility assessment, nutrient intake from supplement use was not documented in the repeat FFQ administration. Data on supplemental vitamin C intake was only used in the validation part of the study.

Nutrient content and frequency computation

For the FFQ, intake of vitamin C, vitamin E, carotenoids and flavonoids was calculated for each subject for each FFQ administration. For the analysis of dietary data for these nutrients, the recorded usual portion size for each food item was converted to grams, using the South African Food Quantities Manual (Langenhoven *et al*., 1991b) and the Food Photo Manual (Senekal & Steyn, 2004). The vitamin C and vitamin E content of foods was determined using Foodfinder III (Nutritional Intervention Research Unit and Research Information Systems Division of the Medical Research Council, South Africa: Medical Research Council, 2002). For the determination of carotenoid content, Foodfinder III was used for most foods. For foods for which no carotenoid values were indicated in Foodfinder, the US database for carotenoids was used (Mangels *et al*., 1993). Total carotenoid intake was calculated by adding the intake of the following carotenoids: ß-carotene, a-carotene, lutein, lycopene and cryptoxanthin. For the determination of the flavonoid content of foods, the US database for flavonoids (U.S.Department of Agriculture, 2003) was used as Foodfinder III does not include flavonoid content data. In order to calculate the total intake of a nutrient, the estimated portion size in grams was multiplied by the frequency of intake and this value was then multiplied by the nutrient content value of each food item. This was transformed to reflect nutrient intake per day by dividing the total nutrient intake by seven (if eaten weekly) or 28 (if eaten monthly). This process was repeated for each food item and the total daily intake of vitamin C, vitamin E, carotenoids and flavonoids was calculated by adding the data from

each food item. Vitamin C and E intake was expressed as mg/day and carotenoid and flavonoid intake as µg/day respectively.

To compute the frequencies of intake per month for the food items on the 28-day dietary record, the number of marks for each food on the record was counted. Some food items were combined into a single category, for example, oranges and "naartjies" were combined into a citrus category, orange juice and other fruit juices into a fruit juice category, potatoes and sweet potatoes into a combined potato category and tomatoes and tomato sauces into a combined tomato group. To quantify the nutrient intake of the 28-day dietary record, portion sizes for each food item was based on either a mean value derived from the portion sizes indicated on the FFQ (first and second administration) or a standard portion size when no portion size was available from the FFQs (Langenhoven *et al*., 1991b; Senekal & Steyn, 2004).

The vitamin C content of each supplement was determined by analysing the nutritional information supplied on the supplement containers and/or dietary supplement company websites. This quantity was multiplied by the dosage and frequency of intake and the value was then transformed to reflect nutrient intake per day by dividing the total vitamin C intake by seven (if taken weekly) or 28 (if taken monthly). The vitamin C intake from each separate supplement was finally added together to obtain total daily supplement vitamin C intake for each subject if applicable. This value was added to the vitamin C intake estimated by FFQ 1, FFQ 2 and FFQmean and the 28-day dietary record respectively, to provide a total vitamin C intake value for comparison with plasma vitamin C levels.

Biomarker assessment procedures

During the four week validation study, subjects were required to provide three blood samples for the assessment of plasma vitamin C. This was done at the beginning of the study period (baseline), two weeks after baseline and four weeks after baseline. Blood samples were prepared for vitamin C analysis and were subsequently analysed for vitamin C levels according to the method of (Wei *et al*., 1996) (see Chapter 4 for details of these methods).

Statistical analysis

Data were analysed using the Statistical Programme for Social Sciences (SPSS) version 14 for Windows *(SPSS 14.0, Chicago, Ill 2006*). As dietary intake data did not follow a normal distribution, non-parametric statistical tests were performed.

Reproducibility

For the assessment of reproducibility, data regarding vitamin C, vitamin E, carotenoid and flavonoid intakes derived from the two FFQ administrations was used. Firstly, means, standard deviations, medians and 25th and 75th percentiles of nutrient intakes for the study sample were calculated for descriptive purposes. Differences in nutrient intakes derived from the two FFQ administrations were then analysed using the Wilcoxon sign-rank test. The presence of no statistically significant differences between the two methods is an indication of satisfactory reproducibility of the FFQ. The association between the two FFQs was further determined using Spearman rank correlation coefficients for each nutrient. In this case, a significant correlation coefficient (*r*) that is moderate to high is desired as this indicates satisfactory reproducibility. The agreement between the two FFQ administrations for each nutrient was further assessed by plotting the mean intake of the two administrations of the FFQ against the difference in intake from the two FFQs for each subject using Bland-Altman plots (Bland & Altman, 1986). The limits of agreement (LOA) were computed using the mean difference ± 2SD (Bland & Altman, 1986). Spearman rank correlation coefficients between the mean of the FFQs and the difference were computed to determine whether proportional bias was present (Bland & Altman, 1986). The criteria that can be applied to indicate satisfactory reproducibility based on Bland-Altman plots include the following: narrow limits of agreement, mean differences close to zero, data points that are scattered close to the line of the mean difference, and no proportional bias (Bland & Altman, 1986; Bakker *et al*., 2003). Lastly, the distributions of the intakes estimated from the two FFQ administrations were divided into quartiles and the number of respondents falling into the same or adjacent quartiles on each administration was determined. The higher the percentage of subjects being classified into the same and adjacent quartiles on both administrations, the more satisfactory the reproducibility.

Validity

Data used in statistical analysis included food item intake frequencies and nutrient intake obtained from FFQ 1, FFQ 2, the mean of FFQ 1 and FFQ 2 (= FFQ mean), and the 28-day dietary record as well as three plasma vitamin C values. The dietary data derived from

the 28-day dietary record was compared to all three FFQ values as the literature regarding which FFQ administration to use is not consistent.

Firstly, the mean and standard deviation (mean ± SD) of frequency of food item intake was calculated for descriptive purposes. The mean, standard deviation, median and 25th and 75th percentile of nutrient intake from FFQ 1, FFQ 2 and FFQmean (= three FFQ values) and the 28-day dietary record were calculated for descriptive purposes. Differences between frequencies of food item intake as well as nutrient intakes, derived from the three FFQ values and the dietary record were analysed using the Friedman test with the Bonferroni post hoc test. Spearman rank correlation coefficients were computed to determine the association between the frequency of food item intake derived from the 28-day dietary record and each of the three FFQ values as well as the association between nutrient intake derived from the 28-day dietary record and each of the three FFQ values. Bland-Altman plots (Bland & Altman, 1986) were used to describe the agreement between the 28-day dietary record and each of the three FFQ values for vitamin C and carotenoid intake. The distributions of the intakes estimated from the dietary record, FFQ 1, FFQ 2 and the mean FFQ were divided into quartiles and the percentage agreement of the classification between the dietary record and the respective three FFQ values was determined. Criteria for validity assessment using these methods are the same as the criteria mentioned above for reproducibility assessment.

For the assessment of plasma vitamin C levels the mean ± SD of the plasma vitamin C levels obtained from the three time points was compared using the Friedman test with the Bonferroni post hoc test. The vitamin C values obtained from the three time points were then combined to provide a mean plasma vitamin C value for each subject. The mean plasma vitamin C was then correlated with the vitamin C intake derived from the FFQ values as well as the 28-day dietary record using the Spearman rank correlation test.

The method of triads (Kaaks, 1997) was used to determine the VC (validity coefficient) between the true unknown intake and intake estimated from the 28-day dietary record (reference dietary method), the three FFQ values (test method), as well as the mean plasma levels (biomarker) (Figure 2). Firstly, the Pearson's correlation coefficient between each of the methods was computed. The VCs between the true dietary intake and the dietary intake estimated from the different dietary assessment methods were then estimated using the correlation coefficients generated using Formulas 1 - 3. This process

was repeated three times, each time using a different one of the three FFQ values, namely FFQ 1, FFQ 2 and the FFQ mean.

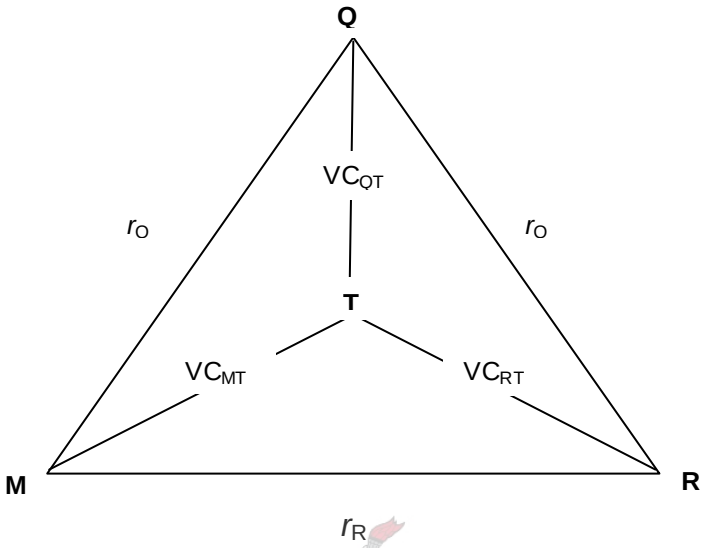


Figure 2: diagrammatic representation of the method of triads used to estimate the validity coefficients (VC) between the true unknown dietary intake (T) and intake estimated by the FFQ (Q), biomarker (M), and dietary record (R). r_{RM}, r_{QR} and r_{QM} are the correlation coefficients between the different methods (adapted from Ocke and Kaaks (1997) and Andersen *et al*. (2005).

$$VC_{QT} = \sqrt{[(r_{QR} \times r_{QM})/r_{RM}]} \qquad \text{Formula 1}$$

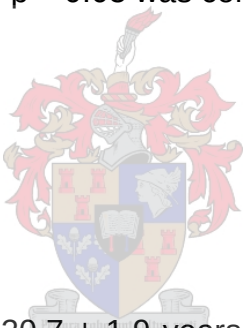
$$VC_{RT} = \sqrt{[(r_{QR} \times r_{RM})/r_{QM}]} \qquad \text{Formula 2}$$

$$VC_{MT} = \sqrt{[(r_{QM} \times r_{RM})/r_{QM}]} \qquad \text{Formula 3}$$

Confidence intervals were constructed for each repetition using empirical percentiles (2.5th and 97.5th) for the replicates of estimated VCs from 1000 bootstrap samples (Ocke & Kaaks, 1997). Bootstrap sampling involves the repeated drawing of samples from the group of subjects participating in the study (Ocke & Kaaks, 1997). Sampling is done with replacement, which allows each case to be drawn more than once or not at all in each of

the samples. The method of triads is then applied to each sample and the empirical distributions of the estimated validity coefficients can be used to determine the confidence intervals. This was done using the Univariate procedure of SAS software. The VC obtained for each assessment method (questionnaire, record and biomarker) indicates the agreement between that method and the true unknown dietary intake (Kaaks, 1997). The higher the value of the VC the better the agreement between the dietary assessment method and the true intake. However, in some cases the VC and confidence intervals are greater than one. This is known as a Heywood case (Dunn, 1989) and when this occurs it is common practice to set the VC and confidence intervals at one (Ocke & Kaaks, 1997; Kabagambe *et al*., 2001; Andersen *et al*., 2005). In the method of triads, Heywood cases occur when the product of two or three sample correlations is larger than the third (Ocke & Kaaks, 1997). In the presence of a high number of Heywood cases, results need to be interpreted with caution.

For all statistical analyses, a value of $p < 0.05$ was considered to be statistically significant.



Results

Sample characteristics

The mean age of the subjects was 20.7 ± 1.9 years. Mean BMI (body mass index) was 27.0 ± 3.7 kg/m^2. Dietary supplement use was reported by 68.4% of subjects.

Reproducibility of the FFQ

A comparison of the nutrient intake obtained from the two administrations of the FFQ is shown in Table 1. Dietary intakes were lower for all the nutrients with the repeat FFQ, significantly so for Vitamin C and flavonoid intake. Spearman Rank correlation coefficients for the nutrients assessed by the two FFQ administrations are also shown in Table 1. Correlation coefficients ranged from 0.51 for vitamin E to 0.68 for flavonoid. All correlations were statistically significant.

Table 1: Comparison of daily nutrient intakes (mg/day) derived from repeated FFQs (n = 38)

	FFQ	Mean ± SD	Median (P25-P75)	Spearman rank correlation coefficients
Vitamin C	1	115.4 ± 71.8*	91.2 (65.3-152.2)	0.54 (p = 0.000)
	2	94.6 ± 73.2	82.0 (55.3-113.0)	
Vitamin E	1	6.9 ± 5.4	5.6 (3.7-9.4)	0.51 (p = 0.001)
	2	5.7 ± 4.9	4.3 (3.2-8.1)	
Carotenoid	1	7.7 ± 6.5	6.5 (2.4-10.5)	0.68 (p = 0.000)
	2	6.3 ± 4.6	5.3 (2.0-8.3)	
Flavonoid	1	74.0 ± 79.4*	51.9 (24.0-91.5)	0.68 (p = 0.000)
	2	56.0 ± 57.3	41.7 (17.0-66.3)	

FFQ 1 = first FFQ administration; FFQ 2 = second FFQ administration

*Statistically significant difference between FFQ 1 and FFQ 2 as determined by the Wilcoxon sign-rank test

The percentage of respondents classified into similar or adjacent quartiles of intake for the two administrations of the FFQ is reported in Table 2. For all nutrients, over 75% of respondents were classified in the same or adjacent quartiles by both administrations. Only vitamin C showed extreme misclassification with 5% of individuals being ranked in the first quartile on one FFQ administration and in the fourth quartile with the other FFQ administration.

Table 2: Classification of subjects into the same and adjacent quartiles of intake for the two administrations of the FFQ (n = 38).

Nutrient	% in same quartile	% within one quartile	% within two quartiles	% within three quartiles
Vitamin C	50	32	13	5
Vitamin E	39	37	24	0
Carotenoid	39	47	13	0
Flavonoid	45	42	13	0

Bland-Altman plots for the nutrients are depicted in Figures 3 – 6 and the actual values are presented Table 3. As can be seen from Figures 3 – 6 and Table 3, almost all observations lay within the limits of agreement. However, the LOA were relatively wide for all nutrients. For vitamin C, the LOA corresponded to more than one and a half times the RDA (90mg/day, Food and Nutrition Board (2000), while the LOA for vitamin E corresponded to just less than the RDA (15mg/day, Food and Nutrition Board (2000). No RDA's have been established for carotenoids and flavonoids. Although the mean difference was close to zero, the scatter was not satisfactory. It is clear from Figures 3 – 6

that significant proportional bias was present for vitamin C, which is also reflected in the by significant Spearman rank correlation coefficients. Therefore, for vitamin C differences in intake are dependant on the magnitude of intake i.e. at lower intakes FFQ 2 underestimated intake, while at higher intakes, FFQ 2 overestimated intake. The Bland-Altman plot for vitamin E showed a different relationship between the two administrations, with the variation between the FFQs increasing in both directions as the magnitude of intake increased (Figure 4).

Table 3: Mean differences (d), limits of agreement (LOA) (d ± 2SD), % observations lying outside the LOA and the presence of proportional bias as calculated by the Bland-Altman method between the first and second FFQ administration (n = 38).

Nutrient	Mean difference (d) (FFQ2– FFQ1)	LOA	% > (d + 2SD)	% < (d – 2SD)	p value for correlation coefficient*	LOA vs RDA
Vitamin C (mg)	-20.8	-164.5 – 122.8	2.6	2.6	0.02	>1xRDA (90mg/day)
Carotenoids (mg)	-1.4	-13.1 – 10.3	2.6	2.6	0.15	RDA ne
Vitamin E (mg)	-1.2	-12.5 – 10.1	2.6	0	0.73	˜ RDA (15mg/day)
Flavonoids (mg)	-18.0	-102.2 – 66.3	2.6	0	0.06	RDA ne

LOA = limits of agreement; RDA = recommended dietary allowances; ne = not established

* Spearman rank correlation between the mean (FFQ 1 and FFQ 2) and the difference (FFQ 2 – FFQ 1). Significant correlation coefficients indicate proportional bias.

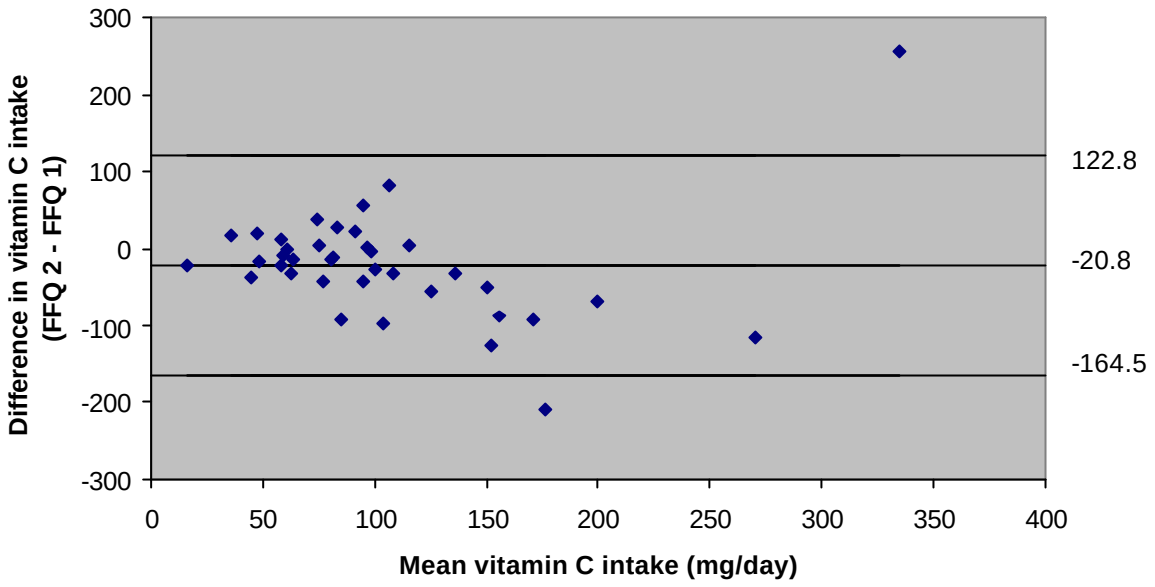


Figure 3: Bland-Altman plot of vitamin C (mg/day) intake derived from the two FFQ administrations showing the mean difference and limits of agreement (d±2SD).

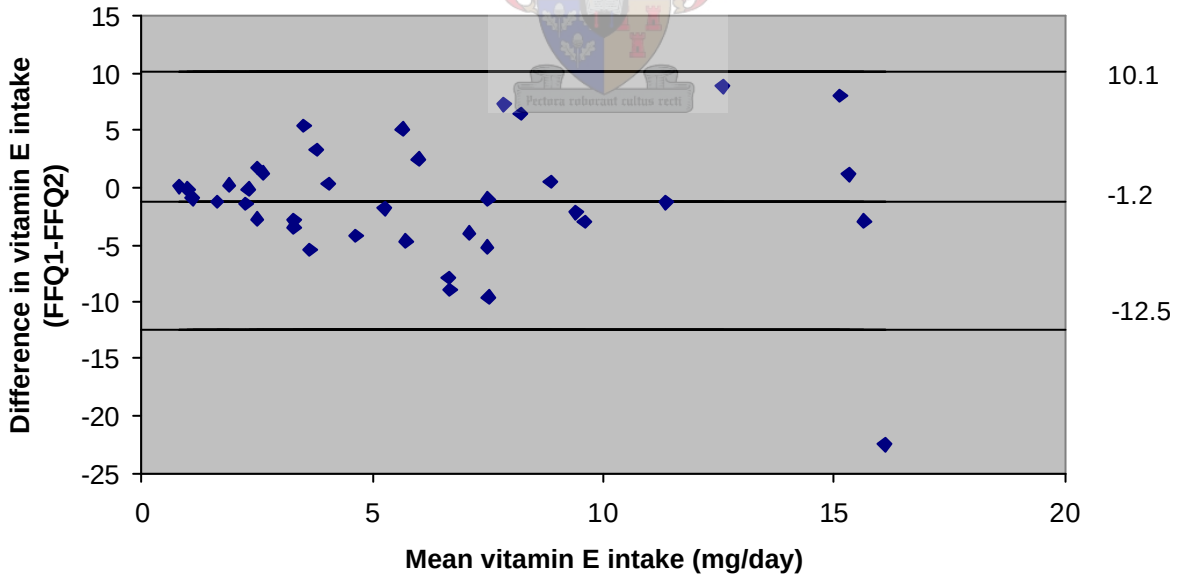


Figure 4: Bland-Altman plot of vitamin E intake (mg/day) derived from the two FFQ administrations showing the mean difference and limits of agreement (d±2SD).

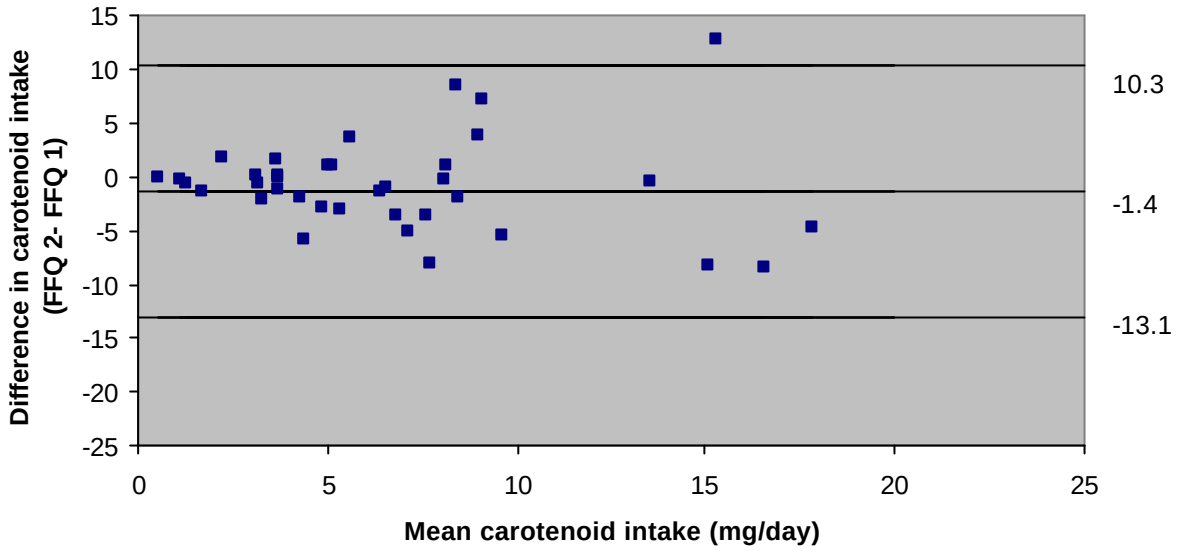


Figure 5: Bland-Altman plot of carotenoid intake (mg/day) derived from the two FFQ administrations showing the mean difference and limits of agreement (d±2SD).

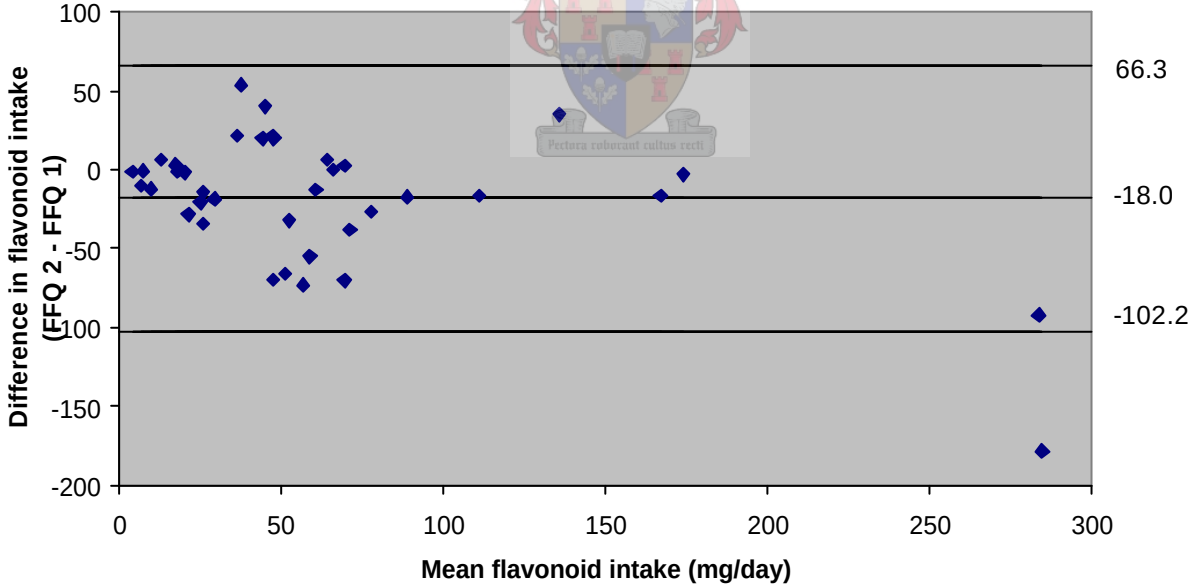


Figure 6: Bland-Altman plot of flavonoid intake (mg/day) derived from the two FFQ administrations showing the mean difference and limits of agreement (d±2SD).

Relative validity of the FFQ

The results of the comparison between the mean frequency of food intake derived from the 28-day dietary record, FFQ 1, FFQ 2 and the FFQmean are shown in Table 4. Food intakes were similar across the board for most foods, except for fruit salad, pumpkin/butternut and potatoes, which were significantly lower for the 28-day dietary record.

Table 4: Mean±SD of reported frequency of intake of food items (times/month) derived from the 28-day dietary record and FFQ 1, FFQ 2 and FFQmean values (n = 18).

Food item	FFQ 1	FFQ 2	FFQ mean	Dietary record
Citrus fruit	6.9 ± 7.8	5.4 ± 8.9	6.2 ± 6.5	3.1 ± 3.8
Strawberry	0.6 ± 1.7	1.4 ± 2.9	1.0 ± 1.8	1.72 ± 2.9
Apple	11.3 ± 7.7	9.9 ± 9.9	10.6 ± 7.3	8.3 ± 7.7
Banana	7.9 ± 5.6	5.8 ± 4.7	6.8 ± 4.5	6.8 ± 7.0
Fruit salad	3.7 ± 6.0*	0.6 ± 1.3	2.1 ± 3.4*	0.6 ± 1.0
Avocado	2.7 ± 4.3	1.9 ± 3.3	2.3 ± 3.1	2.3 ± 3.5
Fruit juice	11.9 ± 10.5	12.2 ± 10.0	12.0 ± 7.7	18.3 ± 13.0
Carrot	6.7 ± 5.6	8.1 ± 6.4	7.4 ± 4.7	5.8 ± 3.1
Pumpkin	6.5 ± 4.3*	4.2 ± 3.0	5.3 ± 3.0*	3.7 ± 2.8
Peas	4.2 ± 5.3	2.4 ± 2.7	3.3 ± 3.0	1.3 ± 1.4
Cauliflower	4.8 ± 4.4	3.6 ± 4.0	4.2 ± 3.6	2.3 ± 2.3
Broccoli	4.1 ± 4.4	4.2 ± 4.3	4.2 ± 3.6	3.1 ± 2.8
Green beans	2.9 ± 3.5	1.8 ± 1.9	2.4 ± 2.3	1.2 ± 1.3
Mixed salad	7.2 ± 6.7	4.9 ± 3.3	6.1 ± 3.6	4.4 ± 4.3
Tomato	22.3 ± 15.7	18.6 ± 16.9	20.5 ± 15.1	18.1 ± 12.2
Mixed vegetable	6.4 ± 4.6	4.0 ± 4.5	5.2 ± 4.0	3.9 ± 3.7
Spinach	0.8 ± 1.4	1.1 ± 2.1	0.9 ± 1.3	0.8 ± 1.9
Potato	29.5 ± 18.0*	22.3 ± 8.8*	25.9 ± 10.6*	16.1 ± 7.8

Citrus includes oranges and naartjies; fruit juice includes orange juice and other fruit juices; pumpkin includes pumpkin and butternut; tomato includes tomatoes, tomato sauces; potato includes potatoes and sweet potatoes.

* Indicates statistically significant difference between FFQ and the 28-day dietary record for the food item ($p<0.05$ as assessed by Friedman test and Bonferroni post hoc)

The Spearman correlation coefficients for the frequencies of intake of individual food items derived from the 28-day dietary record and each of the three FFQ values ranged from 0.03 (non-significant) to 0.93 (Table 5). For all food items, the strongest correlation was found between the 28-day dietary record and FFQ 2. In the fruit group, correlations for frequency of intake derived from the 28-day dietary record and each of the three FFQ values were

strongest for apples and bananas; while the frequency of intake of fruit juice and fruit salad did not show a significant correlation. The frequency of intake of broccoli, cauliflower and tomato intake showed the strongest correlation in the vegetable group, with the frequency of intake of green beans and mixed vegetables not being significantly correlated between the 28-day dietary record and each of the three FFQ values respectively.

Table 5: Spearman rank correlations for frequency of intake of specific food items between the 28-day dietary record and each of the three FFQ values (n = 18)

	Correlation coefficient (*r*) between 28-day dietary record and…					
Food item	**FFQ 1**		**FFQ 2**		**FFQ mean**	
	r	**p**	***r***	**p**	***r***	**p**
Citrus fruit	0.09	0.736	0.62	0.006	0.33	0.179
Strawberry	0.12	0.639	0.57	0.013	0.61	0.007
Apple	0.58	0.012	0.79	0.000	0.80	0.000
Banana	0.48	0.042	0.68	0.002	0.73	0.001
Fruit salad	0.03	0.915	0.23	0.368	0.11	0.652
Avocado	0.29	0.250	0.85	0.000	0.64	0.004
Fruit juice	-0.12	0.629	0.17	0.498	0.14	0.584
Carrot	0.26	0.306	0.55	0.018	0.54	0.022
Pumpkin	0.45	0.060	0.76	0.000	0.69	0.001
Peas	0.37	0.136	0.68	0.002	0.50	0.034
Cauliflower	0.52	0.028	0.93	0.000	0.77	0.000
Broccoli	0.50	0.034	0.86	0.000	0.64	0.004
Green beans	0.08	0.748	0.46	0.054	0.25	0.311
Mixed salad	0.09	0.735	0.73	0.001	0.29	0.247
Tomato	0.77	0.000	0.92	0.000	0.83	0.000
Mixed vegetable	0.26	0.299	0.36	0.149	0.30	0.227
Spinach	0.05	0.831	0.61	0.007	0.44	0.068
Potato	-0.09	0.695	0.49	0.039	0.09	0.716

Citrus includes oranges and naartjies; fruit juice includes orange juice and other fruit juices; pumpkin includes pumpkin and butternut; tomato includes tomatoes, tomato sauces; potato includes potatoes and sweet potatoes.

Table 6 presents a comparison of the vitamin C and carotenoid intakes derived from the 28-day dietary record and each of the three FFQ values. Vitamin C intakes were similar for all dietary intake assessments. Carotenoid intake values derived from FFQ 1, FFQ 2 and the FFQmean respectively, were all three significantly higher than the intake estimated from the dietary record. The Spearman rank correlation coefficients between the nutrient intakes derived from the 28-day dietary record and each of the three FFQ values are

presented in Table 7. For vitamin C, only the correlation between the second FFQ administration and the dietary record was significant. The intake of carotenoids derived from the 28-day dietary record was significantly correlated with the intake derived from all three FFQ values, with the strongest correlation coefficient being between the dietary record and the second FFQ administration (0.80).

Table 6: Comparison of daily vitamin C and carotenoid intake (mg/day) derived from the 28-day dietary record and each of the three FFQ values (n = 18)

		Mean ± SD	Median (P25 – P75)
Vitamin C	DR	89.5 ± 38.6	89.5 (60.9-105.4)
	FFQ 1	137.3 ± 88.1	113.9 (64.5-209.3)
	FFQ 2	120.2 ± 94.4	104.7 (69.2-124.0)
	FFQ mean	128.8 ± 77.0	110.8 (71.8-159.2)
Carotenoid	DR	5.8 ± 3.1	5.7 (3.3-6.8)
	FFQ 1	9.8 ± 7.9*	7.3 (4.0-12.7)
	FFQ 2	8.1 ± 4.9*	7.1 (4.3-11.3)
	FFQ mean	8.8 ± 5.3*	8.3 (4.7-13.9)

DR = 28-day dietary record; FFQ 1 = first FFQ administration; FFQ 2 = second FFQ administration; FFQ mean = mean of FFQ 1 and FFQ 2

* Statistically significantly different from DR using Friedman test and Bonferroni post hoc test ($p<0.05$)

Table 7: Spearman rank correlation coefficients for vitamin C and carotenoids between the 28-day dietary record and each the three FFQ values (food only) (n = 18)

	Dietary record correlation with…		
	FFQ 1	FFQ 2	FFQ mean
Vitamin C	0.27 (p = 0.284)	0.48 (p = 0.043)	0.23 (p = 0.358)
Carotenoids	0.53 (0.025)	0.80 (p = 0.000)	0.71 (p = 0.001)

FFQ 1 = first FFQ administration; FFQ 2 = second FFQ administration; FFQ mean = mean of FFQ 1 and FFQ 2

The ability of each of the FFQ values to assign individuals into the same or adjacent quartiles of nutrient intake relative to the 28-day dietary record is shown in Table 8. For both vitamin C and carotenoids, over 60% of individuals were classified into the same or adjacent quartiles by the dietary record and the respective FFQ value. Agreement was

lowest for vitamin C between the 28-day dietary record and the first FFQ administration (61%), and highest between the dietary record and the second FFQ for carotenoid (94%). The lowest degree of misclassification for both nutrients was between the dietary record and the second FFQ administration. Extreme misclassification varied from 0% to 17%, with carotenoid intake estimated by the dietary record and the FFQ mean showing the greatest degree of misclassification (17%).

Table 8: Classification of subjects into quartiles of intake for the 28-day dietary record versus each of the three FFQ values (n = 18).

	% in same quartile	% within one quartile	% within two quartiles	% within three quartiles
Vitamin C				
DR vs FFQ 1	33	28	33	6
DR vs FFQ 2	33	33	33	0
DR vs FFQ mean	39	22	28	11
Carotenoid				
DR vs FFQ 1	44	33	11	11
DR vs FFQ 2	50	44	6	0
DR vs FFQ mean	39	44	0	17

DR = 28-day dietary record; FFQ 1 = first FFQ administration; FFQ 2 = second FFQ administration; FFQ mean = mean of FFQ 1 and FFQ 2

Table 9 presents the actual values of the Bland-Altman tests for vitamin C and carotenoids for the 28-day dietary record and each of the three FFQ values. The graphs are included in Addendum 3. For both nutrients, only one data point was positioned outside the limits of agreement. The LOA were relatively wide for both nutrients and for all three FFQ values, and in terms of the RDA for vitamin C, lay between approximately one and more than two times the RDA. The scatter of data points was not satisfactory for all plots. The mean difference for vitamin C between the 28-day dietary record and each of the three FFQ values was less than approximately half the RDA. The mean difference was closest to zero between the second FFQ administration and the 28-day dietary record for both nutrients. Proportional bias was present for vitamin C between the 28-day dietary record and FFQ 1 and for carotenoid between the 28-day dietary record and each of the FFQ values.

Table 9: Mean differences (d), limits of agreement (LOA) (d ± 2SD), % observations lying outside the LOA and the presence of proportional bias as calculated by the Bland-Altman method between the 28-day dietary record and each of the FFQ values (n = 18).

Nutrient	Mean difference (DR–FFQ)	LOA	% > (d +2SD)	%< (d – SD)	p value for correlation coefficient*	LOA vs RDA
Vitamin C (mg)						
FFQ 1	-47.9	-221.6 – 125.9	0	2.6	0.001	>1X RDA (90mg/day)
FFQ 2	-30.9	-220.9 – 159.5	0	2.6	0.36	>1X RDA (90mg/day)
FFQ mean	-39.3	-192.2 – 113.7	0	2.6	0.08	>1X RDA (90mg/day)
Carotenoids (mg)						
FFQ 1	-3.8	-18.9 – 11.3	0	2.6	0.03	RDA ne
FFQ 2	-2.3	-10.0 – 5.4	0	2.6	0.04	RDA ne
FFQ mean	-3.1	-12.1 – 6.0	0	0	0.01	RDA ne

LOA = limits of agreement; RDA = recommended dietary allowances; ne = not established

* Spearman rank correlation between the mean (DR and FFQ) and the difference (DR – FFQ). Significant correlation coefficients indicate proportional bias.

The mean ± SD of plasma vitamin C levels measured at the three time points during the validation study were as follows: baseline = 0.92 ± 0.3 mg/dl; two weeks post baseline = 0.91 ± 0.3 mg/dl; and four weeks post baseline = 0.94 ± 0.3 mg/dl. There were no significant differences for plasma vitamin C levels between the three time points (p = 0.28 from Friedman test). Table 10 presents the correlations between vitamin C intakes according to the different dietary assessment methods and the mean plasma vitamin C levels. No significant correlations were evident.

Table 10: Spearman rank correlations between vitamin C intakes (food + supplements) derived from the 28-day dietary record as well as from FFQ 1, FFQ 2 and the FFQmean with plasma vitamin C levels (n = 18)

	Mean plasma vitamin C* correlation with…			
	FFQ 1	FFQ 2	FFQ mean	Dietary record
Vitamin C	0.32 p = 0.197	0.10 p = 0.693	0.21 p = 0.395	0.14 p = 0.581

*Mean of three values for each individual

Finally, the validity coefficients (VCs) and confidence limits for vitamin C for each dietary assessment method (28-day dietary record, FFQ and plasma) for each of the three FFQ values according to the method of triads are shown in Table 11. The VCs ranged from 0.29 to 1.00 and were lower for plasma vitamin C than for the FFQ 1, FFQ 2, FFQmean and the dietary record vitamin C values, which were similar. Confidence limits were also the widest for plasma vitamin C. Heywood cases, represented by VCs larger than one and that are thus set at one, were present for some of the validity coefficients.

Table 11: Validity coefficients (VC) with 95% bootstrap confidence intervals for vitamin C derived from FFQ 1, FFQ 2 and the FFQmean, the 28-day dietary record (DR) and actual plasma levels (n = 18).

	VC*	95% CI*
FFQ 1		
DR vs T	0.86	0.57 – 1.00
FFQ 1 vs T	1.00	0.59 – 1.00
Plasma vs T	0.34	0.04 – 0.80
FFQ 2		
DR vs T	1.00	0.60 – 1.00
FFQ 2 vs T	0.84	0.37 – 1.00
Plasma vs T	0.29	0.03 – 0.76
FFQ mean		
DR vs T	0.92	0.61 – 1.00
FFQ mean vs T	0.99	0.57 – 1.00
Plasma vs T	0.32	0.04 – 0.77

DR = 28-day dietary record; T = true unknown intake, FFQ 1 = first FFQ administration, FFQ 2 = second FFQ administration, FFQ mean = mean of FFQ 1 and FFQ 2.

*Validity coefficients and confidence intervals > 1 (Heywood cases) were set to 1. These values may thus in actual fact be higher than one?

Discussion

The aims of this study were to develop and validate a quantified FFQ that measures intake of vitamin C, vitamin E, carotenoids and flavonoids in a young adult male population. The final FFQ consisted of 81 food items divided into a fruit and fruit juice section, a vegetable section and an “other” section. The short-term reproducibility was assessed by administering the FFQ twice within a one month period. The reference methods that were used to assess the validity of the FFQ in terms of frequency of food item intake and

vitamin C (mg/day) and carotenoid intake (mg/day) was a 28-day close-ended dietary record and repeated plasma vitamin C levels.

For the assessment of reproducibility of the FFQ in this study several statistical methods were used, including comparison of mean differences, correlation coefficients, Bland-Altman plots and cross-classification of individuals according to quartile distributions of intake. When considering the difference between mean values, the first administration of the FFQ generally gave higher estimates of nutrient intakes than the second questionnaire, with intakes being significantly different for vitamin C and flavonoids. This trend of lower nutrient intakes estimated from repeated FFQs has been reported by several other studies (Pietinen *et al*., 1988; Munger *et al*., 1992; Elmstahl *et al*., 1996; Jackson *et al*., 2001), although the opposite has also been observed (Macintyre *et al*., 2001a; Fornes *et al*., 2003). It has been suggested that the overestimation of nutrient intakes associated with a first FFQ administration may be due to participants having a more realistic idea of their diets and thus being able to quantify their intake more accurately with the repeat FFQ administration (Pietinen *et al*., 1988). In the present study the month of recording prior to the second administration of the FFQ in a sub-sample (n = 18) of the reproducibility sample of 38, may have contributed to a higher awareness of actual frequency of intake of food items and thus favouring a more accurate estimate of intake.

With reference to correlation testing in reproducibility studies, correlation coefficients ranging from 0.5 to 0.7 for nutrient intakes measured at recall periods of between one and ten years are generally reported (Willett, 1998b; Johansson *et al*., 2002). The present correlation coefficients ranging from 0.5 (vitamin E and vitamin C) to 0.68 (carotenoids and flavonoids) fall within this range, even though the time interval between the administrations in the present study was shorter. Compared to other short-term reproducibility studies, the present correlation coefficients were in line with what has been reported and accepted as satisfactory by others (Schroder *et al*., 2001; Jackson *et al*., 2001; Macintyre *et al*., 2001a). We therefore feel that our correlation coefficients indicate satisfactory reproducibility for the FFQ.

Reproducibility was further assessed by determining the agreement between the classifications of participants into quartiles of intake based on the two FFQ administrations. The percentage of participants classified in the same or adjacent quartiles in our study ranged from 76% to 87%. This is in line with the range reported by Macintyre *et al*. (2001a).

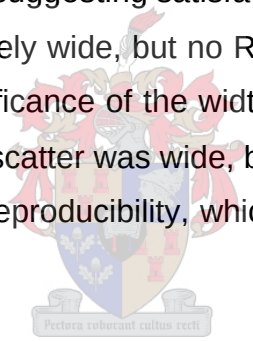
The high levels of agreement in ranking between the two FFQ administrations for all nutrients in the present study suggest that the FFQ is reproducible.

While estimated vitamin C and flavonoid intakes were significantly lower with the second administration of the FFQ, the satisfactory correlation coefficient (0.54 for vitamin C and 0.68 for flavonoid) and the agreement in quartile ranking of individuals between the two FFQ administrations suggests that the entire distribution of vitamin C and flavonoid intake was shifted downwards. A similar pattern, but in the opposite direction, was observed for vitamin C intake by Macintyre *et al*. (2001a). The authors attributed their upward shift in distribution to the increased availability of citrus during the repeat FFQ administration. In the present study, the opposite may have occurred with the repeat FFQ being administered towards the end of the citrus season, which may explain the significantly lower vitamin C intake estimated with FFQ 2.

A statistical test that is now becoming more popular in the assessment of reproducibility and validity of FFQs are Bland-Altman plots (Bland & Altman, 1986). These plots have been referred to as “eyeball tests” as they are used to visualise the mean differences, the limits of agreement between the methods or administrations, and the presence of a relationship or bias between the differences in intake and the mean intake (Bakker *et al*., 2003). As mentioned in the methods, the most desirable outcome would include: 1) narrow limits of agreement; 2) mean differences close to zero, 3) data points that are scattered close to line of the mean difference, and 4) no proportional bias (Bakker *et al*., 2003). However, the judgement of these criteria is to a large extent subjective as it based on judgement by eye and does not rely on statistical tests, except for the judgement regarding proportional bias, which is reflected by a significant Spearman rank correlation coeffcient (Bland & Altman, 1986; Bakker *et al*., 2003). Bland and Altman (1986) point out that the width of the LOA should be judged according to the clinical importance of the differences between the two methods/administrations. However, there are no set criteria against which the clinical importance of the width of the LOA in FFQ validation studies can be judged. Therefore, in the present study, the RDAs (where established) were used to judge the clinical importance of the width of the LOA. The estimations of intake from the two FFQ administrations varied by up to one and a half times the RDA for vitamin C as the mean difference ± 2SD was -164.5 – 122.8 (Food and Nutrition Board, 2000). If a subject were therefore to be classified for adequacy of vitamin C intake based on RDA cut-offs, it would be possible for the intake of vitamin C of the subject to be classified as adequate when

using the vitamin C data derived from the one administration of the FFQ, but as deficient when using the data from the other administration of the FFQ. We feel that this type of scenario would definitely be of clinical importance and therefore suggest that LOA that are wider than one times the RDA should be deemed not satisfactory.

When considering the reproducibility of the FFQ in measuring vitamin C based on Bland-Altman plot outcomes, it did not seem satisfactory, even though all data points were within the LOA and the mean difference close to zero, as the LOA were judged to be clinically significant, the scatter wide and proportional bias was present. Although Macintyre *et al*. (2001a) did not comment on the other criteria, they also observed proportional bias for vitamin C between two FFQ administrations. This outcome of the Bland-Altman plots is not supported by the results of the correlations and the quartile ranking, which pointed to satisfactory reproducibility. For vitamin E, differences were within one RDA, and the LOA were not judged to be clinically significant, the mean difference was close to zero and no proportional bias was present, thus suggesting satisfactory reproducibility. For carotenoids and flavonoids the LOA were relatively wide, but no RDA have been established for these nutrients to assess the clinical significance of the width of the LOA. For both nutrients the mean difference was close to zero, scatter was wide, but no proportional bias was present. These results suggest satisfactory reproducibility, which is in line with the results from the other statistical tests.



When interpreting and applying the results of the Bland-Altman plots it must be borne in mind that, although these plots have been proposed as superior to the correlation assessments, the interpretation thereof in general and in dietary assessment context is subjective and the criteria for good agreement in actual fact not very clear. Furthermore, researchers such as Hopkins (2004) are very critical of the Bland-Altman procedure and warn that it has a fatal flaw in that it may incorrectly indicate systematic differences or bias in the relationship between two measures. Therefore, the fact that at least two out of the four statistical tests conducted for each nutrient indicate satisfactory reproducibility, points to satisfactory reproducibility of the FFQ in the assessment of antioxidant intake. Bearing in mind the limited sample size, we feel that the results do point to reasonable reproducibility.

When considering the validity of a questionnaire, good reproducibility is essential, but this does not necessarily ensure validity. It therefore remains essential that validation

assessments are also carried out (Willett, 1998b). For the purposes of this study, validity was assessed by firstly comparing the mean and frequency estimation of intake of food items, and the vitamin C and carotenoid intake derived from FFQ 1, FFQ 2, and the FFQmean to values generated from a 28-day close-ended dietary record (difference between means, correlation coefficients, Bland-Altman plots and quartile distributions). Secondly, the vitamin C intake derived from FFQ 1, FFQ 2, FFQmean and the 28-day dietary record was correlated with the mean of the three repeat plasma vitamin C assessments. Lastly, a SEM, based on the method of triads, was constructed to assess the association between the vitamin C intake derived from the different assessments and true unknown intake.

The means of reported frequencies of intake of food items (times/month) were similar for most of the food items for the three FFQ values and the 28-day dietary record. In addition, correlation coefficients for the frequencies of intake of food items between each of the three FFQ values and the 28-day dietary record were moderately strong, especially between the dietary record and FFQ 2. If the statement by Flegal and Larkin (1990) that differences in reported food intake frequencies are the most important source of error for group means and for relative ranking of subjects is considered, the present results on estimated frequency of intake, which were similar for the FFQ and the 28-day dietary record, suggest satisfactory validity. These results also create the expectation that the three FFQ values of actual vitamin C and carotenoid intake should be similar to the values derived from the 28-day dietary record.

Comparisons of mean values support this notion for vitamin C, but not for carotenoids. The three FFQ values for vitamin C intake were not significantly different from the 28-day dietary record values. However, carotenoid intake derived from the three FFQ values was significantly higher than the 28-day dietary record values. Several other researchers have observed that FFQs overestimate nutrient intakes when compared with various dietary records (Schroder *et al*., 2001; McKeown *et al*., 2001; George *et al*., 2004). This was especially apparent for vitamin intakes (McKeown *et al*., 2001; Macintyre *et al*., 2001b). The relatively long list of fruit and vegetables in the FFQ may account for this observed overestimation in carotenoid intake (Harlan & Block, 1990; Willett, 1998a). On the other hand, Willett (1998b) points out that the process of recording food intake may lead to a change in eating habits and this may partly explain why intake obtained from dietary records is often lower than the reported FFQ intakes. One of the explanations for the

behavioural modification related to keeping dietary records is the burden that is associated with recording of foods, especially mixed dishes (Willett, 1998b). The present study made use of a close-ended dietary record with relatively low respondent burden compared to open-ended or weighed records. It is thus unlikely that the participants' eating habits would have changed much resulting in lower nutrient estimations from the dietary record compared to the FFQ. However, the fact that the dietary record was a close-ended record that did not include all the food items in the FFQ, could also have contributed to the observed lower nutrient intakes compared to the FFQ.

As far as the correlation assessments are concerned, results from a large number of FFQ validation studies in various populations indicate that the correlation coefficients between FFQ and reference methods range from 0.5 – 0.7 for macro-nutrients and 0.4 – 0.7 for micronutrients (for a summary of studies see (Willett, 1998b). Spearman Rank correlation coefficients for carotenoid and vitamin C intakes between the 28-day dietary record and each of the three FFQ values obtained in the present study ranged from 0.23 (non-significant) to 0.80 (highly significant). Similarly to Willett *et al*. (1985), we observed the best correlation to be between the 28-day dietary record and the second administration of the FFQ. In the present study the second FFQ administration took place at the end of the dietary record and thus gave a measure of intake from the same period as the dietary record, which may explain the improved correlation coefficients. On the other hand, the recording process could have sensitized the subject with regards to actual frequency of intake resulting in better estimation of intake with the second administration.

The results of the agreement of the ranking of subjects according to quartiles of intake for each dietary method support satisfactory validity. Between 61% and 94% of respondents could be classified into the same or adjacent quartiles of intake relative to the 28-day dietary record for each of the three FFQ values, although FFQ 2 resulted in the best comparison. In the present study above 60% (depending on which FFQ value was used) of participants were classified into the same and adjacent quartile of vitamin C intake as the 28-day dietary record, compared to the 95% reported by Macintyre *et al*. (2001b). In terms of classification into the same quartile, the present results (approximately 35%) are however comparable with results reported by McKeown *et al*. (2001) in men (36%) and by George *et al*. (2004) in college women (40%). No similar comparative information is available for carotenoids.

As few studies to date have employed the Bland-Altman plots to analyse validation data, comparative data is also scarce. The Bland-Altman plots for vitamin C and carotenoids in the validation study were judged according to the same criteria mentioned for the assessment of reproducibility. For both vitamin C and carotenoids, proportional bias was present, the scatter of data points was relatively wide and the mean difference was not close to zero. With regards to the LOA, almost all of the data points were within the LOA; however, these were fairly wide for both nutrients. Based on the RDA, the LOA for vitamin C were also judged to be of clinical significance, as the LOA differed by more than once to more than twice the RDA. As no RDA values for carotenoids have been established, this criterion of judgment could not be applied to the Bland-Altman plots for carotenoids. The observed presence of proportional bias and wide LOA have been observed by others for vitamin C (Macintyre *et al*., 2001b) and for ß-carotene (Ambrosini *et al*., 2001). Therefore, to conclude, the agreement between the three FFQ values and the 28-day dietary record according to the Bland-Altman plots was not deemed satisfactory. However, the same comments regarding the judgment and potential flaw of these plots mentioned for the reproducibility, also apply for validity assessments, and this must be borne in mind when interpreting results.

When assessing the association of the three FFQ values and the 28-day dietary record vitamin C intake values with plasma vitamin C values (as biomarker), no significant correlation was found. Porrini *et al*. (1995) and Kobayashi *et al*. (2003) also reported no correlation, while Willett *et al*. (1983); Bolton-Smith *et al*. (1991); Boeing *et al*. (1997); Schroder *et al*. (2001) and McKeown *et al*. (2001) report significant, albeit only modest, correlation coefficients between vitamin C intake obtained from FFQ and dietary records respectively and plasma vitamin C levels. The small sample size in our study may have contributed to the lack of a significant association between plasma levels of vitamin C and vitamin C intake estimates. Indeed, in the main study, where the present FFQ was used in a study with a larger sample, the association between vitamin C intake estimated from the FFQ and plasma vitamin C levels was significant ($r = 0.21$; $p = 0.03$) (Chapter 4). It must also be borne in mind that plasma vitamin C levels are tightly controlled by saturable/dose dependent absorption and renal excretion and are closely related to vitamin C intakes below 100mg/day (Levine *et al*., 1996). The mean vitamin C intake from foods and supplements observed in this study was above 100mg/day and therefore, at these observed high intakes, plasma vitamin C levels may not correspond to greater intakes, which may explain the non-significant correlation coefficient.

A more recent approach to assessing the validity of dietary measurement instruments is structural equation modelling (SEM) (Kaaks *et al*., 1994). The method of triads is an example of such a model and can be used when dietary intake information is available from a test instrument (e.g. a FFQ), a reference method (e.g. a dietary record) and a biomarker of intake (e.g. plasma levels of a nutrient) (Kaaks, 1997), which was the case in the present study. Validity coefficients (VCs) generated for the FFQ and the dietary record were comparable, which suggests that the two methods estimate dietary intake similarly when compared to the true unknown intake. The VCs for plasma vitamin C were lower (0.29 – 0.34), suggesting that plasma vitamin C is not as accurate as the dietary assessments in estimating true vitamin C intake. This was not expected as biomarkers are usually seen as the best indicator of the truth. However, plasma vitamin C levels reflect short-term intakes (last few days) while the FFQ and 28-day dietary record reflect longer term intake. It may therefore be more appropriate to use leukocyte vitamin C levels as a biomarker as these reflect longer term intake (Shils *et al*., 1999). Published data regarding validity coefficients for vitamin C is scarce. Ocke and Kaaks (1997), applied the method of triads to vitamin C data from the EPIC validity studies. The reported validity coefficient for plasma (0.39) was slightly higher than what was observed in the present study, while the questionnaire validity coefficient was lower (0.34) than for the present FFQ. In the EPIC studies, the reference method was the mean of twelve 24-h recalls and the VC for this method was 1.01, which was similar to the VC for the 28-day dietary record used in this study. Similar to the findings in the present study, Ocke and Kaaks (1997) also reported the presence of Heywood cases. Heywood cases may be caused by random sampling fluctuations in the observed correlation coefficients (Ocke & Kaaks, 1997). Random variances in the sample are influenced by sample size, and to stabilize the random fluctuations sample sizes above 120 are recommended (Kabagambe *et al*., 2001). The small sample size used in the present study may thus have contributed to the presence of Heywood cases. The use of a larger sample is therefore advised to correct for random fluctuations. Violations of the underlying model assumptions may also result in Heywood cases, indicating biased validity coefficients (Ocke & Kaaks, 1997). Such model assumptions include linear relations between the measurements and the true intake and independence of random errors between the measurements (Ocke & Kaaks, 1997; Kaaks, 1997). The assumption that errors are not correlated may not be true in the present case as the FFQ and the dietary record may have some sources of error in common , e.g food composition tables (Willett, 1998b). The validity coefficients for the FFQ and the dietary

record may thus be overestimated and should be interpreted as the upper limits of the true validity coefficients (Kaaks, 1997).

In summary it can be said that while Bland-Altman plots suggest that validity was not completely satisfactory, all the other statistical tests that were conducted point to satisfactory validity of our FFQ in assessing dietary antioxidant intake.

Conclusions and recommendations

In order to assess dietary antioxidant intake, a FFQ was developed that measured vitamin C, vitamin E, carotenoid, and flavonoid intake. This FFQ was then assessed for reproducibility and validity. For this reproducibility and validity testing, the sample size used was relatively small when compared to other studies and this must be taken into account when interpreting the results.

In this study, several statistical tests were used to assess the reproducibility and validity of the FFQ as no single statistical test can provide the answer regarding reproducibility and validity. In addition, the interpretation of the Bland-Altman test relies on subjective judgement and this must be borne in mind when analysing the results. For the purposes of this study, we used RDA values as cut-offs to judge the width of the LOAs, and have shown that these indicators can be successfully used to judge the clinical significance of the width of the LOA.

While results from Bland-Altman plots do not indicate satisfactory reproducibility for vitamin C, the reproducibility of the FFQ was deemed satisfactory for the other nutrients assessed, based on the Bland-Altman method. Satisfactory reproducibility for the present FFQ for all nutrients was however indicated by similar nutrient intake means, moderately to strong correlation coefficients and a high percentage of individuals classified correctly into quartiles of intake on both administrations of the FFQ. Thus, these results point to satisfactory reproducibility for the present FFQ.

The validity of the FFQ was assessed using the same statistical techniques as the reproducibility testing. In addition, a SEM (method of triads) was constructed to assess validity further. Satisfactory validity was indicated by similar frequency of intake of food items between the 28-day dietary record and the three FFQ values, satisfactory correlation

coefficients and quartile classifications as well as moderate to strong validity coefficients. However, in terms of the Bland-Altman plots, the agreement between the three FFQ values and the 28-day dietary record was not deemed completely satisfactory. The fact that the results of the other statistical tests conducted suggest satisfactory validity, points to satisfactory validity of the FFQ in assessing vitamin C and carotenoid intake.

It must be borne in mind that the validity of the present FFQ was only tested in terms of vitamin C and carotenoids and this does not necessarily imply validity for vitamin E and flavonoid intake, because the most important food sources, especially for vitamin E, are not similar. It is therefore recommended that the validity of the FFQ in terms of vitamin E and flavonoids be determined in future studies. The lack of an observed association between plasma vitamin C levels and the intake of vitamin C may have been as a result of the small sample size. Therefore, in conclusion, despite the limitations set by the small sample size, the FFQ demonstrated satisfactory reproducibility and validity and can thus be used for its intended purpose.

References

Ambrosini GL, de Klerk NH, Musk AW & Mackerras D (2001) Agreement between a brief food frequency questionnaire and diet records using two statistical methods. *Public Health Nutr* **4,** 255-264

Andersen LF, Veierod MB, Johansson L, Sakhi A, Solvoll K & Drevon CA (2005) Evaluation of three dietary assessment methods and serum biomarkers as measures of fruit and vegetable intake, using the method of triads. *Br J Nutr* **93,** 519-527

Bakker I, Twisk JW, van Mechelen W, Mensink GB & Kemper HC (2003) Computerization of a dietary history interview in a running cohort; evaluation within the Amsterdam Growth and Health Longitudinal Study. *Eur J Clin Nutr* **57,** 394-404

Benzie IF (2003) Evolution of dietary antioxidants. *Comp Biochem Physiol A Mol Integr Physiol* **136,** 113-126

Bland JM & Altman DG (1986) Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **1,** 307-310

Boeing H, Bohlscheid-Thomas S, Voss S, Schneeweiss S & Wahrendorf J (1997) The relative validity of vitamin intakes derived from a food frequency questionnaire compared to 24-hour recalls and biological measurements: results from the EPIC pilot study in Germany. European Prospective Investigation into Cancer and Nutrition. *Int J Epidemiol* **26 Suppl 1,** S82-S90

Bolton-Smith C, Casey CE, Gey KF, Smith WC & Tunstall-Pedoe H (1991) Antioxidant vitamin intakes assessed using a food-frequency questionnaire: correlation with biochemical status in smokers and non-smokers. *Br J Nutr* **65,** 337-346

Cao G, Booth SL, Sadowski JA & Prior RL (1998) Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *Am J Clin Nutr* **68,** 1081-1087

Decker EA & Clarkson PM (2000) Dietary sources and bioavailbality of essential and non-essential antioxidants. In *Handbook of Oxidants and Antioxidants in Exercise*, pp. 323-358 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Dunn G (1989) *Design and analysis of reliability studies: the statistical evaluation of measurement errors.* New York: Oxford University Press.

Elmstahl S, Gullberg B, Riboli E, Saracci R & Lindgarde F (1996) The Malmo Food Study: the reproducibility of a novel diet history method and an extensive food frequency questionnaire. *Eur J Clin Nutr* **50,** 134-142

Flegal KM & Larkin FA (1990) Partitioning macronutrient intake estimates from a food frequency questionnaire. *Am J Epidemiol* **131,** 1046-1058

Food and Nutrition Board IoM (2000) *Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids.* Washington, DC: National Academic Press.

Fornes NS, Stringhini ML & Elias BM (2003) Reproducibility and validity of a food-frequency questionnaire for use among low-income Brazilian workers. *Public Health Nutr* **6,** 821-827

George GC, Milani TJ, Hanss-Nuss H & Freeland-Graves JH (2004) Development and validation of a semi-quantitative food frequency questionnaire for young adult women in the southwestern United States. *Nutrition Research* **24,** 29-43

Harlan LC & Block G (1990) Use of adjustment factors with a brief food frequency questionnaire to obtain nutrient values. *Epidemiology* **1,** 224-231

Hopkins WG (2004) Bias in Bland-Altman but not regression validity analyses. *Sportsci* **8,** 42-46

Horwath C (1990) Food frequency questionnaires: a review. *Australian Journal of Nutrition and Dietetics* **47,** 71-76

Jackson M, Walker S, Cade J, Forrester T, Cruickshank JK & Wilks R (2001) Reproducibility and validity of a quantitative food-frequency questionnaire among Jamaicans of African origin. *Public Health Nutr* **4,** 971-980

Johansson I, Hallmans G, Wikman A, Biessy C, Riboli E & Kaaks R (2002) Validation and calibration of food-frequency questionnaire measurements in the Northern Sweden Health and Disease cohort. *Public Health Nutr* **5,** 487-496

Kaaks R, Riboli E, Esteve J, van Kappel AL & van Staveren WA (1994) Estimating the accuracy of dietary questionnaire assessments: validation in terms of structural equation models. *Stat Med* **13,** 127-142

Kaaks RJ (1997) Biochemical markers as additional measurements in studies of the accuracy of dietary questionnaire measurements: conceptual issues. *Am J Clin Nutr* **65,** 1232S-1239S

Kabagambe EK, Baylin A, Allan DA, Siles X, Spiegelman D & Campos H (2001) Application of the method of triads to evaluate the performance of food frequency

questionnaires and biomarkers as indicators of long-term dietary intake. *Am J Epidemiol* **154,** 1126-1135

Kobayashi M, Sasaki S & Tsugane S (2003) Validity of a self-administered food frequency questionnaire used in the 5-year follow-up survey of the JPHC Study Cohort I to assess carotenoids and vitamin C intake: comparison with dietary records and blood level. *J Epidemiol* **13,** S82-S91

Kumanyika S, Tell GS, Fried L, Martel JK & Chinchilli VM (1996) Picture-sort method for administering a food frequency questionnaire to older adults. *J Am Diet Assoc* **96,** 137-144

Kumanyika SK, Tell GS, Shemanski L, Martel J & Chinchilli VM (1997) Dietary assessment using a picture-sort approach. *Am J Clin Nutr* **65,** 1123S-1129S

Langenhoven M, Kruger M, Gouws E & Faber M (1991a) *MRC Food Composition Tables*, Parow: Medical Research Council

Langenhoven M, Kruger M, Gouws E & Faber M (1991b) *MRC Food Quantities Manual*, Parow: Medical Research Council

Levine M, Conry-Cantilena C, Wang Y, *et al* (1996) Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci U S A* **93,** 3704-3709

Macintyre UE, Venter CS & Vorster HH (2001a) A culture-sensitive quantitative food frequency questionnaire used in an African population: 1. Development and reproducibility. *Public Health Nutr* **4,** 53-62

Macintyre UE, Venter CS & Vorster HH (2001b) A culture-sensitive quantitative food frequency questionnaire used in an African population: 2. Relative validation by 7-day weighted records and biomarkers. *Public Health Nutr* **4,** 63-71

Mangels AR, Holden JM, Beecher GR, Forman MR & Lanza E (1993) Carotenoid content of fruits and vegetables: an evaluation of analytic data. *J Am Diet Assoc* **93,** 284-296

McKeown NM, Day NE, Welch AA, Runswick SA, Luben RN, Mulligan AA, McTaggart A & Bingham SA (2001) Use of biological markers to validate self-reported dietary intake in a random sample of the European Prospective Investigation into Cancer United Kingdom Norfolk cohort. *Am J Clin Nutr* **74,** 188-196

Munger RG, Folsom AR, Kushi LH, Kaye SA & Sellers TA (1992) Dietary assessment of older Iowa women with a food frequency questionnaire: nutrient intake, reproducibility, and comparison with 24-hour dietary recall interviews. *Am J Epidemiol* **136,** 192-200

Ocke MC & Kaaks RJ (1997) Biochemical markers as additional measurements in dietary validity studies: application of the method of triads with examples from the European Prospective Investigation into Cancer and Nutrition. *Am J Clin Nutr* **65,** 1240S-1245S

Pietinen P, Hartman AM, Haapa E, Rasanen L, Haapakoski J, Palmgren J, Albanes D, Virtamo J & Huttunen JK (1988) Reproducibility and validity of dietary assessment instruments. I. A self-administered food use questionnaire with a portion size picture booklet. *Am J Epidemiol* **128,** 655-666

Pietta PG (2000) Flavonoids as antioxidants. *J Nat Prod* **63,** 1035-1042

Porrini M, Gentile MG & Fidanza F (1995) Biochemical validation of a self-administered semi-quantitative food-frequency questionnaire. *Br J Nutr* **74,** 323-333

Prior RL (2003) Fruits and vegetables in the prevention of cellular oxidative damage. *Am J Clin Nutr* **78,** 570S-578S

Rankin G & Stokes M (1998) Reliability of assessment tools in rehabilitation: an illustration of appropriate statistical analyses. *Clin Rehabil* **12,** 187-199

Schroder H, Covas MI, Marrugat J, Vila J, Pena A, Alcantara M & Masia R (2001) Use of a three-day estimated food record, a 72-hour recall and a food-frequency questionnaire for dietary assessment in a Mediterranean Spanish population. *Clin Nutr* **20,** 429-437

Senekal M & Steyn NP (2004) *Food Photo Manual*, Parow: Medical Research Council.

Shils ME, Olson JA, Shike M & Ross AC (1999) *Modern Nutrition in Health and Disease*, 9th ed. Philadelphia: Lippincott Williams & Wilkins.

Steyn NP, Nel JH & Casey A (2003) Secondary data analyses of dietary surveys undertaken in South Africa to determine usual food consumption of the population. *Public Health Nutr* **6,** 631-644

Stipanuk MH (2000) *Biochemical and Physiological Aspects of Human Nutrition*, 1st ed. Philadelphia: W.B. Saunders Company.

Thompson FE & Byers T (1994) Dietary assessment resource manual. *J Nutr* **124,** 2245S-2317S

U.S.Department of Agriculture (2003) *USDA database for the flavonoid content of selected foods*, Beltsville (MD): U.S. Department of Agriculture.

Wei Y, Ota RB, Bowen HT & Omaye ST (1996) Determination of human plasma and leukocyte ascorbic acid by microtiter plate assay. *Nutritional Biochemistry* **7,** 179-186

Willett WC (1998a) Invited commentary: comparison of food frequency questionnaires. *Am J Epidemiol* **148,** 1157-1159

Willett WC (1998b) *Nutritional Epidemiology*, 2nd ed. New York: Oxford University Press.

Willett WC, Sampson L, Stampfer MJ, Rosner B, Bain C, Witschi J, Hennekens CH & Speizer FE (1985) Reproducibility and validity of a semiquantitative food frequency questionnaire. *Am J Epidemiol* **122,** 51-65

Willett WC, Stampfer MJ, Underwood BA, Speizer FE, Rosner B & Hennekens CH (1983) Validation of a dietary questionnaire with plasma carotenoid and alpha-tocopherol levels. *Am J Clin Nutr* **38,** 631-639

Zulkifli SN & Yu SM (1992) The food frequency method for dietary assessment. *J Am Diet Assoc* **92,** 681-685

CHAPTER 4

ASSOCIATION BETWEEN ANTIOXIDANT STATUS AND MnSOD ALA-9VAL POLYMORPHISM IN TRAINED MALE ATHLETES (RUGBY PLAYERS) AND SEDENTARY MALE STUDENTS CONTROLLED FOR ANTIOXIDANT INTAKE: AN EXPLORATORY STUDY

Introduction

Free radicals, including reactive oxygen species (ROS), are continuously produced as part of normal human metabolism (Spitzer, 1995). Although the production of these highly reactive molecules may be harmful and has been associated with various pathophysiological conditions such as the process of ageing and chronic degenerative diseases controlled amounts of these molecules are tolerated by cells and may be physiologically useful in various biological processes, including cell signalling, gene expression, and the body's immune response (Suzuki *et al*., 1997; Halliwell, 1998; Allen & Tresini, 2000; Jackson *et al*., 2002). To protect against free radical attack and subsequent oxidative damage, antioxidant defence systems have developed (Halliwell & Gutteridge, 1999; Benzie, 2000). These systems include both enzymatic and non-enzymatic antioxidants that work as a complex unit to minimize the generation and counter-act the potential oxidative damaging effects of ROS (Benzie, 2000).

The balance between ROS production and antioxidants has been referred to as antioxidant status (Papas, 1996). A disturbance in this pro-oxidant-antioxidant balance in favour of the former leading to potential damage has been defined as oxidative stress (Sies, 1991). Oxidative stress can result from either depletion of antioxidants and/or due to an excess production of ROS (Halliwell, 1998). Physical exercise has been shown to increase the generation of free radicals, including ROS (Davies *et al*., 1982; Ashton *et al*., 1998). In addition, several studies have reported that exercise resulted in increases in markers of oxidative stress in humans, including lipid peroxidation, protein oxidation and DNA oxidation products (Dillard *et al*., 1978; Hartmann *et al*., 1995; Niess *et al*., 1996; Alessio *et al*., 2000; Lee *et al*., 2002). Others have reported decreases in antioxidant status and antioxidant levels following exercise, which points to a compromised antioxidant defence due to increased ROS production (Duthie *et al*., 1990; Lee *et al*., 2002; Groussard *et al*., 2003; Quindry *et al*., 2003). Findings regarding exercise-induced oxidative stress are however not consistent and some do not report increases in oxidative stress markers or decreases in antioxidant status in response to exercise (Duthie *et al*., 1990; Viguie *et al*., 1993; Rokitzki *et al*., 1994b; Mastaloudis *et al*., 2001).

Although some forms of exercise may increase ROS and subsequently result in oxidative stress, regular exercise training has been associated with an improvement in antioxidant status (Brites *et al*., 1999; Evelson *et al*., 2002; Cazzola *et al*., 2003). Currently there is no

clear explanation for this apparent paradox, but there is growing evidence that the antioxidant system is capable of adapting to chronic exposure to oxidants, which may be the case with exercise training (Oberley *et al*., 1987; Ji, 1998; Powers & Lennon, 1999). The majority of studies have reported that endurance training results in the up-regulation of SOD and GPX activity in skeletal muscle in rats (for summary of studies see Ji, 1998; Powers & Lennon, 1999). Human studies comparing oxidative stress markers and antioxidants in trained and sedentary subjects have produced equivocal results. Plasma total antioxidant status as well as individual nonenzymatic plasma antioxidants were found by some to be higher in trained subjects compared to untrained subjects (Brites *et al*., 1999; Evelson *et al*., 2002; Cazzola *et al*., 2003). In contrast, others have reported no differences in plasma antioxidant levels between trained and untrained subjects (Robertson *et al*., 1991; Rokitzki *et al*., 1994a; Balakrishnan & Anuradha, 1998).

These discrepancies in findings may be explained to some extent by study design and subject related factors, including differences in the training status of subjects as well as the dietary antioxidant intake. The effect of dietary antioxidant intake on antioxidant status is often poorly controlled for, and may therefore have influenced some of the study findings.

A further factor that may influence antioxidant status is inter-individual genetic variation (Sen & Goldfarb, 2000). Human genetic variation is quite common and single nucleotide polymorphisms (SNP's) are responsible for the majority of the variants (Forsberg *et al*., 2001; Morgenstern, 2004). Although some single nucleotide polymorphisms in genes encoding antioxidant enzymes have been described, the influence of these on antioxidant status and susceptibility to oxidative stress in an exercise training context is unknown (Forsberg *et al*., 2001). A polymorphism in the gene encoding the MnSOD enzyme, involving an Alanine (GCT) - Valine (GTT) amino acid change, was described by Rosenblum *et al*. (1996)[2]. The variant allele of the gene is quite common, with similar allele frequencies of about 50% occurring for the two alleles at nucleotide position -28 in the Caucasian population (Ambrosone *et al*., 1999). Rosenblum *et al*., 1996 suggested that the MnSOD Ala allele is associated with decreased protection against superoxide radicals in the mitochondria and thus increased oxidative stress, as the transport of the enzyme into the mitochondria, where it is biologically active, seems to be affected. The

[2] Note alternative numbering for the MnSOD polymorphism: DNA change: C to T change at base 47 (47C→T); Protein change: Alanine to Valine amino acid change at codon 16 (Ala16Val)

effect of MnSOD genotype on training related antioxidant status has to our knowledge not been investigated yet.

Based on the inconsistent results observed in studies investigating antioxidant status and oxidative stress in relation to exercise training, it is not clear whether athletes have greater antioxidant needs and at this point in time there is not sufficient evidence to recommend increased antioxidant intakes by athletes, despite the fact that this is a common practice (Froiland *et al*., 2004; Kristiansen *et al*., 2005). More research is needed that considers as many as possible of the confounding factors in order to better identify those in need of additional antioxidants. Therefore, the aim of this study was to investigate the association between the MnSOD Ala-9Val (-28C→T) polymorphism, the total antioxidant status and selected plasma indicators of antioxidant status in trained male athletes (rugby players) and sedentary male students while controlling for dietary intake of the major antioxidants.

Materials and methods

Study design

A cross-sectional study design was used to compare antioxidant status in trained and sedentary male subjects with different MnSOD genotypes, while controlling for dietary antioxidant intake.

Study sample and sampling

Sample size calculation for optimal statistical power resulted in a total sample size (including trained and sedentary group) of 329 to 341 (personal communication, 2005, Dr. C. Lombard, Institute for Biostatistics, Medical Research Council). As it was not feasible within the scope of this study to attain the sample size, the study should be deemed as an exploratory study.

For the trained subjects, male volunteers from the university rugby club were recruited. The definition of trained subjects was based on other similar studies, where subjects who trained between five and eight hours a week and played matches once a week for at least the season, were included (Brites *et al*., 1999; Evelson *et al*., 2002; Chang *et al*., 2002; Schippinger *et al*., 2002). For the purposes of this study, rugby players training at least 1½ hours a day three days a week and participating in matches at least once a week during the season for the past month, were included.

The sedentary subject group consisted of male students enrolled at the university. The definition of sedentary subjects was based on the guidelines for physical activity and health formulated by the US Department of Health and Human Services, which recommends 30 minutes or more of moderate-intensity physical activity on all, or most, days of the week (US Department of Health and Human Services. Physical activity and health: a report of the Surgeon General, 1996). Therefore, for this study, male students who did not meet these requirements were included (a student who did 30 minutes of moderate intensity physical activity two times or less per week qualified for participation).

Only healthy subjects between the ages of 18-25 who did not smoke more than 10 cigarettes per day were included. As dietary supplement use in common among athletes and students in general (Froiland *et al*., 2004; Kristiansen *et al*., 2005; Seele & Senekal, 2005), both dietary supplement users and non-users took part in the study. Supplement use was controlled for in the assessments.

Subjects were recruited over a four-month period between August and November over two consecutive years. The months correspond with the middle and end of the rugby season, which ensured that the rugby players were most likely to be fit. In addition the possible effect of seasonal variation on dietary intake was also accommodated in this way. The recruitment procedure for the rugby players involved an introductory lecture to all members of the university rugby club in which the aims and requirements of the study were briefly explained. A similar procedure was followed for the sedentary students, with introductory lectures given to residents of the university male residences at meal times, followed by an invitation to participate in the study.

Volunteers for the two different groups were subjected to a screening interview. Information regarding the type, frequency, duration and intensity of physical exercise or sport during a typical week; the presence of chronic diseases or illness; and present and past smoking habits was obtained during this session in order to determine whether subjects met the inclusion criteria of this study. Note that the physical activity levels, health and smoking related information documented during the screening interview were not further quantified and analysed for the present study.

The subjects were required to give written informed consent and the study was approved by the Ethics Committee for Human Research of the University of Stellenbosch.

Experimental methods and procedures

Blood sampling

For the analysis of plasma antioxidant status and MnSOD genotyping blood samples were collected from each subject. For the rugby players, blood samples were taken before a training session. Rugby players were encouraged to refrain from exercising for at least 24 hours before the blood drawing to prevent any effects of acute exercise on circulating levels of antioxidant nutrients. For the sedentary subjects, blood was drawn at rest. Blood samples for the analysis of plasma vitamin C, plasma carotenoids and total plasma antioxidant capacity were collected in lithium heparin tubes protected from light and kept on ice. The blood samples were centrifuged within an hour of collection at 4500 rpm for 15 minutes at 4^{o}C. Plasma fractions were aliquoted into Eppendorfs. Plasma for carotenoids and for total antioxidant status analysis was stored at -70^{o}C until analysis within three months. Plasma for the vitamin C analysis was prepared as follows: the plasma layer was transferred to an Eppendorf and an equal volume of 10% trichloroacetic acid (TCA) was added in order to stabilise samples. Each tube was vortexed for 3 minutes and centrifuged at 4500 rpm for 15 minutes at 4^{o}C. The supernatant was removed, placed in an Eppendorf and frozen at -70^{o}C until analysis 24 hours later. Blood samples for MnSOD genotyping was collected in EDTA tubes and kept at 3^{o}C until analysis.

Survey instrument (questionnaire) and administration thereof

Each subject was required to attend a face to face interview with the primary researcher during which information regarding dietary antioxidant intake, dietary supplement use, sociodemographics and weight status was collected (Addendum 1). The interview took place within one week of the blood drawing.

Dietary antioxidant intake

Subjects were required to provide information regarding their dietary antioxidant intake over the past month. For these purposes a quantified food frequency questionnaire was developed to determine intake of vitamin C, vitamin E, carotenoids and flavonoids (Addendum 1). The development and validation of this questionnaire is described in the first paper of this thesis (Chapter 3). The picture-sort method (Kumanyika *et al*., 1996) was used to administer the FFQ. Subjects were requested to sort the food cards (Senekal & Steyn, 2004) into three separate piles based on the frequency of consumption over the previous month, namely never, sometimes or frequently during the past month. The cards corresponding to foods never eaten were placed aside and no further information

regarding those foods was collected. Food items included in the FFQ that were not represented by a photograph, such as vitamin C containing sweets, were asked about separately. Subjects were then required to give detail regarding the exact frequency of consumption (per day / per week / per month) and the usual portion size of the food items that were consumed sometimes or frequently. Visual aids were used to ensure accurate portion size estimation. Life size line drawings depicting small, medium or large servings of fruit and some vegetable items were used (Senekal & Steyn, 2004) as well as a three-dimensional amorphous flour model of half a cup. For the latter, subjects could indicate portion size as half the size, the same size, one-and-a-half times the size or twice the size of the standard portion size. In addition, life size line drawings of different sizes of cups, glasses, bowls and spoons were used (Senekal & Steyn, 2004).

Dietary analysis

The vitamin C and E content of foods was determined using Foodfinder III (Nutritional Intervention Research Unit and Research Information Systems Division of the Medical Research Council, South Africa: MRC, 2002). For the determination of carotenoid content, Foodfinder III was used for most foods. Carotenoid content for foods for which no values were indicated in Foodfinder III, the US database for carotenoids was used (Mangels *et al*., 1993). Total carotenoid intake was calculated by adding the intake of the following carotenoids: ß-carotene, a-carotene, lutein, lycopene and cryptoxanthin. For the determination of the flavonoid content of foods, the US database for flavonoids (U.S.Department of Agriculture, 2003) was used as Foodfinder III does not include flavonoid content data. For the analysis of dietary data, usual portion size for each food item was converted to grams, using the South African MRC Food Quantities Manual (Langenhoven *et al*., 1991) and the Food Photo Manual (Senekal & Steyn, 2004). In order to calculate the total intake of a particular nutrient, the estimated portion size was converted to grams, which was then multiplied by the frequency of intake and the nutrient content/100g of each food item. This was transformed to reflect nutrient intake per day by dividing the total nutrient intake by seven (if eaten weekly) or 28 (if eaten monthly). This process was repeated for each food item and the total daily intake (mg/day) of vitamin C, E, carotenoids and flavonoids was calculated by adding the data from each food item.

Dietary supplement use

For the purposes of this study dietary supplements were defined as products containing one or more vitamins, minerals, herbals and/or botanicals.

Amino acid, protein and carbohydrate supplements were also included in the definition.

Subjects who indicated that they had taken a dietary supplement during the previous month were requested to specify the type of supplement(s) they had used, as well as the dosage, frequency and duration of use. The vitamin C, vitamin E, carotenoid and flavonoid content of each supplement was determined by analysing the nutritional information supplied on the supplement containers and/or dietary supplement company websites. This quantity was multiplied by the dosage and frequency of intake for each nutrient and the value was transformed to reflect nutrient intake per day by dividing the total nutrient intake by 7 (if taken weekly) or 28 (if taken monthly). The individual nutrient intake from individual supplements was finally added together to obtain total daily supplement nutrient intake for each subject where applicable. The supplement nutrient intake was added to the nutrient intake obtained from food to provide a total nutrient intake value.

Sociodemographic related information

Subjects were requested to provide information regarding their birthdates and ages as part of the questionnaire.

Weight status

Weight status assessment was done for descriptive purposes only and was measured using the body mass index (BMI). For these purposes the height and weight of subjects was measured. The weight of the subjects was measured, to the nearest 0.1kg, in light clothing and without shoes using a calibrated electronic scale. Subjects were measured while standing in the centre of the scale without support and with weight distributed evenly on both feet, while looking directly in front of them. Height without shoes was measured to the nearest 0.1cm with a stadiometer. Subjects stood with their feet together and heels, buttocks, scapulae and back of the head touching the vertical surface of the stadiometer; the head was placed in the Frankfort horizontal plane. Subjects were then asked to inhale deeply and hold their breath before the measurement was taken. The body mass index (BMI) was calculated by dividing weight (kg) by the square of height (m^2) (Bastow, 1982).

Biochemical methods

Plasma total antioxidant capacity

Total antioxidant capacity of plasma was measured using the Oxygen Radical Absorbance Capacity (ORAC) assay, which measures the capacity of antioxidants to directly quench

free radicals produced by a biological radical source (Prior & Cao, 1999; Huang *et al*., 2005). The principle of the ORAC assay is based on the reaction between a fluorescent probe and free radicals. Free radical damage to the probe results in a change in fluorescence intensity, and the change of intensity is an index of the degree of free radical damage. In the presence of antioxidants, free radical damage is inhibited, which is reflected in protection against the change of probe fluorescence. The inhibition of free radical damage by the antioxidant is a measure of its antioxidant capacity against the free radical (Huang *et al*., 2002). The ORAC assay was first developed by Cao and co-workers (Cao *et al*., 1993) and then improved by a number of researchers (Naguib, 2000; Ou *et al*., 2001; Huang *et al*., 2002).

The determination of ORAC in the present study was done according to the method described by Huang *et al*., (2002) and Ou *et al*., (2001). Plasma was thawed and diluted 150 times with pH 7.4 phosphate buffer. The following solutions were used: 75mM phosphate buffer, Trolox standard solutions of 6.25µM, 12.5 µM, 25 µM and 50 µM, 53mM 2,2´-azobis(2-amidinopropane)dihydrochloride (AAPH) and 8.16×10^{-5} mM fluorescein solution (Huang et al 2002). For quality control purposes plasma from a volunteer was aliqouted into Eppendorfs and a sample was used in every run. For the analysis of sample 150 µl fluorescein solution was pipetted into the wells of a black 96 well microplate, followed by triplicates of 25 µl of Trolox standards and plasma respectively. The plate was incubated in a Biotek FL_X800 Microplate Fluorescence Reader at 37^0C for 10 minutes before AAPH was solution was added to initiate the reaction. The fluorescence intensity was measured every minute for 35 min at 37^oC using fluorescence filters for an excitation wavelength of 485±20nm and an emission wavelength of 530±25nM. As the reaction progresses, fluorescein is consumed and the FL intensity decreases. In the presence of antioxidant, the FL decay is inhibited.

ORAC data was calculated as follows: The area under the kinetic curve (AUC) of the samples and standards was calculated using Formula 1 below.

$$AUC = 0.5 + f_1/f_0 + \ldots f_i/f_0 + f_{34}/f_0 + 0.5(f_{35}/f_0) \quad \text{[Formula 1]}$$

where f_0 = initial fluorescence reading at 0 minutes and

f_i = fluorescence reading at time i.

The net AUC was calculated using Formula 2.

$$\text{Net AUC} = AUC_{sample} - AUC_{blank} \quad \text{[Formula 2]}$$

A standard curve was obtained by plotting the net AUC of each Trolox standard against the Trolox concentrations of the standard. ORAC values for samples were calculated using the regression equation between TROLOX concentration and net AUC and were expressed as micromole Trolox equivalents per litre. Normal ORAC values that have been reported range between 7780 ± 467 µmol Trolox equivalents/l (Ou et al., 2001) and 9024.7 µmol Trolox equivalents/l (Huang et al., 2002).

Plasma vitamin C

For the measurement of plasma vitamin C levels the method of Wei *et al*., (1996) was followed, which involves a microtiter plate assay that measures the ascorbic acid content in plasma using the 2,4-dinitrophenylhydrazine reaction with ketonic acid groups of dehydroascorbic acid. For this assay, a Biotek EL800 Universal Microplate Reader was used together with untreated 96-well microplates. The reagents that were used included a 10% TCA solution, a DNPH/thiourea/copper (DTC) solution and a 65%H_2SO_4 solution. The TCA-stabilised plasma was thawed and centrifuged at 14000 rpm for 30 minutes at 4^oC. Three 100µl aliquots of each sample were placed into separate Eppendorfs. Standard ascorbic acid solutions varying from 0.0 to 2.0 µg of ascorbic acid/100µl were prepared in Eppendorfs. 20 µl of DTC solution was added to all samples and standards. Tubes were gently vortexed and thereafter incubated in a water bath at 37^oC for two hours. Samples were vortexed every 30 minutes during the incubation period. After incubation, samples were placed on ice and 150 µl ice-cold H_2SO_4 was added. Samples were vortexed and kept in the dark at room temperature for one hour with gentle vortexing after the first 30 minutes. Samples were transferred to a 96-well plate and read on a Biotek EL800 Universal Microplate Reader at 515nm. Means were calculated for triplicate readings and sample vitamin C concentrations were obtained from the standard curve. Vitamin C concentrations were expressed as mg/dl. For quality control purposes, plasma from a volunteer was prepared as necessary, aliqouted into Eppendorfs and a sample was used in every run. Normal plasma vitamin C levels are between 0.4mg/dl and 1.5mg/dl, with values below 0.2mg/dl indicating deficiency (Shils *et al*., 1999).

Plasma carotenoid

Carotenoid levels in plasma or serum are ideally measured using high performance liquid chromatographic (HPLC) approaches (Craft, 1992). As this technique has not been set up in our laboratory, carotenoid levels in plasma in the present study were colorimetrically analysed based on the method of (Neeld & Pearson, 1963). A 0.5mg/ml ß-carotene stock

solution was prepared by dissolving 12.5 mg of all-trans ß-carotene in a few milliliters of chloroform. The solution was brought to exactly 25 ml with hexane. From this stock solution, an intermediate standard was prepared by diluting 250 µl of the stock solution in 24.75 ml hexane. The intermediate solution was used to prepare working standards of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µg ß-carotene/ml by diluting the intermediate solution further with the appropriate volumes of hexane. Plasma samples were thawed and plasma proteins were precipitated with ethanol. This was done by mixing 100 µl of plasma with 100 µl 95% ethanol in an Eppendorf after which 150 µl of hexane was added and the solution vortexed for two minutes to extract the carotenoids. Solutions were then centrifuged for 7 minutes at 3000 rpm to achieve a clean phase separation. 100 µl of the hexane extract of each sample as well as 100 µl of each standard solution was pipetted into a 96-well microplate in triplicate and read against a hexane blank on a Biotek EL800 Universal Microplate Reader at 450 nm. Calculation of plasma carotenoid levels involves calculating a carotene factor (FC) using the Formula 3.

$$FC_{450} = (\mu g \text{ carotene/ml})/A_{450} \qquad \text{[Formula 3]}$$

where A_{450} is the absorbance of the standard at 450 nm.

Formula 4 was then used to calculate the plasma values for carotenoids for each sample:

$$\text{Total carotenoids (as } \mu g \text{ ß-carotene/dl)} = A_{450} \times FC_{450} \times 150 \qquad \text{[Formula 4]}$$

where FC_{450} is the constant determined for each laboratory and 150 accounts for dilution factors.

A quality control sample was included in every run. No cut-off values or normal levels of plasma carotenoid values have as yet been published. However, the developers of this method reported carotenoid values of between 63.5µg/dl and 180.6µg/dl for human plasma (Neeld & Pearson, 1963).

MnSOD genotyping

MnSOD genotyping was done by GeneCare Molecular Genetics (Pty) Ltd. using a real-time polymerase chain reaction (PCR) method.

Statistical analysis

Data was analysed using the Statistical Programme for Social Sciences (SPSS) version 14 for Windows *(SPSS 14.0, Chicago, Ill 2006*). Means, standard deviations, medians and

25th and 75th percentiles were calculated as appropriate for applicable variables for descriptive purposes. Sociodemographic and anthropometric differences between rugby players and sedentary subjects were analysed using the unpaired student t-test. The Pearson's Chi-squared test was used to assess significant differences in supplement use and genotype distribution between the two groups. Genotype distribution within the total group and for the rugby and sedentary groups separately was analysed for Hardy-Weinberg equilibrium and deviations were assessed using Pearson's Chi-squared test.

Spearman rank correlation coefficients were calculated to determine association between dietary intake and plasma concentrations of vitamin C and carotenoid respectively.

To determine the effect of activity group (rugby players versus sedentary subjects) and MnSOD genotype (Ala/Ala, Ala/Val, Val/Val) on plasma antioxidant concentrations while controlling for dietary intake, three univariate general linear models (GLM) were constructed for plasma vitamin C, plasma carotenoids and total plasma antioxidant status as dependent variables in each case. Activity group and MnSOD genotype were the independent variables and dietary antioxidant intake was entered as a covariate. When plasma vitamin C was the dependent variable, dietary vitamin C intake was entered as covariate, while carotenoid intake was the covariate for plasma carotenoid. For total antioxidant status as dependent variable, dietary intake of vitamin C, vitamin E, carotenoids and flavonoids were entered as covariates. Differences were considered significant at values of $P < 0.05$.

Results

Subjects

The trained group included 76 rugby players, while the control group included 39 sedentary students. The characteristics of the study population are displayed in Table 1. Both groups were of similar age. The trained group weighed significantly more and had a higher BMI than the sedentary group. Dietary supplement use was non-significantly higher in the rugby players compared to the sedentary students, with about three-quarters of rugby players and two-thirds of sedentary subjects reporting dietary supplement use in the past month.

Table 1: Characteristics of the study sample

	Rugby players (n = 76)	Sedentary students (n = 39)	p value*
Age (yrs) (mean±SD)	20.6 ± 1.8	20.6 ± 1.5	0.84
Height (m) (mean±SD)	1.83 ± 0.07	1.80 ± 0.07	0.05
Weight (kg) (mean±SD)	92.3 ± 13.3	79.5 ± 12.5	0.00
BMI (kg/m^2) (mean±SD)	27.5 ± 3.6	24.4 ± 3.0	0.00
Supplement use (n (%))	56 (73.7)	25 (64.1)	0.29 (Chi-square)

* Independent sample t-test except where otherwise indicated

BMI = Body mass index

Genotype distribution

Table 2 shows the distribution of the MnSOD genotype for the rugby players and sedentary group as well as for the total group. Both groups displayed similar genotype distributions (Pearsons Chi-square, p=0.81), with an allele frequency of about 0.5. All distributions were in agreement with the Hardy-Weinberg equilibrium (Addendum 4).

Table 2: MnSOD genotype distribution

	Rugby players (n =76)	Sedentary students (n = 39)	Total group (n = 115)
Ala/Ala	19 (25.0)	11 (28.2)	30 (26.1)
Val/Ala	40 (52.6)	18 (46.2)	58 (50.4)
Val/Val	17 (22.4)	10 (25.6)	27 (23.5)

Values are n (%)

Dietary antioxidant intake

The dietary intakes from foods alone and from foods combined with dietary supplements of vitamin C, vitamin E, carotenoids and flavonoids are displayed in Table 3. Dietary intakes for all nutrients were similar for trained and sedentary subjects. Nutrient intake from supplements was also similar between the groups. For the rugby players, the contribution of dietary supplements to intake for the various nutrients was as follows: 50% for vitamin C, 44% for vitamin E and 2% for carotenoids. No flavonoid containing supplements were used. A similar pattern was observed for the sedentary group for vitamin C, carotenoid and flavonoid intake with supplements contributing 52%, 1% and 0% respectively to intake. Supplements contributed slightly less to vitamin E intake in this group (28%) than in the

rugby players. The relationship between dietary vitamin C and carotenoid intake and their respective plasma concentrations is shown in Table 4. Dietary vitamin C intake displayed a significant correlation with plasma vitamin C levels for the total group only. Carotenoid intake and plasma carotenoid showed a significant association only in the rugby group. However, these correlations need to be interpreted with caution as the correlation coefficients (*r)* were on the low side.

Table 3: Dietary antioxidant intakes (mg/day) in rugby players and sedentary controls.

	Rugby players (n = 76)		**Sedentary subjects (n = 38)**	
	Total *	**Food only**	**Total ***	**Food only**
Vitamin C				
Mean ± SD	252.1 ± 232.7	127.2 ± 74.6	219.6 ± 234.6	104.7 ± 61.5
Median (P 25^{th}–75^{th})	190.2 (96.9–315.0)	106.7 (69.3–164.7)	150.5 (83.9–220.1)	91.5 (60.9–129.8)
Vitamin E				
Mean ± SD	12.3 ± 15.8	6.9 ± 5.6	7.9 ± 6.2	5.7 ± 4.4
Median (P 25^{th} – 75^{th})	8.9 (3.9–13.8)	6.1 (2.8–9.0)	5.3 (2.7–11.2)	4.4 (2.5–8.8)
Carotenoids				
Mean ± SD	8.6 ± 7.9	8.4 ± 8.0	8.1 ± 4.7	8.0 ± 4.8
Median (P 25^{th} – 75^{th})	6.7 (3.8–9.7)	6.6 (3.6–9.5)	6.5 (4.4–11.2)	6.5 (4.3–11.2)
Flavonoids				
Mean ± SD	76.9 ± 77.0	76.9 ± 77.0	107.4 ± 156.0	107.4 ± 156.0
Median (P 25^{th} – 75^{th})	61.3 (29.5–90.0)	61.3 (29.5–90.0)	58.2 (28.0–100.8)	58.2 (28.0–100.8)

* Intake from food + supplements

Table 4: Spearman rank correlation coefficients (*r*) between dietary vitamin C intake and plasma vitamin C concentrations and dietary carotenoid intake and plasma carotenoid concentrations.

	Vitamin C		**Carotenoid**	
	r	**p**	***r***	**p**
Total group*	0.21	0.03	0.17	0.08
Rugby subjects (n = 76)	0.17	0.14	0.24	0.04
Sedentary subjects **	0.23	0.16	0.11	0.51

* n = 115 for vitamin C and 114 for carotenoid

** n = 39 for vitamin C and 38 for carotenoid

Antioxidant status

Plasma vitamin C and carotenoid levels were significantly higher in the rugby players compared to the sedentary subjects ($p<0.05$) (Figs 1 and 2). For both groups, the plasma vitamin C and carotenoid values were within the expected ranges respectively (dotted lines in Figures 1 and 2). Plasma total antioxidant capacity, measured by the ORAC assay, was similar in both groups of subjects, but tended to be higher in the rugby players ($p = 0.09$) (Fig 3). The observed ORAC values were in line with the expected values. As shown in Figures 1 – 3, the MnSOD genotype did not have a significant effect on plasma total antioxidant capacity or on plasma vitamin C and carotenoid levels in either subject group, with or without control for dietary intake.

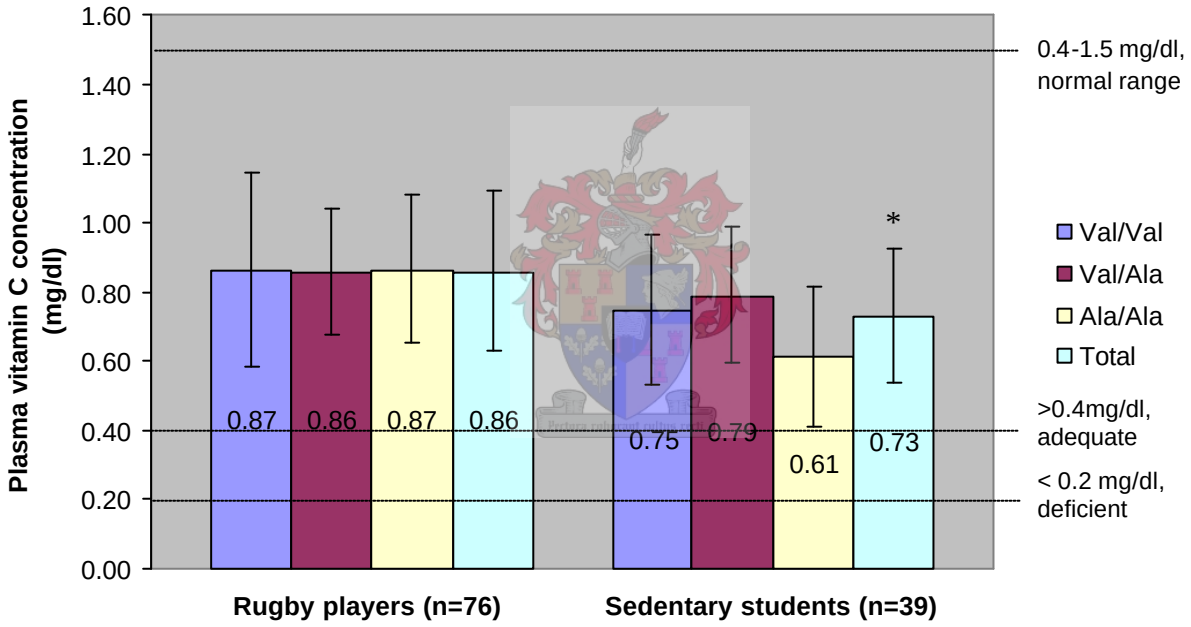


Figure 1: Plasma vitamin C concentration (mg/dl) according to MnSOD genotype and physical activity group. *Significant difference between total rugby group and total sedentary group ($p = 0.005$, univariate GLM). Dotted lines indicate adequacy values for plasma vitamin C levels (Shils *et al*., 1999).

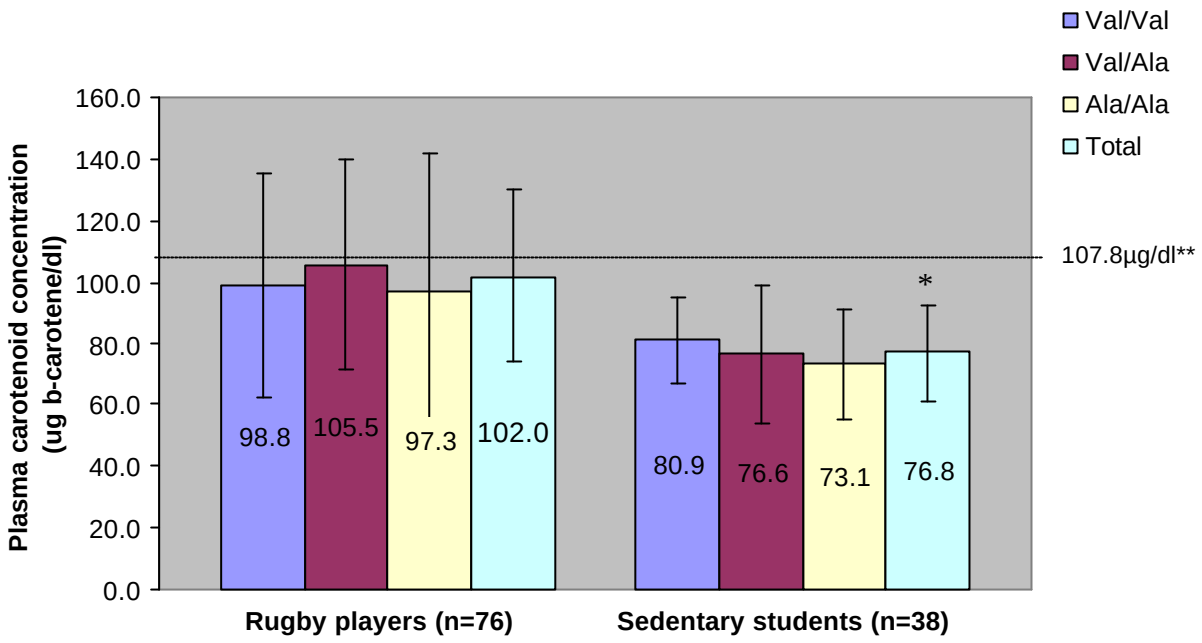


Figure 2: Plasma carotenoid concentration (µg ß-carotene/dl) according to MnSOD genotype and physical activity group.

* Significant difference between total rugby group and total sedentary group (p = 0.001, univariate GLM). ** Dotted line indicates estimated normal level as reported (Neeld & Pearson, 1963)

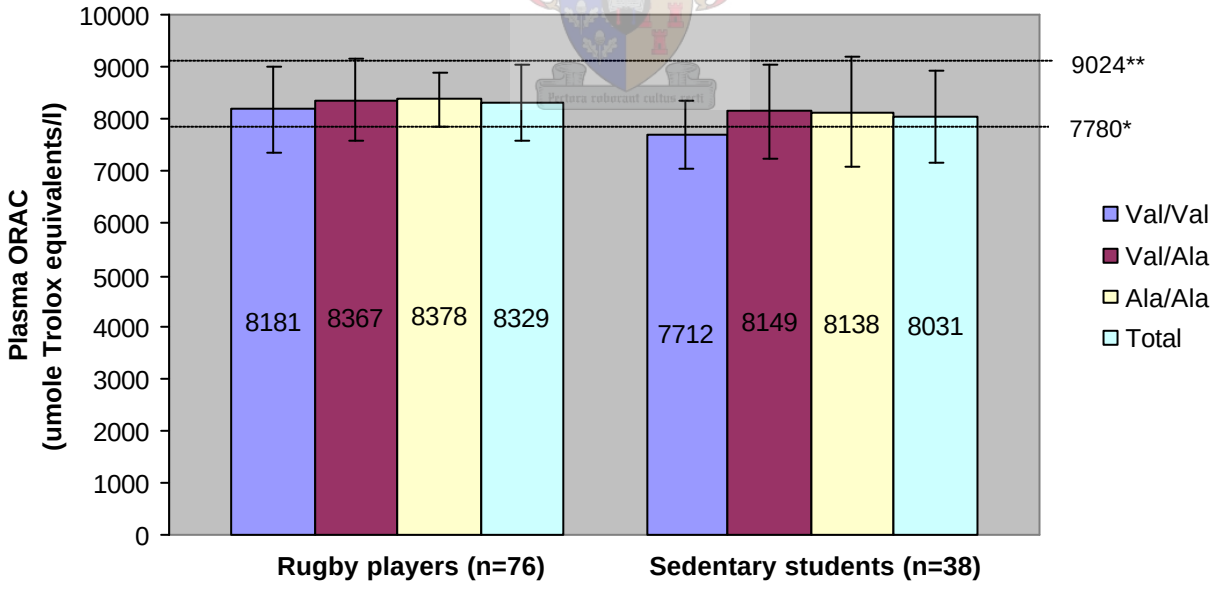


Figure 3: Total plasma antioxidant capacity (µmol Trolox equivalents/l) as measured by the ORAC assay according to MnSOD genotype and physical activity group. Dotted lines indicate estimated normal level as reported by Ou *et al*., (2001) (*) and Huang *et al*., (2002) (**).

Discussion

In order to examine the link between antioxidant status and exercise training in more depth, the association between physical training, markers of plasma antioxidant status (plasma vitamin C, plasma carotenoids and plasma total antioxidant status) and genotype (MnSOD Ala-9Val polymorphism) was investigated while controlling for dietary antioxidant intake.

As the sample size was smaller than the sample calculated for optimal statistical power, the present study should be viewed as an exploratory study. As it is the first study to examine the mentioned relationship, the results provide further insight into a very contentious field of research.

In this study all three indicators of plasma antioxidant status point to a possible more satisfactory antioxidant status in trained subjects compared to sedentary controls. This statement can be motivated as follows:

Plasma vitamin C for both groups were within the normal range (0.4 – 1.5 mg/dl, (Shils *et al*., 1999), indicating adequate vitamin C status and possibly vitamin C based antioxidant status. However, rugby players showed better plasma vitamin C levels compared to sedentary students in this study. Similarly to the findings in this study, others have also reported higher plasma vitamin C levels in soccer players (Brites *et al*., 1999; Cazzola *et al*., 2003) and rugby players (Evelson *et al*., 2002) compared to sedentary controls. However, this finding is not universal as other studies have reported that plasma vitamin C levels were not different between athletes and controls (Robertson *et al*., 1991; Fogelholm *et al*., 1992; Rokitzki *et al*., 1994a; Watson *et al*., 2005). With regards to vitamin C intake as an indicator of vitamin C status, mean intake (from foods and dietary supplements) in our study population was similarly high for both groups and was approximately two and a half times the current recommended dietary allowance (RDA) of 90mg/day for males (Food and Nutrition Board, 2000). When vitamin C intake was calculated from foods only, estimated intake was considerably less but was still above the RDA. Research by others indicates that vitamin C intake in athletes varies considerably and in male athletes is reported to range from 90 to 520 mg per day (Fogelholm *et al*., 1992; Cazzola *et al*., 2003; Peake, 2003; Rousseau *et al*., 2004; Lukaski, 2004; Watson *et al*., 2005). Our data thus supports the view that athletes generally consume adequate amounts of vitamin C even if supplements are not taken. Therefore, in this study, vitamin C status as assessed by

plasma vitamin C and dietary vitamin C intake was adequate in both groups, with rugby players showing an improved plasma vitamin C status compared to sedentary students.

In this study vitamin E status was only assessed in terms of dietary intake. There was no difference in vitamin E intake between the groups, although the mean vitamin E intake was approximately 80% of the RDA of 15mg/day (Food and Nutrition Board, 2000) for the rugby players and only 50% for sedentary controls. This does suggest that rugby players may have a better dietary vitamin E status compared to sedentary students. Reported vitamin E intakes in our study were similar to what was reported by others (Cazzola *et al*., 2003; Rousseau *et al*., 2004). The results of our study point to the possibility that vitamin E based antioxidant status may not be optimal in both study groups based on inadequate dietary vitamin E intake according to the RDA.

With regards to plasma carotenoid adequacy, no reference values for normal ranges exist. Plasma levels in this study were however in line with values reported by other researchers in the general population (Neeld & Pearson, 1963). In this study, rugby players had higher plasma carotenoid levels than sedentary students. No other studies were found that compared plasma *carotenoid* levels between trained individuals and sedentary controls. However, Watson *et al*., (2005) reported that plasma *ß-carotene* was higher in athletes than in sedentary controls, which is in line with the finding in the present study. Concerning carotenoid intake, there is currently no RDA available and comparative data on the dietary intake of carotenoids in athletes is scarce. In this study, reported carotenoid intakes for rugby players were in line with the values reported by Rousseau *et al*., (2004) for athletes, but slightly higher than the reported values for sedentary controls. Therefore, in this study plasma carotenoid levels and carotenoid intake, suggest that carotenoid status is adequate for both groups, with the trained group showing better plasma carotenoid levels and thus possibly improved carotenoid based antioxidant status.

When considering flavonoid intake as an indicator of flavonoid related antioxidant status, flavonoid intake in rugby players and sedentary controls was similar in this study. As no RDA values for flavonoid intake have as yet been established, the adequacy of intake in this study is not clear, but the intake does correspond to the reported range of intake in various populations (Pietta, 2000).

As the above indicators of plasma antioxidant status point to a more satisfactory plasma antioxidant status in rugby players, based on individual dietary antioxidants, the total plasma antioxidant status should reflect this difference. The observed ORAC values, which reflect total plasma antioxidant capacity, in this study were in line with values reported by others for plasma samples from the general population (Ou *et al*., 2001; Huang *et al*., 2002). Furthermore, although not statistically significant, plasma total antioxidant capacity was higher in the rugby players in our study compared to the controls. This tendency may have become statistically significant with an increase in sample size. Plasma antioxidant capacity was also reported to be higher in soccer players (Brites *et al*., 1999; Cazzola *et al*., 2003) and rugby players (Evelson *et al*., 2002) when compared to sedentary controls. In contrast, Watson *et al*., (2005) reported that male athletes had lower antioxidant capacity than the sedentary male controls. However, it must be borne in mind that the methodology for total antioxidant capacity assessment varies between these studies. It has been shown that results from these various assays do not necessarily correlate and are therefore not necessarily automatically directly comparable (Cao & Prior, 1998). It could thus be said that the findings of this study support the fact that that plasma antioxidant status may be more satisfactory in athletes compared to sedentary controls.

When considering the effect of physical activity on antioxidant status, it has to be considered that acute exercise may cause transient increases in plasma antioxidants with returns to pre-exercise levels at rest (Duthie *et al*., 1990; Mastaloudis *et al*., 2001; Aguilo *et al*., 2003; Groussard *et al*., 2003). This may be attributed to the mobilization of antioxidant vitamins and other biologically active compounds such as carotenoids, from tissue pools and their transfer through the plasma to sites undergoing oxidative stress (Packer *et al*., 1989; Peake, 2003). Watson *et al*., (2005) suggested that this could account for the increases in circulating antioxidants seen in those exercising regularly as the antioxidants mobilised during acute exercise might spill over during the recovery into rest periods. In this study, rugby players were requested to refrain from exercising for 24 hours before the blood drawing to control for the acute effect of exercise on circulating levels of antioxidants. Thus, the finding that plasma levels of antioxidants were higher in rugby players than in sedentary controls is likely to be a “real” increase in plasma levels of antioxidants. If, however, the finding is due to the spill-over effect of acute exercise on circulating antioxidant levels, then the plasma levels of antioxidants may not be an accurate reflection of overall antioxidant status in athletes. The exercise-induced fluctuations in plasma antioxidant levels that have been reported in other studies may

necessitate additional assessments of antioxidant body stores, for example leukocytes for vitamin C status, as they may provide a better reflection of antioxidant status over the middle or long term (Mayne, 2003). Whether the mobilisation of antioxidants from body stores and their transfer through plasma actually depletes body stores is not clear and requires further investigation. If a depletion of antioxidant body stores does indeed occur, this would indicate that athletes may have a greater antioxidant requirement in order to replenish body stores.

As mentioned in the introduction, a wide range of genes encoding antioxidant enzymes may play a role in oxidative stress susceptibility and antioxidant status, which could possibly explain the larger than expected inter-individual variation in oxidative stress markers in athletes as well as sedentary controls (Watson *et al*., 2005). The polymorphism investigated in this study was the MnSOD Ala-9Val polymorphism, which has previously been linked to cancer (Rosenblum *et al*., 1996; Ambrosone *et al*., 1999; Woodson *et al*., 2003). The MnSOD genotype distribution, which has not yet been reported in a South African population, was 23.5% for Val/Val and 50.4% for Val/Ala and 26.1% for Ala/Ala, in our study, which is similar to what has been reported in other Caucasian populations (Ambrosone *et al*., 1999; Van Landeghem *et al*., 1999; Mitrunen *et al*., 2001). In the present study population, a specific MnSOD genotype was not associated with decreased plasma levels of carotenoids or vitamin C and total plasma antioxidant status. While the sample size may have been a factor contributing to the lack of an association in this study, Tamimi *et al*., (2004) also did not find a significant interaction between MnSOD genotype and plasma antioxidant levels in their study that examined the role of MnSOD genotype in breast cancer risk in 1205 women. The researchers in the last mentioned study did not provide an explanation for their finding. We speculate thus that, in individuals with adequate dietary antioxidant intake, as was the case in the present study, the variant allele of the MnSOD gene does not result in increased risk of oxidative stress as the levels of antioxidants and the antioxidant capacity may be sufficient to counter the production of free radicals and ROS.

In the assessment of the association between plasma antioxidant status, physical activity and genotype the confounding effect of differences in dietary antioxidant intake should always be considered as plasma antioxidant vitamin levels are generally influenced by the dietary antioxidant intake (Block *et al*., 2001; Record *et al*., 2001; Cazzola *et al*., 2003; Nelson *et al*., 2003; Anlasik *et al*., 2005). In our study, there were weak indications that

dietary antioxidant intake were reflected in plasma antioxidant levels. With regards to vitamin C, the correlation between dietary vitamin C intake and plasma vitamin C levels was statistically significant albeit weak. It must be borne in mind that plasma vitamin C levels are tightly controlled by saturable/dose dependent absorption and renal excretion (Shils *et al*., 1999). According to Levine *et al*., (1996) plasma vitamin C concentration as a function of daily oral dose follows a steep sigmoidal curve in healthy young men, with the steep portion of the curve lying between 30 mg and 100 mg of oral vitamin C daily. Similarly, Rousseau *et al*., (2004) reported that plasma ascorbic acid was closely related to vitamin C intakes when the latter was less than 100mg/day in male athletes, but only a minimal increase in plasma ascorbic acid concentration was observed at intakes above this dose. In our study over 70% of subjects had intakes above 100mg/day and therefore, at these observed high intakes, plasma vitamin C levels may not correspond to greater intakes, explaining the weak correlation.

The association between dietary carotenoid intake and plasma carotenoid was significant but with a low correlation coefficient (r value) within the rugby group and non-significant within the sedentary and total group respectively. Significant associations between plasma levels and intakes of various individual carotenoids have been reported by others, although in these studies correlation coefficients were also moderately low (Bolton-Smith *et al*., 1991; Brady *et al*., 1996; El Sohemy *et al*., 2002). Although the results could reflect an actual lack of association between plasma and intake levels in the present study, the following methodological issues need to be considered when interpreting the results: Incomplete nutrient databases may have resulted in inaccurate carotenoid intake estimations, thereby reducing the likelihood of an association between dietary intake and plasma levels. Furthermore, the method used to analyse plasma carotenoid concentrations does not specify which carotenoids are assessed, and it is therefore not clear whether these correspond to the carotenoids that were included in the intake assessment. Lastly, the use of the HPLC analytical method (Craft, 1992) would possibly also have provided more accurate estimates of plasma levels.

In the present study, dietary antioxidant intake, which was measured using a specially designed and validated FFQ, was similar in the two groups. Therefore, in this study, dietary antioxidant intake did not seem to have a confounding effect on the tested association between plasma antioxidant status, physical activity and MnSOD genotype. This observation is also supported by the fact that the control for dietary antioxidant intake

did not change the outcomes of the association between the different variables in a general linear model (GLM).

Finally the implications of the results of this study regarding dietary and supplementary antioxidant requirements for athletes should be considered as dietary supplement use was found to be widespread for both subject groups. Approximately three-quarters of the rugby players and two-thirds of the sedentary students reporting use in the past month. A meta-analysis of 51 studies investigating supplement use amongst athletes, showed an overall prevalence of supplement use of 46% in 10 274 male and female athletes (Sobal & Marquart, 1994). More recent studies have reported prevalence of supplement use amongst university athletes to be above 80% (Froiland *et al*., 2004; Kristiansen *et al*., 2005). These studies however included sports drinks as supplements, which were not included in our study. Supplement use among sedentary students in this study was similar to the reported prevalence of 62% in a survey of 400 students attending the same university as the current subjects (Seele & Senekal, 2005). Despite the adequate vitamin C intake from food, the use of vitamin C containing supplements was common and contributed 50% to total vitamin C intake for the total study group. Regarding the other antioxidant nutrients investigated, supplements contributed 1.2% to carotenoid intake and 39% to vitamin E intake while no flavonoid containing supplements were used in this study.

Despite dietary intake being similar among rugby players and sedentary students as well dietary supplement use being equally prevalent in rugby players and sedentary students, plasma vitamin C and carotenoid concentrations were higher in the rugby players. Findings such as these have led several researchers to reflect on the need for antioxidant supplementation in athletes. This contention is based on two questions: Firstly, do athletes have a lower antioxidant status and therefore an increased antioxidant need, and secondly, is antioxidant supplementation beneficial? Findings from the present study and others suggest that most athletes have an adequate antioxidant intake and demonstrate adequate or above adequate antioxidant status as measured by vitamin C, vitamin E and carotenoids (Robertson *et al*., 1991; Fogelholm *et al*., 1992; Rokitzki *et al*., 1994a; Lukaski, 2004). In addition, individuals who train regularly may have an enhanced antioxidant status, as was shown in the present study and in other studies that have compared antioxidant status in athletes and sedentary controls (Brites *et al*., 1999; Evelson *et al*., 2002; Cazzola *et al*., 2003). Regarding antioxidant supplementation, results from studies examining the efficacy of antioxidant supplementation in reducing exercise-induced oxidative damage are

equivocal and inconclusive. While studies generally report that antioxidant supplementation results in increases in plasma levels of antioxidants (Papas, 1996; Block *et al*., 2001; Record *et al*., 2001; Nelson *et al*., 2003), this does not necessarily result in decreases in exercise-induced oxidative stress (McCall & Frei, 1999; Williams *et al*., 2006). Our findings thus support the notion that athletes do not necessarily have greater antioxidant requirements and that there is not sufficient evidence to recommend antioxidant supplements to persons exercising regularly.

Although only included as a descriptive variable, the higher BMI of rugby players, which was observed in this study, must be mentioned. The mean BMI of the rugby players corresponded to the overweight category (SASSO, 2003). However, in athletes with well developed musculature, and thus a higher lean body mass, BMI may lead to misclassification of the athlete as overweight or obese as BMI does not necessarily reflect body composition, lean body mass and fat mass effectively (Jonnalagadda *et al*., 2001; Prentice & Jebb, 2001). Therefore, the higher observed BMI in rugby players is most likely a result of higher lean body mass due to their exercise training.

Conclusions and recommendations

Rugby players who were engaged in regular physical activity displayed a higher plasma antioxidant capacity in terms of plasma vitamin C and carotenoid levels when compared to sedentary male students despite similar dietary intakes. While this finding points to an actual improvement in antioxidant status, the mechanism responsible for the improvement is not clear and further research is needed.

MnSOD genotype did not affect markers of plasma antioxidant status or total plasma antioxidant status when dietary antioxidant intake was controlled for. We recommend increasing our sample size to further investigate the influence of MnSOD genotype as well as other antioxidant enzyme gene variations on antioxidant status. It is also recommended that the effect of antioxidant enzyme genetic variants on other markers of oxidative damage, e.g. lipid, protein and DNA oxidation by-products be investigated.

The fact that rugby players displayed increased plasma vitamin C and carotenoid levels compared to sedentary controls, despite similar dietary intakes, supports the notion that athletes generally do not necessarily have increased requirements for antioxidant vitamins.

Despite the observed high prevalence of supplement use in our study as well as others, there is currently not enough conclusive evidence to recommend antioxidant supplementation in athletes. Once results have been confirmed, these results should be considered when formulating dietary recommendations for athletes.

References

Aguilo A, Tauler P, Pilar GM, Villa G, Cordova A, Tur JA & Pons A (2003) Effect of exercise intensity and training on antioxidants and cholesterol profile in cyclists. *J Nutr Biochem* **14,** 319-325

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

Allen RG & Tresini M (2000) Oxidative stress and gene regulation. *Free Radic Biol Med* **28,** 463-499

Ambrosone CB, Freudenheim JL, Thompson PA, *et al* (1999) Manganese superoxide dismutase (MnSOD) genetic polymorphisms, dietary antioxidants, and risk of breast cancer. *Cancer Res* **59,** 602-606

Anlasik T, Sies H, Griffiths HR, Mecocci P, Stahl W & Polidori MC (2005) Dietary habits are major determinants of the plasma antioxidant status in healthy elderly subjects. *Br J Nutr* **94,** 639-642

Ashton T, Rowlands CC, Jones E, Young IS, Jackson SK, Davies B & Peters JR (1998) Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. *Eur J Appl Physiol Occup Physiol* **77,** 498-502

Balakrishnan SD & Anuradha CV (1998) Exercise, depletion of antioxidants and antioxidant manipulation. *Cell Biochem Funct* **16,** 269-275

Bastow MD (1982) Anthropometrics revisited. *Proc Nutr Soc* **41,** 381-388

Benzie IF (2000) Evolution of antioxidant defence mechanisms. *Eur J Nutr* **39,** 53-61

Block G, Norkus E, Hudes M, Mandel S & Helzlsouer K (2001) Which plasma antioxidants are most related to fruit and vegetable consumption? *Am J Epidemiol* **154,** 1113-1118

Bolton-Smith C, Casey CE, Gey KF, Smith WC & Tunstall-Pedoe H (1991) Antioxidant vitamin intakes assessed using a food-frequency questionnaire: correlation with biochemical status in smokers and non-smokers. *Br J Nutr* **65,** 337-346

Brady WE, Mares-Perlman JA, Bowen P & Stacewicz-Sapuntzakis M (1996) Human serum carotenoid concentrations are related to physiologic and lifestyle factors. *J Nutr* **126,** 129-137

Brites FD, Evelson PA, Christiansen MG, Nicol MF, Basilico MJ, Wikinski RW & Llesuy SF (1999) Soccer players under regular training show oxidative stress but an improved plasma antioxidant status. *Clin Sci (Lond)* **96,** 381-385

Cao G, Alessio HM & Cutler RG (1993) Oxygen-radical absorbance capacity assay for antioxidants. *Free Radic Biol Med* **14,** 303-311

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

Cazzola R, Russo-Volpe S, Cervato G & Cestaro B (2003) Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. *Eur J Clin Invest* **33,** 924-930

Chang CK, Tseng HF, Hsuuw YD, Chan WH & Shieh LC (2002) Higher LDL oxidation at rest and after a rugby game in weekend warriors. *Ann Nutr Metab* **46,** 103-107

Craft NE (1992) Carotenoid reversed-phase high-performance liquid chromatography methods: reference compendium. *Methods Enzymol* **213,** 185-205

Davies KJ, Quintanilha AT, Brooks GA & Packer L (1982) Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun* **107,** 1198-1205

Dillard CJ, Litov RE, Savin WM, Dumelin EE & Tappel AL (1978) Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. *J Appl Physiol* **45,** 927-932

Duthie GG, Robertson JD, Maughan RJ & Morrice PC (1990) Blood antioxidant status and erythrocyte lipid peroxidation following distance running. *Arch Biochem Biophys* **282,** 78-83

El Sohemy A, Baylin A, Kabagambe E, Ascherio A, Spiegelman D & Campos H (2002) Individual carotenoid concentrations in adipose tissue and plasma as biomarkers of dietary intake. *Am J Clin Nutr* **76,** 172-179

Evelson P, Gambino G, Travacio M, Jaita G, Verona J, Maroncelli C, Wikinski R, Llesuy S & Brites F (2002) Higher antioxidant defences in plasma and low density lipoproteins from rugby players. *Eur J Clin Invest* **32,** 818-825

Fogelholm GM, Himberg JJ, Alopaeus K, Gref CG, Laakso JT, Lehto JJ & Mussalo-Rauhamaa H (1992) Dietary and biochemical indices of nutritional status in male athletes and controls. *J Am Coll Nutr* **11,** 181-191

Food and Nutrition Board IoM (2000) *Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids.*, Washington, DC: National Academic Press.

Forsberg L, de Faire U & Morgenstern R (2001) Oxidative stress, human genetic variation, and disease. *Arch Biochem Biophys* **389,** 84-93

Froiland K, Koszewski W, Hingst J & Kopecky L (2004) Nutritional supplement use among college athletes and their sources of information. *Int J Sport Nutr Exerc Metab* **14,** 104-120

Groussard C, Machefer G, Rannou F, Faure H, Zouhal H, Sergent O, Chevanne M, Cillard J & Gratas-Delamarche A (2003) Physical fitness and plasma non-enzymatic antioxidant status at rest and after a wingate test. *Can J Appl Physiol* **28,** 79-92

Halliwell B (1998) Free radicals and oxidative damage in biology and medicine: An introduction. In *Oxidative stress in skeletal muscle*, pp. 1-27 [Reznick AZ, Packer L, Sen CK, Holloszy JO and Jackson MJ, editors]. Basel: Birkhäuser Verlag.

Halliwell B & Gutteridge JMC (1999) *Free Radicals in Biology and Medicine*, 3rd ed. New York: Oxford University Press.

Hartmann A, Niess AM, Grunert-Fuchs M, Poch B & Speit G (1995) Vitamin E prevents exercise-induced DNA damage. *Mutat Res* **346,** 195-202

Huang D, Ou B, Hampsch-Woodill M, Flanagan JA & Prior RL (2002) High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J Agric Food Chem* **50,** 4437-4444

Huang D, Ou B & Prior RL (2005) The chemistry behind antioxidant capacity assays. *J Agric Food Chem* **53,** 1841-1856

Jackson MJ, Papa S, Bolanos J, *et al* (2002) Antioxidants, reactive oxygen and nitrogen species, gene induction and mitochondrial function. *Mol Aspects Med* **23,** 209-285

Ji LL (1998) Antioxidant enzyme response to exercise and training in the skeletal muscle. In *Oxidative Stress in Skeletal Muscle*, pp. 103-125 [Reznick AZ, Packer L, Sen CK, Holloszy JO and Jackson MJ, editors]. Basel: Birkhäuser Verlag.

Jonnalagadda SS, Rosenbloom CA & Skinner R (2001) Dietary practices, attitudes, and physiological status of collegiate freshman football players. *J Strength Cond Res* **15,** 507-513

Kristiansen M, Levy-Milne R, Barr S & Flint A (2005) Dietary supplement use by varsity athletes at a Canadian university. *Int J Sport Nutr Exerc Metab* **15,** 195-210

Kumanyika S, Tell GS, Fried L, Martel JK & Chinchilli VM (1996) Picture-sort method for administering a food frequency questionnaire to older adults. *J Am Diet Assoc* **96,** 137-144

Langenhoven M, Kruger M, Gouws E & Faber M (1991) *MRC Food Quantities Manual*, Parow: Medical Research Council, 1991.

Lee J, Goldfarb AH, Rescino MH, Hegde S, Patrick S & Apperson K (2002) Eccentric exercise effect on blood oxidative-stress markers and delayed onset of muscle soreness. *Med Sci Sports Exerc* **34,** 443-448

Levine M, Conry-Cantilena C, Wang Y, *et al* (1996) Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci U S A* **93,** 3704-3709

Lukaski HC (2004) Vitamin and mineral status: effects on physical performance. *Nutrition* **20,** 632-644

Mangels AR, Holden JM, Beecher GR, Forman MR & Lanza E (1993) Carotenoid content of fruits and vegetables: an evaluation of analytic data. *J Am Diet Assoc* **93,** 284-296

Mastaloudis A, Leonard SW & Traber MG (2001) Oxidative stress in athletes during extreme endurance exercise. *Free Radic Biol Med* **31,** 911-922

Mayne ST (2003) Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. *J Nutr* **133 Suppl 3,** 933S-940S

McCall MR & Frei B (1999) Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic Biol Med* **26,** 1034-1053

Mitrunen K, Sillanpaa P, Kataja V, Eskelinen M, Kosma VM, Benhamou S, Uusitupa M & Hirvonen A (2001) Association between manganese superoxide dismutase (MnSOD) gene polymorphism and breast cancer risk. *Carcinogenesis* **22,** 827-829

Morgenstern R (2004) Oxidative stress and human genetic variation. *J Nutr* **134,** 3173S-3174S

Naguib YM (2000) A fluorometric method for measurement of oxygen radical-scavenging activity of water-soluble antioxidants. *Anal Biochem* **284,** 93-98

Needld JB & Pearson WN (1963) Macro- and micromethods for the determination of serum vitamin A using trifluoroacetic acid. *J Nutr* **79,** 454-462

Nelson JL, Bernstein PS, Schmidt MC, Von Tress MS & Askew EW (2003) Dietary modification and moderate antioxidant supplementation differentially affect serum carotenoids, antioxidant levels and markers of oxidative stress in older humans. *J Nutr* **133,** 3117-3123

Niess AM, Hartmann A, Grunert-Fuchs M, Poch B & Speit G (1996) DNA damage after exhaustive treadmill running in trained and untrained men. *Int J Sports Med* **17,** 397-403

Oberley LW, St Clair DK, Autor AP & Oberley TD (1987) Increase in manganese superoxide dismutase activity in the mouse heart after X-irradiation. *Arch Biochem Biophys* **254,** 69-80

Ou B, Hampsch-Woodill M & Prior RL (2001) Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J Agric Food Chem* **49,** 4619-4626

Packer L, Almada AL, Rothfuss LM & Wilson DS (1989) Modulation of tissue vitamin E levels by physical exercise. *Ann N Y Acad Sci* **570,** 311-321

Papas AM (1996) Determinants of antioxidant status in humans. *Lipids* **31 Suppl,** S77-S82

Peake JM (2003) Vitamin C: effects of exercise and requirements with training. *Int J Sport Nutr Exerc Metab* **13,** 125-151

Pietta PG (2000) Flavonoids as antioxidants. *J Nat Prod* **63,** 1035-1042

Powers SK & Lennon SL (1999) Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle. *Proc Nutr Soc* **58,** 1025-1033

Prentice AM & Jebb SA (2001) Beyond body mass index. *Obes Rev* **2,** 141-147

Prior RL & Cao G (1999) In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radic Biol Med* **27,** 1173-1181

Quindry JC, Stone WL, King J & Broeder CE (2003) The effects of acute exercise on neutrophils and plasma oxidative stress. *Med Sci Sports Exerc* **35,** 1139-1145

Record IR, Dreosti IE & McInerney JK (2001) Changes in plasma antioxidant status following consumption of diets high or low in fruit and vegetables or following dietary supplementation with an antioxidant mixture. *Br J Nutr* **85,** 459-464

Robertson JD, Maughan RJ, Duthie GG & Morrice PC (1991) Increased blood antioxidant systems of runners in response to training load. *Clin Sci (Lond)* **80,** 611-618

Rokitzki L, Hinkel S, Klemp C, Cufi D & Keul J (1994a) Dietary, serum and urine ascorbic acid status in male athletes. *Int J Sports Med* **15,** 435-440

Rokitzki L, Logemann E, Sagredos AN, Murphy M, Wetzel-Roth W & Keul J (1994b) Lipid peroxidation and antioxidative vitamins under extreme endurance stress. *Acta Physiol Scand* **151,** 149-158

Rosenblum JS, Gilula NB & Lerner RA (1996) On signal sequence polymorphisms and diseases of distribution. *Proc Natl Acad Sci U S A* **93,** 4471-4473

Rousseau AS, Hininger I, Palazzetti S, Faure H, Roussel AM & Margaritis I (2004) Antioxidant vitamin status in high exposure to oxidative stress in competitive athletes. *Br J Nutr* **92,** 461-468

SASSO (Southern African Society for the Study of Obesity) (2003) *Guidelines for the prevention and management of overweight and obesity in South Africa*, Johannesburg: SASSO.

Schippinger G, Wonisch W, Abuja PM, Fankhauser F, Winklhofer-Roob BM & Halwachs G (2002) Lipid peroxidation and antioxidant status in professional American football players during competition. *Eur J Clin Invest* **32,** 686-692

Seele M & Senekal M (2005) Dietary supplement use and associated factors among university students. *SAJCN* **18,** 17-30

Sen CK & Goldfarb AH (2000) Antioxidants and physical exercise. In *Handbook of oxidants and antioxidants in exercise*, pp. 297-320 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Senekal M & Steyn NP (2004) *Food Photo Manual*, Parow: Medical Research Council.

Shils ME, Olson JA, Shike M & Ross AC (1999) *Modern Nutrition in Health and Disease*, 9th ed. Philadelphia: Lippincott Williams & Wilkins.

Sies H (1991) *Oxidative Stress II. Oxidants and Antioxidants*, pp. London: Academic Press.

Sobal J & Marquart LF (1994) Vitamin/mineral supplement use among athletes: a review of the literature. *Int J Sport Nutr* **4,** 320-334

Spitzer JA (1995) Active oxygen intermediates--beneficial or deleterious? An introduction. *Proc Soc Exp Biol Med* **209,** 102-103

Suzuki YJ, Forman HJ & Sevanian A (1997) Oxidants as stimulators of signal transduction. *Free Radic Biol Med* **22,** 269-285

Tamimi RM, Hankinson SE, Spiegelman D, Colditz GA & Hunter DJ (2004) Manganese superoxide dismutase polymorphism, plasma antioxidants, cigarette smoking, and risk of breast cancer. *Cancer Epidemiol Biomarkers Prev* **13,** 989-996

U.S.Department of Agriculture (2003) *USDA database for the flavonoid content of selected foods*, Beltsville (MD): U.S. Department of Agriculture.

Van Landeghem GF, Tabatabaie P, Kucinskas V, Saha N & Beckman G (1999) Ethnic variation in the mitochondrial targeting sequence polymorphism of MnSOD. *Hum Hered* **49,** 190-193

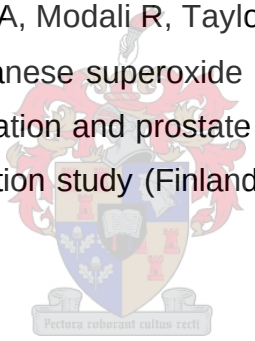
Viguie CA, Frei B, Shigenaga MK, Ames BN, Packer L & Brooks GA (1993) Antioxidant status and indexes of oxidative stress during consecutive days of exercise. *J Appl Physiol* **75,** 566-572

Watson TA, MacDonald-Wicks LK & Garg ML (2005) Oxidative stress and antioxidants in athletes undertaking regular exercise training. *Int J Sport Nutr Exerc Metab* **15,** 131-146

Wei Y, Ota RB, Bowen HT & Omaye ST (1996) Determination of human plasma and leukocyte ascorbic acid by microtiter plate assay. *Nutritional Biochemistry* **7,** 179-186

Williams SL, Strobel NA, Lexis LA & Coombes JS (2006) Antioxidant requirements of endurance athletes: implications for health. *Nutr Rev* **64,** 93-108

Woodson K, Tangrea JA, Lehman TA, Modali R, Taylor KM, Snyder K, Taylor PR, Virtamo J & Albanes D (2003) Manganese superoxide dismutase (MnSOD) polymorphism, alpha-tocopherol supplementation and prostate cancer risk in the alpha-tocopherol, beta-carotene cancer prevention study (Finland). *Cancer Causes Control* **14,** 513-518



CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

1. General discussion

Research has shown that some forms of acute exercise may result in increases in ROS production, which may place additional pressure on antioxidant defences, thus resulting in oxidative stress. On the other hand, regular exercise training has been shown by some researchers to result in the improvement of antioxidant defences, suggesting that the antioxidant system is able to adapt to exercise-induced ROS production and so reduce the risk of oxidative stress. While it has been shown that antioxidant enzymes are capable of adapting to the exercise-induced increase in ROS production (summarised by Ji (1998) and Powers and Sen (2000), the effect of exercise training on antioxidant capacity and oxidative stress is not so clear. Studies that support the notion that antioxidant defence systems are enhanced with exercise training have shown that concentrations of antioxidants and total antioxidant status are higher in individuals who train regularly while markers of oxidative stress are lower compared to sedentary individuals (Brites *et al*., 1999; Evelson *et al*., 2002; Cazzola *et al*., 2003). On the other hand, some studies have reported that markers of oxidative stress and antioxidant status were similar between trained individuals and sedentary controls (Robertson *et al*., 1991; Rokitzki *et al*., 1994). One potential confounding factor in studies investigating training induced antioxidant improvement is dietary antioxidant intake, from food plus supplements, which is often not controlled for adequately. Circulating levels of antioxidants are influenced by dietary intake and differences observed between trained individuals and sedentary controls may in part be due to differences in antioxidant intake (Block *et al*., 2001; Cazzola *et al*., 2003; Anlasik *et al*., 2005). Part of the problem of controlling for dietary intake is the difficulty in assessing dietary intake largely due to limitations of available dietary assessment methods (Willett, 1998).

In order to address the potential confounding effect of dietary antioxidant intake in studies investigating antioxidant status in athletes, the first objective of the study was to develop a quantified FFQ that specifically measures intake of the major dietary antioxidants, namely vitamin C, vitamin E, carotenoids and flavonoids. For these purposes, foods that were good sources of these nutrients and that were commonly consumed were identified and added to a food list. The FFQ included an open frequency category and portion sizes.

The reproducibility and relative validity of the FFQ was assessed using several statistical approaches as the judgement of agreement between two methods or two administrations

is a rather subjective one and no single statistical test can provide the answer (Willett, 1998; Rankin & Stokes, 1998). To assess reproducibility of the FFQ it was administered twice with a one month interval between administrations. Various statistical tests are available to assess FFQ reproducibility, including comparison of means and standard deviations, correlation coefficients, cross-classification of intake according to quartiles of intake and Bland-Altman plots (Bland & Altman, 1986; Willett, 1998). Although the intake obtained from the second administration of the FFQ was lower than the first for some nutrients, the FFQ demonstrated satisfactory reproducibility as was shown by moderate to strong correlation coefficients between administrations and a high percentage of individuals classified within the same or adjacent quartile of intake on both administrations. Bland-Altman plots (Bland & Altman, 1986) were used to further assess reproducibility. While these plots are able to visually represent the relationship between two administrations or methods, it must be borne in mind that the final interpretation and judgement regarding the agreement is subjective and does not rely on statistical tests (Bland & Altman, 1999; Bakker *et al*., 2003). Generally, differences that are within the limits of agreement (LOA) (d±2SD) are in agreement, provided that these differences are not clinically relevant (Bland & Altman, 1986). However, the judgement regarding the width of the LOA and thus the clinical significance thereof is subjective and no criteria have been developed for dietary studies. Therefore, we propose using the RDA values (where established) as criteria to assess the width of the LOA. Nutrient intakes of approximately one times the RDA are generally regarded as sufficient for most individuals. The LOA in a Bland-Altman plot are derived from the intakes obtained from two administrations of a dietary method or two separate dietary methods. If one administration/method provides a value of 50% of the RDA and the other 200% of the RDA for the same individual, this individual will be classified as deficient according to the one method, but as more then adequate using the other method. Therefore, using the RDA as a means of assessing the clinical relevance of the width of the LOA found for nutrient intakes between two administrations or methods seems very appropriate. In the present study, although most of the data points lay within the LOA, the latter were relatively wide (more than once the RDA for vitamin C), and may thus result in misclassification of vitamin C status. Furthermore, although the mean difference was close to zero, the scatter was wide and proportional bias was present, indicating poor reproducibility according to the Bland-Altman plots. This finding is however in contrast to the results of the other statistical tests, which suggest satisfactory reproducibility for vitamin C. For vitamin E, carotenoids and flavonoids almost all data points were within the LOA, with none judged to be clinically significant based on

the RDA; the scatter of points was wide, but the mean differences were close to zero and no proportional bias was present. These Bland-Altman plot results indicate satisfactory reproducibility for the FFQ for vitamin E, carotenoids and flavonoids, which is in line with the results from the other statistical tests for these nutrients.

The validity study involved two administrations of the FFQ (FFQ 1, FFQ 2) separated by one month, during which a 28-day close-ended dietary record was kept by the subjects. Three blood samples were taken during the month; namely at baseline, two weeks post baseline and four weeks post baseline. The validity of the FFQ was assessed by comparing the frequencies of intake of food items derived from three FFQ values (FFQ 1, FFQ 2 and the FFQmean) to frequencies derived from the 28-day close-ended dietary record. Furthermore, vitamin C and carotenoid intake derived from each of the three FFQ values was compared with the intake derived from the 28-day close-ended dietary record as well as repeated plasma vitamin C levels. The same statistical tests used to assess reproducibility are applicable in FFQ validation studies. In addition, the use of biomarkers enables structural equation modelling, such as the method of triads, to be used in the assessment of FFQ validity (Kaaks *et al*., 1994; Kaaks, 1997).

For all of the statistical tests used to assess the validity of the FFQ, on both the frequency of intake of specific food items as well the nutrient intake, the second administration of the FFQ (FFQ 2) showed better agreement with the 28-day dietary record than the first administration (FFQ 1) or the mean of the administrations (FFQmean). This has been observed by others and could be due to the second administration of the FFQ measuring intake during the same period as the dietary record (Willett *et al*., 1985). On the other hand, the recording process could have sensitized the subject with regards to actual frequency of intake resulting in better estimation of intake with the second administration (Willett, 1998).

In the present study, frequencies of intake of foods as well as vitamin C intake estimated by the FFQ (especially FFQ 2) were similar and correlated well with the frequencies and intakes determined by the 28-day dietary record. The three FFQ values were also able to classify a high percentage of individuals correctly according to quartiles of intake based on the 28-day dietary record. Validity of the FFQ in measuring vitamin C and carotenoid intake was further assessed using Bland-Altman plots (Bland & Altman, 1986). While almost all of the data points were within the limits of agreement (LOA), these were wide and for vitamin C were judged as clinically significant based on the RDA. Furthermore, for

both nutrients, the scatter was wide, the mean difference not close to zero and proportional bias was present, thus suggesting that validity was not satisfactory based on the Bland-Altman method.

In order to further assess the validity of the FFQ, plasma vitamin C levels were used as a biomarker in the SEM according to the method of triads (Kaaks, 1997). In this method, the vitamin C intake estimated from the three methods (three FFQ values respectively, 28-day close-ended dietary record and plasma levels) is compared to the true unknown vitamin C intake by computing validity coefficients (VC) (Kaaks, 1997). Plasma vitamin C demonstrated the lowest correlation with the true unknown intake, while the three FFQ values and the 28-day dietary record showed similar VCs of above 0.8, suggesting that both methods are valid assessment tools for estimating dietary vitamin C intake. The presence of Heywood cases (VC > 1) indicate that the VC for the three FFQ values and the 28-day dietary record may be overestimated and should thus be interpreted as the upper limits of the true validity coefficients (Kaaks, 1997).

To summarize, the validity and reproducibility of the FFQ according to the Bland-Altman method was not completely satisfactory, however the other statistical tests used indicate satisfactory reproducibility and validity. From these results, it is evident that several statistical tests need to be used in reproducibility and validity testing. Bearing in mind the limitation of the sample size, the developed FFQ showed satisfactory reproducibility and validity and could thus be used for its intended purpose in the second part of the study.

The second part of the study investigated the association between antioxidant status and the MnSOD Ala-9Val polymorphism in trained male athletes (rugby players) and sedentary male students while controlling for dietary antioxidant intake.

Rugby players in the present study demonstrated significantly higher plasma levels of vitamin C and carotenoids, while total plasma antioxidant status tended to be higher compared to sedentary students. Plasma levels of vitamin C as well as dietary vitamin C intake were within the normal range for both groups, which indicates adequate vitamin C status and possibly adequate vitamin C based antioxidant status. Dietary vitamin E intake was slightly below the RDA for both groups, pointing to the possibility that vitamin E status may not be optimal. Flavonoid and carotenoid intakes were in line with reported ranges. These findings suggest that antioxidant status is more satisfactory in rugby players

compared to sedentary students, which is in line with the finding that antioxidant status is enhanced with regular exercise training (Brites *et al*., 1999; Evelson *et al*., 2002; Cazzola *et al*., 2003).

It has been suggested that the increased plasma levels of antioxidants found in athletes may be due to a spill-over effect of the exercise-induced mobilisation of antioxidants from tissue pools and their transfer through the plasma to sites undergoing oxidative stress (Packer *et al*., 1989; Peake, 2003; Watson *et al*., 2005). However, in this study rugby players were requested to refrain from exercising for 24 hours before the blood drawing to control for this effect of acute exercise on circulating levels of antioxidants. Thus, the finding that plasma levels of antioxidants were higher in rugby players than in sedentary controls is likely to be an actual increase in plasma levels of antioxidants.

A factor that has not yet been investigated in the context of exercise training and antioxidant status is genotype. Variations in a wide range of genes encoding antioxidant enzymes may play a role in oxidative stress susceptibility and thus antioxidant status (Forsberg *et al*., 2001). Therefore, in order to investigate the effect of MnSOD genotype on antioxidant status in trained and sedentary individuals, while dietary antioxidant intake was controlled for, DNA samples were screened for the MnSOD Ala-9Val polymorphism. The genotype distribution, which has not yet been reported in a South African population, was similar to what has been reported in other Caucasian populations (Ambrosone *et al*., 1999; Van Landeghem *et al*., 1999; Mitrunen *et al*., 2001). In the present study population, a specific MnSOD genotype was not associated with decreased plasma levels of vitamin C, carotenoids or total plasma antioxidant status when dietary intake was controlled for. This finding may have been influenced by the sample size in this study, however, a similar result was reported by Tamimi *et al*. (2004) with reference to MnSOD genotype and cancer. At this stage it is therefore not clear whether MnSOD genotype does influence antioxidant status in athletes. We speculate that, in individuals with adequate dietary antioxidant intake, as was the case in the present study, the variant allele of the MnSOD gene does not result in increased risk of oxidative stress as the levels of antioxidants and the antioxidant capacity may be sufficient to counter the production of free radicals and ROS.

As plasma antioxidants are generally influenced by the dietary antioxidant intake, the possible confounding effect thereof in the association between plasma antioxidant status, genotype and physical activity must be considered. In our study there were weak

indications that dietary intake was reflected in plasma levels for vitamin C for the total group, but not for carotenoids. Dietary intake of vitamin C, vitamin E, carotenoids and flavonoids was similar in both groups, which points to the fact that differences observed in plasma antioxidant levels between rugby players and sedentary students can most probably not be directly attributed to differences in antioxidant intake.

The finding that rugby players in this study showed higher plasma vitamin C and carotenoid levels than sedentary controls, despite similar dietary intake has certain implications regarding antioxidant requirements in athletes and the need for antioxidant supplementation. Research, including ours, has shown that dietary supplement use (including antioxidant containing supplements) is widespread among athletes and students in general (Sobal & Marquart, 1994; Schroder *et al*., 2002; Froiland *et al*., 2004; Kristiansen *et al*., 2005; Seele & Senekal, 2005). Whether such supplementation by athletes is indeed necessary to reduce exercise-induced oxidative stress is questionable as there is no conclusive evidence pointing to a greater antioxidant requirement by athletes (Sharpe, 1999; ADA, 2000; Williams *et al*., 2006). Research has shown that antioxidant status in athletes in general is adequate, which was also found in this study. In addition we, and others, have shown that individuals who train regularly may have a better antioxidant status compared to sedentary controls. Therefore, despite the high prevalence of supplement use among athletes, there is currently not sufficient conclusive evidence to recommend antioxidant supplementation in athletes.

2. Conclusions and recommendations

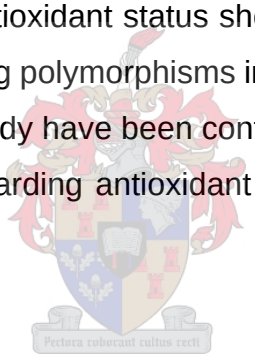
The FFQ, which was developed for this study, showed satisfactory reproducibility and validity for measuring antioxidant intake in a young adult male population. Results from this research indicate that rugby players undergoing regular physical activity have a higher antioxidant status, as measured by plasma vitamin C and plasma carotenoid concentrations, compared to sedentary students despite similar dietary antioxidant intake. The presence of a specific MnSOD genotype did not influence plasma antioxidant status when dietary antioxidant intake was controlled for. Dietary supplement use was widespread among both groups and contributed significantly to vitamin C and vitamin E intake. Therefore, results from this study support the finding that regular exercise training does not result in increased antioxidant requirements. The association between

antioxidant status and MnSOD genotype in rugby players and sedentary students seemed not to be influenced by dietary antioxidant intake.

Based on these findings, the following are recommended with reference to:

1. FFQ development and validation:
    - Several statistical tests should be used in the assessment of FFQ reproducibility and validity.
    - The RDA values should be considered as a criterion to judge the agreement between two methods according to Bland-Altman plots.

2. Antioxidant assessment
    - The use of dietary supplements and their contribution to total dietary intake should be considered when assessing dietary intake.
    - The effect of genotype on antioxidant status should be researched further including additional commonly occurring polymorphisms in antioxidant enzymes
    - Once the results from this study have been confirmed, results should be used in the formulation of guidelines regarding antioxidant intake and dietary supplement use by athletes.



3. References

ADA (2000) Position of the American Dietetic Association, Dietitians of Canada, and the American College of Sports Medicine: Nutrition and athletic performance. *J Am Diet Assoc* **100,** 1543-1556

Ambrosone CB, Freudenheim JL, Thompson PA, *et al* (1999) Manganese superoxide dismutase (MnSOD) genetic polymorphisms, dietary antioxidants, and risk of breast cancer. *Cancer Res* **59,** 602-606

Anlasik T, Sies H, Griffiths HR, Mecocci P, Stahl W & Polidori MC (2005) Dietary habits are major determinants of the plasma antioxidant status in healthy elderly subjects. *Br J Nutr* **94,** 639-642

Bakker I, Twisk JW, van Mechelen W, Mensink GB & Kemper HC (2003) Computerization of a dietary history interview in a running cohort; evaluation within the Amsterdam Growth and Health Longitudinal Study. *Eur J Clin Nutr* **57,** 394-404

Bland JM & Altman DG (1986) Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **1,** 307-310

Bland JM & Altman DG (1999) Measuring agreement in method comparison studies. *Stat Methods Med Res* **8,** 135-160

Block G, Norkus E, Hudes M, Mandel S & Helzlsouer K (2001) Which plasma antioxidants are most related to fruit and vegetable consumption? *Am J Epidemiol* **154,** 1113-1118

Brites FD, Evelson PA, Christiansen MG, Nicol MF, Basilico MJ, Wikinski RW & Llesuy SF (1999) Soccer players under regular training show oxidative stress but an improved plasma antioxidant status. *Clin Sci (Lond)* **96,** 381-385

Cazzola R, Russo-Volpe S, Cervato G & Cestaro B (2003) Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. *Eur J Clin Invest* **33,** 924-930

Evelson P, Gambino G, Travacio M, Jaita G, Verona J, Maroncelli C, Wikinski R, Llesuy S & Brites F (2002) Higher antioxidant defences in plasma and low density lipoproteins from rugby players. *Eur J Clin Invest* **32,** 818-825

Forsberg L, de Faire U & Morgenstern R (2001) Oxidative stress, human genetic variation, and disease. *Arch Biochem Biophys* **389,** 84-93

Froiland K, Koszewski W, Hingst J & Kopecky L (2004) Nutritional supplement use among college athletes and their sources of information. *Int J Sport Nutr Exerc Metab* **14,** 104-120

Ji LL (1998) Antioxidant enzyme response to exercise and training in the skeletal muscle. In *Oxidative Stress in Skeletal Muscle*, pp. 103-125 [Reznick AZ, Packer L, Sen CK, Holloszy JO and Jackson MJ, editors]. Basel: Birkhäuser Verlag.

Kaaks R, Riboli E, Esteve J, van Kappel AL & van Staveren WA (1994) Estimating the accuracy of dietary questionnaire assessments: validation in terms of structural equation models. *Stat Med* **13,** 127-142

Kaaks RJ (1997) Biochemical markers as additional measurements in studies of the accuracy of dietary questionnaire measurements: conceptual issues. *Am J Clin Nutr* **65,** 1232S-1239S

Kristiansen M, Levy-Milne R, Barr S & Flint A (2005) Dietary supplement use by varsity athletes at a Canadian university. *Int J Sport Nutr Exerc Metab* **15,** 195-210

Mitrunen K, Sillanpaa P, Kataja V, Eskelinen M, Kosma VM, Benhamou S, Uusitupa M & Hirvonen A (2001) Association between manganese superoxide dismutase (MnSOD) gene polymorphism and breast cancer risk. *Carcinogenesis* **22,** 827-829

Packer L, Almada AL, Rothfuss LM & Wilson DS (1989) Modulation of tissue vitamin E levels by physical exercise. *Ann N Y Acad Sci* **570,** 311-321

Peake JM (2003) Vitamin C: effects of exercise and requirements with training. *Int J Sport Nutr Exerc Metab* **13,** 125-151

Powers SK & Sen CK (2000) Physiological antioxidants and exercise training. In *Handbook of oxdidants and antioxidants in exercise*, pp. 221-242 [Sen CK, Packer L and Hänninen O, editors]. Amsterdam: Elsevier.

Rankin G & Stokes M (1998) Reliability of assessment tools in rehabilitation: an illustration of appropriate statistical analyses. *Clin Rehabil* **12,** 187-199

Robertson JD, Maughan RJ, Duthie GG & Morrice PC (1991) Increased blood antioxidant systems of runners in response to training load. *Clin Sci (Lond)* **80,** 611-618

Rokitzki L, Hinkel S, Klemp C, Cufi D & Keul J (1994) Dietary, serum and urine ascorbic acid status in male athletes. *Int J Sports Med* **15,** 435-440

Schroder H, Navarro E, Mora J, Seco J, Torregrosa JM & Tramullas A (2002) The type, amount, frequency and timing of dietary supplement use by elite players in the First Spanish Basketball League. *J Sports Sci* **20,** 353-358

Seele M & Senekal M (2005) Dietary supplement use and associated factors among university students. *SAJCN* **18,** 17-30

Sharpe P (1999) Oxidative stress and exercise: need for antioxidant supplementation? *Br J Sports Med* **33,** 298-299

Sobal J & Marquart LF (1994) Vitamin/mineral supplement use among athletes: a review of the literature. *Int J Sport Nutr* **4,** 320-334

Tamimi RM, Hankinson SE, Spiegelman D, Colditz GA & Hunter DJ (2004) Manganese superoxide dismutase polymorphism, plasma antioxidants, cigarette smoking, and risk of breast cancer. *Cancer Epidemiol Biomarkers Prev* **13,** 989-996

Van Landeghem GF, Tabatabaie P, Kucinskas V, Saha N & Beckman G (1999) Ethnic variation in the mitochondrial targeting sequence polymorphism of MnSOD. *Hum Hered* **49,** 190-193

Watson TA, MacDonald-Wicks LK & Garg ML (2005) Oxidative stress and antioxidants in athletes undertaking regular exercise training. *Int J Sport Nutr Exerc Metab* **15,** 131-146

Willett WC (1998) *Nutritional Epidemiology*, 2 ed. New York: Oxford University Press.

Willett WC, Sampson L, Stampfer MJ, Rosner B, Bain C, Witschi J, Hennekens CH & Speizer FE (1985) Reproducibility and validity of a semiquantitative food frequency questionnaire. *Am J Epidemiol* **122,** 51-65

Williams SL, Strobel NA, Lexis LA & Coombes JS (2006) Antioxidant requirements of endurance athletes: implications for health. *Nutr Rev* **64,** 93-108

ADDENDUM 1

SURVEY INSTRUMENT (QUESTIONNAIRE)

SUBJECT INFORMATION

Code | | | |

Section A: Sociodemographic information

Date: _____

1. Name: _____ Surname: _____

2. Date of birth: | | | | | | | | |

d d m m y y y y

3. Age: _____

4. Population group: _____

5. Occupation: _____

6. Residence:

University residence	
Flat or student digs	
At home	

Section B: Health, physical activity and lifestyle information

7. Do you currently smoke daily?

Yes	
No	

8.1 If yes, for how long have you been smoking? _____

8.2 If yes, how many cigarettes on average do smoke per day? _____

9. If no, do you smoke occasionally (socially)?

Yes	
No	

9.1 If yes, how many days a week, on average, do you smoke? _____

9.2 How many cigarettes, on average, do you smoke per day/per occasion? ____

10. If no, did you smoke at any point?

Yes	
No	

10.1 If yes, when did you stop smoking?_____

10.2 On average, how many cigarettes did you smoke per day? _____

11. Physical activity and training during a typical week:

Activity	Frequency (Days per week)	Duration (h or min per session)	Description of activity

12. On average, how active are you at work/university?

Sitting most of the time, little walking or standing.	
Less sitting, more walking and standing but no hard physical labour.	
Very little sitting, mostly walking and/or hard physical labour.	

13. In what rugby position do you usually play?

Forward	
Backline	
N.A.	

Section C: Dietary supplement use

14.1 Have you taken any dietary supplements during the past month?

Yes	
No	

14.2 Supplement use information:

Product name	Supplement type	Nutrient content	Frequency of use			Amount/ dosage	Duration of use
			Per day	Per week	Per month		

15. How does this month relate to your usual month as far as the following are concerned?

	Usual	Less	More	Specify
Activity levels				
Dietary intake				
Supplement intake				
Stress levels				

Section D: Food frequency questionnaire

FOOD ITEM	DESCRIPTION PREPARATION	PORTION SIZE				FREQUENCY			
		1x std	½x std	1½x std	2x std	Per day	Per week	Per month	Never/ seldom
FRUITS									
ORANGE									
NAARTJIE									
OTHER CITRUS (grapefruit)									
MANGO									
PAW-PAW									
GUAVA									
STRAW-BERRIES									
KIWIS									
PEACHES									
WATER-MELON, SPANSPEK									
APPLES									
BANANAS									
FRUIT SALAD									
GRAPES									
AVOCADO PEAR									
ORANGE JUICE									
GRAPE JUICE									
OTHER FRUIT JUICES									
VEGETABLES									
COOKED CARROTS									
RAW CARROTS (Carrot salad, sticks)									
SWEET POTATO									

FOOD ITEM	DESCRIPTION PREPARATION	PORTION SIZE				FREQUENCY			
		1x std	½x std	1½x std	2x std	Per day	Per week	Per month	Never/ seldom
PUMPKIN, BUTTERNUT SQUASH									
BEETROOT									
PEAS									
CAULI-FLOWER									
BROCCOLI									
BRUSSEL SPROUTS									
GREEN BEANS									
BEAN, POTATO, ONION MIX									
COOKED CABBAGE									
MIXED SALAD (lettuce, tomato, cucumber, onion)									
PEPPERS (green, red, yellow)									
TOMATO – RAW									
TOMATO – COOKED									
TOMATO & ONION MIX									
TOMATO SAUCE									
MIXED VEGETABLES									
COOKED SPINACH									
RAW SPINACH									
FRIED ONIONS									
RAW ONIONS									

FOOD ITEM	DESCRIPTION/ PREPARATION	PORTION SIZE				FREQUENCY			
		1x std	½x std	1½x std	2x std	Per day	Per week	Per month	Never/ seldom
COOKED POTATO, MASH									
POTATO CHIPS									
FRIED/ROAST POTATO									
BAKED BEANS									
STIRFRIES									
VEGETABLE STEWS									
VEGETABLE JUICES									
OTHER									
MIXED NUTS (almonds, cashews, hazelnuts)									
PEANUTS									
PEANUT BUTTER									
SOFT MARGARINE									
SUNFLOWER OIL									
SALAD DRESSING (French)									
SALAD DRESSING (Mayo)									
FRIED, SCRAMBLED EGG									
FRIED FISH									
VETKOEK									
PIES									
GARLIC									
COOKIES/ BISCUITS/ RUSKS									

FOOD ITEM	DESCRIPTION/ PREPARATION	PORTION SIZE				FREQUENCY			
		1x std	½x std	1½x std	2x std	Per day	Per week	Per month	Never/ seldom
HEALTH BAR, MUESLI BAR									
PRONUTRO									
MUESLI									
OTHER VITAMIN ENRICHED BREAKFAST CEREAL (fruit loops)									
VIT. ENRICHED SWEETS (supa-C's, vita C's)									
DRIED FRUIT/ FRUIT ROLL									
TEA (black/green)									
LUCOZADE GAME									
VITAMIN C ENRICHED JUICE (e.g.Clifton)									
RED/ WHITE WINE									
BEER, CIDERS									
SPIRITS									

Section E: Anthropometric measurements

Height (m)	
Weight (kg)	

ADDENDUM 2

28-DAY CLOSE-ENDED DIETARY RECORD

Dietary record

NAME: _____

INSTRUCTIONS
1. Write in the date of each day as you go along.
2. Keep the diary with you at all times.
3. When you eat something, take out the diary and check if the food item is on the list. If it is, make a mark (✓) in the block corresponding to the day and food item in question.
4. If you eat the same item again later in the day, make another mark (✓) in that particular block.
5. Repeat this procedure every day for every food item that you eat.
6. You do not have to worry about portion size.

	Mon	Tue	Wed	Thur	Fri	Sat	Sun
Oranges							
Naartjies							
Orange juice							
Other fruit juice							
Banana							
Apples							
Strawbe rries							
Fruit salad							
Tomatoes							
Tomato and onion mix							
Tomato sauce							
Potatoes (any type)							
Sweet potato							
Broccoli							
Cauliflower							
Pumpkin/ butternut							
Carrots							
Peas							
Green beans							
Mixed vegetables							
Spinach							
Mixed salad							
Avocado							
Vitamin C sweets (Vita C, Supa C, bioplus)							

ADDENDUM 3

BLAND-ALTMAN PLOTS FOR VITAMIN C AND CAROTENOIDS ESTIMATED FROM THE FFQ AND 28-DAY DIETARY RECORD

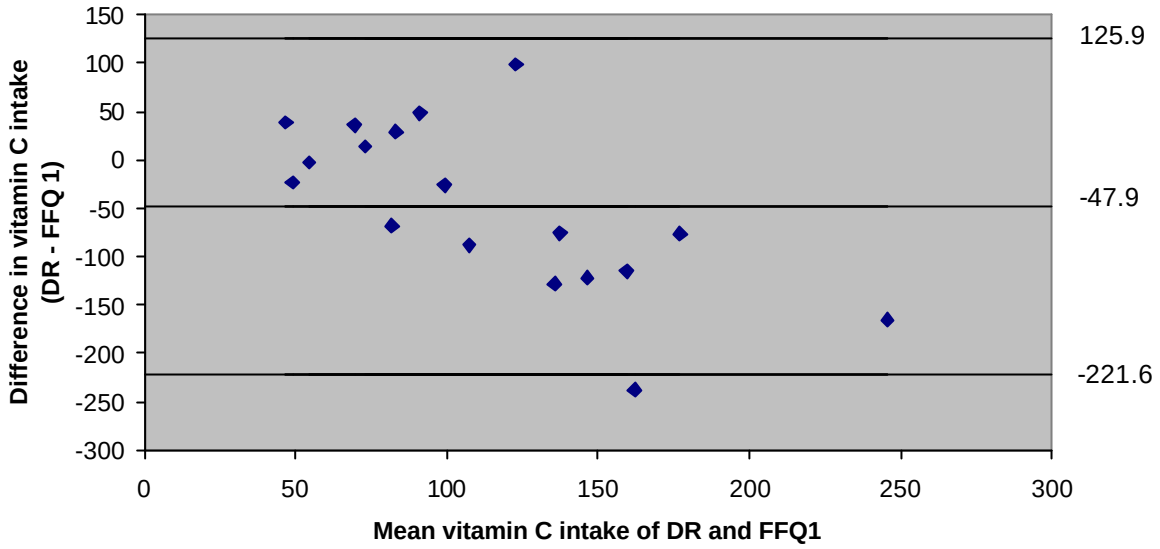


Figure 1: Bland-Altman plot of vitamin C intake (mg/day) illustrating agreement between the dietary record and FFQ 1 showing the mean difference and limits of agreement (d±2SD). DR = 28-day dietary record; FFQ 1 = first administration of the FFQ.

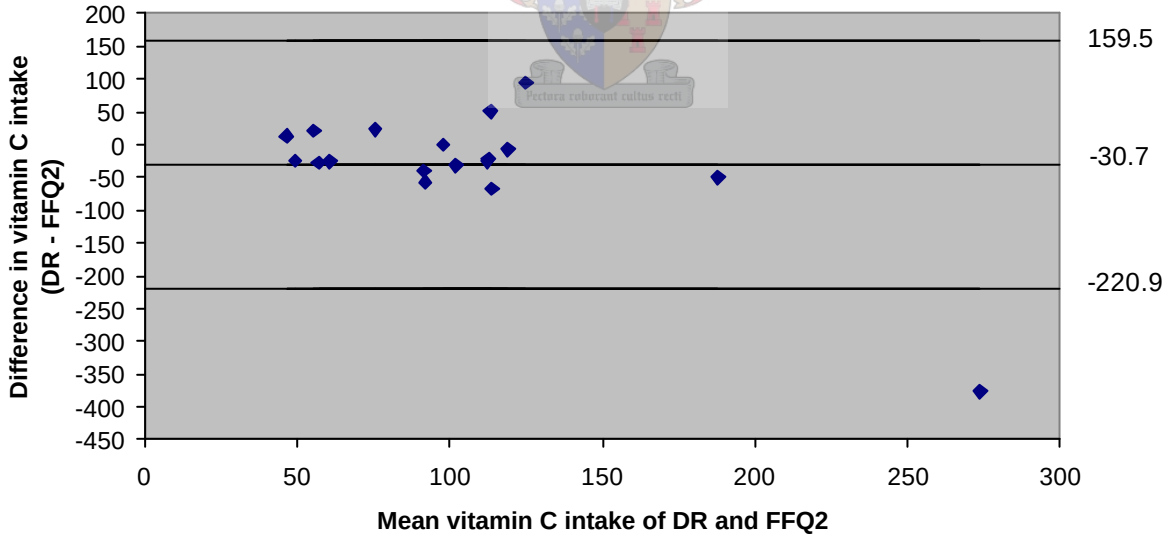


Figure 2: Bland-Altman plot of vitamin C intake (mg/day) illustrating agreement between the dietary record and FFQ 2 showing the mean difference and limits of agreement (d±2SD). DR = 28-day dietary record; FFQ 2 = second administration of the FFQ.

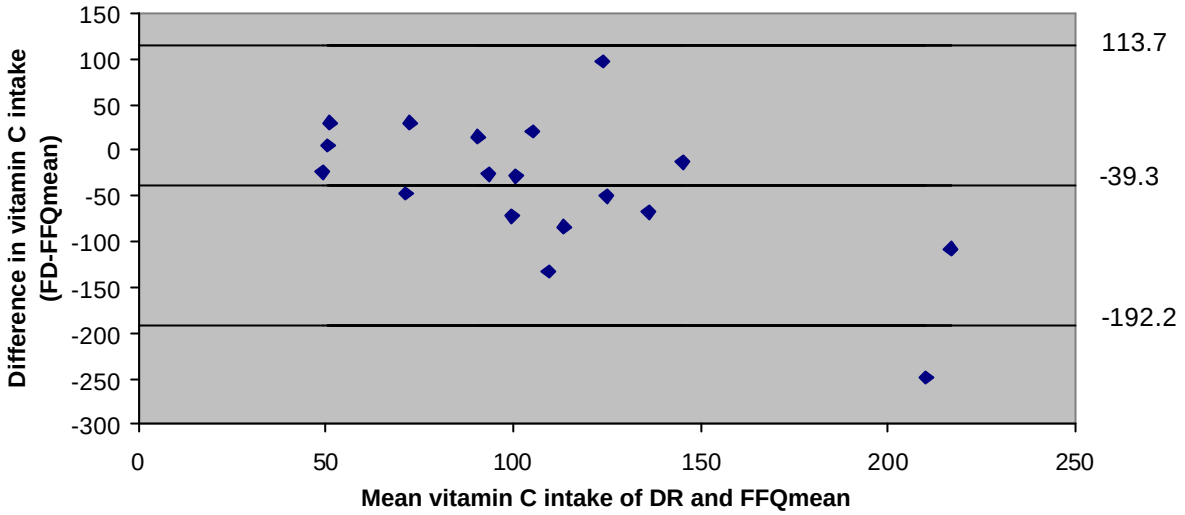


Figure 3: Bland-Altman plot of vitamin C (mg/day) intake illustrating agreement between the dietary record and FFQmean showing the mean difference and limits of agreement (d±2SD). DR = 28-day dietary record; FFQ mean = mean of the two administrations of the FFQ.

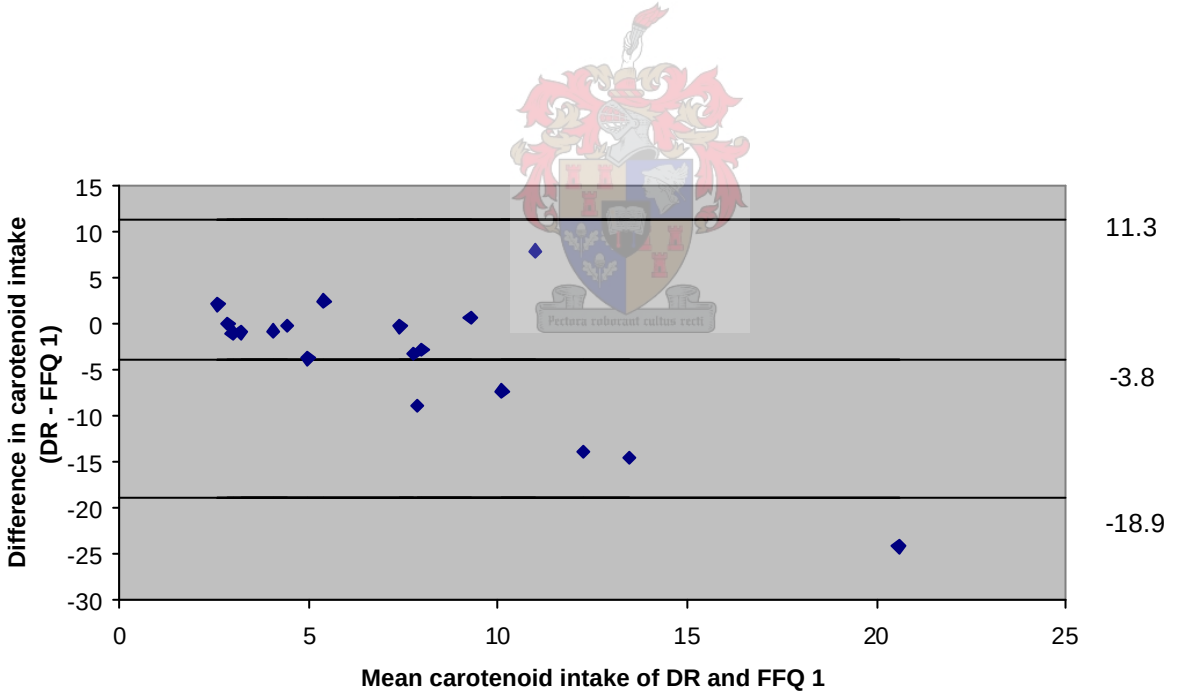


Figure 4: Bland-Altman plot of carotenoid intake (mg/day) illustrating agreement between the dietary record and FFQ 1 showing the mean difference and limits of agreement (d±2SD). DR = 28-day dietary record; FFQ 1 = first administration of the FFQ.

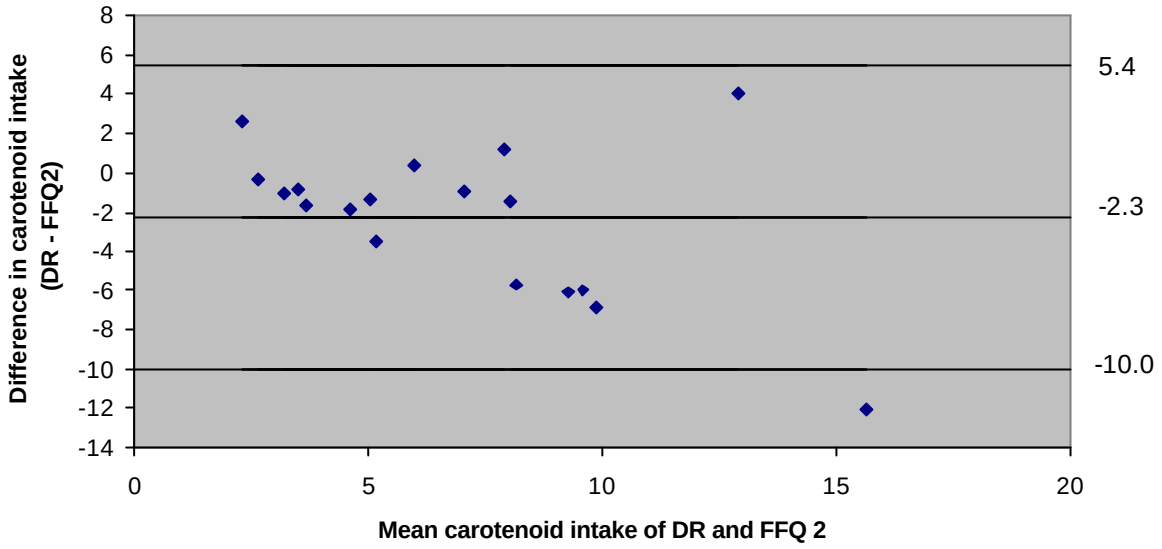


Figure 5: Bland-Altman plot of carotenoid intake (mg/day) illustrating agreement between the dietary record and FFQ 2 showing the mean difference and limits of agreement (d±2SD). DR = 28-day dietary record; FFQ 2 = second administration of the FFQ.

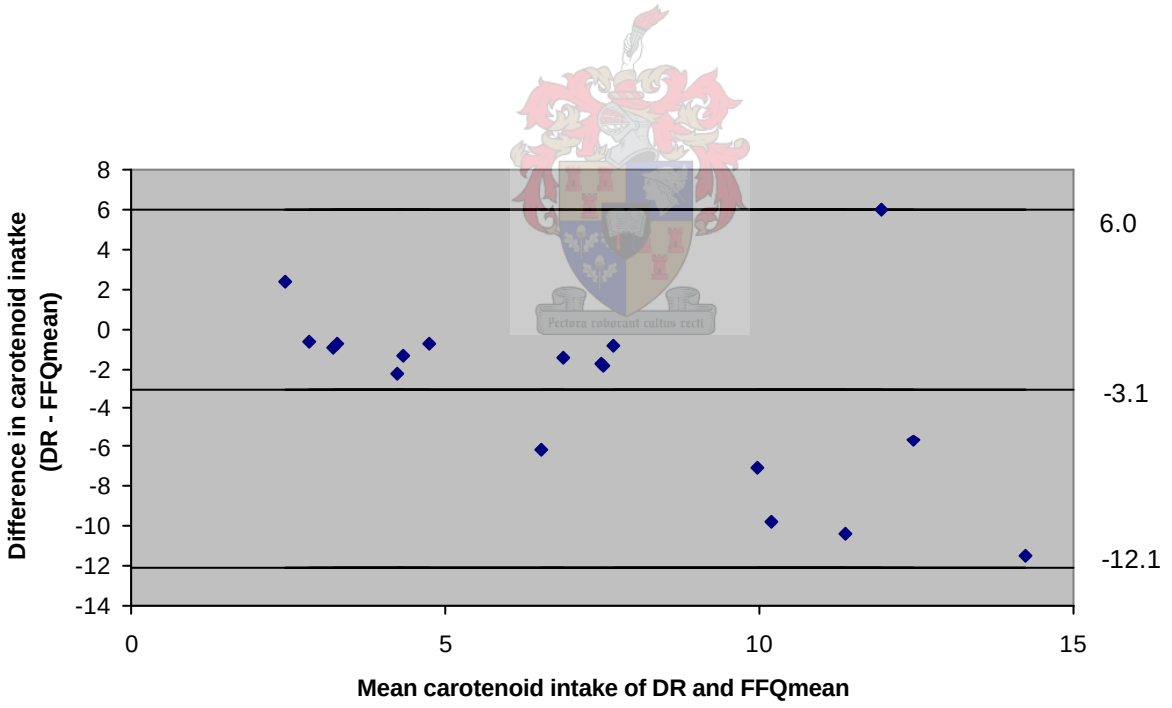


Figure 6: Bland-Altman plot of carotenoid intake (mg/day) illustrating agreement between the dietary record and FFQ 2 showing the mean difference and limits of agreement (d±2SD). DR = 28-day dietary record; FFQ mean = mean of the two administrations of the FFQ.

ADDENDUM 4

HARDY-WEINBERG EQUILIBRIUM CALCULATIONS

Table 1: Hardy-Weinberg equilibrium calculations for MnSOD genotype frequencies for rugby players, sedentary subjects and total group.

Group	Rugby players (n=76)			Sedentary subjects (n=39)			Total group (n=115)		
Genotype	A/A	A/V	V/V	A/A	A/V	V/V	A/A	A/V	V/V
Observed frequency	19	40	17	10	18	11	30	58	27
Expected frequency*	20.0	38.0	18.0	9.3	19.5	10.3	30.3	57.5	27.3
Pearson's Chi-square**		0.22			0.23			0.01	

A = Ala, V = Val

* for genes with two alleles (A and V) with allele frequencies of p and q, respectively, Hardy-Weinberg equilibrium predicts genotype frequencies of p^2 for A/A, 2pq for A/V and q^2 for V/V (http://en.wikipedia.org/wiki/Hardy-Weinberg_principle; accessed 2006).

Expected frequency of p and q calculated as follows:

$$p = \frac{2x(\text{observed A/A}) + \text{observed A/V}}{2x(\text{observed A/A} + \text{observed A/V} + \text{observed V/V})}$$

expected frequency of A/A = p^2 x n

similarly,

$$q = \frac{2x(\text{observed V/V}) + \text{observed A/V}}{2x(\text{observed A/A} + \text{observed A/V} + \text{observed V/V})}$$

expected frequency of V/V = q^2 x n

And expected frequency of A/V = 2pq x n

** Calculated as: $X^2 = ? \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$

df = 1 and 5% significance level = 3.84. Therefore, all groups are in Hardy-Weinberg equilibrium ($X^2 < 3.84$).