In situ and *in vivo* modification by palm fruit-derived carotenes and tocotrienols on the oxidative response to commonly-prescribed doses of oral iron

BY

Monica Orozco

A Thesis
Submitted to the Faculty of Graduate Studies
In partial fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Interdepartmental Program of Food and Nutritional Sciences
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

In Situ and in Vivo Modifiation by Palm Fruit-derived Carotenes and Tocotrienols on the Oxidative Response to Commonly Prescribed Doses of Oral Iron

BY

Monica Orozco

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree Of

Doctor of Philosophy

I hereby declare that I am the sole author of this thesis.

I authorize the University of Manitoba to lend this thesis to other institutions or individuals for the purpose of scholarly research.

Monica Orozco

I further authorize the University of Manitoba to reproduce this thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Monica Orozco

ACKNOWLEDGEMENTS

I am deeply grateful with Dr. Noel Solomons for his constant support and guidance. Without his intellectual input, bright ideas and immense patience this study would not have been possible. I would also like to thank Dr. James Friel for his valuable collaboration and academic guidance. To Dr. Klaus Schümann I would like to express my gratitude for enriching this research with his scientific experience and knowledge, and providing me with chemicals difficult to obtain in Guatemala.

In a special way I want to acknowledge Mrs. Ana Luisa de Montenegro from Universidad del Valle de Guatemala for patiently and kindly guiding me through the use of analytical equipment and laboratory techniques. Also to the authorities from Universidad del Valle de Guatemala for allowing me to work in their laboratories. To Raquel Campos I would like to express my appreciation for sharing her time and knowledge to help me with the dietary assessment and analysis presented in this work. I thank the Malaysian Palm Oil Board and the Hildegard Grunow Foundation for their financial support as well as the INF-EMF program for funding my tuition at the University of Manitoba.

Finally, I am grateful with my parents Max and Dyna and my husband Richard for their unconditional emotional support in times when things got difficult, and especially with my baby boy Rafael for brightening up my days and making this experience worthwhile.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

2,3 DHBA	2,3-dihydroxybenxoic acid
2,5 DHBA	2,5-dihydroxybenzoic acid
8-OHdG	8-hydroxy-2'-deoxyguanosine
AI	Adequate Intakes
CTCMC	Carotino® Tocotrienol Carotenoid Mixed Concentrate
DNA	Deoxyribonucleic acid
DRI	Dietary Reference Intakes
HPLC	High Performance Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MDA	Malondialdehyde
RDA	Recommended Dietary Allowances
ROS	Reactive Oxygen Species
RPO	Red Palm Oil
SD	Standard Deviation
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid-Reactive Substances

ABSTRACT

Iron is an essential nutrient and a potent dietary oxidant, capable of generating free radicals via Fenton chemistry that cause oxidative damage at molecular and cellular levels. Excessive amounts of free radicals increase systemic and *in situ* oxidative stress. The main objective of this study was to create an *in vivo* oral-iron-challenge response in healthy adults with 120 mg/day of supplemental iron for seven days to produce detectable alterations in two urinary and one fecal oxidation biomarkers: 8-OHdG (8-hydroxy-2'-deoxyguanosine) in urine, TBARS (Thiobarbituric Acid-Reactive Substances) in urine and the products of hydroxyl radical attack on salicylic acid in feces; as well as to determine if supplementation with antioxidants present in the Carotino® Tocotrienol Carotenoid Mixed Concentrate (CTCMC) will attenuate the effects of the oral-iron challenge. In total, a convenience sample of 17 Guatemalan, healthy male adults, aged 21 – 35 years, participated in the two phases of the study, five in the first phase (pilot study) and twelve in the second phase (intervention trial). Subjects underwent several cycles of supplementation, in which they received different treatments, separated by washout periods, They included, variously: iron alone (FE); iron mixed with refined palm oil (FEoil); and iron in palm oil combined with one of two concentrations of CTCMC (0.4 g and 0.8 g/ 5 ml refined palm oil) (CTCB and CTCA treatments, respectively). Subjects in the pilot study received FE and FEoil treatments, whereas the participants in the second phase took FEoil, CTCB and CTCA treatments. Urine and fecal sample were collected at different, assigned points throughout the pilot study and intervention trial. Urinary TBARS and 8-OHdG were assayed to evaluate

systemic oxidation, and the production of hydroxylated products in fecal matter was assessed to measure *in situ* colonic oxidative stress. In summary, the findings demonstrate that doses of 120 mg of supplemental iron alone or with palm oil did not alter the production of the urinary biomarkers 8-OHdG and TBARS above habitual levels; however, it did increase the generation of hydroxylated, reactive-oxygen-species products in the feces. The latter is provisionally attributed to a depleting of the intrinsic fecal antioxidant capacity state. CTCA and CTCB treatments suppressed the urinary excretion of both TBARS and 8-OHdG below usual levels. Both CTCMC doses induced different suppression responses in the urinary and fecal biomarkers of oxidation; these discrepancies prevent us from drawing conclusions about the dose-dependant action of the CTCMC. In conclusion, we observed in these individuals a significant protective effect of consuming the palm-oil derived CTCMC against iron-induced colonic oxidative stress, and secondarily on habitual systemic oxidation; these effects are presumably due to its content of mixed carotenoids and tocotrienol as dietary antioxidants.

1.0 INTRODUCTION

Iron is an essential micronutrient, vital for human life. It plays an important role in oxygen transport and multiple enzymatic reactions; given its chemical properties, however, it is also involved in several *in vivo* oxidative reactions at molecular and cellular levels. Its deficiency is among the ten leading global risk factors in terms of attributable disease burden, and may lead to anemia. (McLean *et al*, 2007) Low-income populations are the most affected mainly because of their low intakes of meat, poultry and fish, large consumption of staples rich in inhibitors of iron absorption, and high prevalence of parasitic infections leading to blood losses.

According to the World Health Organization, in the year 2000, about 2 billion people suffered from anemia, of which approximately 30% is due to iron deficiency. Children below 5 years of age, pregnant women and women in reproductive age are the most affected groups (Department of Reproductive Health and Research (RHR), World Health Organization (WHO), 1998). In Guatemala, the statistics show prevalences of anemia as high as 39.1% for pregnant women and 35.4% for women in reproductive age (Pan American Health Organization (PAHO), 2005). In certain areas, it has been reported that 45% of women had serum ferritin levels less than or equal to 9 ng/ml (iron deficiency) (Franzetti *et al*, 1984).

Several strategies to combat iron deficiency have been shaped throughout the years with different degrees of success. The most commonly used approach to prevent and treat iron deficiency and iron deficiency anemia is oral supplementation with iron.

Therapeutic treatment consists of a daily dosage of up to 300 mg of iron for adults, whereas the prophylactic dosage for pregnant women consists of several options, beginning at 30 mg in developed populations to up to 60 to 120 mg/day in developing nations. (Stolzfus and Dreyfuss, 1999). In Guatemala, the governmental norm states that non-pregnant women between 15 and 19 years old (regardless of their iron status), should receive 60 mg of elemental iron in the form of ferrous sulphate weekly, whereas pregnant and post-partum women should receive 120 mg weekly. (Ministerio de Salud Pública, 2006)

Recent observations have suggested that these approaches may not be completely safe. It is well known that excess iron may cause intoxication or lead to conditions such as hemosiderosis (Food and Nutrition Board, 2001). However, several studies have shown that prophylactic doses of 120 mg/day of supplemental iron produce altered oxidative responses when administered on a daily basis (Casanueva and Viteri, 2003; Schümann *et al*, 2005), presumably due to the ability of iron to catalyze the production of reactive oxygen species. In the long term, enhanced oxidative stress poses a risk to human health and may lead to illnesses such as cancer, including colorectal cancer (Lund *et al*, 1999), cardiovascular disease (Poredos, 2006; Yao *et al*, 2005) and gastric injury (Nur *et al*, 2005). Studies showed that exposure to 120 mg of elemental iron per week caused *in vivo* oxidation (Schümann *et al*, 2005) and DNA damage (Walter *et al*, 2002).

This evidence suggests that the untargeted prophylactic supplementation programs used in Guatemala may not be an adequate approach, given the potential long-term detrimental effects of supplemental iron (Schümann *et al*, 2007) and the indiscriminate nature of the recommendation.

The main purpose of this dissertation was to create an *in vivo* oral-iron-challenge response in healthy male adults with 120 mg of supplemental iron to produce detectable alterations in the following free radical/oxidation urinary and fecal biomarkers: urinary 8-OHdG (8-hydroxy-2'-deoxyguanosine); urinary TBARS (Thiobarbituric Acid-Reactive Substances); and the products of hydroxyl radical attack on salicylic acid (2,5 dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid and catechol) in feces. It was also intended to determine if a combination of natural antioxidants from the Carotino Tocotrienol-Carotene Mixed Concentrate® (CTCMC) will help attenuate the effects of prophylactic doses of 120 mg iron on the oxidative status of these subjects and if this product acts in a dose-dependent manner. The CTCMC is a derivate from the native, red palm oil and contains large amounts of natural antioxidants of lipid (carotenoids, tocotrienols, and tocopherols) nature.

We hypothesized that supplementation with 120 mg of iron per day for seven days would cause enhanced oxidation (measured as changes in fecal and urinary markers of oxidative stress) in the study subjects and that the antioxidants present in the CTCMC would attenuate the oxidative effects of supplemental iron. We also wanted to test the hypothesis that the CTCMC acts in a dose-dependent manner to modify oxidation by iron.

The lipidic nature and thickness of the CTCMC required us to mix it with vegetable oil to facilitate its consumption and this might interfere with our observations. For this reason we considered necessary to compare the alterations produced by an oral-iron-challenge alone and an oral iron-in-oil-challenge in the oxidation biomarkers described above, in order to establish if the oil has a protective effect against oxidation that might be produced by iron supplementation. We hypothesize that the reduction in the iron-generated oxidative response is due to the antioxidant properties of the supplemented CTCMC and not to the added vegetable oil.

A second part of this research involved the elucidation of the sites in which oxidation occurs. Similar studies have failed to determine whether oxidation is a systemic process or if it takes place within the intestines. Mechanisms as to how large doses of oral iron affect the oxidative status of humans are still unclear. Research has focused on the properties of the supplemental carotenoids and tocopherols (Nur *et al*, 2005; Darley-Usmar and Starke-Reed, 2000; Upritchard *et al*, 2003), but little in known about the potential health benefits of the natural antioxidants found on the CTCMC. Moreover, intervention studies have failed to demonstrate a consistent effect of antioxidant supplementation on chronic disease. Scientific research with this product is scarce (Nagesundram *et al*, 2000) and there is a lack of human studies.

2.0 LITERATURE REVIEW

2.1 Oxidative stress

Normal metabolism generates potentially harmful species such as reactive oxygen, nitrogen and chlorine species (free radicals), as by-products that can also act as physiological mediators and signaling molecules (Vertuani *et al*, 2004). Despite their recent notoriety, free radicals and their derivates have important functions in normal metabolism; among them are the regulation of vascular tone and oxygen tension, signal transduction in several membrane receptors (mainly in lymphocytes) and maintenance of redox homeostasis (Droge, 2002).

There is a delicate balance between the beneficial and detrimental effects of reactive species; their generation should not exceed the capacity of the tissue to metabolize them (Acworth and Bailey, 1995). Usually, their levels are controlled by the antioxidant defense system, composed by antioxidants and pro-antioxidants. When reactive species are produced in amounts that exceed the body's antioxidant defense system, they produce oxidative stress (Acworth and Bailey, 1995).

2.1.1 Reactive oxygen species (ROS)

These molecules play a relevant role in biological systems since oxygen is a readily-available reactive element that participates in a vast range of metabolic processes (e.g, respiration, phagocytosis, and enzymatic processes (van der Logt *et al*, 2005; Ferrari and Torres, 2003). There are several types of reactive oxygen species: the superoxide anion ($O_2^{-\bullet}$) which is highly reactive and short-lived; hydrogen peroxide

(H_2O_2), nitric oxide ($NO^{\bullet}$) and the most reactive one, the hydroxyl free radical ($HO^{\bullet}$) (Acworth and Bailey, 1995).

The superoxide anion ($O_2^{-\bullet}$) is commonly formed by the enzyme-mediated reduction of triplet-state molecular oxygen ($^3O_2$). The enzymes participating in this process can be NAD(P)H oxidases or xanthine oxidase (Droge, 2002). The enzyme Cu-Zn superoxide dismutase (SOD) converts superoxide into hydrogen peroxide (H_2O_2). On one hand, certain reduced transition metals (iron or copper) can convert hydrogen peroxide into the highly reactive hydroxyl radical ($HO^{\bullet}$). On the other hand, the enzymes catalase and glutathione peroxidase can convert hydrogen peroxide into water. (Droge, 2002)

Exogenous sources such as high-impact energy (thermal, microwave, radioactive), metals (cadmium, copper, iron, mercury, zinc, etc.) and toxic agents (alcohol, pesticides, cigarettes, air pollutants, etc.) (van der Logt *et al*, 2005; Ferrari and Torres, 2003), can also increase the production of ROS. Psychological stress (Nur *et al*, 2005), infectious diseases (hepatitis C (Marotta *et al*, 2007), dengue fever (Klassen *et al*, 2004)), obesity, diabetes and aging have also been linked to the generation of reactive oxygen species (Darley-Usmar and Starke-Reed, 2000). During episodes of infection, oxidative stress is used by the host´s cells as a defense mechanism against microorganisms (Klassen *et al*, 2004).

The role of free radicals in aging has been a well-established theory that has now been extended to include both reactive oxygen and nitrogen species. The original

concept that the overwhelming oxidative stress depleted antioxidants and, thus, damaged intracellular components is being modified as reactive oxygen species are now recognized to play an essential role in signal transduction (Darley-Usmar and Starke-Reed, 2000).

2.1.2 Mechanisms of Oxygen Damage

Lipids, proteins and DNA are vital components of the cell. They are all vulnerable to oxidative damage; especially if the body's natural defenses are compromised (Acworth and Bailey, 1995). Excessive amounts of free radicals may promote lipid peroxidation, mutagenesis, DNA strand breaks, oncogene activation, and tumor suppressor gene inhibition (Kabat and Rohan, 2007).

2.1.2.1 Lipid oxidation

Lipids include a wide variety of different compounds such as fatty acids, phospholipids, waxes, steroids and terpene-based compounds. Polyunsaturated fatty acids make up the cell membrane's lipid bilayer; consequently, they regulate the membrane fluidity. When they are attacked by hydroxyl free radicals, they generate lipid peroxides and oxidized fatty acids (Acworth and Bailey, 1995; Huycke and Gaskins, 2004); this process is called lipid peroxidation (Gutteridge, 1995). As a consequence, polyunsaturated fatty acids become more hydrophilic and this alters the membrane structure, disturbing the normal membrane function (Acworth and Bailey, 1995). This process is initiated by ROS generated during peroxide formation from methylated fatty acids. Lipid peroxidation is a chain reaction that produces a

continuous supply of free radicals that perpetuate further peroxidation (Mayes, 1994). Breakdown products of this process include electrophilic aldehydes such as malondialdehyde (MDA), 4-hydroxy-2-nonenal, and 4-oxo-2-nonenal, all of which can also form mutagenic DNA adducts (Huycke and Gaskins, 2004).

Lipid peroxidation is commonly used as a gauge to measure the participation of free radicals in toxicology and disease (Gutteridge, 1995). This chain reaction process involves three stages: initiation; propagation; and termination (Gutteridge, 1995; Mayes, 1994). During initiation, the free radical removes a hydrogen atom from the lipid molecule, generating a highly reactive peroxyl radical (ROO·) which is capable of removing another hydrogen atom from another fatty acid, causing a chain reaction (Gutteridge, 1995; Mayes, 1994). Peroxidation continues (propagation) until all the substrate is used or a chain-breaking molecule such as an antioxidant takes action to finish the process (termination) (Gutteridge, 1995).

Iron or iron complexes found *in vivo,* which are capable of sustaining Fenton chemistry can be lipid peroxidation promoters. Iron (II) can react with lipid peroxides to give alkoxyl radicals (RO·), whereas iron (III) produces peroxyl and alkoxyl radicals. Both species stimulate lipid peroxidation by removing hydrogen atoms from the free fatty acids found in lipid membranes (Gutteridge, 1995).

Lipid peroxidation can cause cancer, inflammation, atherosclerosis, and aging, among others. (Mayes, 1994). Arachidonic acid is the precursor of prostaglandins, thromboxanes and leukotrienes, which act as hormones affecting blood flow, ion

transport and synaptic transmission (Acworth and Bailey, 1995). Excessive peroxidation of arachidonic acid may interfere with the synthesis of these hormones (Acworth and Bailey, 1995). Another negative effect of the peroxidation of fatty acids is that the free-radical metabolites inhibit certain enzymes that disrupt the processes within the membrane and within the cell (Acworth and Bailey, 1995).

A commonly used assay for screening and monitoring lipid peroxidation is the quantification of thiobarbituric acid (TBA) (Simic *et al*, 1992). TBA reacts with malondialdehyde (MDA) to form a 1:2 adduct (see Figure 1) that can be measured by fluorometry or spectrophotometry (Badcock *et al*, 1997). Biological specimens contain thiobarbituric acid reactive substances (TBARS) that include lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress (OXItek, 2008).

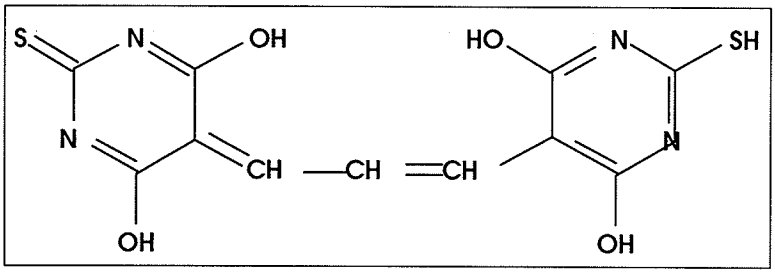

Figure 1. Malondialdehyde and TBA adduct.

2.1.2.2 Protein oxidation

Protein oxidation enhances protein catabolism and clearance, which may have negative effects on normal functioning of the organism. There are several forms of protein oxidation, e.g. the formation of protein carbonyls, attack on certain amino acids

and oxidation of sulfhydryl groups (Acworth and Bailey, 1995). It has been established that hydroxyl radical attack generates very stable hydroxylated derivatives of the amino acids, leucine and valine, and the aromatic amino acids phenylalanine and tyrosine (*o*-tyrosine and *m*-tyrosine) (Fu *et al*, 1998). Protein hydroxides resulting from the reduction of protein hydroperoxides by free radicals can produce further radicals that might react with other biological molecules, and propagate oxidative damage (Fu *et al*, 1998).

2.1.2.3 DNA oxidation

DNA contains the genetic code of all living organisms. Free radical attacks on DNA may cause oxidation and chemical modification of the nitrogenated bases, which can lead to point mutations (Acworth and Bailey, 1995). They may also produce single- and double-stranded DNA breaks and deletions (Chung *et al*, 2007). These mutations if transcribed and translated will lead to modified protein structure, which can cause altered functionality and disease (Acworth and Bailey, 1995). Several ROS are capable of producing DNA damage, but hydrogen peroxide is the only one that is stable enough to diffuse into cells and generate hydroxyl radicals in the presence of transition metals (Huycke and Gaskins, 2004). It has also been observed that ROS may lead to alterations on nuclear and cytoplasmic signal transduction pathways (van der Logt *et al*, 2005). It has been established that an imbalance between oxidative DNA damage and repair is largely responsible for the development of cancer (Yeum *et al*, 2007).

8-hydroxy-2'deoxyguanosine (8-OHdG) is one of the most commonly used markers to assess oxidative DNA damage given its sensitivity to detect very low levels of oxidative damage (Chung *et al*, 2007). This molecule is the oxidized form of the nucleoside 2'-deoxyguanosine present in DNA (Chung *et al*, 2007) (See Figure 2). Oxidation in the C-8 of guanine results in a mutation that produces a G-to-T transversion that may lead to mutagenesis (Wong *et al*, 2005). This method measures this DNA oxidative product in human DNA samples and urine (Mayne, 2003) resulting from the repair process in which excised 8-OHdG adduct is excreted into the urine (Wong *et al*, 2005). 8-OHdG has been found to be elevated in smokers, cancer or chronic renal failure patients (Chung et al, 2007). Two Japanese studies by Tsukuhara and colleagues demonstrated that patients with diabetes and atopic dermatitis also have significantly higher levels of DNA oxidation (see Table 1).

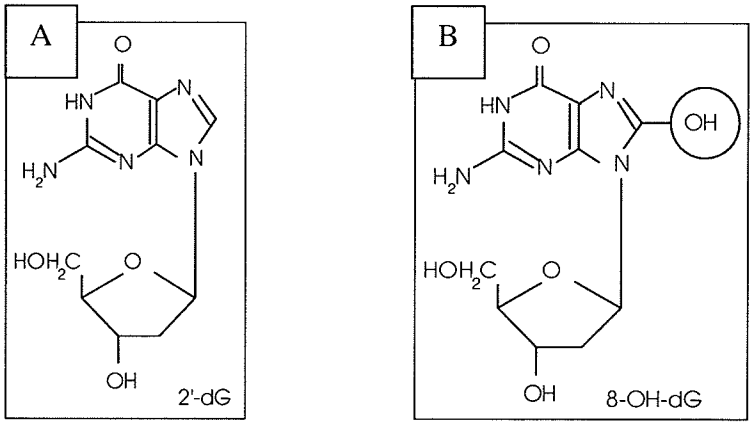

Figure 2. A 2'deoxyguanosine molecule (A) and the DNA adduct 8-hydroxy-2'deoxyguanosine (8-OHdG) (B).

Table 1. Reported values of 8-hydroxy 2'deoxyguanosine (8-OHdG) in the urine of healthy and diseased subjects

Reference	Country	Subjects	Value	p-value
Tsukuhara, *et al*, 2003 a	Japan	60 healthy subjects aged 4.2–21 y	11.9 ± 5.2 ng/mg Cr	p<0.005
		38 patients with diabetes Type 1 aged 3.8 – 21.3 y	15.4 ± 6.7 ng/mg Cr	
Tsukuhara, *et al*, 2003 b	Japan	28 healthy children aged 1.5–10 y	17.0 ± 4.9 ng/mg Cr	p<0.05
		13 patients with acute exacerbation of atopic dermatitis aged 1.5 – 10 y	31.9 ± 18.9 ng/mg Cr	

2.2 Protection against oxidants

The antioxidant defenses control levels of reactive species by working as a coordinated system. Hence, deficiencies in one component may affect the efficiency of the others. In this network, some of the components act as direct antioxidants whereas others act indirectly either by modulation of direct agents or by regulation of the biosynthesis of antioxidant proteins (Vertuani *et al*, 2004). Several components of this system are micronutrients (e.g. vitamins C and E), dependent upon dietary micronutrients (e.g. Cu- Zn and Mn superoxide dismutase), or produced by specific endogenous pathways (Vertuani *et al*, 2004). There are at least three major mechanisms to control the normal oxygen tension and keep homeostasis.

2.2.1 Enzymatic protection

Superoxide dismutase (SOD), present in the cytosol and mitochondria, is responsible for decreasing superoxide levels to $<10^{-11}$ M. Catalase is an enzyme found in peroxisomes and it acts breaking down hydrogen peroxide to oxygen and water.

Glutathione peroxidase is found in the mitochondria and cytosol and reduces organic hydroperoxides and hydrogen peroxide into water and a glutathione derived product (Acworth and Bailey, 1995). Patients with type 1 diabetes and nephrotic syndrome have reduced levels of glutathione peroxidase (Varvarovska *et al*, 2003; Kamireddy et al, 2002) (See Table 2), indicative of impaired antioxidant protection (Varvarovska *et al*, 2003) In the case of nephrotic syndrome, these patients also showed an almost a four-fold increase in SOD levels (see Table 3). It can be noted that the reported values for healthy children vary greatly between the published reports. A study by Siemianowicz and colleagues in 2003 demonstrated an interesting association between family history of CVD and levels of GPx. In their experience, GPx levels were reduced in a group of children with a history of cardiovascular disease (see Table 2); this observation was important given the fact that dimished serum antioxidant enzymes can precipitate the progression of atheroesclerosis.

Table 2. Reported values of glutathione peroxidase (GPx) in healthy and non-healthy subjects

Reference	Country	Subjects	Value	p-value
Ozbay, et.al, 2002	Turkey	58 healthy children, 9-14 y	64.7 ± 4.4 U/g Hb	-
Kamireddy, *et al*, 2002	India	10 healthy children, 6-13 y	12.0 mg/g Hb	P<0.01
		15 children suffering nephrotic syndrome, aged 6-13 y	5.81 mg/g Hb	
Varvarovska, *et al*, 2003	Czech Republic	30 healthy children 2 – 19 y	58.4 ± 14.2 U/g Hb	p<0.05
		50 subjects with Type 1 diabetes, 2.5 – 19.5 y	58.1 ± 13.1 U/g Hb	
Siemianowicz, *et al*, 2003	Poland	18 healthy children without a family history of CVD* 6 –15 y	37 U/g Hb	P<0.00001
		22 healthy children with family history of CVD, aged 7 – 15 y	28 U/g Hb	

*CVD = Cardiovascular Disease

Table 3. Reported values of Superoxide Dismutase (SOD) in healthy and diseased subjects

Reference	Country	Subjects	Value	p-value
Ozbay, *et al*, 2002	Turkey	58 healthy children, 9-14 years old	1631 ± 551 U/g Hb	-
Kamireddy, *et al*, 2002	India	10 healthy children 6-13 years old	7729.5 U/g Hb	p<0.001
		15 children suffering nephrotic syndrome, aged 6-13 years	27,968.2 U/g Hb	
Siemianowicz, *et al*, 2003	Poland	18 healthy children without a family history of CVD* 6 –15 years	820 U/g Hb	Not significant
		22 healthy children with family history of CVD, aged 7 – 15 years	750 U/g Hb	

*CVD = Cardiovascular Disease

2.2.2 Antioxidants

Antioxidants are naturally-occurring compounds that have the ability to react with free radicals and neutralize them (Acworth and Bailey, 1995). Epidemiological evidence indicates that high plasma concentrations of exogenous antioxidants are associated with lower risk of cardiovascular disease and certain types of cancer (Winklhofer-Roob *et al*, 2003). In supplementation studies in humans, alpha-tocopherol decreases lipid peroxidation (low-density lipoprotein [LDL] oxidation and F2-isoprostanes) and platelet aggregation and adhesion as well as being anti-inflammatory (Jialal and Devaraj, 2003). Current research shows that either carotenoid-rich fruits or vegetables, a combination of antioxidants or a combination of carotenoids are more

efficient in protecting against DNA damage than single antioxidant supplementation (Yeum, *et al*, 2007).

2.2.2.1 Carotenoids

Carotenoids consist of a large family of plant pigment molecules, which include the carotenes and xanthophylls (Wildman, 2001). These liposoluble pigments are responsible for the yellow and orange colors of some fruits and vegetables. Their nutraceutical role in humans is related to molecular protection against free radical attack, since they appear to quench reactive oxygen species that would be harmful to biological membranes and other molecules (Wildman, 2001). When the partial pressure of oxygen is low, beta-carotenes stabilize free organic peroxides within their structure of conjugated double bonds (Mayes, 1994). They provide protection against cancer (Nelson *et al*, 2003) and may decrease the incidence of some degenerative diseases (Tyssandier *et al*, 2003). It has also been observed that they offer antioxidant protection by scavenging DNA-damaging free radicals and modulating DNA repair mechanisms (Astley *et al*, 2004). Since beta-carotene is efficient at low concentrations, it complements vitamin E's antioxidant properties (Mayes, 1994).

Several epidemiological studies indicate that carotenoid-rich diets are associated with lower risk of contracting certain types of cancer, heart disease and other important diseases (Minguez-Mosquera *et al*, 2002). A randomized crossover study by Kiokias and Gordon (2003), in which 32 healthy non-smoking subjects consumed a natural carotenoid mixture consisting of α- and β-carotenes, lycopene, bixin, lutein and paprika

carotenoids for 3 weeks, demonstrated that the consumption of this mixture lowered the increase in oxidative stress.

There are various factors that affect the effectiveness of the antioxidant action. Among these are the presence of oxygenated functional groups in the structure of the pigment, the conditions of the medium where the pigment acts and the nature of the prooxidant substance (Minguez-Mosquera *et al*, 2002). These factors may cause a self oxidazing effect in place of the expected antioxidant beneficial one (Minguez-Mosquera *et al*, 2002).

As far as it can be ascertained, the carotenoids are not cyto- or genotoxic in either large acute doses or in chronic supplementation. Bioconversion to retinol is regulated so there is no danger of vitamin A toxicity (Benade, 2003; Wildman, 2001). One of the most commonly encountered effects in individuals consuming large quantities of carrot or carrot products is carotenodermia or yellowing of the skin. This condition is harmless, can be rapidly reversed by ceasing the intake (Wildman, 2001).

2.2.2.2 Vitamin E

Vitamin E is present in plants in 8 different forms: four tocopherols and four tocotrienols (termed α-, β-, γ- and δ-), with similar antioxidant potential (Schwedhelm *et al*, 2003; Zingg and Azzi, 2004). Tocopherols have a saturated hydrocarbon side chain while tocotrienols possess three unsaturated carbon chains (Nur *et al*, 2005). Tocopherols are present in polyunsaturated vegetable oils and in the germ of cereal

seeds, whereas tocotrienols are found in the aleurone and subaleurone layers of cereal seeds and in palm oils. (Yoshida *et al*, 2003)

In higher organisms, only α-tocopherol is preferentially retained; this suggests a specific mechanism for the uptake for this analogue (Zingg and Azzi, 2004). These compounds are highly active free radical scavengers that protect unsaturated membrane lipids from oxidation (Acworth and Bailey, 1995). It seems to be that its major function is as a non-specific chain-breaking antioxidant (Food and Nutrition Board, 2000). During recent years new roles for vitamin E have been determined. At the post-translational level, α-tocopherol inhibits protein kinase C, 5-lipoxygenase and phospholipase A2 and activates protein phosphatase 2A and diacylglycerol kinase (Zingg and Azzi, 2004). Some genes (e. g. scavenger receptors, α-TTP, α-tropomyosin, matrix metalloproteinase-19 and collagenase) are modulated by α-tocopherol at the transcriptional level. Alpha-tocopherol also inhibits cell proliferation, platelet aggregation and monocyte adhesion. (Zingg and Azzi, 2004)

Although α- and β-tocopherol are considered to be the more biologically active forms of vitamin E, recent evidence suggests that the tocotrienols may be more potent than the tocopherols in preventing both cardiovascular disease and cancer (Lee *et al*, 2003). It has been observed that tocotrienol supplementation beneficially influences the course of carotid atherosclerosis in human (Khanna *et al*, 2003). Dietary tocotrienols incorporate into circulating human lipoproteins, where they react with peroxyl radicals as efficiently as the corresponding tocopherol isomers. Yoshida and his group (2003)

compared the antioxidant activity of tocopherols and tocotrienols and found that the corresponding tocotrienols exerted the same reactivities toward radicals and the same antioxidant activities against lipid peroxidation *in vitro*, but tocotrienols were more readily transferred between membranes and incorporated into them than tocopherols. Also Nur and collaborators (2005) demonstrated that both tocotrienols and tocopherols significantly protected the gastric mucosa of stressed rats against free radical damage, but only tocotrienols were efficient in normalizing the alterations of gastric acidity and gastrin levels caused by increased oxidative stress.

In general terms, vitamin E can modulate some of the negative effects that ROS have on normal physiologic function. It has been demonstrated that high doses of vitamin E increase the resistance of LDL to oxidation, enhance antioxidant capacity, and reduce F2-isoprostane concentrations in persons under increased oxidative stress due to disease (Upritchard *et al*, 2003).

Upritchard and colleagues (2003) demonstrated that the consumption of food products with moderate amounts of vitamin E and carotenoids significantly improved the antioxidant status and biomarkers of oxidative stress in healthy individuals. They assessed the effect of consuming 25 g per day of two spreads containing 43 and 111 mg α-tocopherol, and 0.45 and 1.24 mg caroteonoids respectively, and found an antioxidant dose-dependent effect in the reduction of the concentrations of F(2α)-isoprostane (a biomarker for assessing plasma lipid peroxidation). *In vivo* studies with rats also

provided strong evidence about the benefits of supplemental tocopherols E and their ability to inhibit colon cancer cell growth (Stone *et al*, 2004).

It was previously reported that achieving increasing concentrations of tocopherols and carotenoids in plasma through diet is difficult, because it requires a substantial increase in the consumption of vegetable oils, fruits and vegetables (Upritchard *et al*, 2003). Therefore, a fortified food with tocopherols and carotenoids could provide a safe and effective way to achieve a desirable antioxidant status. There is no evidence of adverse effects from the consumption of vitamin E naturally occurring in foods (Food and Nutrition Board, 2000).

2.3 Iron

Iron is an essential nutrient that serves multiple tasks such as oxygen transport and as a co-factor of iron-dependent metalloenzymes (Schümann *et al*, 2005). It is also involved in the normal functioning of our natural resistance to infection by regulating phagocytic cells and tissue fluids (Bullen *et al*, 2006). In order to accomplish the latter, iron should exist in very low concentrations of its free ionic form. This is achieved by the unsaturated iron-binding proteins transferrin and lactoferrin which are dependent of the tissue's pH and oxygenation levels (Bullen *et al*, 2006).

2.3.1 Iron metabolism

On average, total body iron ranges from 2.5 g in women to 4.0 g in men (Yip, 2001). It can be found in two forms:

– Functional iron involved in metabolic and enzymatic processes. This pool makes up to 2/3 of total body iron in men, and is found mainly in form of hemoglobin.

– Storage iron serving in storage and transport. In iron-adequate men, approximately 1/3 of total body iron is found in form of iron stores, compared to women whose iron stores comprise only 1/8 of the total body iron (Yip, 2001).

Iron balance and metabolism are regulated by intake, stores and loss. The mucosal intestinal surface is the main regulatory system for iron absorption, but at the same time, iron intake is also dependent of the quantity and bioavailability of dietary iron. The type of food and the interaction between iron and the uptake mechanisms at the intestinal mucosa determine the organism's physiological iron needs. The absorption of dietary iron may vary from less than 1% to more than 50%. (Yip, 2001)

2.3.2 Iron absorption and regulation

Iron absorption is dependent of the amount of iron consumed, its bioavailability, as well as on the iron stores and the rate of erythrocyte production of the organism (Yip, 2001). Mammals do not possess mechanisms for eliminating excess iron; the only way of doing so is through blood loss or pregnancy (Kabat and Rohan, 2007). Because of this, iron stores increase with age (Kabat and Rohan, 2007). In women, iron levels are stable during reproductive years, increase during menopause, and reach a plateau at about age 60 (Kabat and Rohan, 2007).

Under normal conditions, with replete iron stores, it is important to maintain adequate levels of circulating iron and stores and to prevent the entry of additional iron

to protect the organism from iron overload (Wish, 2006). When non-heme dietary Fe^{3+} reaches the duodenum and proximal jejunum, it is reduced to Fe^{2+} by the cytochrome b-like ferrireductase (Dcytb) and then crosses the lumen through the divalent metal transporter-1 (DMT1) (Horl, 2007). Iron concentration strongly affects the expression of both Dcytb and DMT1. Hypoxia, anemia, and iron deficiency up-regulate intestinal iron absorption, while iron overload or inflammation/infection inhibits it (Horl, 2007). After it has been absorbed, it is transported to the liver for storage. When needed, it is converted into functional forms such as heme and the iron-sulfur cluster; this change is accompanied with a transition from ferrous to ferric ions. (Taketani, 2005)

Due to iron's toxicity, organisms have developed a complex regulatory system that consists of a series of transport and storage proteins that control circulating iron levels (Kabat and Rohan 2007). Most of the body's iron is bound to proteins that prevent it from causing oxidative damage (Kabat and Rohan, 2007). Ferritin is an iron storage protein involved in the regulation of iron availability (Kabat and Rohan, 2007; Koorts and Viljoen, 2007). This acute-phase protein has a cage-like structure capable of hosting up to 4,500 iron atoms (Kabat and Rohan, 2007) and is up-regulated in conditions such as uncontrolled cellular proliferation, oxidative stress, and by infectious and inflammatory processes (Koorts and Viljoen, 2007). The intracellular iron content is regulated by cellular uptake and binding of low molecular labile iron by ferritin (Taketani, 2005).

Another important iron-related protein is transferrin, which is responsible for transporting iron in the blood stream and distributing it to the cells (Kabat and Rohan, 2007). Transferrin recognizes transferrin receptors at the cell's membrane, binds to them and enters the cell *via* endocytosis (Taketani, 2005). The increased production of reactive oxygen species and cytokines stimulates the synthesis of ferritin causing a decline on the bioavailability of iron, which results in the control of cellular proliferation and a decrease in the production of reactive oxygen species (Koorts and Viljoen, 2007). Other factors such as infectious or inflammation processes can also disturb iron homeostasis (Kabat and Rohan, 2007).

The peptide hepcidin is produced by the liver and controls iron homeostasis by mediating iron absorption and mobilization (Wish, 2006). It inhibits the release of iron by the iron exporter ferroportin located on the intestinal enterocytes and also in the intracellular vesicular compartment of tissue macrophages (Horl, 2007). Hepcidin is primarily produced in response to acute-phase reactions, and to a lesser extent it is synthesized depending on the degree of hepatic iron storage (Horl, 2007; Wish, 2006). If iron stores are high, hepcidin production increases, inhibiting iron uptake at the gastrointestinal tract and the placenta of pregnant women (Wish, 2006).

2.3.3 Iron deficiency and supplementation strategies

Iron deficiency has been defined as an "iron supply not sufficient to meet the need for functional iron after storage iron has been depleted" (Yip, 2001). However, at a cellular level, iron deficiency can result with an insufficient release of stored iron, even

if there is an adequate intake and stores (Yip, 2001). Estimates based on World Health Organization (WHO) criteria indicated that around 600–700 million people worldwide have marked iron deficiency anemia (IDA), and the majority of these people live in developing countries (World Health Organization, 2004). In developed countries, the prevalence of iron deficiency anemia is much lower and usually varies between 2% and 8% (World Health Organization, 2004). In women, IDA can cause impaired immune response, reproductive failure, risk of premature delivery, poor pregnancy outcomes and maternal death during childbirth (Galloway and McGuire, 1994; Walter *et al*, 2002; Huma *et al*, 2007), whereas young children are at risk of impaired cognitive development (Walter *et al*, 2002), growth retardation and a reduction in physical activity (Huma *et al*, 2007).

Food fortification, supplementation, and dietary diversification are the most common strategies used to combat iron deficiency around the world (Clift, 1997); disease control measures are important as well (Huma *et al*, 2007). Iron supplementation campaigns in developing countries target high-risk groups such as infants, pre-school children and pregnant and post-partum women. Strategies developed to address this problem are diverse and the recommendations for iron supplementation vary according to the entity that designs them. The Department of Reproductive Health and Research (RHR) from the World Health Organization (WHO) (1998) suggests supplementation strategies in areas of high prevalence of iron deficiency anemia with 400 mg ferrous sulphate (150 mg of elemental iron) per day or once a week plus 250 μg folate for 4 months for pregnant and lactating women. In areas

of low prevalence 1 tablet of ferrous sulphate daily may be sufficient, but in these areas another approach is to give iron only if anemia is diagnosed or suspected. (RHR, WHO, 2005). Some programs use capsules with 60 mg of iron and 25 mg of folic acid twice a day for pregnant and post-partum women (Global Micronutrient Project, 2001). The International Nutritional Anemia Consultative Group's (INACG) recommended therapeutic treatment consists of a daily dosage of up to 300 mg of iron for adults, whereas the prophylactic dosage for pregnant women consists of several doses of 30 mg in prosperous populations to doses of 30 to 120 mg/day in developing nations (Stolzfus and Dreyfuss, 1999).

Oral supplemental iron is absorbed better if it is taken without food. The most common side effects of oral iron therapy include constipation, diarrhea, nausea, and abdominal pain (Food and Nutrition Board, 2001; Horl, 2007). People with iron deficiency have enhanced intestinal iron absorption, which declines when normal iron levels are achieved (Horl, 2007).

2.3.4 Iron toxicity

Free available iron is usually derived from iron overload, free heme compounds, or hypoxia in injured tissue (Bullen *et al*, 2006). It can exist in two oxidation states in an aqueous solution: as Fe^{2+} (ferrous form) or as Fe^{3+} (ferric form). The relative ease in which these changes occur allows iron to catalyze a series of redox reactions, mainly processes related to oxygen and energy metabolism (Yip, 2001). On the other hand, this ability can be potentially harmful given the oxidative nature of iron (Yip, 2001).

Ferrous ions can react with hydrogen peroxide formed during the respiration process at the mitochondria producing hydroxyl radicals that can damage cell lipids, proteins and DNA (Taketani, 2005). For this reason, living organisms have a complex homeostatic system of carrier and transport proteins as well as antioxidant molecules that ensures an adequate iron pool and controls the possible damage of redox reactions to cellular fatty acids, proteins and nucleic acids (Yip, 2001; Cairo and Pietrangelo, 2000). The production of such proteins (transport and enzymes) is regulated by the expression of several genes that contain iron-responsive sequences (Cairo and Pietrangelo, 2000).

Iron and its binding proteins are related to immune regulation, affecting it negatively with deficient or excessive iron concentrations (Kabat and Rohan, 2007). Under conditions of iron overload, the distribution of T-lymphocytes is affected by suppressing the action of helper T cells (CD4). Macrophages and monocytes anti-tumor functions are also restrained, weakening immune mechanisms for cancer control (Kabat and Rohan, 2007).

Most of the *in vivo* oxidative processes at the cellular level are mediated by iron through Fenton-type chemical reactions (Schümann *et al*, 2005):

(1) $Fe^{3+} + O_2 \rightarrow Fe^{2+} + O_2\cdot$

(2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OH\cdot + OH^-$

The free radicals formed in these reactions cause peroxidation or cross-linking of membrane lipids and intracellular compounds, which can cause cell aging and apoptosis (Yip, 2001). Under normal conditions, the organism antioxidant system has the ability

to neutralize the reactive oxygen species generated by such processes (Acworth and Bailey, 1995). But the regulation of iron availability is the main mechanism in which the cell keeps ROS levels under control since an adequate chelation of iron still allows the relatively safe and necessary occurrence of redox reactions without an excessive production of ROS (Cairo and Pietrangelo, 2000).

Recent evidence has supported the hypothesis that excess amounts of iron have detrimental effects on human health (Lund *et al*, 1999; Schümann *et al*, 2005) and have been associated with several pathologies such as cancer, inflammation, diabetes, liver and heart disease (Fisher and Naughton, 2004). It has also been pointed out that high concentrations of iron may be implicated in the growing incidence of colon cancer in developed countries (Lund *et al*, 1998) due to cereal fortification, widespread use of iron-containing supplements and high intakes of red meats (Kabat and Rohan, 2007).

Results from several studies in humans and rats (Lund *et al* 1998, Lund *et al*, 2001) and the National Health and Nutrition Examination Study (NHANES) revealed a significant association among high dietary iron, high body iron stores, and colon cancer (Lund *et al*, 2001). Possible mechanisms to explain these observations are that reactive oxygen species generated by residual iron increase lipid peroxidation in intestinal mucosa and DNA damage in colonocytes (Lund *et al*, 2001). Supportive evidence includes studies in rats showing that administration of high-iron supplements result in increased accumulation of intestinal and hepatic non-heme iron, increased lipid peroxidation, mitochondrial malfunction (Walter *et al*, 2002), and the augmentation of

tumor incidence (Nelson *et al*, 1989). It has been suggested that the residual iron present in the small intestine may enter the colon and participate, together with intraluminal bacteria, in Fenton-type reactions, increasing the production of hydrogen peroxide and hydroxyl radicals at the mucosal surface. Here it can cause DNA damage, which may lead to tumor promotion (Lund *et al*, 1998; 2001). Commensal bacteria in the colon may also be involved in the generation of reactive oxygen species that may damage epithelial cell DNA, increasing the burden of oxidative stress in the colon (Huycke and Gaskins, 2004). Furthermore, cancer cells have high iron requirements and increased levels of transferrin receptors (Kabat and Rohan, 2007) suggesting that iron may also support cancer growth.

Evidence also indicates that excessive intakes and storage of iron affects negatively the physiological function and risk of chronic disease, as well as favors the virulence of pathogens (Schümann *et al*, 2005).

2.3.5 Interactions with other substances

Alcohol consumption has been associated with several types of cancer. It has been demonstrated that alcohol may amplify iron's detrimental effects by increasing blood ferritin levels and other markers of iron overload, triggering the release of free iron from ferritin stores (Kabat and Rohan, 2007). It has been proposed that acetaldehyde produced from ethanol metabolism generates hydrogen peroxide and superoxide anions that participate with Fe (II) in Fenton-type reactions (Kabat and Rohan, 2007).

The benefits of vitamin C as an antioxidant have been widely confirmed (Fisher and Naughton, 2004). Often, iron supplements are taken in conjunction with vitamin C in order to increase shelf-life of the preparation and to aid absorption, but in high doses, vitamin C can act as a pro-oxidant rather than an antioxidant (Fisher and Naughton, 2004). Ascorbate has the ability to reduce Fe^{3+} to Fe^{2+}, promoting the formation of hydroxyl radicals *in vitro* if H_2O_2 is available (Proteggente *et al*, 2000). It has been observed that uncontrolled interaction between iron salts and vitamin C leads to oxidative stress (Fisher and Naughton, 2004).

2.4 Red Palm Oil (RPO) and Carotino Tocotrienol-Carotene Mixed Concentrate® (CTCMC)

Palm oil is obtained from the mesocarp of the tropical plant *Elaeis guineensis* (Nagesundram *et al*, 2000). In its natural unprocessed form it has a deep red color provided by the liposoluble carotenes, tocopherols and tocotrienols present in it (Kritchevsky, 2000); it also contains free fatty acids, trace metals, impurities and other compounds that affect its shelf stability and palatability (Nagesundram *et al*, 2000).

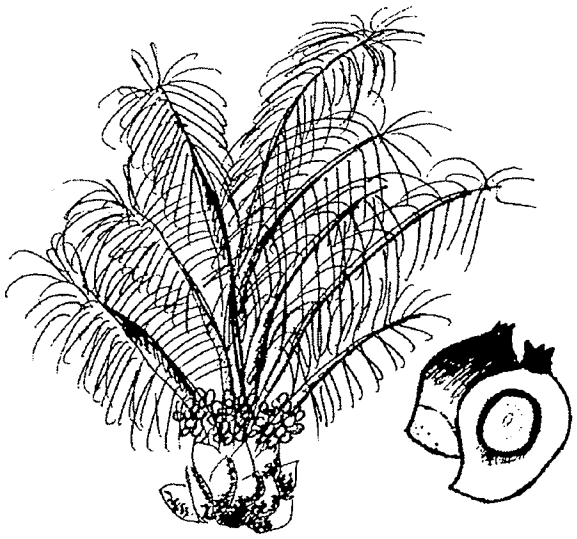

Figure 3: Palm tree (*Elaeis guineensis*) and palm fruit

Palm oil has been historically used as a food for over 5000 years (Scrimshaw, 2000). Worldwide, it is used in the form of oil, margarine and shortenings for cooking and it is also blended with other oils to produce a wide variety of products (Scrimshaw, 2000).

2.4.1. Red Palm Oil

The Palm Oil Research Institute of Malaysia (PORIM) patented a refining short-path distillation method that eliminates the undesirable compounds that alter its stability and flavor while preserving most of the palm oil's carotenoid and vitamin E content (Bayorh *et al*, 2005). As a result, RPO constitutes a rich natural source of carotenoids (500 – 700 ppm). Approximately 50% comprises β-carotene together with large amounts of vitamin E (700 ppm) in the form of tocopherols (70%) and tocotrienols (30%) (Boateng *et al*, 2006), that confer it powerful antioxidant properties. Previous

studies demonstrated that red palm oil inhibited endogenous lipid peroxidation (Esterhuyse, 2005), improved blood pressure and arterial thrombosis, (Bayorh *et al*, 2005) and was associated with a reduction in oxidative stress in Dahl salt-sensitive rats by attenuating superoxide production (Baryorh *et al*, 2005).

RPO contains a mixture of saturated fatty acids (48%), monounsaturated fatty acids (42%) and polyunsaturated fatty acids (10%) (Esterhuyse *et al*, 2005). Despite the fact that it contains a high percentage of saturated fatty acids, several clinical trials have shown that palm oil does not raise serum total cholesterol and LDL cholesterol as expected (Esterhuyse *et al*, 2005; Kruger *et al*, 2007); instead, it has a protective effect by increasing HDL-cholesterol (Zeba *et al*, 2006). One possible explanation is that palm oil does not contain myristic acid, which is associated with blood lipid increase (Bayorh *et al*, 2005). Other studies have suggested that the monounsaturations with oleic acid on the triacylglycerol's sn2-position are greatly responsible for the palm oil's beneficial effects (Bayorh *et al*, 2005).

In several studies, carotenoids have been supplemented successfully in order to improve vitamin A status (Mahapatra and Manorama, 1997; van Stuijvenberg *et al*, 2001; Zagre *et al*, 2003, Benade, 2003; Zeba *et al*, 2007), and anti-oxidative status (Nelson *et al*, 2003). Red palm oil has the advantages of being a highly bioconvertible form of alpha- and beta-carotene, having a long shelf life and a higher cost/benefit ratio when compared to other approaches such as high-dose-vitamin A supplements and fortification of foods with retinyl ester fortificants (Benade, 2003). The main advantage of supplementing with bioconvertible carotenoids to achieve an improvement in the

vitamin A status is that there is no risk of toxicity, since the organism regulates the bioconversion to retinol. Provitamin A carotenoids of RPO are highly bioavailable because they are found in an oily base and lack a plant matrix that may interfere with its absorption (Zeba *et al*, 2006) Chronic dosing with supplements or foods needs to be done until the plasma concentration reaches a plateau. This normally takes several weeks when supplementing with modest amounts (15 mg/day) and may increase β-carotene plasma concentrations up to tenfold (Faulks and Southon, 2001).

In addition to the carotenoids, tocopherols and tocotrienols present in RPO, this oil also contains sterols (sitosterols, stigmasterols and campesterol), phospholipids, glycolipids, squalene and other natural carotenoids found in smaller quantities, such as lycopene, lutein, and phytoene, that may help reduce lipid oxidation, oxidative stress and free radical damage to cells (Boateng *et al*, 2006). Several experiments have demonstrated the beneficial effects of the phytochemical-rich fraction of RPO against several diseases (Boateng *et al*, 2006). A study by Boateng and colleagues showed that diets containing 7% and 14% of RPO were effective in inhibiting the formation of azoxymethane (AOM) induced aberrant crypt foci (ACF) in a mouse model. The AFC is a pre-neoplasic lesion that appears on the surface of rodent colonocytes after treatment with colon carcinogens such as AOM. The ACF model has been used as a biomarker to screen for colon tumorigenesis with considerable success (Boateng *et al*, 2006).

2.4.2 Carotino Tocotrienol-Carotene Mixed Concentrate®

The CTCMC is a deep red paste derived from the refining process of palm oil. It contains high concentrations of carotenes (75,000 ppm of α- and β- carotenes), which are equivalent to 27562 Retinol Activity Equivalents (RAE) per gram of concentrate. It also contains large amounts of natural tocotrienols and lycopene, which have been demonstrated to have powerful antioxidant properties (Kioskas and Gordon, 2003; Boateng *et al* 2006). The composition of the CTCMC is shown in Table 4.

Table 4. Carotino Tocotrienol-Carotene Mixed Concentrate composition

Unsaponifiable matter	41.3%
• Provitamin A carotenoids	7.5%
• Lycopene	0.6%
• Tocotrienols	6.3%
Saponifiable matter	58.1%
Moisture	0.6%

To our knowledge, there is a lack of scientific studies related to this product. Given the fact that the CTCMC has 150 times more carotenes and 300 times more tocotrienols than RPO, it is expected that its antioxidant benefits excel by far those from RPO. With this research it is intended to exploit the high antioxidant content of this derivative of palm oil and evaluate its effectiveness in attenuating the systemic or *in situ* intestinal oxidation caused by normal doses of oral iron used in treating or preventing IDA.

3.0 MATERIALS AND METHODS

3.1 Subjects

A convenience sample of seventeen healthy male adults participated in the study: five of them in the first phase (pilot study) and twelve in the second phase (intervention trial). Sample size selection was based on a study by Schümann *et al*, 2005 with three German volunteers. In the mentioned study, significant increases on several systemic oxidation biomarkers were observed in these subjects using the same supplementation scheme applied in this work. In a similar study, Lund and colleagues (1999) used a sample of 18 men and women to test the effects of oral iron supplementation on the free radical-generating capacity of feces from these healthy volunteers.

Subjects were recruited in Guatemala City, Guatemala at the local state university. A preliminary meeting was held to inform the interested persons about the objectives and methodology of the study. Assistants were given a short questionnaire about health status and smoking habits that allowed making a pre-screening of possible participants in the study (Appendix 1).

Pre-selected subjects who signed the informed consent form (Appendices 2 and 3) were asked to give a blood sample for initial routine hematological screening to ensure that no participant was anemic. Those who complied with the inclusion criteria and did not present any condition stated on the exclusion criteria were invited to participate in the study and awarded a monthly monetary compensation until the end of the supplementation trial.

- Inclusion criteria: Informed written consent and willingness to provide all the blood, feces and urine samples required for this study.
- Exclusion criteria: Smokers. Anemia, history of gastrointestinal disorders, intolerance to iron supplements, hematological disorders, consumption of other nutritional supplements, including iron supplements in particular. Presents or develops an inflammatory or infectious condition during the conduct of the study.

3.1.1 Ethical approval

Ethical approval from the Bannatyne Campus Research Ethics Board at the University of Manitoba and the Human Subjects Committee of CeSSIAM was obtained. Subjects could not participate on the study unless they had signed the informed written consent form (Appendix 4).

3.2 Experimental design and Methods

Experimental Design:

This was a longitudinal metabolic study with repeated measures in which each subject was its own control. No separate control group was included in the experimental design. The supplementation schedule is the same one as the one used in the study by Schümann *et al*, 2005, who found significant alterations in several urinary biomarkers when supplying 120 mg of iron during 7 days. This group also found that a washout period of 14 days was enough to eliminate the effects of 7 days of supplementation with iron on specific urinary biomarkers. We were guided by Lund *et*

al (1999) for our duration of washout period on the fecal side. In those British subjects after a supplementation period of 14 days with 19 mg elemental iron/day, a significant increase in the free radical-generating capacity of the feces was detected. Fourteen days of washout were sufficient to decrease the free radical generation capacity to baseline.

The selection of systemic urinary oxidation biomarkers was done on the basis that Schümann and colleagues found that from a series of potential urinary biomarkers, only TBARS, 8-OHdG and isoprostanes showed consistent responses to the iron challenge. A validated HPLC-based method proposed by Owen *et al* (2000) was used to assess *in situ* luminal oxidation. This method was chosen because of its high precision and ease. The number of urine and fecal samples collected and analyzed, together with the sample size, depended on the available funding. Given the budget constraints, it was not possible to analyze urine and fecal samples for every day of the study. Urine samples were processed in duplicate, whereas fecal samples could be analyzed once. Key samples were chosen to reflect the oxidative conditions of the subjects during all the treatment phases. To avoid discomforts and inconveniences to the participants, blood sampling was only done at the beginning and end of the study to evaluate hemoglobin concentrations. The interest in hemoglobin status was as a screening test to eliminate the enrollment of anemic subjects into the study. Anemic subjects would likely have a greater affinity (Yip, 2001) for iron absorption and the systemic and fecal exposures would be more variable within the group period. We repeated the hematological screening at the end of the study to confirm stability throughout the period of study, which included an additional 2520 mg of supplemental iron.

The experiment consisted of two phases as described below:

- Phase 1 (PILOT STUDY):

The first phase allowed testing and implementing the laboratory methods used in the next phase. It also helped determining if the proposed supplementation schedules and doses were adequate to produce detectable responses on the free-radical/oxidation markers in urine and the fecal matrix. The CTCMC used throughout Phase 2 needed to be diluted in an oily matrix to facilitate its ingestion, refined palm oil was chosen for this purpose. For this reason, the effect of the oil matrix on the oxidative response produced by the supplemental iron was also assessed. Treatments provided to the subjects during phase 1 are shown in Table 5.

Table 5. Treatments administered in the first phase (pilot phase)

Treatment	**Iron (mg in form of $FeSO_4$)**	**Water (*ad libitum*)**	**Refined palm oil (ml)**
FE	120 mg	Yes	0
FEoil	120 mg	Yes	5

This phase consisted of two consecutive 21-day cycles with 2 treatments carried over a period of 43 days. Each 7-day treatment period was separated by a 14-day washout interlude to eliminate the effects of supplementation on the oxidation biomarkers and return to baseline levels. A summarized scheme of the supplementation design is shown in Figure 4 and a detailed version with sample codes, procedures and collection days is shown in Table 7.

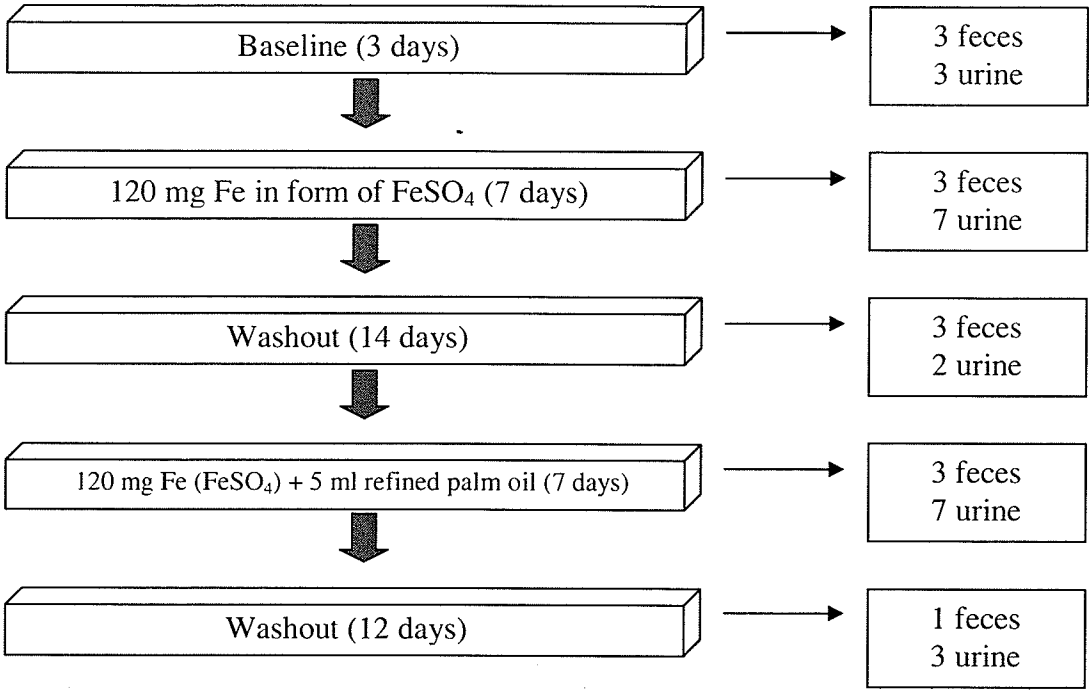

Figure 4. Schematic summarized supplementation scheme and sample collection design used in the pilot study

Table 7. Supplementation and sample collection scheme used in the pilot study

Cycle	Time elapsed	Phase	Collection day	Oral dose	Procedure schedule	Sample name
Cycle 1: Treatment FE (Iron Challenge)	Day 1	Baseline	Day 1	Water	Collect morning urine, feces and blood samples; take water; breakfast	BSL1
	Day 2		Day 2		Collect morning urine and feces sample, take water	BSL2
	Day 3		Day 3			BSL3
	Day 4	Supplementation with iron ($FeSO_4$) + water	Day 4	$FeSO_4$ + water	Collect morning urine, take $FeSO_4$, breakfast	FE1
	Day 5		Day 5			FE2
	Day 6		Day 6			FE3
	Day 7		Day 7			FE4
	Day 8		Day 8		Collect morning urine and feces sample; take $FeSO_4$, breakfast	FE5
	Day 9		Day 9			FE6
	Day 10		Day 10			FE7
	Day 11	Washout period and urine sample collection				
	Day 12					
	Day 13					
	Day 14		Day 11	Water	Collect morning urine	WO1
	Day 15					
	Day 16					
	Day 17					
	Day 18		Day 12	Water	Collect morning urine	WO2
	Day 19					
	Day 20					
	Day 21					
	Day 22	Washout period and feces sample collection	Day 13		Collection of feces sample	WO3
	Day 23		Day 14			WO4
	Day 24		Day 15			WO5
Cycle 2: Treatment FEoil (Supplementation with Fe + refined palm oil)	Day 25	Supplementation with iron ($FeSO_4$) + palm oil	Day 16	$FeSO_4$ + refined palm oil	Collect morning urine, take $FeSO_4$, breakfast	FEoil1
	Day 26		Day 17			FEoil2
	Day 27		Day 18			FEoil3
	Day 28		Day 19			FEoil4
	Day 29		Day 20		Collect morning urine and feces sample; take $FeSO_4$ + oil; breakfast	FEoil5
	Day 30		Day 21			FEoil6
	Day 31		Day 22			FEoil7
	Day 32	Washout period and urine sample collection				
	Day 33					
	Day 34					
	Day 35		Day 23	Water	Collect morning urine	WO6
	Day 36					
	Day 37					
	Day 38					
	Day 39		Day 24	Water	Collect morning urine	WO7
	Day 40					
	Day 41					
	Day 42					
	Day 43	Sample collection	Day 25		Collection of feces, urine and blood samples, breakfast	WO8

Phase 2 (INTERVENTION TRIAL):

The main objective of this phase was to determine if supplementation with dietary carotenoids, tocopherols and tocotrienols from the CTCMC caused a reduction in urinary (indicators of systemic oxidation) and fecal (intraluminal oxidation in the bowel) oxidation markers in response to an oral iron challenge. A dose-response effect of the CTCMC on the oxidative response using two doses of CTCMC was also evaluated.

This phase consisted of three consecutive 21-day cycles with different treatments: FEoil, CTCB or CTCA on each cycle. Treatment FEoil was administered on the first cycle of supplementation to all subjects, while the administration of treatments CTCB and CTCA was randomized on the second and third cycles. This phase had duration of 63 days. Treatment preparations (Table 6) were made one day earlier and stored in dark flasks until consumed the next day. Summarized and detailed supplementation schemes are shown in Figure 5 and Table 8.

Table 6. Treatments administered in the second phase (intervention trial)

Treatment	**Iron** (mg in form of $FeSO_4$)	**Water** (*ad libitum*)	**Refined palm oil (ml)**	**CTCMC (g)**	**Pro-vitamin A carotenoids (mg)**	**Tocopherols and tocotrienols (mg)**
FEoil	120 mg	Yes	5	0	0	0
CTCB	120 mg	Yes	5	0.4	30	36.8
CTCA	120 mg	Yes	5	0.8	60	73.6

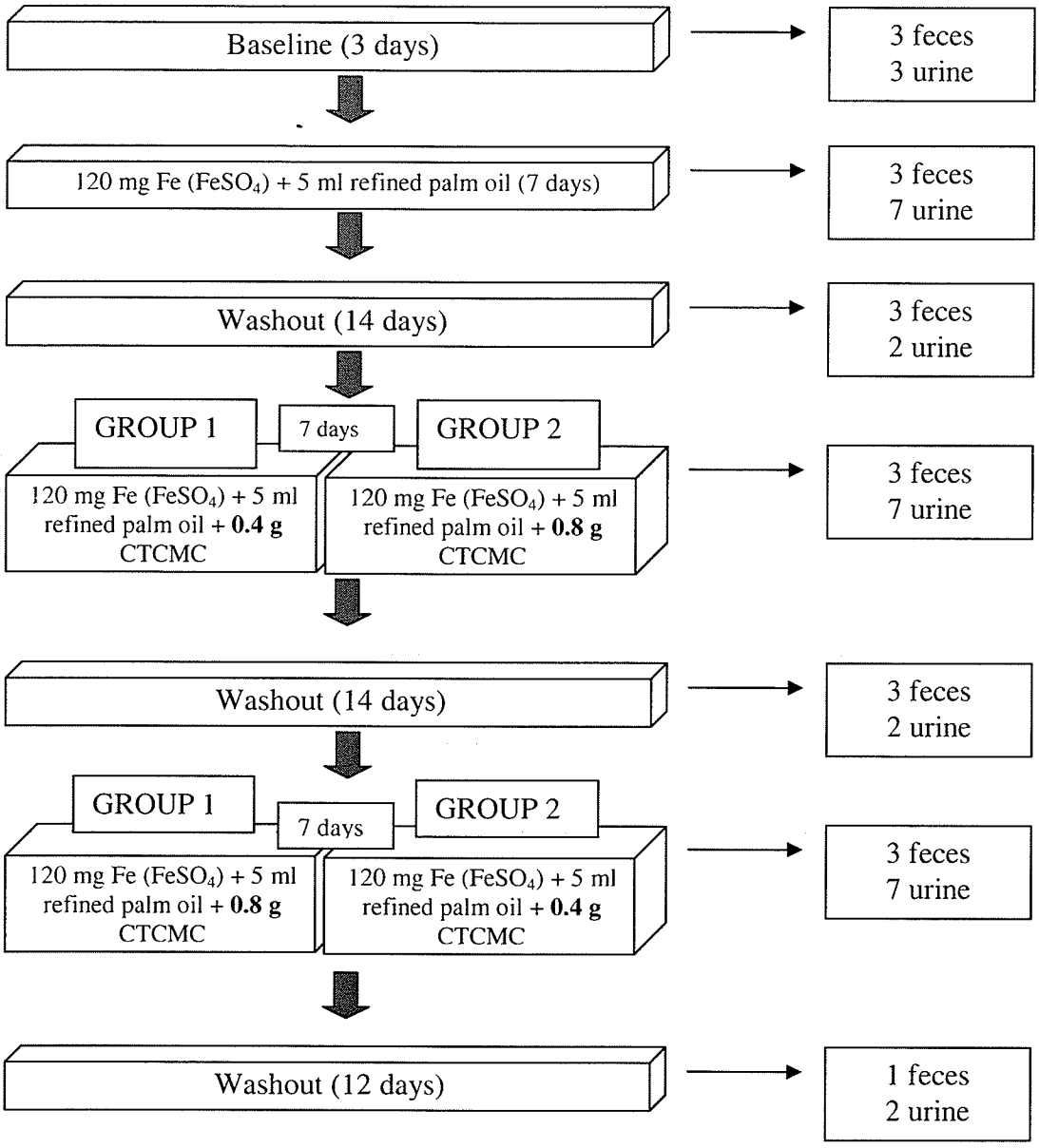

Figure 5. Schematic summarized supplementation scheme and sample collection design used in the intervention trial

Table 8. Supplementation schedule for Phase 2

Cycle	Time elapsed	Phase	Collection day	Oral dose	Procedure schedule	Sample name
Cycle 1: Treatment FEoil (Supplementation with iron + refined palm oil)	Day 1	Baseline	Day 1	Water	Collect morning urine, feces sample and baseline blood sample; take water; breakfast	BSL1
	Day 2		Day 2		Collect morning urine and feces sample, take water	BSL2
	Day 3		Day 3			BSL3
	Day 4	Supplementation with iron ($FeSO_4$) + refined palm oil	Day 4	$FeSO_4$ + oil	Collect morning urine, take $FeSO_4$, breakfast	FEoil1
	Day 5		Day 5			FEoil2
	Day 6		Day 6			FEoil3
	Day 7		Day 7			FEoil4
	Day 8		Day 8		Collect morning urine and feces sample; take $FeSO_4$; breakfast	FEoil5
	Day 9		Day 9			FEoil6
	Day 10		Day 10			FEoil7
	Day 11	Washout period and urine sample collection				
	Day 12					
	Day 13					
	Day 14		Day 11	Water	Collect morning urine	WO1
	Day 15					
	Day 16					
	Day 17					
	Day 18		Day 12	Water	Collect morning urine	WO2
	Day 19					
	Day 20					
	Day 21					
	Day 22	Washout period and feces sample collection	Day 13		Collection of feces sample	WO3
	Day 23		Day 14			WO4
	Day 24		Day 15			WO5
Cycle 2: Treatment CTCMC Supplementation with Fe + refined palm oil + CTCMC	Day 25	Supplementation with iron ($FeSO_4$) + refined palm oil + low dose CTCMC or high dose CTCMC	Day 16	$FeSO_4$ + refined palm oil + CTCMC	Collect morning urine, take $FeSO_4$, breakfast	CTCA1 CTCB1
	Day 26		Day 17			CTCA2 CTCB2
	Day 27		Day 18			CTCA3 CTCB3
	Day 28		Day 19			CTCA4 CTCB4
	Day 29		Day 20		Collect morning urine and feces sample; take $FeSO_4$ + oil;	CTCA5 CTCB5
	Day 30		Day 21			CTCA6 CTCB6

	Day 31		Day 22		breakfast	CTCA7 CTCB7
	Day 32	Washout period and urine sample collection				
	Day 33					
	Day 34					
	Day 35		Day 23	Water	Collect morning urine	WO6
	Day 36					
	Day 37					
	Day 37					
	Day 39		Day 24	Water	Collect morning urine	WO7
	Day 40					
	Day 41					
	Day 42					
	Day 43	Washout period and feces sample collection	Day 25		Collection of feces samples	WO8
	Day 44		Day 26			WO9
	Day 45		Day 27			WO10
Cycle 3: Treatment CTCMC Supplementation iron + refined palm oil + CTCMC	Day 46	Supplementation with iron ($FeSO_4$) + refined palm oil + high dose CTCMC or low dose CTCMC	Day 28	$FeSO_4$ + refined palm oil + CTCMC	Collect morning urine, take $FeSO_4$, breakfast	CTCA1 CTCB1
	Day 47		Day 29			CTCA2 CTCB2
	Day 48		Day 30			CTCA3 CTCB3
	Day 49		Day 31			CTCA4 CTCB4
	Day 50		Day 32		Collect morning urine and feces sample; take $FeSO_4$ + oil; breakfast	CTCA5 CTCB5
	Day 51		Day 33			CTCA6 CTCB6
	Day 52		Day 34			CTCA7 CTCB7
	Day 53	Washout period and urine sample collection				
	Day 54					
	Day 55					
	Day 56		Day 35	Water	Collect morning urine	WO11
	Day 57					
	Day 58					
	Day 59					
	Day 60		Day 36	Water	Collect morning urine	WO12
	Day 61					
	Day 62					
	Day 63					
	Day 64	Washout period and sample collection	Day 37		Collection of feces and blood samples, breakfast	WO13

3.3 Sample Collection

3.3.1 Blood collection

Venous blood collection for initial and final screening was obtained by a trained technician. Samples were collected in tubes with heparin (BD Vacutainer®, Franklin Lakes, New Jersey, USA), refrigerated and transferred to the clinical laboratory at the Sanatorio Nuestra Señora del Pilar (Guatemala City) for further analysis. Hemoglobin, red blood cell count, total hematocrit and mean red cell volume were measured with standard photometric methods using a Coulter MAXM Hematology Analyzer (GMI, Inc., Ramsey, Minnesota, USA).

3.3.2 Urine collection

Morning urine samples were self-collected at home by the subjects, stored in special 50 ml plastic urine collectors (Amsino International Inc., Pomona, California, USA) and transported to the study site in iceboxes with Blue ice® gel. The samples were separated in aliquots, stored in 1.5 ml tubes (Safe-lock®, Eppendorf) and frozen at -50°C (Forma Scientific Inc., International, Marietta, OH, USA) until analyzed for quantification of 8-OHdG, TBARS and creatinine.

3.3.3 Feces collection

On days of collection, subjects were asked to collect a feces sample in a sealed 50 ml fecal collection plastic container (Amsino International Inc., Pomona, California, USA), according to their normal bowel habit and to transport it in an icebox with Blue ice® gel to the study site. Samples were transferred to properly labeled plastic bags and

frozen at -50°C (Forma Scientific Inc., International, Marietta, OH, USA) until analyzed for determination of total hydroxylated products and non-heme iron.

3.3.4 Oral dosing

Mixtures containing all the elements of the treatment were prepared one day before and ingested by the participants in fasting state. Ferrous sulphate 125 mg/ml, 25 mg iron (Fer-In-Sol®, Mead Johnson Nutricionales, Bristol-Myers Squibb, Ecuador), commercial refined palm oil (Olmeca®, Guatemala) and the Carotino® Tocotrienol-Carotenoid Concentrated Mix (Carotino® SDN. BHD., Malaysia) were used for these preparations. Subjects were allowed to drink water *ad libitum.* After 1 hour they were given a small breakfast at the study site.

3.4 Chemical and dietary analysis

3.4.1 Free radical/oxidation markers analysis for assessment of systemic oxidation and *in situ* luminal oxidation

These analyses were performed at the Universidad del Valle de Guatemala laboratories by Monica Orozco, except for the creatinine in urine assessment for which urine samples were sent for analysis to the clinical laboratories of Sanatorio Nuestra Señora del Pilar in Guatemala City, Guatemala. All of the urine and feces samples collected were analyzed with the following assays:

3.4.1.1 Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG)

The commercial BIOXYTECH® 8-OHdG-EIA™ Kit Quantitative Assay for 8-hydroxy-2'-deoxyguanosine (Catalog Number 21026) from OXIS Health Products Inc., Portland, Oregon, USA, was used. This is a competitive in vitro enzyme-linked immuno-sorbent assay used for quantitative measurement of the oxidative DNA adduct, 8-hydroxy-2'-deoxyguanosine (8-OHdG).

In this assay the 8-OHdG present in a sample or standard competes with 8-OHdG bound to the pre-coated microtiter plate for the 8-OHdG monoclonal antibody binding sites. Antibodies bound to the 8-OHdG in the sample are washed away, leaving the ones bound to 8-OHdG coated on the plate. An enzyme-labeled secondary antibody is then added to bind with the monoclonal antibody that remains in the plate. A washing step removes the unbound enzyme-labeled secondary antibody. Finally, a chromogen is added to develop color in proportion to the amount of antibody bound to the plate.

Materials included in the commercial kit:

- 8-OHdG microtiter plate precoated with 8-OHdG (8×12 wells, split type)
- Primary antibody: monoclonal antibody specific for 8-OHdG
- Primary antibody dilution buffer: phosphate buffer saline
- Secondary antibody: HRP-conjugated antibody
- Secondary antibody dilution buffer: phosphate buffer saline
- Chromogen: 3,3',5,5'-tetramethylbenzidine
- Chromogen dilution buffer: hydrogen peroxide/citrate-phosphate buffer saline
- Washing buffer (5x): concentrated phosphate buffer saline

- Stop solution: 1M phosphoric acid
- Standards 1-6: 0.5, 2.0, 8.0, 20.0, 80.0, 200.0 ng/ml 8-OHdG
- Plate seal adhesive sheet for covering plate

Urine samples were thawed and centrifuged at 3000 rpm for 2 minutes (Centrifuge 5415C, Eppendorf, Brinkmann Instruments, Inc. NY, USA). The primary antibody was reconstituted with the primary antibody dilution buffer. Fifty microliters of sample or standard were added per well using a micropipette (Reference®, Eppendorf, Weetbury, NY, USA). Fifty microliters of reconstituted primary antibody were added to each well except for the blank, using a multichannel pipette (50-200 mL, Eppendorf Research® multichannel pipette, Weetbury, NY, USA). The plate was sealed tightly with the plate seal adhesive sheet provided with the kit, shaken from side to side to mix fully and incubated at 37°C for 1 hour (Shake' N' Bake® hybridization oven Model 136400, Boekel Scientific, Feasterville, Pennsylvania, USA). After the incubation period, the contents were poured off the plate and 250 mL of diluted washing buffer were pipetted into each well. The plate was washed thoroughly by agitation, inverted and blotted against a clean paper towel to remove any remaining washing buffer. The wash process was repeated twice. The secondary antibody was reconstituted with the secondary antibody dilution buffer and 100 mL were added to each well. The plate was sealed tightly with the adhesive plate seal, shaken to mix and incubated at 37°C for 1 hour. Chromogen was prepared by diluting it with 100 volumes of chromogen dilution buffer. The washing process was repeated. One hundred milliliters of the diluted chromogen solution were added per well. The plate was shaken from side to side to mix fully and

incubated at room temperature in the dark for 15 minutes. After the incubation period, 100 mL of the stop solution were added, contents were mixed, and the plate was left standing at room temperature for 10 minutes. The absorbance was read at 450 nm (ELx800 absorbance microplate reader, Biotek, with microplate data collection and analysis software Gen5™, Biotek, Biotek Instruments, Inc. Vermont, USA). A standard curve was generated by plotting absorbance vs. log of concentration. The absorbance values obtained for test samples were used to determine the concentration from the calibration curve.

3.4.1.2 Urinary Thiobarbituric Acid Reactive Substances (TBARS)

The commercial OXItek TBARS Assay Kit. ZMC (Catalog number 0801192) from Zeptometrix Corporation, Buffalo, NY, USA, was used for quantifying thiobarbituric acid reactive substances (TBARS) that include lipid hydroperoxides and aldehydes in urine samples. The principle of the system consists of the formation of a malondialdehyde (MDA)-thiobarbituric acid adduct which can be measured by fluorometry or spectrophotometry, and expressed as MDA equivalents. Urine contains lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress and return to normal levels in presence of antioxidants.

Materials included in the commercial kit:

- Thiobarbituric Acid (4 vials/kit): Contains 0.53 grams thiobarbituric acid
- TBARS Diluent 1 (4 x 50 ml/kit): Contains acetic acid
- TBARS Diluent 2 (4 x 50 ml/kit): Contains sodium hydroxide

- SDS Solution (30 ml/kit): Contains sodium dodecyl sulfate
- MDA Standard (10 ml/kit): Contains 100 nmol/ml malondialdehyde bis (dimethyl acetal)
- MDA Diluent (100 ml/kit): Contains sterile deionized water
- Glass marbles

Samples were thawed and centrifuged at 2000 rpm for 2 minutes (Centrifuge 5415C, Eppendorf, Brinkmann Instruments, Inc., NY, USA). Reagents were allowed to reach room temperature. One hundred microliters of sample or standard were added to a properly labeled glass tube, after that, 100 µl of SDS Solution were added to each tube and vortexed (Genie 2® vortex (12-812), Fisherbrand, Scientific Industries, NY, USA). Two and a half milliliters of TBA/Buffer Reagent were added forcefully down the side of each tube. Each tube was covered with a glass marble and incubated at 95°C for 60 min (PREMLAB incubator, Guatemala). After incubation, tubes were cooled down to room temperature in a refrigerator for 10 min. Absorbance was read in a spectrophotometer set at 532 nm (UV-Vis Spectronic 21D spectrophotometer, Spectronic Instruments, Bausch & Lomb, Rochester, NY, USA).

3.4.1.3 Creatinine in urine

Quantification was done at the clinical laboratory of "Sanatorio Nuestra Señora del Pilar", Guatemala City. Creatinine in urine was determined with a kinetic-spectrophotometrically-based assay using a Dimension AR analyzer (Dade-Behring Ltd., Merck, S.A., Guatemala).

3.4.1.4 Generation and detection of products resulting from the hydroxyl radical attack on salicylic acid (2,5 dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid and catechol) in feces for assessment of *in situ* luminal oxidation

Method adapted from Owen, et al, 2000 with some modifications. In this assay, hydroxyl radicals generated by ferric ions chelated with EDTA attack salicylic acid present in an incubation buffer that contains residual iron and antioxidants from the fecal sample. Three hydroxylation products are generated as a result of this reaction: catechol, 2,3 dihydroxybenzoic acid (2,3 DHBA), and 2,5 dihydroxybenzoic acid (2,5 DHBA). These compounds are separated and quantified spectrophotometrically using high performance liquid chromatography (HPLC) with a UV/VIS detector.

3.4.1.4.1 HPLC method validation

Method was validated by determining two analytical quality parameters: limit of detection (LOD) and limit of quantification (LOQ) of the compounds of interest (2,5 dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid and catechol). Both analytical parameters were obtained by running 20 blank samples with the HPLC conditions described in section 3.4.1.4.2. Blank samples consisted of phosphate buffer 100 mM.

Limit of detection is defined as the "minimum concentration or amount of sample that can be detected for a given confidence level" (Skoog, 2001). It was calculated as follows:

$$LOD = (Sm - Sbl)/m$$

$$Sm = Sbl + ks_{bl}$$

Sbl = mean blank signal of 20 measurements
s_{bl} = standard deviation of 20 blank measurements
Sm = minimum distinguishable analytical signal
k = 3
m = slope of the calibration curve using the standard compound of interest

The limit of quantification is the smallest concentration to which a qualitative measurement can be done. It is defined as:

$$LOQ = 10s_{bl}$$ (Skoog, 2001)

3.4.1.4.2 Generation and detection of products resulting from the hydroxyl radical attack on salicylic acid (2,5 dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid and catechol) in feces

Working solutions were prepared as follows:

a) Phosphate buffer 100 mM

To 600 ml dd H_2O, 205.5 ml of a 0.2 M KH_2PO_4 (Merck S.A., Guatemala) stock solution and 94.5 ml of a 0.2 M K_2HPO_4 (Merck S.A., Guatemala) stock solution were added. The pH was adjusted to 6.5.

b) Incubation buffer

The chemicals used to prepare the incubation buffer were all purchased from Merck S.A., Guatemala. The incubation buffer contained phosphate buffer (100 mM),

EDTA) (500 mM), $FeCl_3$ H_2O (50 µM with respect to elemental iron) and salicylic acid (2 mM). Final pH was adjusted to 6.5.

c) Calibration curve dilutions

Fifty milliliters of 5mM stock solutions for the following compounds were prepared: catechol (Sigma Aldrich, Merck S.A., Guatemala), salicylic acid (Merck S.A., Guatemala), 2,5 DHBA (Merck) and 2,3 DHBA (Sigma Aldrich, Merck, S.A., Guatemala). Dilutions of the stock solutions for the four compounds were prepared to get the following concentrations: 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1 mM and 2 mM

For assessing the generation of total hydroxylated products, 10 ml of incubation buffer were added to a 100ml Erlenmeyer flask. One hundred milligrams of fecal sample were weighed (OHAUS® analytical balance, Analytical Plus, Pine Brook, NJ, USA) and placed into the Erlenmeyer flask containing the incubation buffer. Samples were homogenized at room temperature for 1 hour and incubated at 37°C for 21 hours (PREMLAB incubator, Guatemala). After incubation, 1 ml of the sample was transferred into a 1.5 ml microtube (Safe-lock tubes®, Eppendorf) and centrifuged at 10,000 rpm for 5 minutes (Centrifuge 5415C, Eppendorf, Brinkmann Instruments, Inc., NY, USA). Samples were filter-sterilized with 0.20 µm filters (Pyrogen-free disposable filter 0.20 µm, Sartorius, Merck S.A., Guatemala). Twenty microliters of sample were injected directly into the HPLC (High performance liquid chromatograph with UV/VIS detector. Hewlett Packard Series 1100, with Hypersil ODS column 200 x 2.1 mm 5µm).

The mobile phase consisted of 2% acetic acid glacial (Merck S.A., Guatemala) in water (Lichrosolv® water for chromatography, Merck S.A., Guatemala), making up

solvent A; and methanol gradient grade for chromatography (Merck S.A., Guatemala) (solvent B) using the following gradient: 95% A/5% B for 2 min, 75% A/25% B for 8 minutes, 60% A/40% B for 10 minutes, 50% A/50% B for 10 minutes and 0% A/100% B for 10 minutes. The UV/VIS detector was set at 278 nm for 5.5 minutes and then changed to 301 nm until the end of the run. Flow rate was set at 0.5 ml/min. An HP Chemstation® analysis software was used for instrument control and data handling.

3.4.1.5 Determination of non-heme iron in fecal matter

To correlate the free radical production in feces with the presence of residual supplemental iron, non-heme iron was quantified using the commercial spectrophotometrically-based assay Feren-B-Method kit (Bioanalytic, Umrich, Freiburg, Germany). In this assay, a preliminary step in which samples are digested with an acid mixture is followed by the separation of the digested organic matter from the iron compounds by centrifugation. A reducing agent is added to convert ionic iron in the Fe^{2+} form to Fe^{3+}, which reacts with a chromophore to yield a colored complex that can be quantified with spectrophotometry.

Materials included in the commercial kit:

- Iron buffer reagent
- Reducing agent
- Color reagent
- Standard (140 µg/dL Fe^{3+})

An acid mix was prepared by adding 100 ml of 6 M 37% HCl (Merck S.A., Guatemala) to 100 ml 20% trichloroacetic acid (J.T Baker, Soluciones Analíticas S.A., Guatemala). From 300 – 400 mg of feces samples were weighed in 15 ml glass tubes. Five milliliters of the acid mix were added to the sample and vortexed. Samples were incubated 65°C for 20 hours and vortexed after incubation. Afterwards, 1 ml of supernatant was transferred into a 1.5 ml microtube and centrifuged at 3000 rpm for 3 minutes. A 1:10 dilution was prepared by adding 100 µl of supernatant plus 900 µl millipore water.

For the photometric determination of iron, one package of reducing agent was dissolved in one bottle of iron-buffer reagent, yielding solution R1. A blank was prepared by adding 500 µl of R1 solution and 100 µl millipore water in 1.5 ml microtube. Twenty microliters of color reagent solution were added and mixed. After one minute, the sample was vortexed again. The blank was transferred to a cuvette and the photometer (590 nm) was adjusted to zero. For the samples, 500 µl of R1 solution and 100 µl of the diluted sample were placed in a 1.5 ml microtube and vortexed. The color reagent solution (20 µl) was added and vortexed. After one minute, the samples were vortexed again. Samples were transferred to a cuvette (UV-cuvette) and their absorbance was read at 590 nm. For the standard solution (140 µg/dL Fe^{3+}), 500 µl of R1 solution and 100 µl of the iron standard were added in a 1.5 ml microtube and vortexed. Twenty microliters of color reagent solution were added and vortexed. One minute later the standard solution was vortexed again. Standard was transferred to a cuvette (UV-cuvette) and its absorbance was read at 590 nm.

Iron content in fecal samples was calculated as follows:

$$\frac{(\text{absorbance} \times 10.52 \times 5 \times 1000)}{(\text{sample weight (mg)} \times 10)} = \mu g \text{ Fe/ g feces}$$

Iron content in the standard was calculated as:

$$\text{Absorbance} \times 1062 = \mu g/dl$$

3.4.2 Dietary assessment

In order to detect foods rich in iron and antioxidants on the diet or any dietary confounders that may affect iron absorption, a Food Diary was used. Subjects were asked to record all the foods they consumed, including snacks, water and beverages, during a period of one week on each 21-day cycle (weekends as well). In total, subjects from phase 1 recorded 14 days of habitual food consumption, whereas participants from phase 2 recorded 21. Reported food portions were converted into grams and their total iron, fiber, total carotenoids, vitamin E and vitamin C contents were estimated using the USDA online food composition database (available at: http://www.nal.usda.gov/fnic/foodcomp/search/index.html), the Food Composition Table for the Central American area (Menchú *et al*, 1996), and food labels. The information obtained was used to complement the free radical/oxidation markers analysis. Dietary data was collected and entered in a database by Mrs. Raquel Campos, Research Nutritionist from CeSSIAM, and analyzed by Mónica Orozco.

3.5 Data management and statistical analysis

The researcher collected, entered on an electronic spreadsheet and analyzed the personal and medical information of the participants. Samples were coded with a three-digit code to avoid using the names of the participating subjects. The hematological results were made available upon request of the participants.

Each subject served as its own control. During phase 2, CTCB and CTCA treatments were administered randomly to each subject. Lab results for each of the free radical/oxidation markers were treated as repeated measures and analyzed with a repeated measures linear model. Pearson product-moment correlation coefficient (for parametric distributions) and Spearman´s rank correlation coefficient (non-parametric measure of correlation, does not assume normality) and Cohen's kappa scores were used to measure the strength of the associations between fecal non-heme iron and ROS production in fecal matter, and between the two biomarkers of systemic oxidation: TBARS and 8-OHdG. For evaluating the strength of the agreement between the two variables using kappa scores the following criteria was used: Poor agreement = less than 0.20, fair agreement = 0.20 to 0.40, moderate agreement = 0.40 to 0.60, good agreement = 0.60 to 0.80 and very good agreement = 0.80 to 1.00.

Data was analyzed with statistical software (EXCEL 2003 and SPSS 12.0.1 for Windows). Criteria for a response associated with iron and CTCMC exposure was based on the baseline values obtained at the beginning.

4.0 RESULTS AND DISCUSSION

4.1 Demographic and Nutritional Characteristics of the Experimental Subjects

Demographic data of the study population for both phases is shown in Table 9. All participants completed every supplementation cycle and provided, with some exceptions, a total of two blood, 22 urine and 13 fecal samples during the pilot study. During the definite intervention trial 2 blood, 30 urine and 19 fecal samples were collected from each subject. Clinical data from the study is presented in Table 10, showing the final average creatinine values and average change in hemoglobin between baseline (Day 1) and the last day of the intervention trials for both phases (Day 43 in pilot study and Day 64 in second phase). After supplementing the participants with a total of 1680 mg and 2520 mg of iron throughout the pilot study and intervention trial, respectively, it can be observed that there were no significant changes in the hemoglobin concentrations ($p = 0.15$ and $p = 0.29$). These findings can be expected from a population of non-anemic, healthy men.

Table 9. Characteristics of participants in phases 1 and 2: number, mean values, and standard deviations

Characteristic	Phase 1 (pilot study)	Phase 2 (intervention trial)
N	5	12
Age (years) [range]	30.0 (21 – 35)	24.1 (18 – 34)
Weight (kg) [± SD][1]	79.8 ± 11.5	64.4 ± 11.2
BMI (kg/m^2) [± SD][1]	26.7 ± 3.9	22.2 ± 3.4

[1]Standard deviation

Table 10. Average blood hemoglobin at baseline and end and urinary creatinine values at end of the intervention for phases 1 and 2

	First phase (N = 5)			Second phase (N = 12)		
	Baseline	Final	Change	Baseline	Final	Change
Hemoglobin (g/dL) (± SD)[1]	15.8 ± 0.5	16.2 ± 0.9	+0.32 ± 0.6 (p = 0.15)*	15.8 ± 0.5	15.8 ± 0.9	+0.09 ± 0.5 (p = 0.29)*
Creatinine (mg/dL) (± SD)[1]	-	213.6 ± 66.6	-	-	171.1 ± 93.9	-

* Probability associated with a Student's paired t-test, with a two-tailed distribution
[1]Standard deviation

4.1.1 Background diet and inter-study nutrient intake of subjects

The habitual daily iron, fiber, total carotenoids, and vitamin E and C intakes were estimated for the complete pooled study population from phases 1 and 2 (N = 17) on a total of 14 days (2 weeks) during (CTCMC treatment) and away (WO treatment) from iron plus CTCMC supplementation. The purpose of this analysis was to assess the adequacy of the participants' accustomed diets to the Daily Recommended Intakes (DRI's) for these 17 males, 19 to 50 years of age, by comparing it to the recommendations for individuals expressed as Recommended Daily Allowances (RDA's) and Adequate Intakes (AI), or to the population-based recommendation, given as Estimated Average Requirements (EAR's), when available (Food and Nutrition Board, 2004). It was also of interest to know if the act of supplementation would cause any disruptions on the usual nutrient intakes of the study subjects and variations on a day-to-day basis that could be considered confounding effects on the oxidation biomarker measurements. The repeated measures analysis reported compared daily nutrient intakes per week separately. Each week

consisted of 7 consecutive days during both supplementation cycles with the CTCA and CTCB treatments. No statistical analysis could be performed comparing all 14 days of dietary assessment because there were missing data.

In terms of dietary patterns, per se, that is the food items and food sources of the nutrients of interest, the individuals were very different, one from another, and reported a wide variety of different food items. In fact, a total of 501 food and beverage items were reported over the 224 subject-days (out of a goal of 14 x 17 = 238 subject-days) of dietary diary recording.

The number of different items reported (dietary variety) ranged from a low of 3 to a high of 27. Within subjects, the number of items consumed over 24 hours varied up and down from report to report. Pattern description can only be given in qualitative terms. One subject was a lacto-ovo-vegetarian; the remainder ate meat. The consumption of tortillas, the traditional staple of Guatemala, is being substituted by wheat breads. Much of the diet consisted of foods eaten away from home, including "fast foods". Alcoholic beverages and sodas were frequently reported. In terms of diversity, with notable exceptions, consumption of fresh fruits and vegetables was low in the study sample.

4.1.1.1 Background iron intake from the habitual diet

On average, during the days when diet was recorded, the total iron intake of the study population was well above the RDA of 8 mg/day and the EAR of 6 mg/day; meaning that their dietary iron intakes were adequate according to the individual and population-based recommendations (Figure 6). Individually, all of the participants

complied with the RDA and none of them reached the upper tolerable limit (UL) of 45 mg/day. Iron consumption did not vary significantly from day to day during the 14 days on which diet was evaluated.

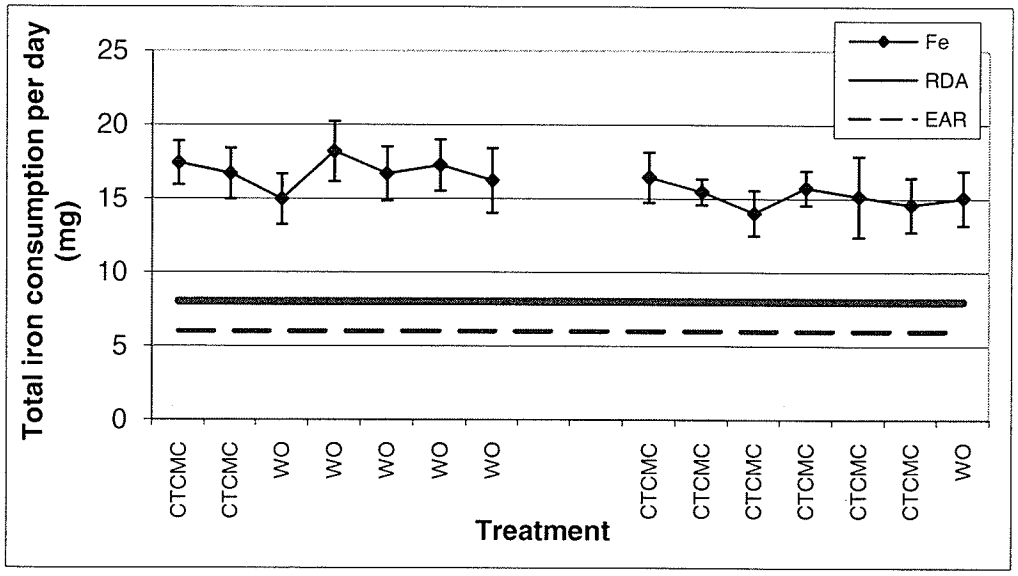

Figure 6. Average total iron consumption during two weeks of the study for all the participants from phases 1 and 2 (N = 17 subjects). CTCMC = Day in which CTCMC treatment was supplied. WO = Washout day, no treatment was supplied.

4.1.1.2 Background intake of dietary carotenoids and other antioxidant constituents of the diet

Average dietary fiber consumption for the complete population was below the RDA on every day that it was evaluated (Figure 7). All of the subjects failed to meet the recommendation for Adequate Intake of 38 g of fiber per day (Food and Nutrition Board, 2004). There are no significant differences between days of CTCMC treatment and washouts.

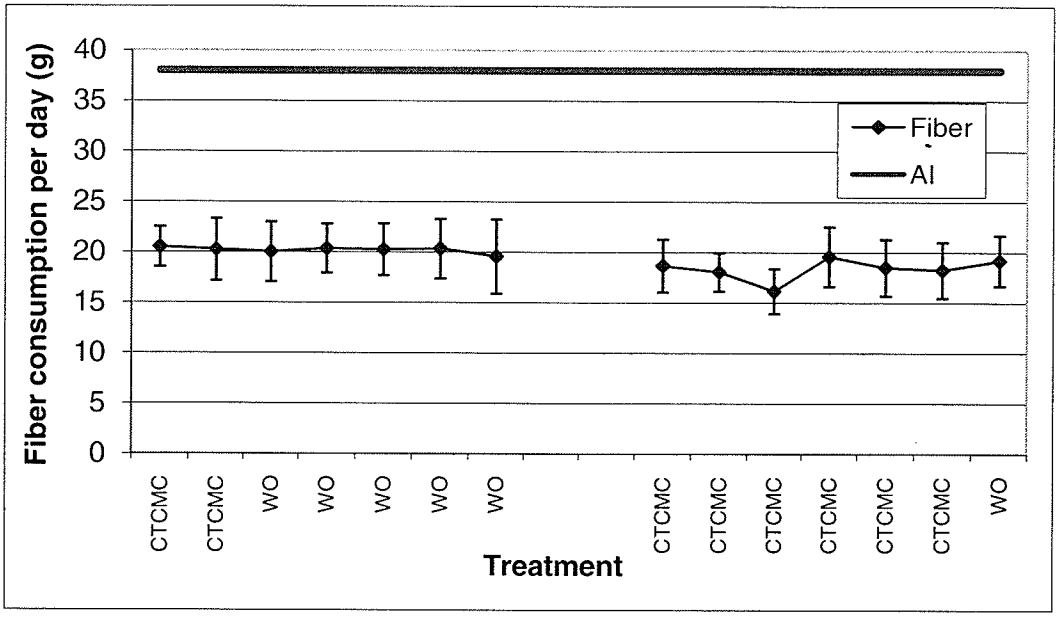

Figure 7. Average total fiber consumption during two weeks of the study for all the participants from phases 1 and 2 (N = 17 subjects). CTCMC = Day in which CTCMC treatment was supplied. WO = Washout day, no treatment was supplied. Adequate Intakes (AI) were used as recommendation to compare the subjects´ fiber consumption.

Day-to-day average vitamin E consumption in the 17 subjects was below the RDA and EAR every day it was assessed during the first 7-day period (Figure 8). Only one subject reached the RDA with an average vitamin E intake of 16.8 mg/day calculated during the 14 days of dietary assessment. In general, there are no significant differences in vitamin E consumption between days of week one but during the second week there are differences among three days. These disparities occur between days when the CTCMC was supplied and might be due to normal variations in diet and not to the experimental supplementation.

It should be noted that the CTCMC supplied either 36.8 mg (CTCB) or 73.6 mg (CTCA) of tocotrienols and tocopherols. As such, the approximately 6 mg of natural

vitamin E (alpha tocopherol) from the diet was six-fold below the lowest dose of antioxidant supplement.

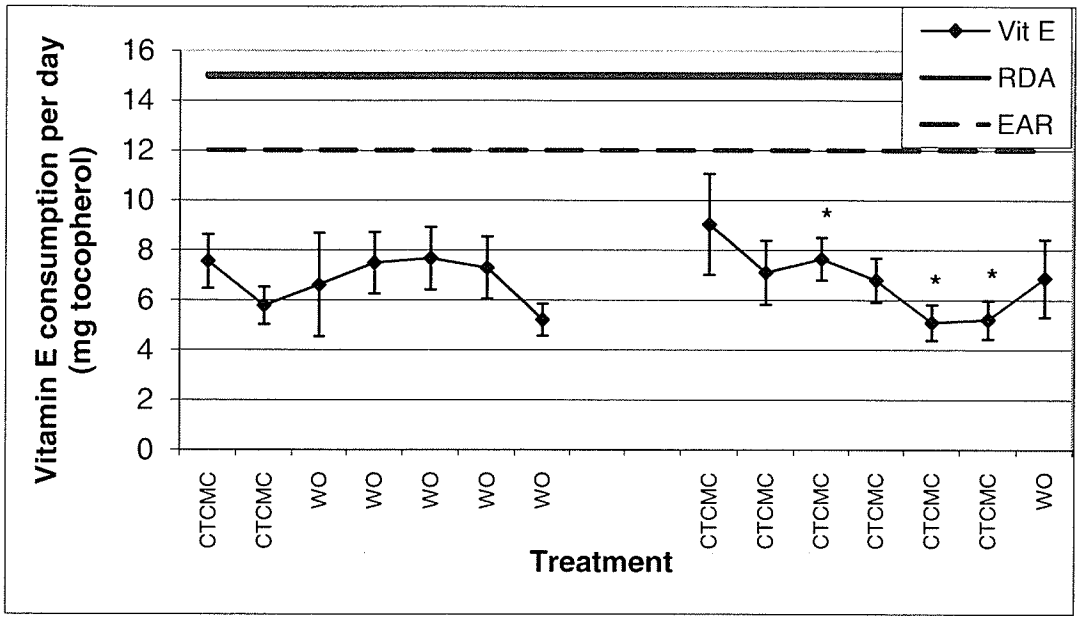

Figure 8. Average vitamin E consumption during two weeks of the study for all the participants from phases 1 and 2 (N = 17 subjects). *Days with statistical differences in reported vitamin E intakes by repeated measures ANOVA. CTCMC = Day in which CTCMC treatment was supplied. WO = Washout day, no treatment was supplied.

There are no dietary recommendations for carotenoid intake. Instead, reported US mean intakes from foods (Lindshiel and Erdman, 2006) were used as a parameter to compare carotenoid consumption in this Guatemalan population. The average total carotenoid intake in US males 19-50 years of age is 16,170 µg/day (16.2 mg/day) (Lindshiel and Erdman, 2006). During the 14-day dietary assessment, the participants in this study barely consumed 500 µg (0.5 mg) of carotenoids per day in average (Figure 9), with the exception of one subject who consumed 14,617 µg (14.6 mg) of carotenoids on one day during the washout period. Total carotenoids consumption from the habitual diet of these subjects is relatively stable throughout time. It is also low and insignificant

compared to the 30,000 and 60,000 µg of carotenoids supplied in one day with the CTCB and CTCA treatments, respectively.

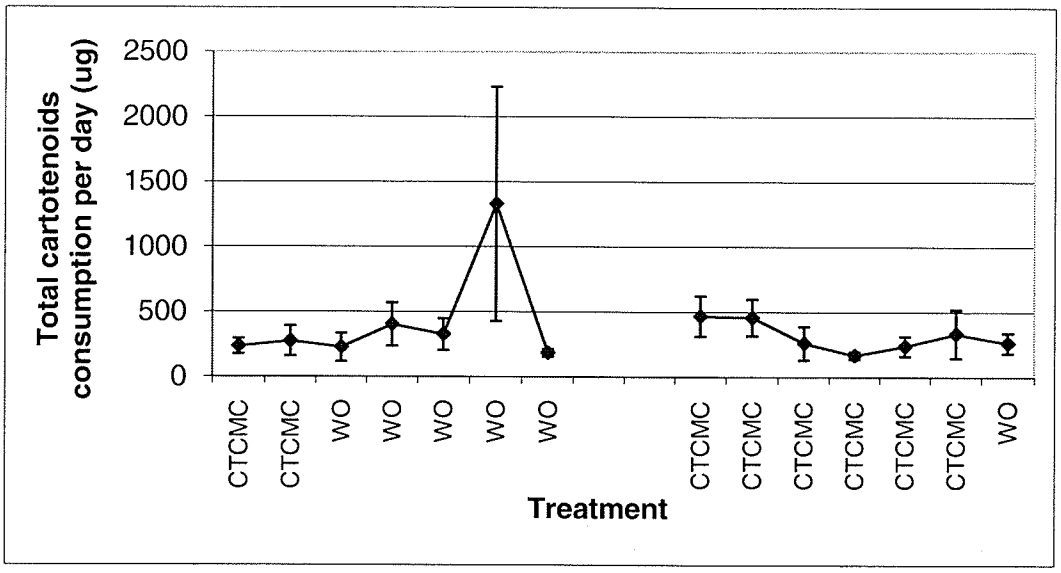

Figure 9. Average carotenoid consumption during two weeks of the study for all the participants from phases 1 and 2 (N = 17 subjects). CTCMC = Day in which CTCMC treatment was supplied. WO = Washout day, no treatment was supplied.

Vitamin C consumption varies widely among subjects (Figure 10). There are significant differences between several days in both weeks but these differences are between days in which the same treatment was administered or between washout days, indicating that the CTCMC treatment had no effect on the habitual vitamin C consumption. The analysis of the individual vitamin C intakes, estimated as an average of the 14 days, indicate that 13 out of 17 individuals did not reach the RDA of 90 mg/day as a personal average.

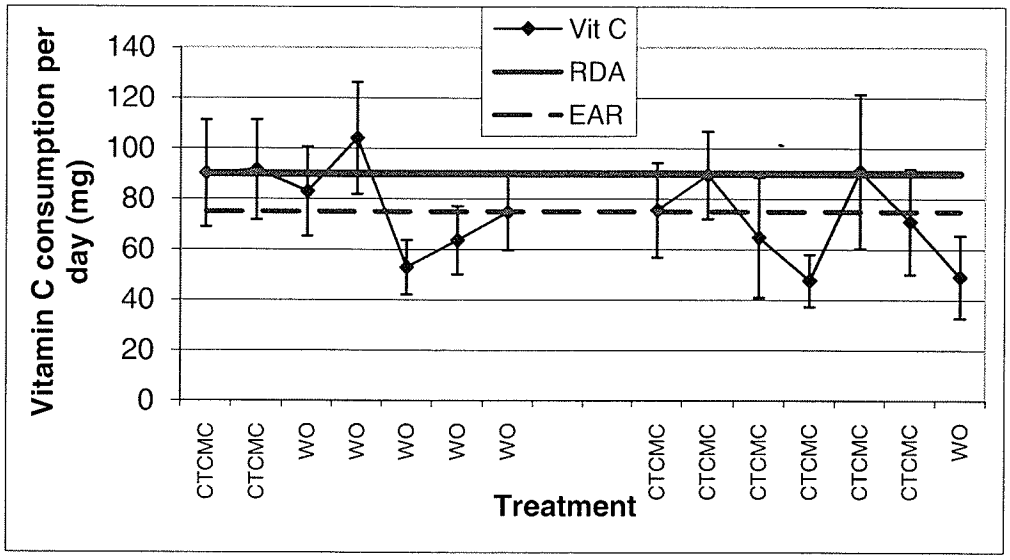

Figure 10. Average vitamin C consumption during two weeks of the study for all the participants from phases 1 and 2 (N = 17 subjects). CTCMC = Day in which CTCMC treatment was supplied. WO = Washout day, no treatment was supplied.

4.1.2 Summary Discussion

The population of men enrolled in the study was apparently healthy, but the influence of a modern mobile lifestyle and the need for "convenience" foods is evident in the dietary records. As a population, our subjects were inadequate in their average intakes of dietary fiber, carotenoids and vitamin E, on the mark for vitamin C, and superior for iron. We have used the US-Canada recommendations (Food and Nutrition Board, 2004) for adequacy of iron intake. The assumption for bioavailability is based on the U.S. diets constituents. The low fiber intake, average ascorbic acid, and moderate meat intake reported here would be consistent with an uptake of dietary iron at North American Standards. The EAR and RDA of the Daily Recommended Intakes (DRI) are therefore appropriate as references.

The average iron intakes were 7 times lower than the 120 mg supplement dosage of iron; eliminating any concern for interference. With the low dietary fiber intake, undigested carbohydrates would not buffer iron in the lower intestine. Dietary alpha-tocopherol was at least six times less than the lowest dosage of tocopherols from the CTCMC, whereas carotenoid consumption from the diet was 60-fold below the concentrations of carotenoids found in the 0.4 g CTCMC. The intake of vitamin C from the diet, then, would not be added to add oxygenated amounts of an antioxidant (ascorbic acid) to confound the effect of the carotene-tocopherol-tocotrienol mixture.

In general, intakes of fiber and iron are the most stable day-to-day with vitamins E and C being the most erratic over time. The days of iron plus CTCMC did not differ from washout days in nutrient intakes.

4.2 Findings related to the hypothesis of oral iron inducing systemic oxidation and Carotino Tocopherol-Carotene Mixed Concentrate® attenuating iron-induced oxidation

Living organisms have developed a series of complex regulatory mechanisms to maintain cell iron homeostasis (Schümann et al, 2007). At the intestinal level, iron absorption is adjusted to the demand of iron by the organism (Schümann et al, 2007), once it has been introduced into the cells, iron binds tightly to a series of proteins dedicated to transporting and storing it. Intracellular iron undergoes a series of processes with the purpose of ensuring an adequate and appropriate distribution of iron throughout the body. During iron turn-over, the body reuses iron from destroyed erythrocytes to form new ones

and distributes new absorbed iron where needed (Schümann et al, 2007). In order to reduce oxidative damage by free iron, maintain full iron stores and guarantee a proper iron absorption/excretion balance, iron homeostasis is tightly regulated.

The following descriptions are of our study to evaluate the impact of supplemental doses of 120 mg Fe/day normally used in the treatment of iron anemia, on the oxidative biology of healthy male adults by evaluating systemic oxidative damage in cell lipids and proteins.

Treatment codes used through the RESULTS and DISCUSSION sections are presented in Table 11 to help the reader associate the treatment with the treatment-phase-associated- biological sample and its assay results.

Table 11. Treatments and treatment codes for phases 1 and 2 of the study

Treatment phase associated sample code	**Treatment description**
BSLN1-BSLN3	Baseline, no iron supplement or CTCMC were administered.
FE1-FE7	120 mg of supplemental iron + water (only participants from phase 1 received this treatment).
FEoil1-FEoil7	120 mg of supplemental iron with water + 5 ml of refined palm oil were administered.
WO1-WO14	Washout period, no iron supplement or CTCMC were administered.
CTCB1-CTCB7	0.4 g of CTCMC + 5 ml of refined palm oil + 120 mg Fe.
CTCA1-CTCA7	0.8 g of CTCMC + 5 ml of refined palm oil + 120 mg Fe.

4.2.1 Oral Iron Alone does not influence Lipid Peroxidation Biomarkers (urinary TBARS)

Reactive iron ions can participate in electron transfer reactions with molecular oxygen, forming superoxide radicals that can contribute to the formation of hydroxyl radicals causing lipid peroxidation in the cell (Gutteridge, 1995). The thiobarbituric acid reactive substances (TBARS) are a group of hydroperoxides and aldehydes, formed during lipid peroxidation that can be measured in biological specimens, converting them into an important indicator of oxidative stress.

4.2.1.1 Findings from the pilot phase

The assessment of urinary TBARS in the pilot study was used to determine if the proposed oral iron challenge would create disturbances in the normal behavior of this biomarker, one that could be indicative of enhanced cell injury at a systemic level. Normal lipid peroxidation levels, evaluated during the days on which no iron was administered, present large fluctuations. These are apparently unaffected by the factor of supplemental iron either alone or with oil (Figure 11). Based on numerical tendency, the administration of ferrous sulphate with oil appeared to increase the production of urinary TBARS (Figures 12 and 13), but there were no significant differences between any of the treatments by repeated measures ANOVA. The inability to demonstrate statistical significance may be due to the large intra-individual variations caused by the fluctuations of lipid peroxidation in these subjects. These findings indicate that oral supplementation with 120 mg of iron during seven days does not have any influence on

the lipid peroxidation in this population, at least as measured by the urinary excretion of biomarker, TBARS.

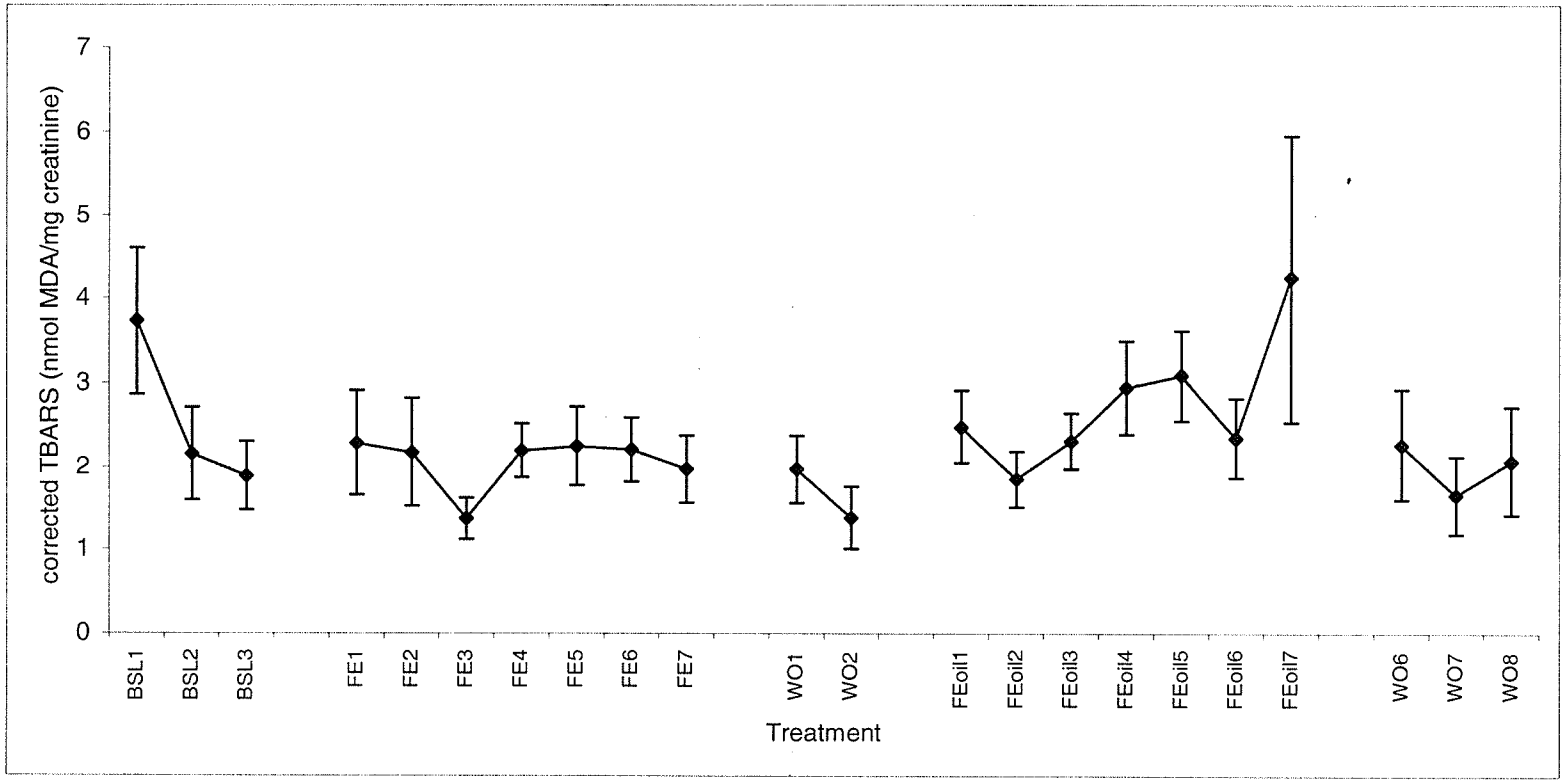

Figure 11. Average creatinine corrected TBARS expressed as nmol MDA/mg creatinine in urinary samples throughout phase 1 (N=5 subjects). Graph is not in real time scale as days on which no samples were collected are not shown. Y-axis error bars represent standard error of the mean (SEM). (BSL = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout). Repeated measures ANOVA show no significant differences between any of the treatment-phase-associated samples.

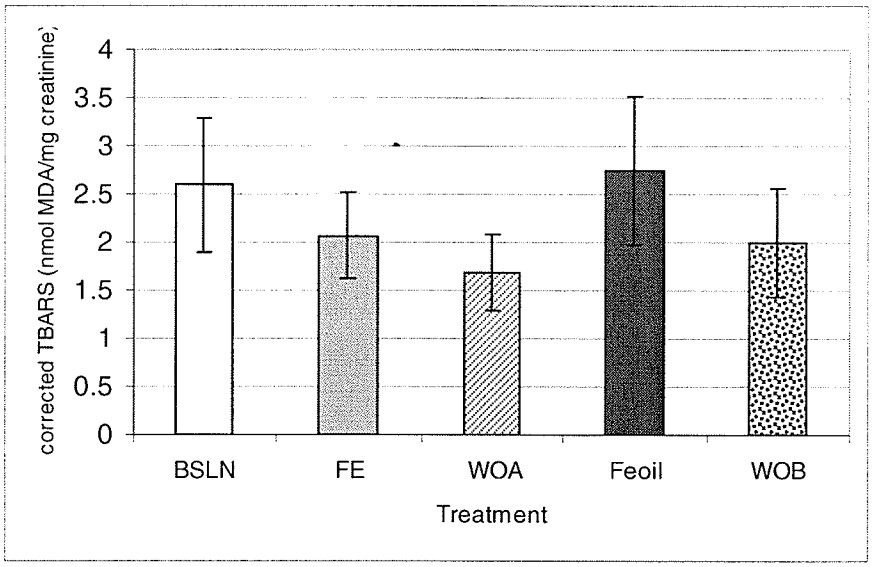

Figure 12. Total pooled average creatinine corrected TBARS for phase 1 (N=5 subjects). Each treatment bar represents the pooled average of all treatment days for the five subjects. Y-axis error bars represent SEM. BSLN = baseline, FE = 120 mg Fe, Feoil = 120 mg Fe + oil, WOA = WO1 + WO2, WOB = WO6 + WO7 + WO8. Because of unequal sample number, no statistical testing was performed.

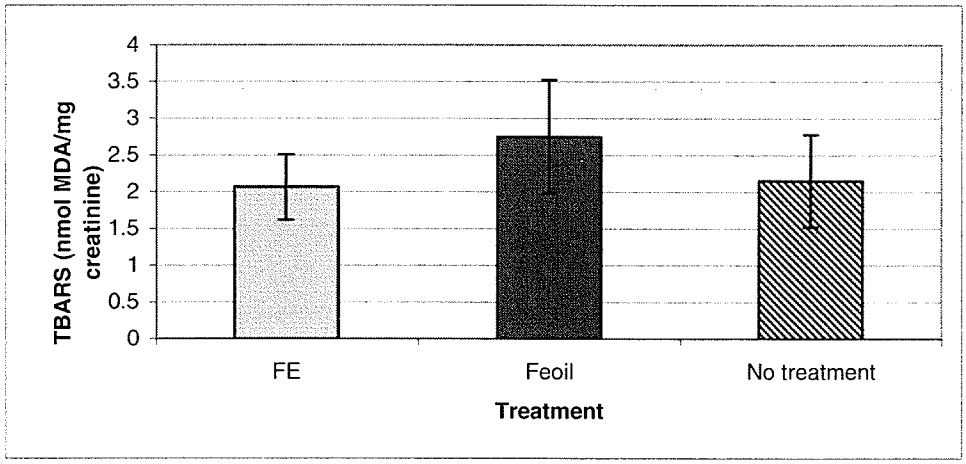

Figure 13. Total pooled average creatinine corrected TBARS for phase 1 (N=5 subjects) by individual treatment. Each treatment bar represents the pooled average of all treatment days for the five subjects. The "No treatment" bar is a pooled average of the three baseline days and all five washout days when no treatment was administered. In order to have the same number of days per treatment (7 days), we randomly eliminated one day were no treatment was administered from each subject to produce a weighed average. FE = 120 mg Fe, Feoil = 120 mg Fe + oil. Repeated measures ANOVA show no significant differences between treatments. Y-axis error bars represent SEM.

4.2.1.2 Findings from the definitive intervention trial

Initially, the definite intervention trial was designed to examine the effects of natural antioxidants from the CTCMC on a hypothesized and expected elevation of TBARS in response to supplemental iron exposure. It was also proposed that the CTCMC would act in a dose-dependent manner in suppressing iron-induced oxidation. To test these hypotheses, subjects were given two concentrations of CTCMC (0.4 and 0.8 g) together with the ferrous sulphate and refined palm oil. The results from this phase show the same fluctuations during baseline and supplementation with aqueous iron and iron in refined oil as in the pilot study (Figures 14, 15 and 16); this confirms the findings that supplemental iron with oil does not increase TBARS production. So, our testing of a hypothesis of attenuation by oral antioxidants of an iron-induced effect on systemic lipid peroxidation was made inoperative by the absence of the expected increase in TBARS to a 7-day course of 120 mg of iron.

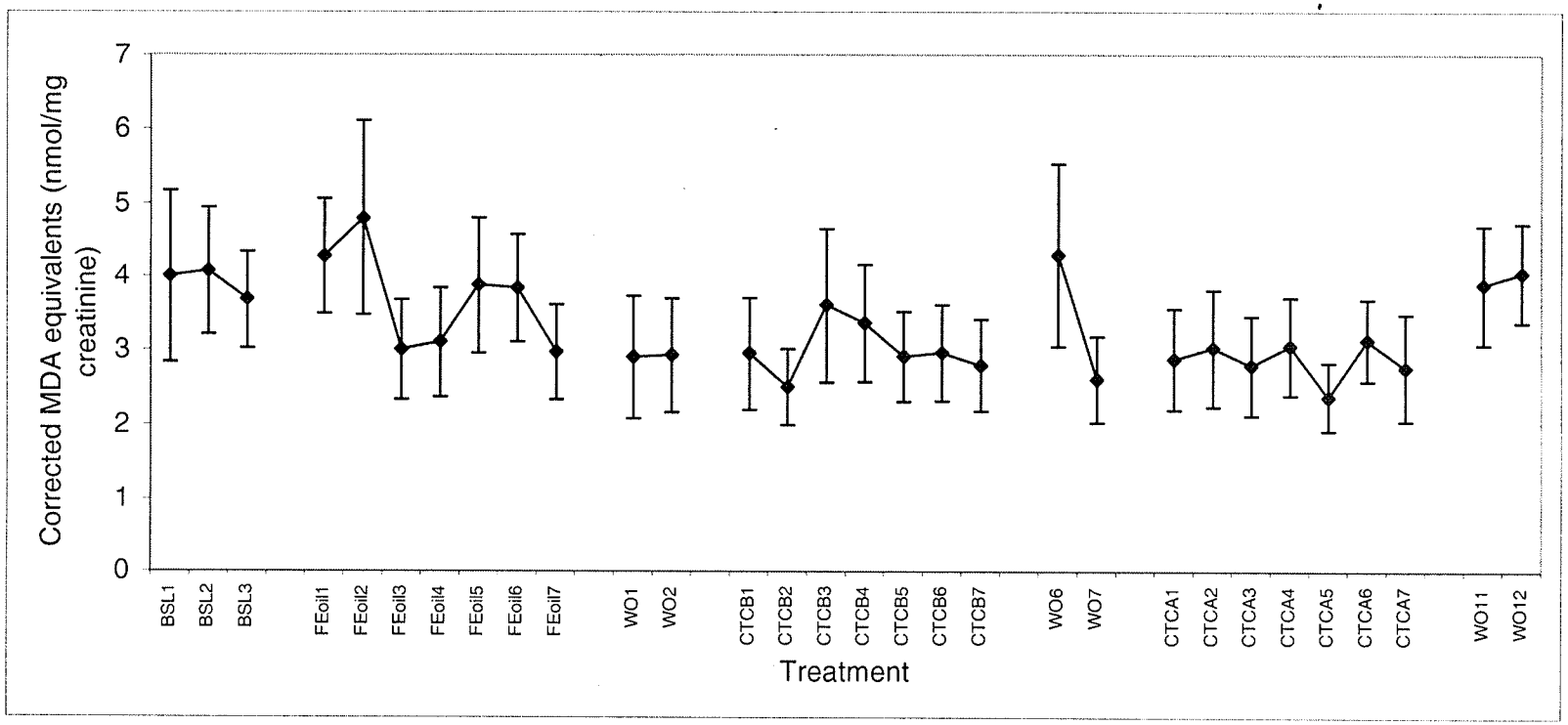

Figure 14. Average creatinine corrected TBARS in urinary samples throughout phase 2 (N = 12 subjects). Graph is not in real time scale as days on which no samples were collected are not shown. Y-axis error bars represent SEM. (BSL = baseline, FEoil = 120 mg Fe + vegetable oil, WO = washout, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC).

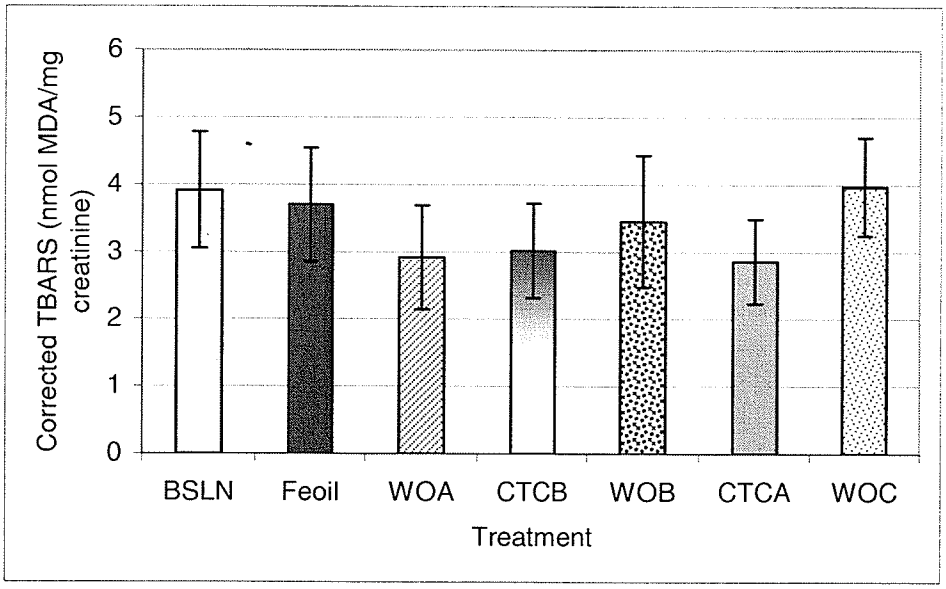

Figure 15. Total average corrected TBARS for phase 2 (N = 12 subjects) by individual treatment. Each treatment bar represents the pooled average of all treatment days for the twelve subjects (BSLN = baseline, Feoil = 120 mg Fe + vegetable oil, WOA = WO1 + WO2, WOB = WO6 + WO7, WOC = WO11 + WO12, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC). Because of unequal sample number, no statistical testing was performed. Y-axis error bars represent SEM.

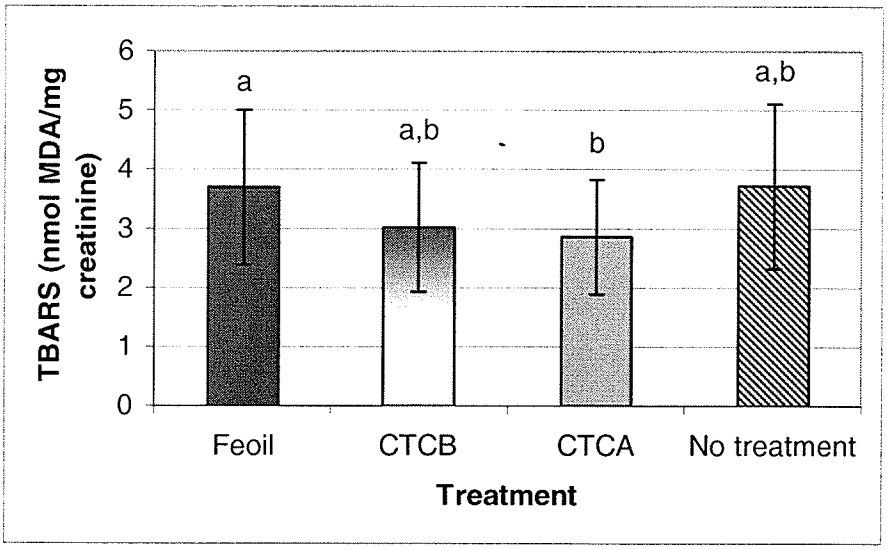

Figure 16. Total average creatinine corrected TBARS for phase 2 (N = 12 subjects) by individual treatment. Each treatment bar represents the pooled average of all treatment days for the twelve subjects (FEoil = 120 mg Fe + vegetable oil, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC). The "No treatment" bar is a pooled average of the three baseline days and all six washout days where no treatment was administered. In order to have the same number of days per treatment (7 days), we randomly eliminated two days on which no treatment was administered from each subject. Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM.

4.2.2 Oral Iron Alone does not influence DNA Oxidation Biomarkers (urinary 8OHdG)

To complement the information obtained from the TBARS analysis, another biomarker of systemic oxidation was used, the urinary 8-OHdG. This indicator reflects the average rate of systemic oxidative DNA damage (Chin *et al*, 2008). In the present study, it was intended to confirm other reports in which iron supplementation was associated with increased DNA damage (Schümann *et al*, 2007). We hypothesized that the ingestion of large doses of highly-bioavailable ferrous sulphate would augment DNA oxidation in this

population. The following sections present our findings on the influence of iron on the usual behavior of this biomarker.

The same urine samples used to assess TBARS were employed to quantify 8-OHdG in order to reduce variability between samples.

4.2.2.1 Findings from the pilot phase

Results form the pilot phase (Figure 17) show the same irregular behavior observed in TBARS. Repeated measures ANOVA demonstrate that supplemental iron in this dose has no significant effect on the excretion of urinary 8-OHdG. Even though the addition of oil to the iron dose tended to increase the value of urinary of 8-OHdG, this elevation was not significant (Figures 18 and 19).

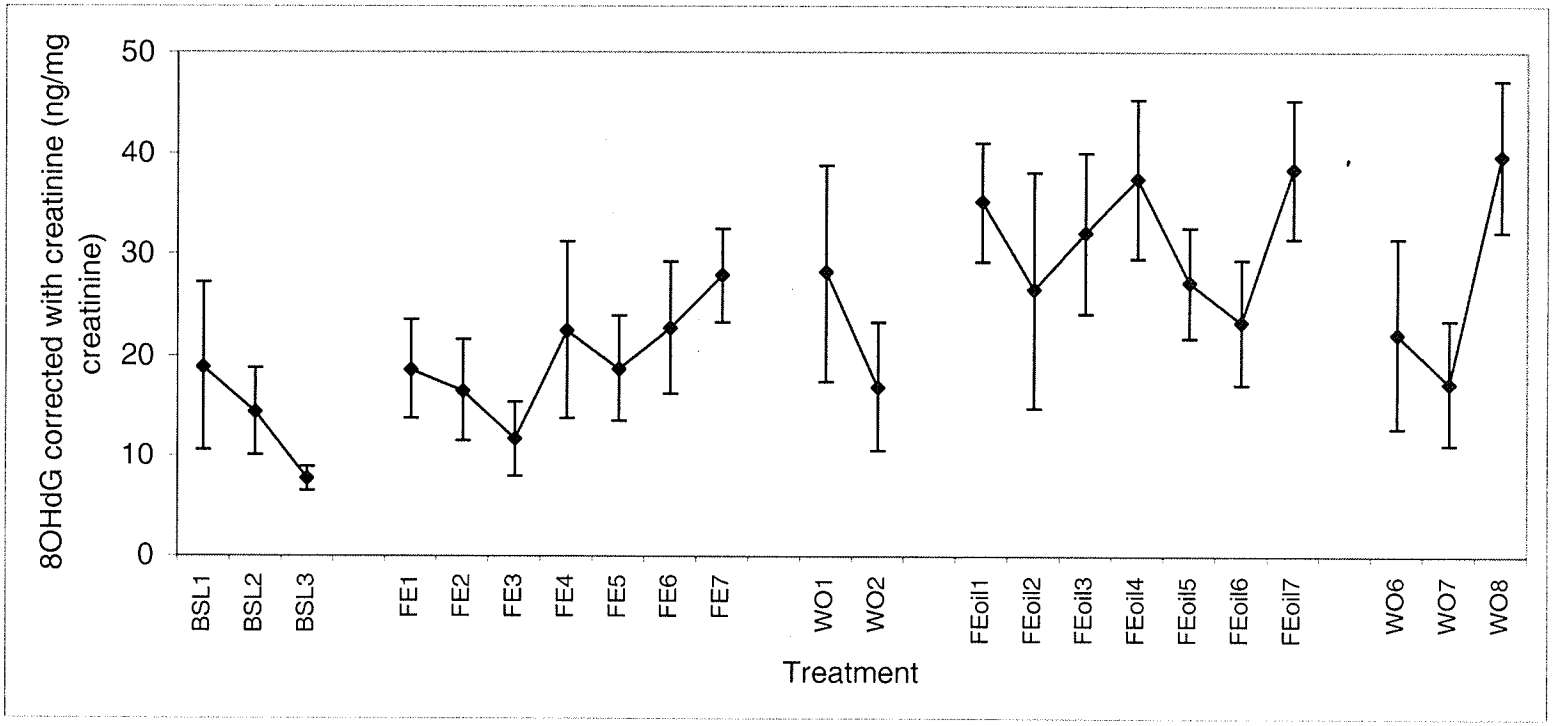

Figure 17. Average creatinine corrected 8-OHdG in urinary samples throughout phase 1 (N = 5 subjects). Graph is not in real time scale, days where no samples were collected are not shown. Y-axis error bars represent SEM. BSL = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout.

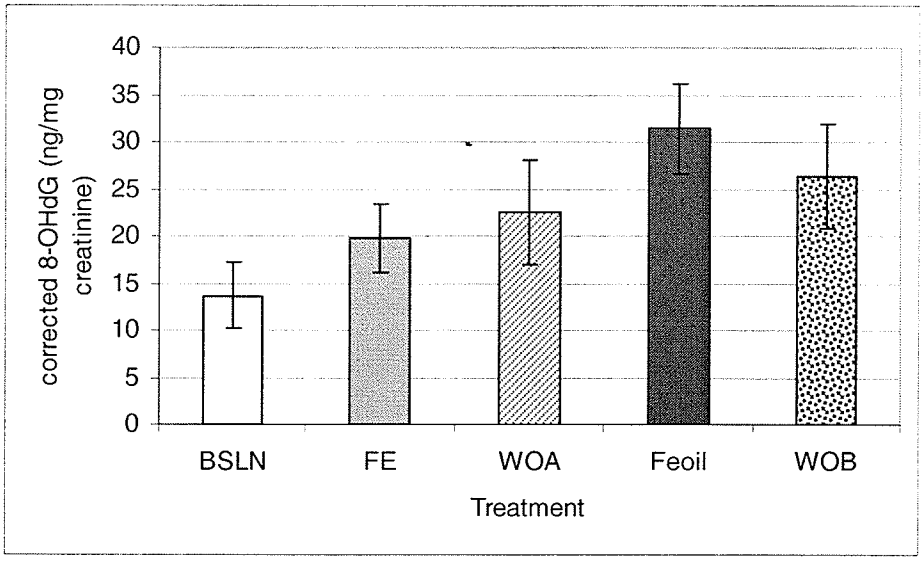

Figure 18. Total creatinine average corrected 8-OHdG for phase 1 (N = 5 subjects). Each treatment bar represents the pooled average of all treatment days for the five subjects (BSLN = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WOA = WO1 + WO2, WOB = WO6 + WO7 + WO8). Because of unequal sample number, no statistical testing was performed. Y-axis error bars represent SEM.

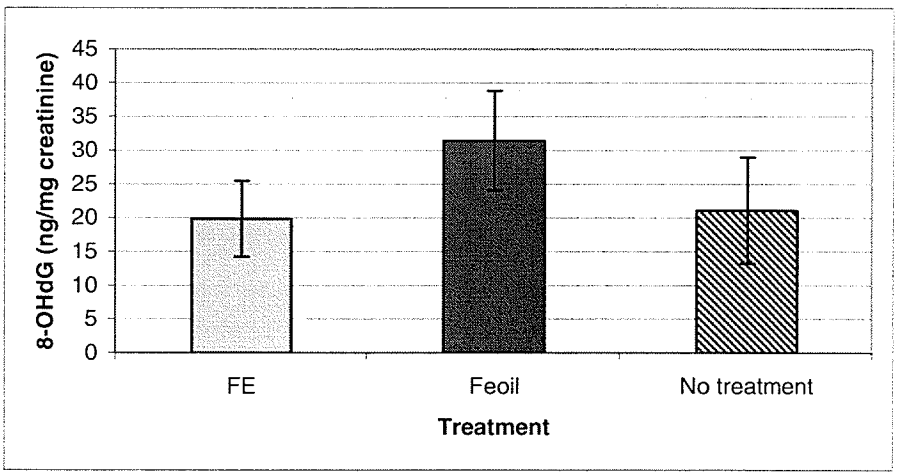

Figure 19. Total pooled average corrected 8-OHdG for phase 1 (N = 5 subjects) per individual treatment. Each treatment bar represents the pooled average of all treatment days for the five subjects (FE = 120 mg Fe, Feoil = 120 mg + vegetable oil). The "No treatment" bar is a pooled average of the three baseline days and the five washout days where no treatment was administered. In order to have the same number of days per treatment (7 days), we randomly eliminated one day on which no treatment was administered from each subject to produce a weighed average. Repeated measures ANOVA show no significant differences between treatments. Y-axis error bars represent SEM.

4.2.2.2 Findings from the definitive intervention trial

Decreased excretion of 8-OHdG may be an indicator of reduced DNA oxidation (Chin *et al*, 2008). According to the observations from this phase, there are no significant differences between days were no treatment was administered and days when iron was supplied that might be indicative of oxidative damage caused by iron. Again, the results from the pilot study are confirmed, reinforcing our findings that supplemental iron in this dose is incapable of influencing systemic oxidation when measured as an increased production of TBARS and 8-OHdG (Figure 20). The CTCMC seems to have a suppressing effect on habitual oxidation though, that not only decreases the levels of 8-OHdG on days with no iron challenge (Figures 21 and 22), but attenuates the inter-subject variations observed during each CTCMC treatment day (observed as a reduction on the standard error).

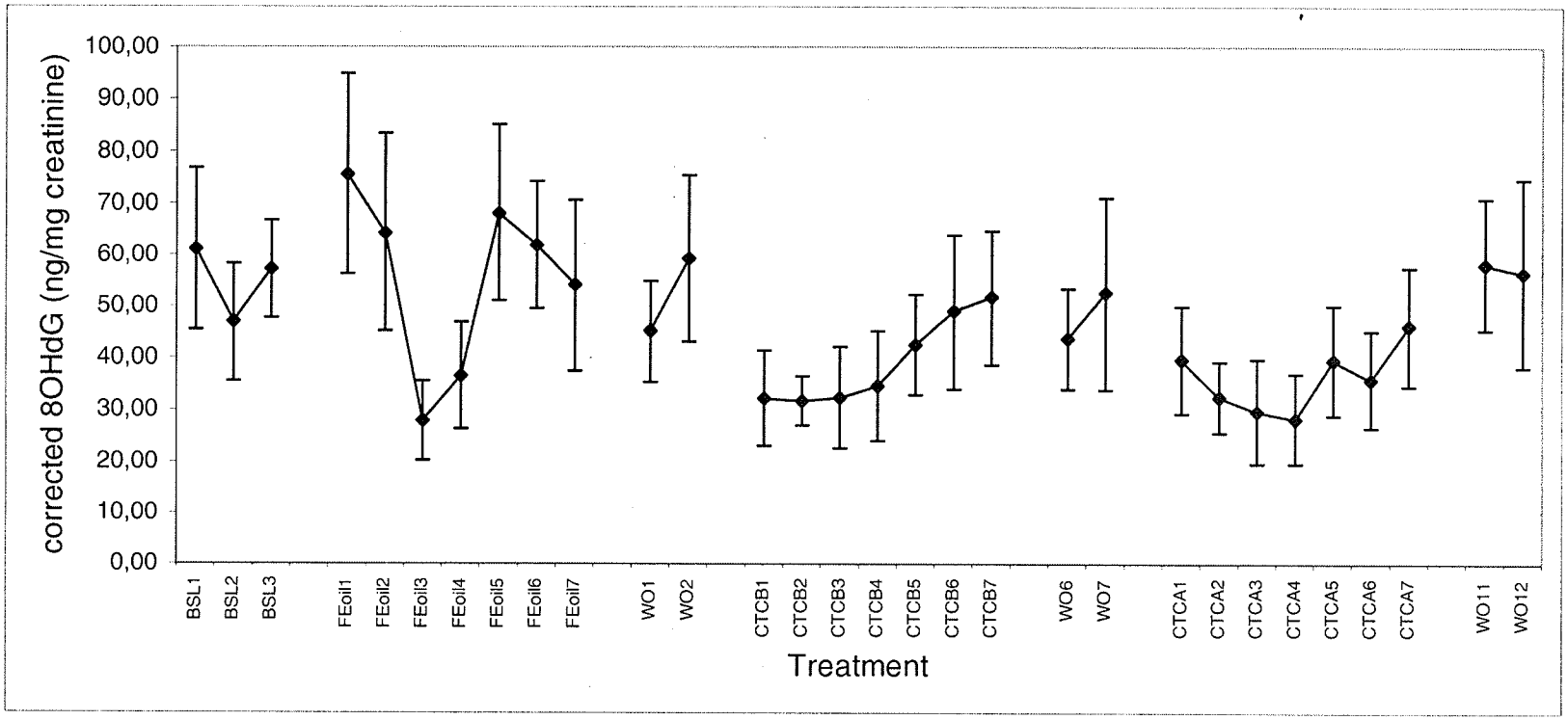

Figure 20. Average creatinine corrected 8-OHdG in urinary samples throughout phase 2 (N = 12 subjects). Graph is not in real time scale, days where no samples were collected are not shown. Y-axis error bars represent SEM. (BSL = baseline, FEoil = 120 mg Fe + vegetable oil, WO = washout, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC).

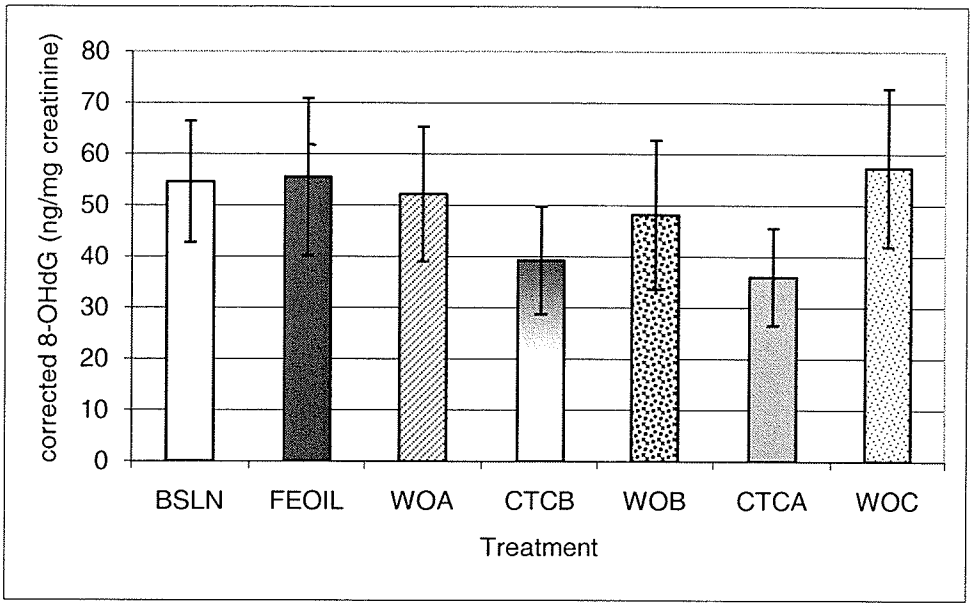

Figure 21. Total average creatinine corrected 8-OHdG for phase 2 (N = 12 subjects) per individual treatment. Y-axis error bars represent SEM. Each treatment bar represents the pooled average of all treatment days for the twelve subjects (BSLN = baseline, FEoil = 120 mg Fe + vegetable oil, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC, WOA = WO1 + WO2, WOB = WO6 + WO7, WOC = WO11 + WO12. Because of unequal sample number, no statistical testing was performed.

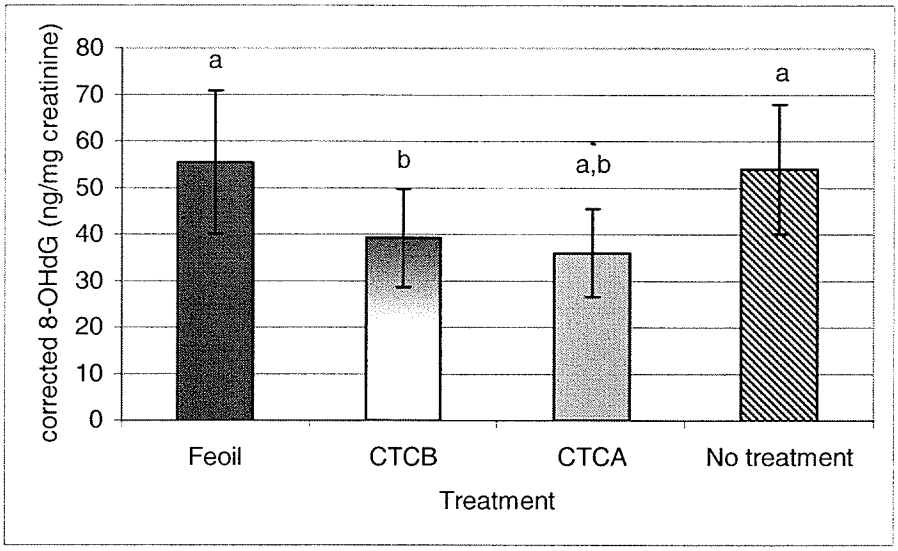

Figure 22. Total average corrected 8-OHdG for phase 2 (N = 12 subjects) per individual treatment. Each treatment bar represents the pooled average of all treatment days for the twelve subjects (FEoil = 120 mg Fe + vegetable oil, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC). The "No treatment" bar is a pooled average of the three baseline days and the six washout days where no treatment was administered. In order to have the same number of days per treatment (7 days), we randomly eliminated two days were no treatment was administered from each subject to produce a weighed average. Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM

4.2.3 Summary Discussion

It has been reported in several studies that iron supplementation increases biomarkers of oxidative stress (TBARS and 8-OHdG) in humans and rats (Schümann *et al*, 2007). In a particular study with three German university students, responses to iron supplementation were observed with doses of 120 mg Fe/day, administered on seven consecutive days. Even though they were not pathological, they were seven-fold above baseline values (Schümann *et al*, 2005).

In the present study, iron supplementation with 120 mg Fe/d alone or with oil had no significant effect on the oxidation biomarkers. When the antioxidant mix from the

CTCMC is introduced into the experimental model, significant attenuating effects of *in vivo* oxidation were observed. The CTCMC was capable of reducing DNA oxidation below normal values measured during days were no treatments were administered, indicating that this product is capable of modifying normal oxidative stress. The apparent disagreement between the concentration of CTCMC that causes a reduction in iron-induced oxidation observed in both TBARS and 8-OHdG assays may be explained again by intra- and inter-individual variations, but at the same time they provide strong evidence that the CTCMC modifies positively the oxidation response to supplemental iron.

The question arises as to why our 7-day oral challenge with 120 mg of iron failed to reproduce the same manner of response in 5 Guatemalan healthy adults that it had produced in three German university students (Schümann *et al*, 2005). Differences in the environmental conditions between both countries could explain the singular responses to the oxidation challenge. Toxicological research studies have confirmed the association between particulate air pollution and increased generation of ROS (Stone *et al*, 2007). It is possible that the Guatemalan subjects, who live in a highly polluted environment, are normally under elevated oxidative stress and may have reached a plateau; in consequence they are unaffected by the iron challenge.

In general, the findings on urinary biomarkers from the small pilot study of five volunteers and two experimental treatments were consistently confirmed in the larger, definitive study of 12 volunteers through three treatments.

4.3 Findings related to the hypothesis of oral iron favoring in situ intra-intestinal oxidation and Carotino Tocopherol-Carotene Mixed Concentrate attenuating iron-induced oxidation

A method by Owen and colleagues (2000) was modified and validated to measure the buffering capacity of fecal matter against free radical production. Feces are capable of sustaining Fenton-type reactions that lead to the formation of highly reactive oxygen species that can potentially harm colonocytes (Lund et al, 1999). The presence of significant concentrations of non-absorbed non-heme iron in the gut may deplete the buffering capacity of the feces and generate overwhelming concentrations of ROS that can increase oxidative damage in the colon.

Catechol, 2,5 DHBA and 2,3 DHBA are chemical compounds that result from the free radical attack to salicylic acid and will serve as markers of increased free radical production in fecal matter. To validate the method by Owen and collaborators and ensure that this fecal biomarker would be adequate for the purposes of this study, two analytical quality parameters were determined: the limit of detection (LOD) and limit of quantification (LOQ) (refer to methods section for definitions); the results are shown in Table 12. Since the limit of quantification for catechol is too high, catechol was excluded from the analysis. Final results are reported as the sum of the concentrations of 2,3 DHBA and 2,5 DHBA instead.

Table 12. Limits of detection (LOD) and of quantification (LOQ) for the three standard compounds used in the determination of total hydroxylated products in feces by HPLC. Parameters were used for the validation of the HPLC method used in phases 1 and 2 of the study.

	Catechol	**2,5 DHBA**	**2,3 DHBA**
LOD (mg/ml)	1.10×10^{-4}	1.64×10^{-4}	1.67×10^{-4}
LOQ (mg/ml)	0.3411	5.49×10^{-3}	0.0100

A major challenge of adapting the original protocol to the local conditions of the laboratory was that the original method used a diode array detector and the available HPLC equipment just had a UV-Vis detector. This was solved by changing the programmed wavelength of the detector from 278 to 301 nm 5.5 minutes after the run had started. This allowed us to detect the peaks for 2,3 and 2,5 DHBA, but interfered with the catechol signal (Figure 24). The latter constituted another reason to eliminate catechol from the final analysis. Figures 23 and 24 show chromatograms of standards and a fecal sample, good resolution was accomplished under these analytical conditions. These results demonstrate that the validated method is capable of detecting free radical production in the fecal matter under these circumstances, and though, can be used as an oxidation fecal biomarker in a human metabolic context. CAMBIAR FIGURAS 22 Y 23!!

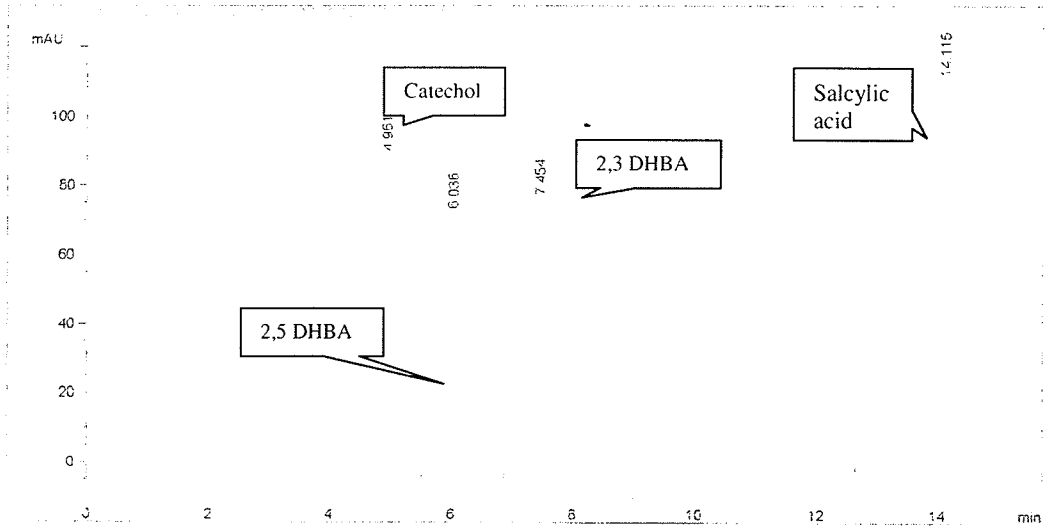

Figure 23. HPLC chromatogram showing the retention times of salicylic acid and the three standard compounds used in the quantification of total hydroxylated products in fecal matrix.

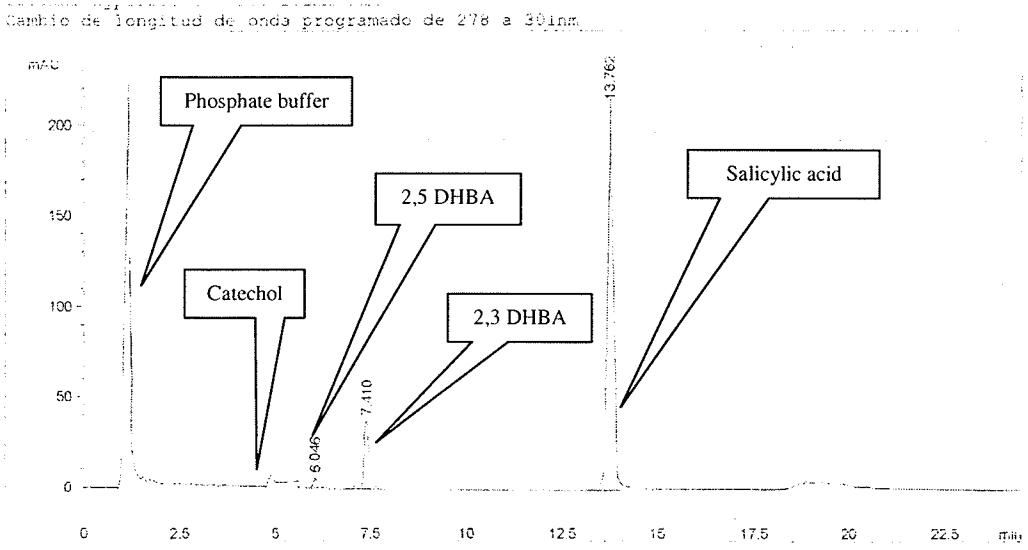

Figure 24. HPLC chromatogram of a feces sample showing the chromatographic peaks of the hydroxylated products of interest: catechol, 2,3 and 2,5 DHBA. Chromatogram also shows the peaks for phosphate buffer and excess salicylic acid.

4.3.1 Pilot studies on the Influence of Lipid Matrix in the Oral Dose

Iron overload due to excessive oral iron intake in healthy individuals is very rare, because iron absorption is tightly regulated by multiple mechanisms in the organism (Yip, 2001, Schümann *et al*, 2007). Iron overload occurs when there is a deficient regulation of gastrointestinal absorption that leads to excess iron accumulation (Yip, 2001). Other type of iron overload disease results from hematological disorders that require frequent blood transfusions or from excess iron accumulation as a consequence of stimulation from severe anemia (Yip, 2001). In healthy persons, iron intestinal uptake is determined by total body iron reserves (Yip, 2001; Hallberg *et al*, 1997); with replete iron stores, the absorption of dietary iron at the intestinal level is decreased and excess residual iron remains in the intestinal lumen (Schümann *et al*, 2007). Some studies have successfully demonstrated that residual iron in the feces is capable of producing free radicals *in vitro* (Owen *et al*, 2000) that possibly increase oxidative stress at the intestinal lumen (Lund *et al*, 1998, 1999, and 2000). One of the purposes of this study was to evaluate whether an *in vivo* oral-iron-challenge with 120 mg of supplemental iron would produce considerable modifications in the normal production of free radicals in the intestines that could be measured in fecal samples.

Results from the individual and average production of free radicals throughout the supplementation study show a shift in the production of free radicals with iron administration, a decline in the washout period and a new raise when subjects received a new series of iron supplement with refined oil (Figures 25 and 26), all of the above points out to a possible iron effect that enhances *in situ* oxidation. Individual responses

from subjects 001, 002, 003, and 005 are very similar under conditions of no iron. When iron is administered, stronger disturbances are noticed, particularly in subject 005. The different individual responses caused by iron may be influenced by highly variable factors such as dietary fiber consumption (Jacobs *et al*, 2007), unabsorbed antioxidants that quench free radical production (Stone *et al*, 2004), residual heme-iron (Tappel, 2007) and presence of residual iron from other sources than the supplement.

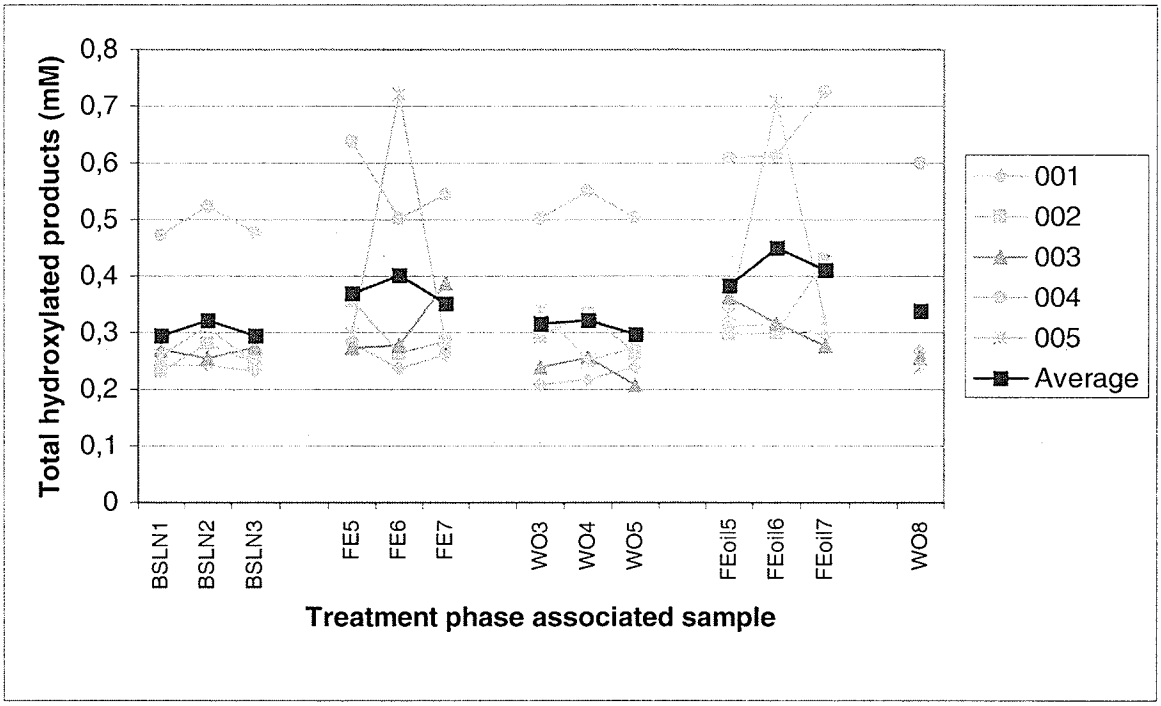

Figure 25. Individual (subject-by-subject) and average production of free radicals measured as total hydroxylated products resulting from the free radical attack on salicylic acid in fecal matrix for phase 1 (N = 5 subjects). Graph is not in real time scale, days where no samples were collected are not shown. Subjects coded from 001 – 005. (BSLN = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout).

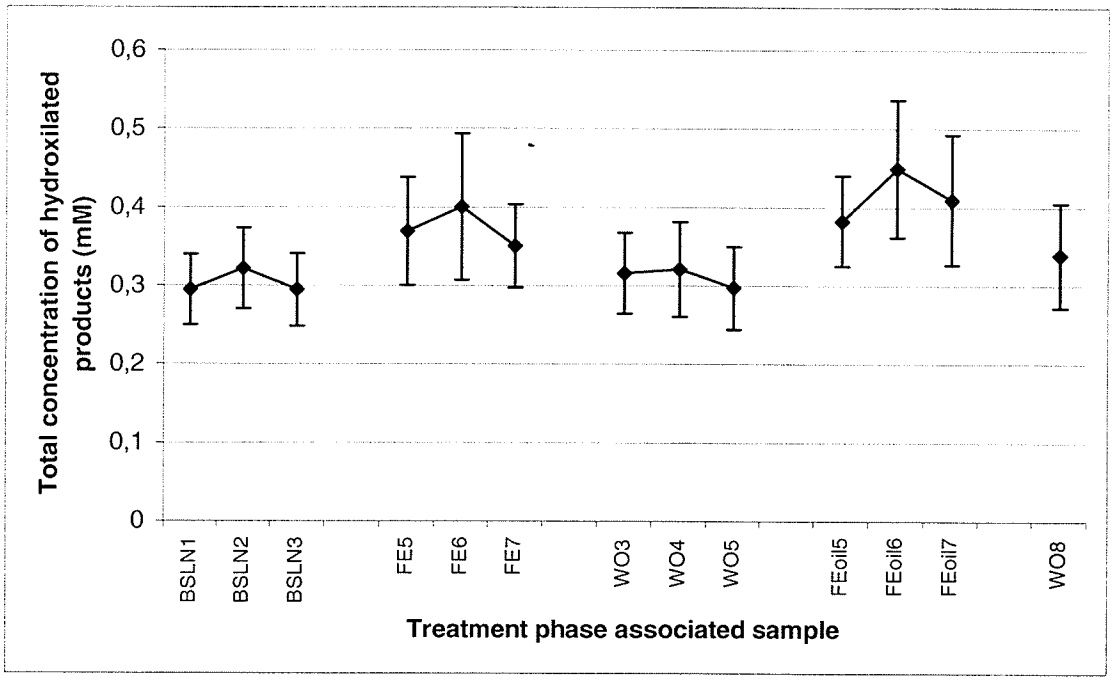

Figure 26. Average production of free radicals measured as total hydroxylated products present in fecal samples of all participants in phase 1 (N = 5 subjects). Graph is not in real time scale, days where no samples were collected are not shown. Y-axis error bars represent SEM. (BSLN = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout).

Analysis of free radical production comparing both iron treatments with baseline (Figure 27) show that ferrous sulphate, alone or with oil, increases the formation of hydroxylated products in the fecal matrix ($P<0.05$), which is indicative of enhanced free radical production. The addition of refined palm oil leads to a significant higher levels of free radical production ($p = 0.043$) than when iron is administered alone (Figure 28). With increased oxidative stress at the intestines, the fecal matrix's buffering capacity is reduced; exposing the intestinal lumen to hydroxyl radical's attack that could cause cell damage.

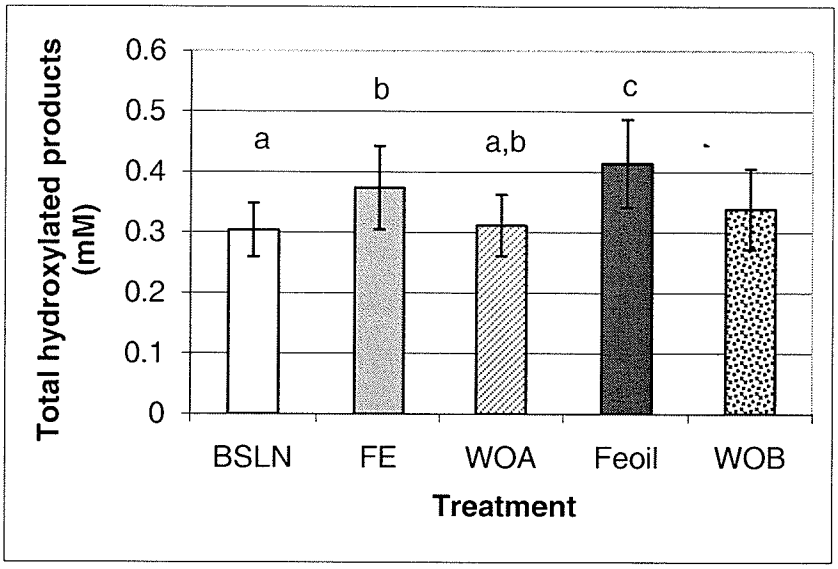

Figure 27. Total pooled average production of ROS per treatment measured as total hydroxylated products present in fecal samples from all participants in phase 1 (N = 5 subjects). Each treatment bar represents the pooled average of all treatment days for the five subjects (BSLN = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WOA = WO3 + WO4 + WO5, WOB = WO8). Repeated measures analysis was done without the WOB treatment day because of unequal sample number. Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM

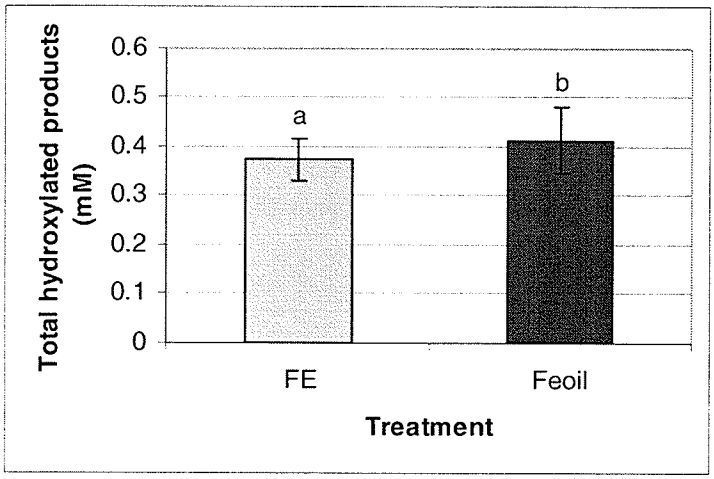

Figure 28. Total pooled average production of ROS per treatment measured as total hydroxylated products present in fecal samples of all participants in phase 1 (N = 5 subjects). Each treatment bar represents the pooled average of all treatment days for the five subjects (FE = 120 mg Fe, Feoil 120 mg Fe + vegetable oil). Y-axis error bars represent SEM. Repeated measures ANOVA indicate that both treatments are significantly different from each other ($p < 0.05$).

The summation of change with respect to baseline expressed through deltas was calculated in two ways: 1. as the difference between an average pooled value from baseline and washout days when no iron was supplied and individual days were iron was administrated (Figure 29), or 2. as a pooled treatment where all the treatment days were averaged and compared to the previously calculated habitual value with no iron supplementation (Figure 30). Changes from baseline in a day-to-day basis are not statistically significant, however, when treatment days are pooled, ROS production is significantly higher during the FEoil treatment.

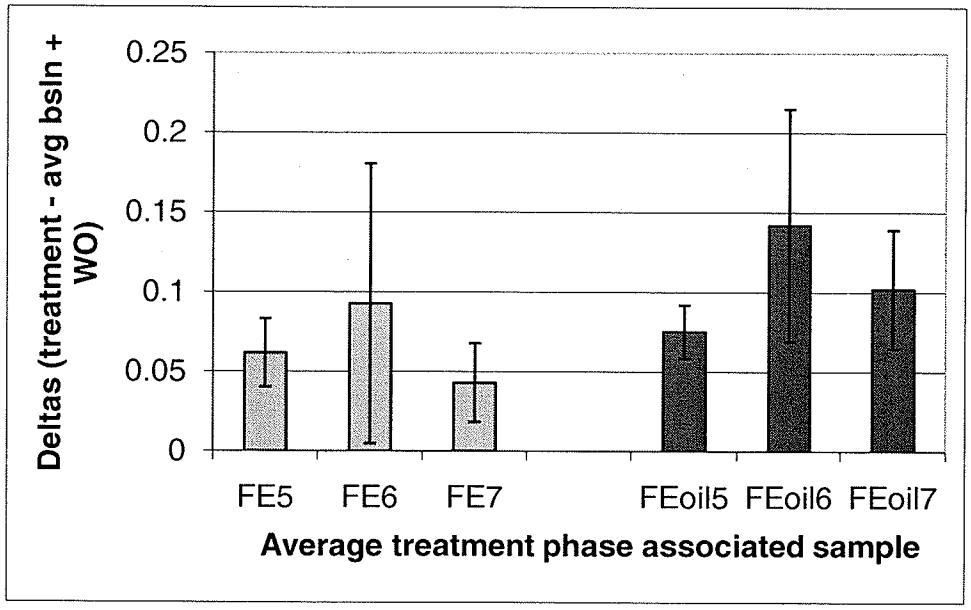

Figure 29. Difference (expressed as deltas) between ROS production in fecal samples collected during FE and FEoil treatments and ROS production in fecal samples of a pooled average of baselines and washouts days for phase 1 (N = 5 subjects). Deltas for each average iron and iron + vegetable oil treatment day (FE5, FE6, FE7, FEoil5, FEoil6 or Feoil7) were calculated as the average of the treatment day minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and three washout samples (WO3, WO4 and WO5). Y-axis error bars represent SEM. Repeated measures ANOVA show no significant differences between treatment days.

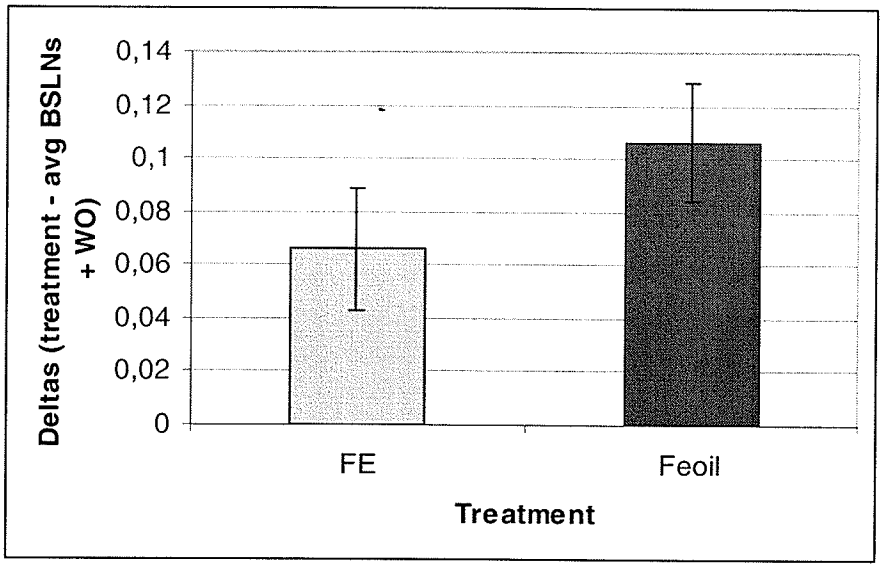

Figure 30. Difference (expressed as deltas) between ROS production in fecal samples collected during FE and FEoil treatments and ROS production in fecal samples of a pooled average of baselines and washouts days for phase 1 (N = 5 subjects). Deltas for each average iron and iron + vegetable oil treatment (FEoil, CTCB and CTCA) were calculated as the average of all treatment days minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and three washout samples (WO3, WO4 and WO5). Y-axis error bars represent SEM. Both treatments are significantly different from each other ($p < 0.05$)

4.3.2 Findings from the Definitive Intervention Trial

Results from the pilot phase helped us understand how large doses of iron affect the normal oxidation buffering capacity of feces from healthy individuals. It has been established that the iron-induced challenge used in this work produces significantly higher amounts of ROS in the fecal matter that can be quantified in a precise way. The following analyses will provide evidence that could support the theory that supplementing antioxidants along with ferrous sulphate will restore the feces normal buffering capacity, depleted by iron supplementation.

In this phase, twelve participants underwent three cycles of supplementation in which they received iron with oil alone or with two concentrations of CTCMC (0.4 and 0.8 g). It was intended to determine if the CTCMC provides antioxidant protection and, if so, if it was in a dose-dependent manner.

A higher basal level of ROS production was recorded on the fourth day of iron supplementation with oil ($p < 0.001$) (Figures 31 and 32), indicative of an iron-stimulated oxidation effect, thus confirming the findings from the pilot study. Eleven days after the last iron + oil dose had been administered, the generation of hydroxylated products during the washout period had dropped significantly ($p < 0.001$) and was even lower than baseline values ($p = 0.01$). It is not known exactly when residual iron from supplementation is cleared from the colon, but a washout period of 14 days has been proven to be sufficient to eliminate the possible effects of experimental iron doses in this trial. The results from the last three days of ferrous sulphate plus CTCMC administration in either concentration of 0.4 or 0.8 g achieve baseline values and do not reach oxidation levels of iron supplementation alone (P=0.002 and P=0.014, respectively), suggesting a protection by CTCMC supplementation against the antioxidant depletion caused by iron supplementation. There is no difference between using a low or a high dose of CTCMC (Figure 33), and this could be beneficial because high amounts of CTCMC are not required to achieve a significant antioxidant effect. With this information it is impossible to determine the minimum required dose of CTCMC necessary to observe antioxidant activity.

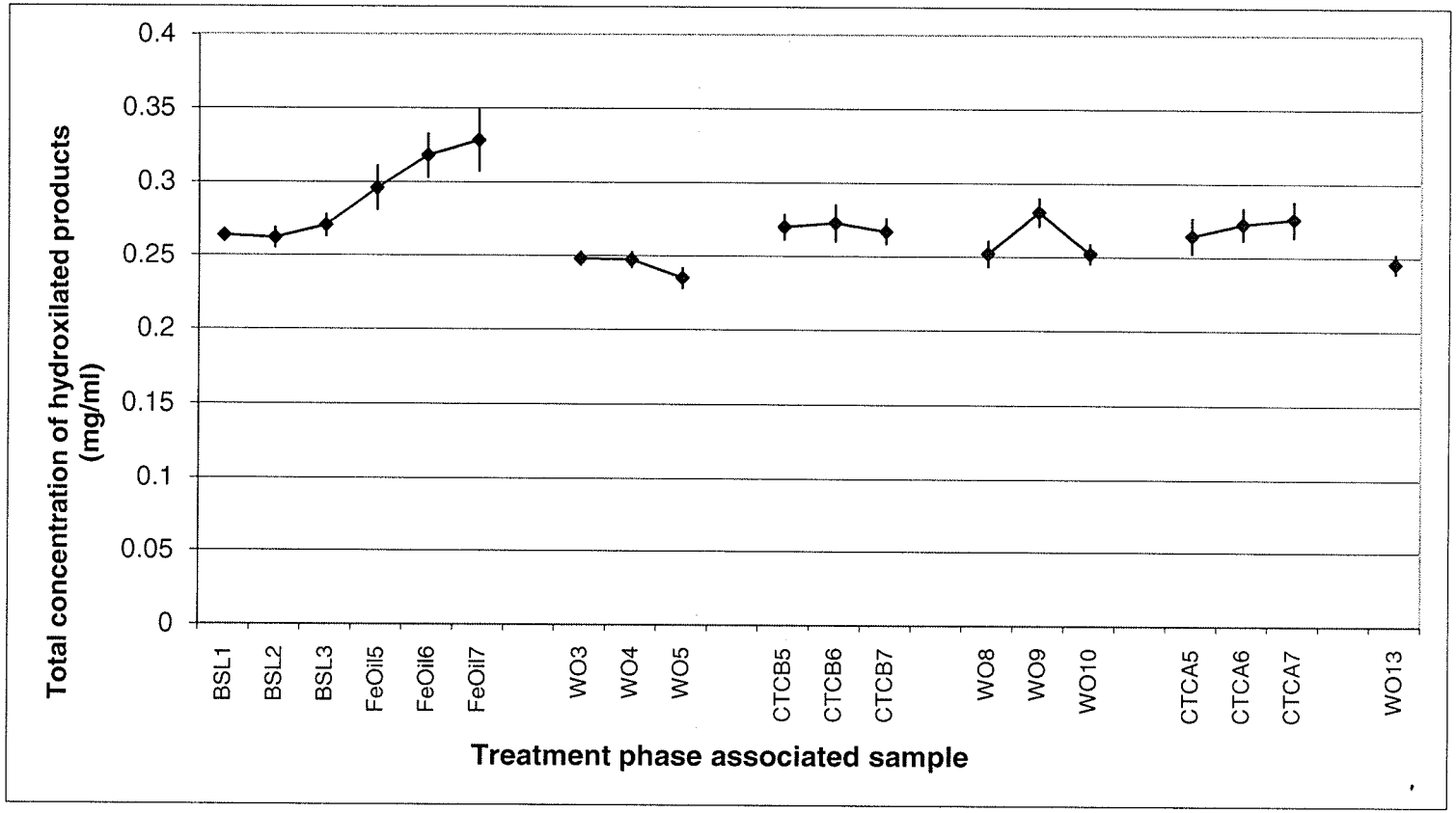

Figure 31. Average production of total hydroxylated products in fecal matrix for participants in phase 2 (N = 12 subjects). Graph is not in real time scale, days where no samples were collected are not shown. Y-axis error bars represent SEM. (BSL = baseline, FEoil = 120 mg Fe + vegetable oil, WO = washout, CTCB = 0.4g CTCMC, CTCA = 0.8 g CTCMC).

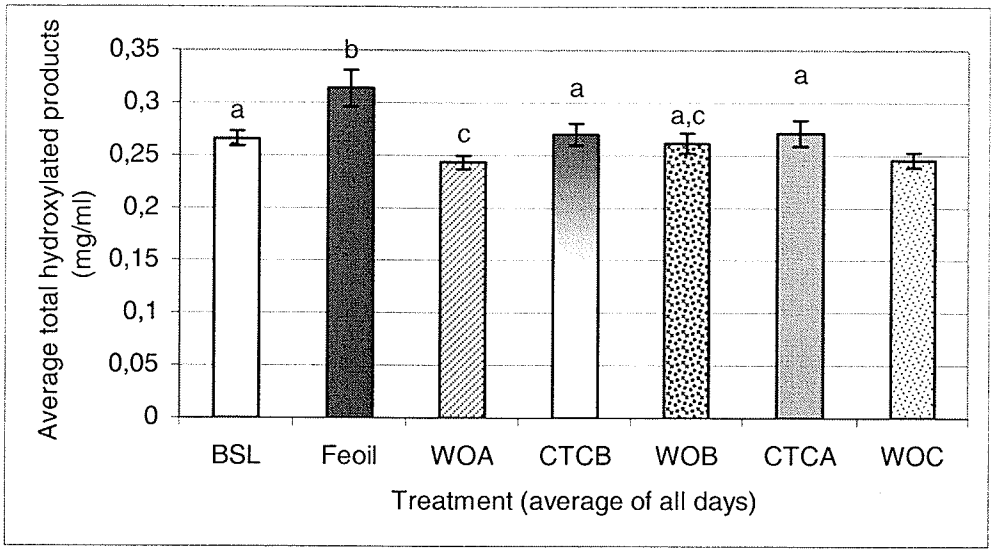

Figure 32. Average total hydroxylated products per individual treatment. Each treatment bar represents the pooled average of all treatment days for the twelve subjects (BSL = baseline, FEoil = 120 mg Fe + vegetable oil, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC, WOA = WO3 + WO4 + WO5, WOB = WO8 + WO9 + WO10, WOC = WO13). Repeated measures analysis was done without the WOC treatment day because of unequal sample number. Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM.

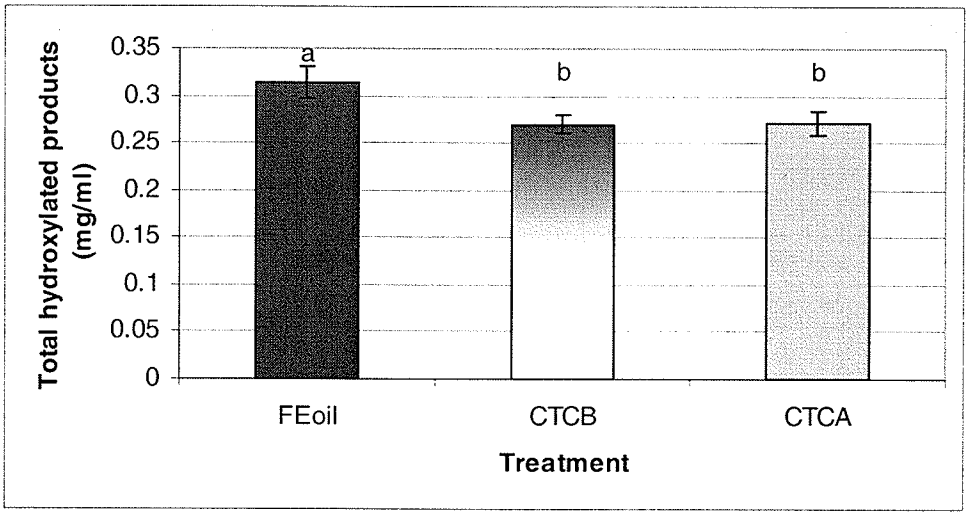

Figure 33. Total pooled average production of ROS measured as total hydroxylated products present in fecal samples of all participants in phase 2 (N = 12 subjects) per treatment. Each treatment bar represents the pooled average of all treatment days for the five subjects (FEoil = 120 mg Fe + vegetable oil, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC). Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM.

While examining individual responses to supplementation (Figure 34), we noticed that individual fluctuations and inter-subject variability were magnified when iron was given to the participants. Antioxidants from the CTCMC seem to not only attenuate these deviations but also flattened them.

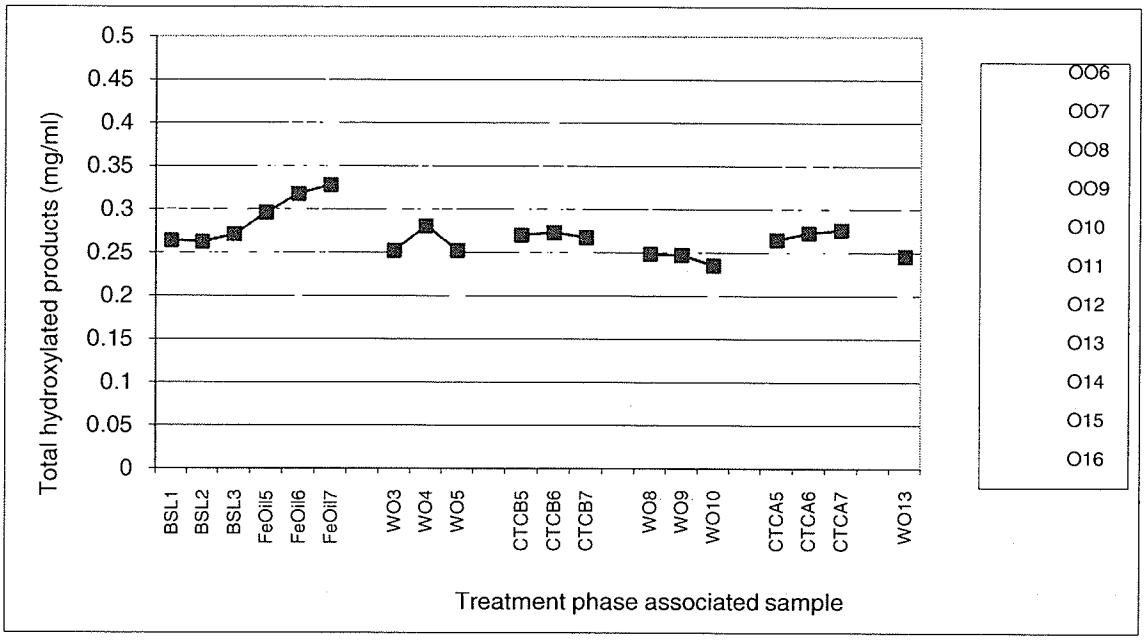

Figure 34. Individual and average production of ROS measured as total hydroxylated products resulting from the free radical attack on salicylic acid in fecal matrix for phase 2 (N = 12 subjects). Graph is not in real time scale, days where no samples were collected are not shown. Series in gray represent individual subjects, whereas the series in black is the average production of free radicals for the 12 participants. (BSL = baseline, FEoil = 120 mg Fe + vegetable oil, WO = washout, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC).

There are no differences in free radical production between individual days, when measured as changes between the iron treatment and days when no iron was supplied (Figure 35), or when the deltas were calculated using a pooled baseline that included washout values as well (Figure 36).

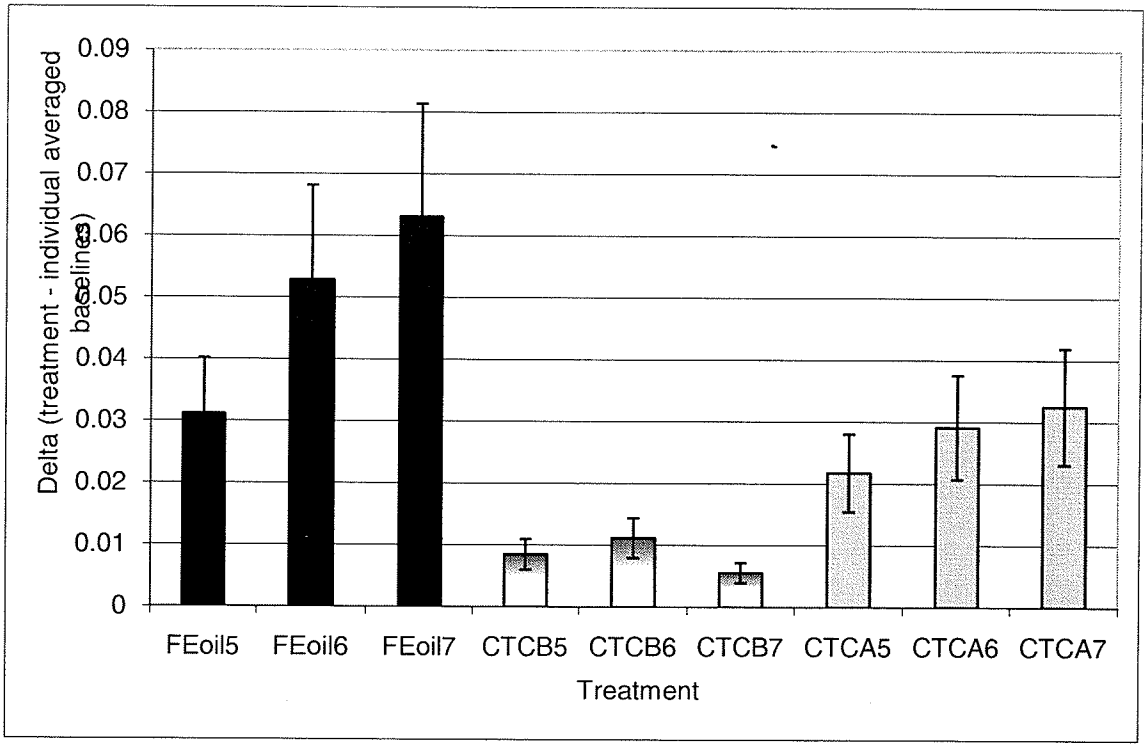

Figure 35. Difference (expressed as deltas) between free radical production in fecal samples collected during FEoil, CTCB and CTCA treatments and the free radical production in fecal samples of baseline or washout days for phase 2 (N = 12 subjects). Deltas for each iron treatment day (FEoil5, FEoil6 or FEoil7) were calculated as the average of the iron treatment minus the average of the three baseline samples (BSLN1, 2 and 3). Deltas for each CTCB treatment day (CTCB5, CTCB6 or CTCB7) were calculated by subtracting the average of each treatment day minus the pooled average of the three washout days WO3, WO4 and WO5. Deltas for CTCA were calculated in the same manner as CTCB, except that the pooled average used was done with washout days WO8, WO9 and WO10. CTCB = 0.4 g CTCMC and CTCA = 0.8 g CTCMC. Y-axis error bars represent SEM.

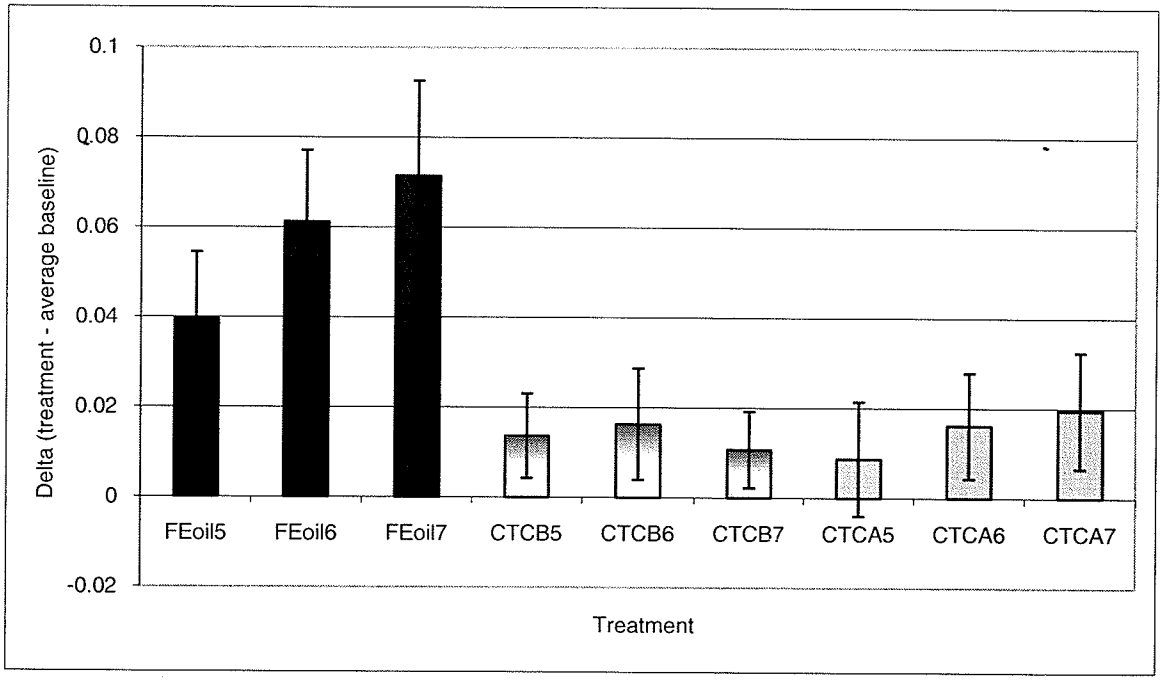

Figure 36. Difference (expressed as deltas) between ROS production in fecal samples collected during FEoil, CTCB, and CTCA treatments and the ROS production in fecal samples of a pooled average of all baseline and washout days for phase 2 (N = 12 subjects). Deltas for each iron and CTCMC treatment day (FEoil5, FEoil6, FEoil7, CTCA5,CTCA6, CTCA7, CTCB5, CTCB6 and CTCB7) were calculated as the average of the treatment day minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and the six washout samples (WO3, WO4, WO5, WO8, WO9, and WO10). CTCB = 0.4 g CTCMC and CTCA = 0.8 g CTCMC. Y-axis error bars represent SEM.

Deltas using pooled values of all treatment days show significant differences between the FEoil and CTCB and CTCA treatments though (Figure 37). Nevertheless, there is no difference in ROS production between the CTCB and CTCA treatments.

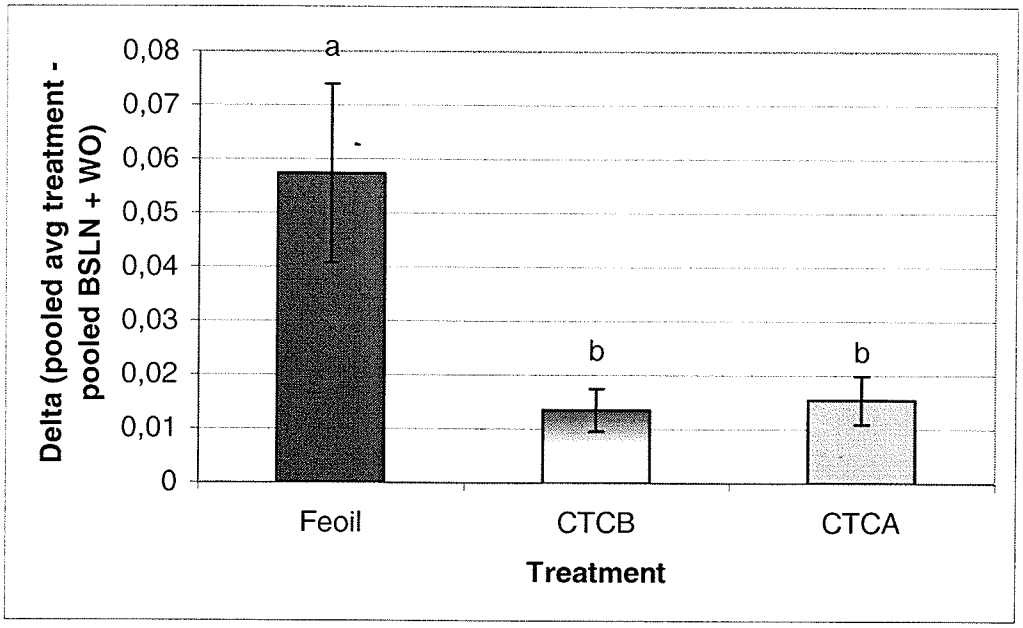

Figure 37. Difference (expressed as deltas) between ROS production in fecal samples collected during FEoil, CTCA and CTCB treatments and ROS production in fecal samples of a pooled average of baselines and washouts days for phase 2 (N = 12 subjects). Deltas for each average FEoil, CTCA and CTCB treatment were calculated as the average of all treatment days minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and the six washout samples (WO3, WO4, WO5, WO8, WO9, and WO10). CTCB = 0.4 g CTCMC and CTCA = 0.8 g CTCMC. Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM.

4.3.3 Summary Discussion

Iron's ease to participate in redox reactions enables it to produce ROS that may be involved in the etiology of several human diseases such as colorectal cancer (Owen *et al*, 2000; Lund *et al*, 1999; 2000), Crohn's disease (Tapia and Araya, 2006), and ulcerative colitis (Seril *et al*, 2005); thus making it a relevant risk factor (Glei *et al*, 2002). High levels of dietary iron are capable of promoting oxidative stress in feces and colonocytes (Stone *et al*, 2004). Iron overload has been associated with an increased risk of colorectal cancer (Knöbel *et al*, 2006). According to Glei and colleagues (2002), iron is taken by

colonocytes and participates in the initiation of oxidative DNA damage. It is believed that the induction of DNA damage is mediated by ROS attack or through lipid peroxidation that may result in the production of promutagenic DNA adducts that could precipitate colon carcinogenesis (Owen *et al*, 2000). Other studies suggest that iron overload could increase the genotoxic risk during the early stages of colorectal carcinogenesis (Knöbel *et al*, 2007).

In the case of Crohn's disease and ulcerative colitis, the iron supplements used to treat the associated anemia commonly observed in these patients, exacerbate the related symptoms, diminish plasma's antioxidant state (Tapia and Araya, 2006), and increase oxidative stress and inflammation injury (Seril *et al*, 2005). Based on these observations, iron supplementation has been associated with a higher risk of colon carcinoma in patients suffering these disorders (Seril *et al*, 2005).

It has been demonstrated throughout this study that in an experimental *in vitro* assay, iron can have acted *in situ* as an antioxidant buffer depleter by producing large concentrations of ROS in the feces and reducing its normal antioxidant capacity state. These findings support the evidence found by other researchers who established that iron is capable of producing cellular effects which result in an enhanced production of intracellular ROS (Knöbel *et al*, 2006). From this we could state that large supplemental doses of iron have profound effects on the gut's ability to resist oxidation and, in consequence, may become vulnerable to the development of cancer.

Another significant finding came from the pilot study, which showed that the addition of refined palm oil to the iron dose significantly increased the production of ROS

in fecal samples, contrary to our speculation that oil might have a protective effect against oxidation produced by iron supplementation. Possible explanations could be: 1. a potential oxidation of the oil in the gut; 2. a delaying effect of oil on intestinal transit; or 3. that oil is reducing iron absorption in the intestine. Without further evidence, we cannot conclude among them.

Most important was the demonstration that the antioxidants from the palm oil-derived CTCMC are capable of replenishing the fecal oxidation buffering capacity and provide antioxidant protection to the intestinal lumen. In order to interpret these findings, we conceptualized a possible mechanism of action. We believe that in basal states, the subjects had a certain amount of antioxidants in the colonic lumen that make up the normal buffering capacity state of the feces. *In vitro*, these antioxidants buffer the ferric iron-induced generation of ROS and thus determine a basal formation of hydroxylated products (Figure 38). Residual iron from the oral supplementation induces *in situ* oxidation that depletes endogenous antioxidants. This is detected as a rise in the production of free-radical split products through the fecal challenge assay by Owen and colleagues (2000) (Figure 39).

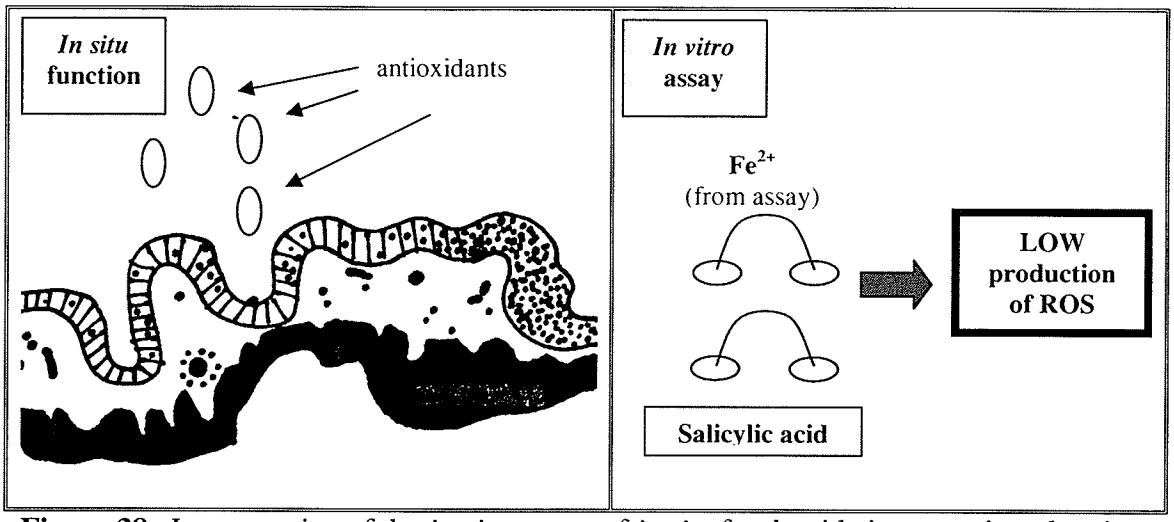

Figure 38. Interpretation of the *in vitro* assay of *in situ* fecal oxidation capacity when iron is not supplied. In the basal states, subjects have a certain quantity of antioxidants shown as gray ovals in the colonic lumen. In a fecal sample *in vitro*, these antioxidants determine the low generation of ROS by buffering the generation of radicals from the salicylic acid by ferric iron.

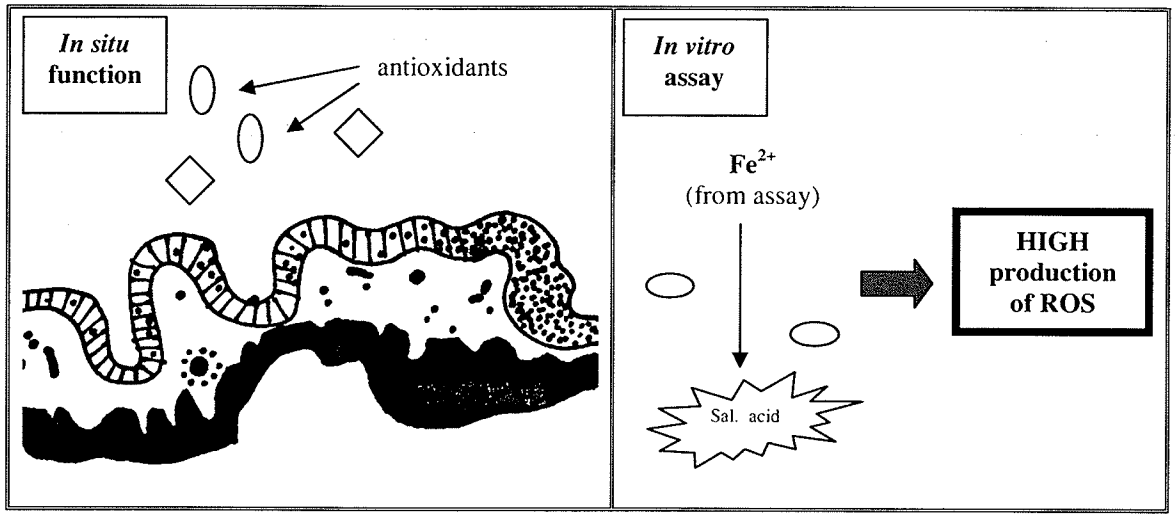

Figure 39. Interpretation of the *in vitro* assay of *in situ* fecal oxidation capacity during iron supplementation. The effect of passage of the residual iron (white rhomboids) from the oral supplementation in the colon induces *in situ* oxidation that depletes the endogenous antioxidants (gray ovals). When the fecal sample is challenged by the assay system, the ferric iron easily attacks the substrate producing more detectable hydroxylated products.

With the simultaneous consumption of ferrous sulphate with the antioxidant-rich CTCMC, residual iron depletes antioxidants, but they are reinforced by the supplement. In the *in vitro* assay, there is residual capacity to dampen and buffer the radical production, as in the baseline state (Figure 40). Our proposed model could be sustained by a study from Stone and colleagues (2004) in which they established that high levels of dietary iron decreased tocopherol levels in rat colonocytes and promoted intracellular oxidative stress. Subsequent supplementation with alpha-tocopherol replenished the vitamin E levels of the rats' colonocytes and feces.

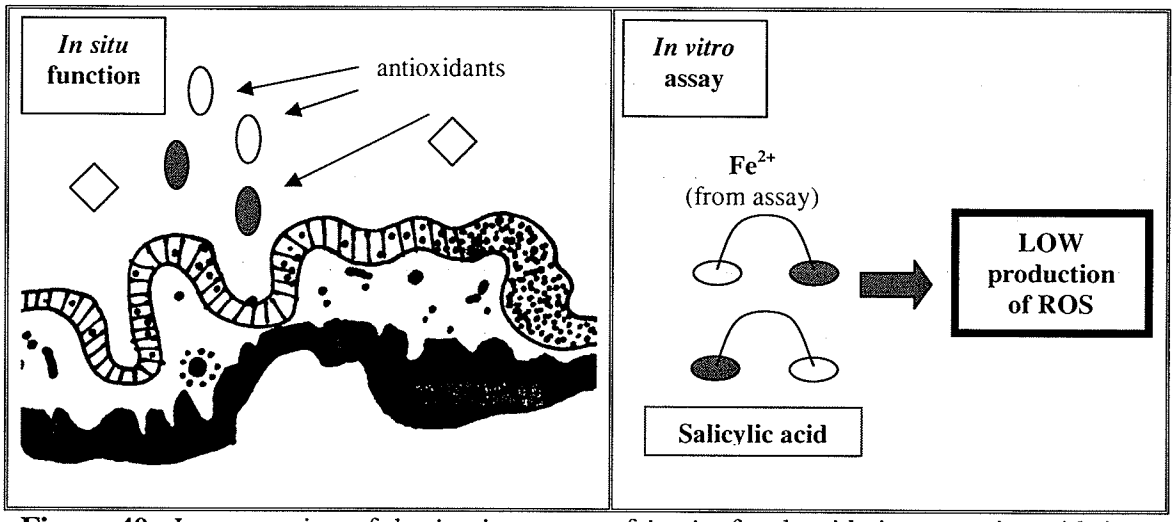

Figure 40. Interpretation of the *in vitro* assay of *in situ* fecal oxidation capacity with iron and CTCMC supplementation. With the passage of iron (white rhomboids), usual antioxidants (gray ovals) are depleted, but they are reinforced by the antioxidants from the supplement (black ovals). In the *in vitro* assay the antioxidant capacity to dampen and buffer the radical production is restored as in the ambient state, which is measured as low concentrations of total hydroxylated products.

To our knowledge, there are no scientific studies that have explored the effects of the CTCMC on colonic oxidative stress, but a study by Boateng and colleagues (2006) with a similar product, red palm oil (RPO), could support our findings and allow us to

get a clearer image of the mechanisms of action of palm-derived antioxidants. Boateng's study showed that antioxidants from RPO were effective in inhibiting the formation pre-neoplastic lesions on the surface of rodent colonocytes after treatment with the carcinogen azoxymethane. (Boateng *et al*, 2006). The authors hypothesize that RPO reduces membrane and cellular deterioration by decreasing lipid peroxidation, oxidative stress and free radical damage. They also report that tocotrienols are involved in inducing apoptosis in colonic epithelial cells by increasing NK cell and β-lymphocyte proliferation.

4.4 Fecal Iron after 7-Day Oral Iron Supplementation

On the one hand, iron absorption at the small intestine increases with low iron stores, iron deficiency, or hemolysis; on the other hand, it decreases with replete iron stores (Schümann *et al*, 2007). At the brush border of the intestine, iron is reduced from ferric to ferrous iron by ferric reductase and taken up from the lumen by the divalent metal transporter 1 (DMT 1) (Conrad and Umbreit, 2000). Afterwards, ferritin present in enterocytes sequesters reduced iron where it is oxidized to the ferric state by the hephaestin protein so that it can bind to transferrin for distribution to the rest of the body (Schümann *et al*, 2007). It has been observed that this regulatory mechanism works effectively under normal circumstances, but fails with acute oral iron intoxication (Schümann *et al*, 2007).

The subjects participating in this study were healthy males with replete iron stores at the beginning and end of the study. Results from the systemic oxidation biomarkers

assays demonstrated that the oral iron they received did not cause any significant oxidation damage in cell lipids and DNA, suggesting that iron might not be taken up across the enterocytes, but rather remain in the intestinal lumen. Furthermore, when assessing ROS production in feces it was demonstrated that the iron supplement does have a detrimental effect on the buffering capacity state of fecal matter in the colon. Considering that 80-90% of ingested iron remains in the gut lumen and can cause substantial damage (Schümann *et al*, 2007), it was necessary to quantify how much non-heme iron remained at the intestines and was excreted by the subjects under these circumstances and if it was correlated with the generation of ROS in the fecal matrix.

4.4.1 Quantitative Aspects of Fecal Iron Concentration

Healthy individuals absorb and excrete around 1 mg of iron per day (Yip, 2001), the remaining fraction of ingested iron that was not absorbed remains in the intestinal lumen and is excreted afterwards (Schümann *et al*, 2007). With 120 mg of iron supplied each day for seven days it is expected that a large fraction of this iron is unabsorbed and remains in the intestinal lumen. In this context, non-heme iron quantification was done to ensure compliance of the participants with the supplementation scheme since we can not quantify the total concentration of iron present in feces due to the limitations of our study protocol. It is expected that this data will also help clarify the mechanisms of oxidation occurring within the intestinal lumen.

4.4.1.1 Findings from the pilot phase

Residual non-heme iron in feces increases with iron supplementation alone ($P<0.05$) and with oil ($P<0.05$) (Figures 41 and 42). Refined palm oil however is not a determining factor in the concentrations of residual non-heme iron that remain in the intestinal lumen (Figure 43). Normal iron concentrations in fecal matter assessed during the baseline period do not show large variations between subjects or days. Once iron is supplied to the participants, however, they increase and larger variations appear among subjects (Figure 41). On day WO3 (11 days of the washout), non-heme iron excretion had still not reached baseline values; this was only accomplished two days later. It is impossible to know if the residual iron quantified on day WO3 came from supplemental iron or from the diet since lower non-heme iron concentrations were detected before, on day FE7 of the iron alone supplementation cycle. On average, fecal iron concentrations measured during the last three days of washout (WO3, 4, and 5) did not decrease to usual levels.

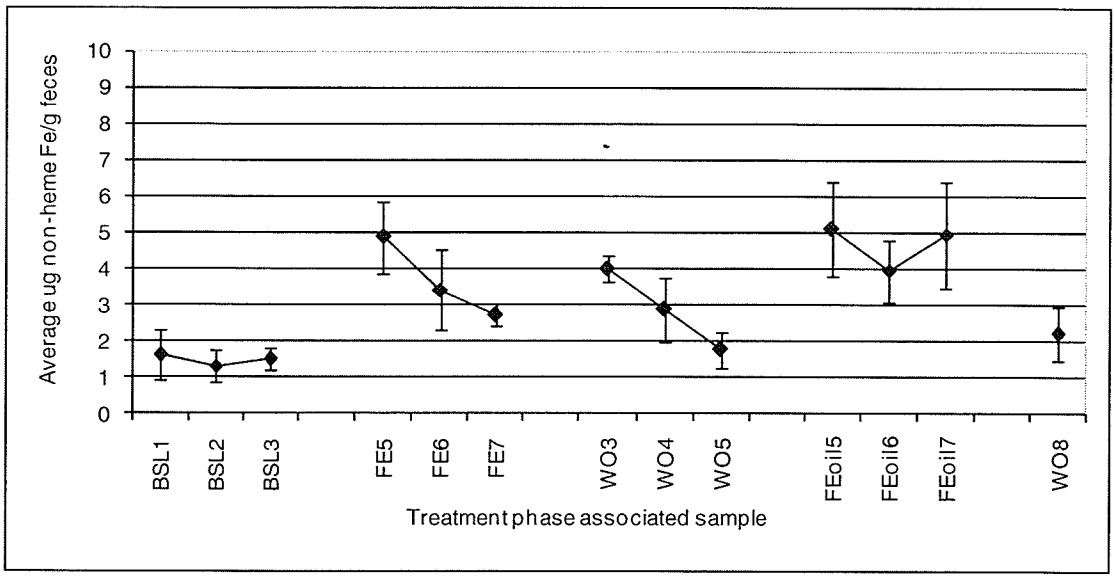

Figure 41. Average residual non-heme iron present in fecal samples of participants in phase 1 (N = 5 subjects), throughout the study. Graph is not in real time scale, days were no samples where collected are not shown. Y-axis error bars represent SEM. (BSL = baseline, FE = 120 mg oil, FEoil = 120 mg Fe + vegetable oil, WO = washout).

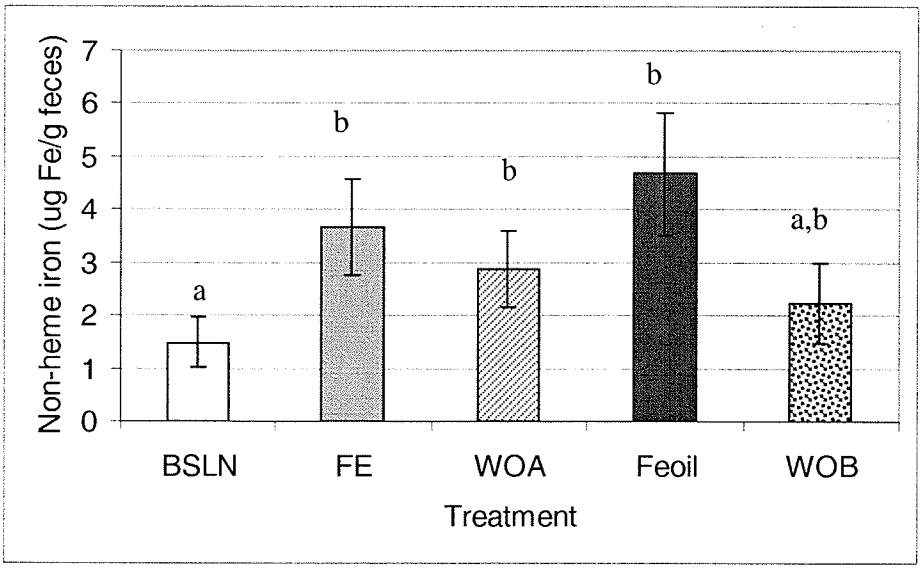

Figure 42. Total average non-heme iron present in fecal samples of all participants in phase 1 (N = 5 subjects). Each treatment bar represents the pooled average of all treatment days for the five subjects (BSL = baseline, FE = 120 mg oil, FEoil = 120 mg Fe + vegetable oil, WOA = WO3 + WO4 + WO5, WOB = WO8 + WO9 + WO10). Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM

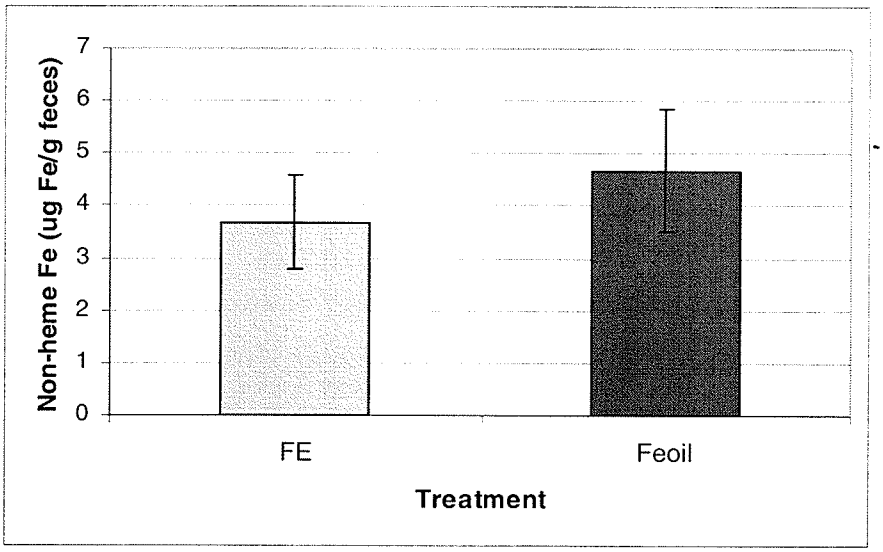

Figure 43. Total pooled average production of non-heme iron present in fecal samples of all participants in phase 1 (N = 5 subjects) per treatment. Each treatment bar represents the pooled average of all treatment days for the five subjects (FE = 120 mg Fe, Feoil = 120 mg Fe + vegetable oil). Repeated measures ANOVA show no significant differences between treatments ($p = 0.098$). Y-axis error bars represent SEM.

The change from habitual residual iron concentrations measured in days when supplemental iron was administrated to days with ongoing iron supplementation was calculated as deltas. Deltas were estimated in two ways: 1. as the average of the treatment day minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and the three washout samples (WO3, WO4 and WO5) (Figure 44) and 2. as a pooled average of all treatment days minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and the three washout samples (WO3, WO4 and WO5) (Figure 45). Either way, no significant differences were found in the concentrations of residual non-heme iron between FE and FEoil treatments.

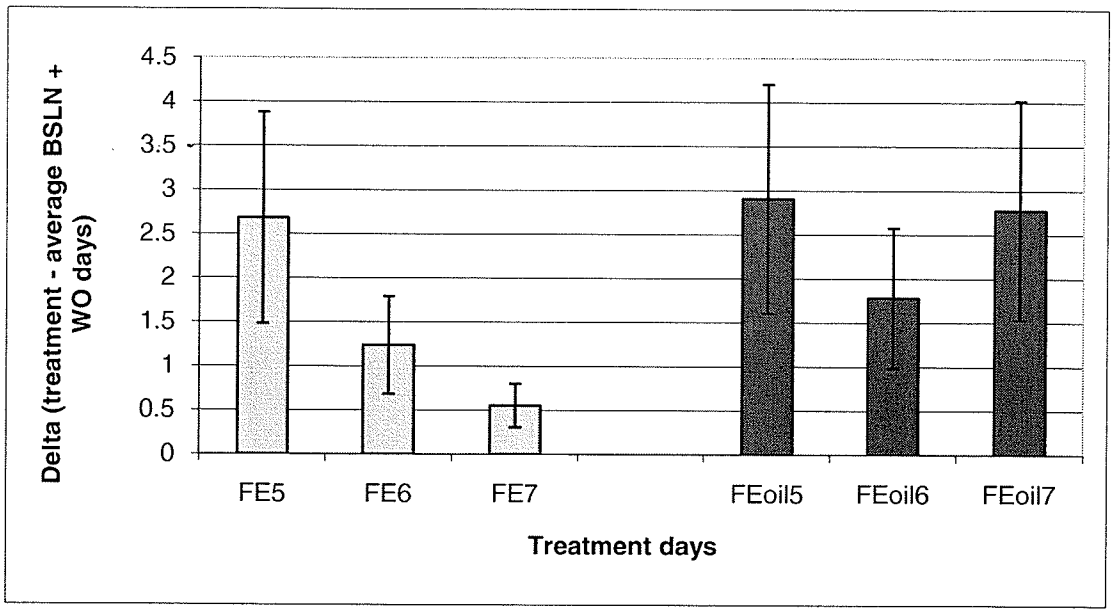

Figure 44. Difference (expressed as deltas) between non-heme iron in fecal samples collected during FE and FEoil treatments and non-heme iron present in fecal samples of a pooled average of baselines and washouts days for phase 1 (N = 5 subjects). Deltas for each average iron and iron + refined palm oil treatment day (FE5, FE6, FE7, FEoil5, FEoil6 or Feoil7) were calculated as the average of the treatment day minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and the three washout samples (WO3, WO4 and WO5). Y-axis error bars represent SEM. Repeated measures ANOVA show no significant differences between treatment days

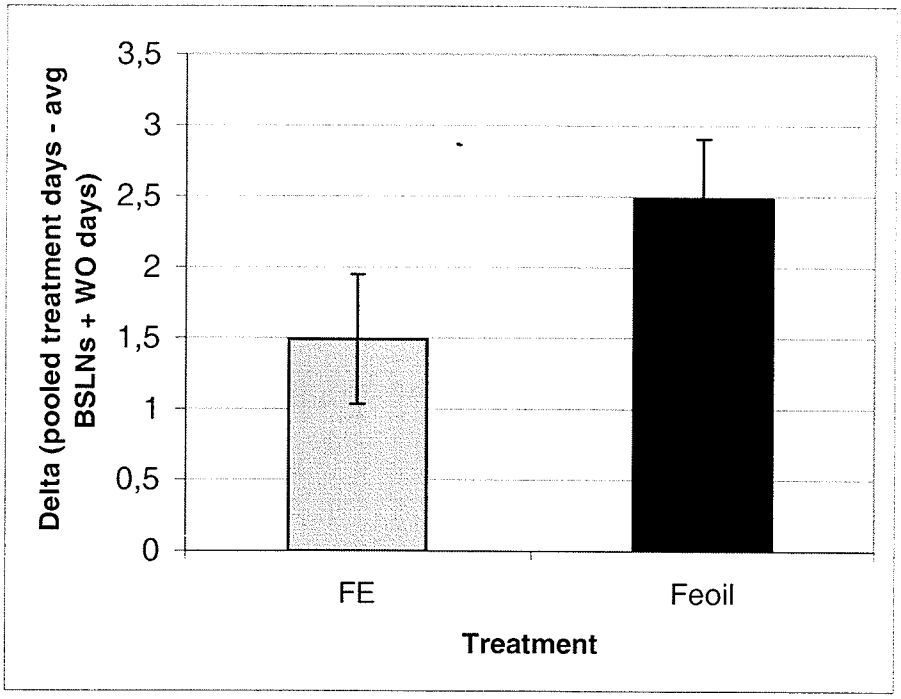

Figure 45. Difference (expressed as deltas) between non-heme iron present in fecal samples collected during FE and FEoil treatments and the non-heme iron concentration in fecal samples of a pooled average of baselines and washouts days for phase 1 (N = 5 subjects). Deltas for each average iron and iron + refined palm oil treatment were calculated as the average of all treatment days minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and the three washout samples (WO3, WO4 and WO5). Y-axis error bars represent SEM. Repeated measures ANOVA show no significant differences between treatments ($p = 0.098$)

4.4.1.2 Findings from the definitive intervention phase

This experiment does not allow examining how an oily matrix could affect overall iron absorption or excretion because 24 hour fecal collections were not obtained and thus, it is impossible to determine the total amount of residual iron in the feces. Non-heme iron quantification at this stage will only confirm the subjects' compliance to the treatment and the effects of iron supplementation on the usual concentrations of residual iron in the intestinal lumen.

The pilot phase demonstrated that the oily medium used to dilute the CTCMC has no influence on the amount of iron present in the colon. This eliminates the possibility that oil is somehow inhibiting iron absorption and in consequence, allowing more iron to stay in the intestinal lumen, increasing ROS production. Results from the definite intervention trial show that non-heme iron excretion is above baseline when iron is supplemented with oil alone or with CTCMC in either concentration (Figure 46). There are no differences between the FEoil and CTCA treatments, or between CTCA and CTCB treatments. There is, however a significantly higher concentration of non-heme iron in the feces during CTCA treatment when compared to the FEoil treatment (Figures 47 and 48). This suggests that a higher concentration of CTCMC may affect residual iron's transit in the intestinal lumen.

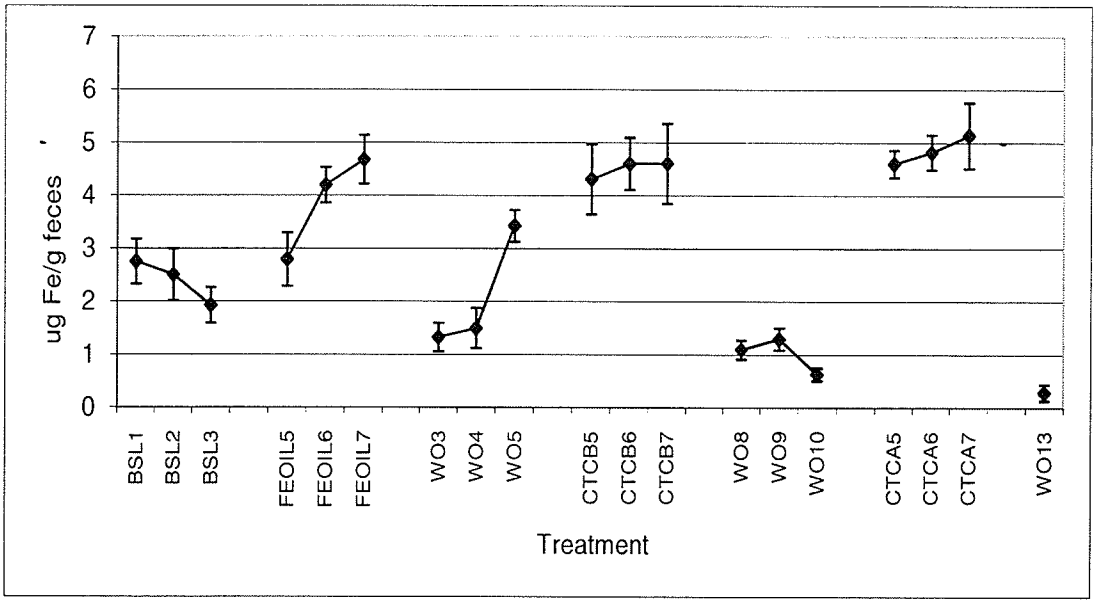

Figure 46. Average residual non-heme iron present in fecal samples of participants in phase 2 (N = 12 subjects), throughout the study. Graph is not in real time scale, days where no samples were collected are not shown. BSL = baseline, FEoil = 120 mg Fe + vegetable oil, WO = washout, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC. Y-axis error bars represent SEM.

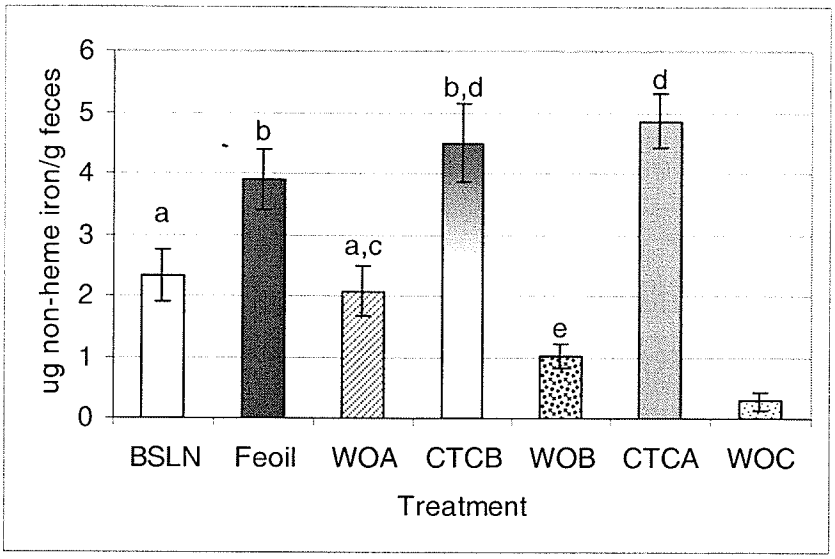

Figure 47. Average total non-heme iron per individual treatment. Each treatment bar represents the pooled average of all treatment days for the twelve subjects (BSL = baseline, FEoil = 120 mg Fe + vegetable oil, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC, WOA = WO3 + WO4 + WO5, WOB = WO8 + WO9 + WO10, WOC = WO13). Repeated measures analysis was done without the WOC treatment day because of unequal sample number. Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM.

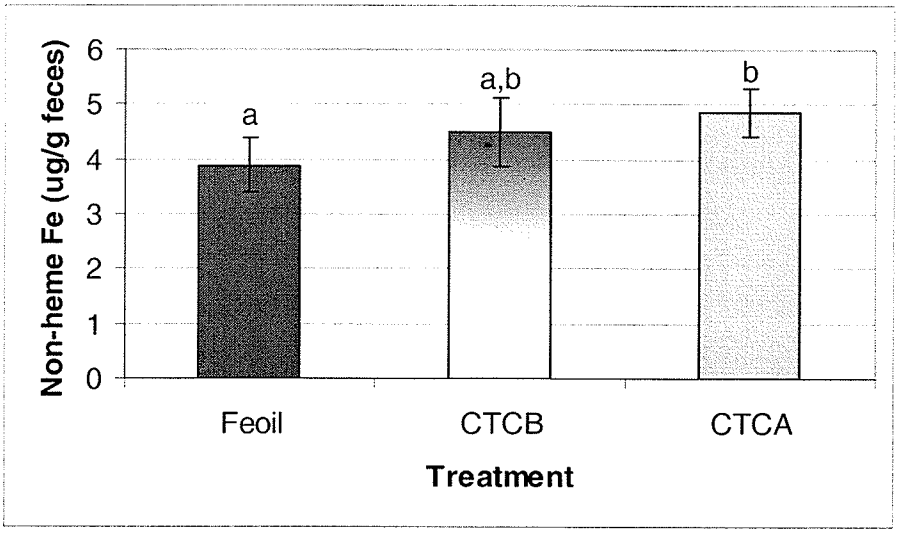

Figure 48. Total pooled average of non-heme iron present in fecal samples of all participants in phase 2 (N = 12 subjects) per treatment (FEoil = 120 mg Fe + vegetable oil, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC). Each treatment bar represents the pooled average of all treatment days for the five subjects. Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM.

Deltas calculated individually for each day are not statistically different from each other, indicating that there are no differences between individual treatment days (Figure 49). The change from baseline values to pooled treatment values (Figure 50) show the same tendency as when the concentration of non-heme iron was estimated as a pooled absolute value, confirming the fact that there is a higher concentration of non-heme iron present in stool samples collected during the CTCA treatment supplementation.

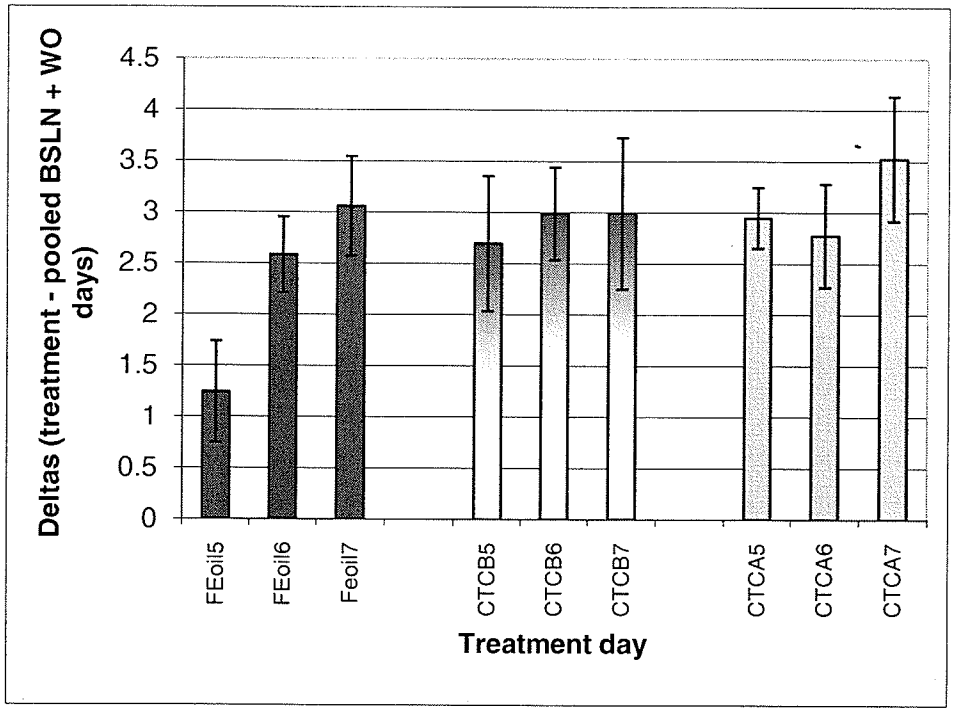

Figure 49. Difference (expressed as deltas) between residual non-heme iron in fecal samples collected during individual FEoil (120 mg Fe + vegetable oil), CTCB (0.4 g CTCMC), and CTCA (0.8 g CTCMC) treatment days and washout days and the free radical production in fecal samples of a pooled baseline for phase 2 (N = 12 subjects). Deltas for all treatment days were calculated as the average of the treatment day minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and 6 washout days (WO3, 4, 5, 8, 9, and 10). Y-axis error bars represent SEM.

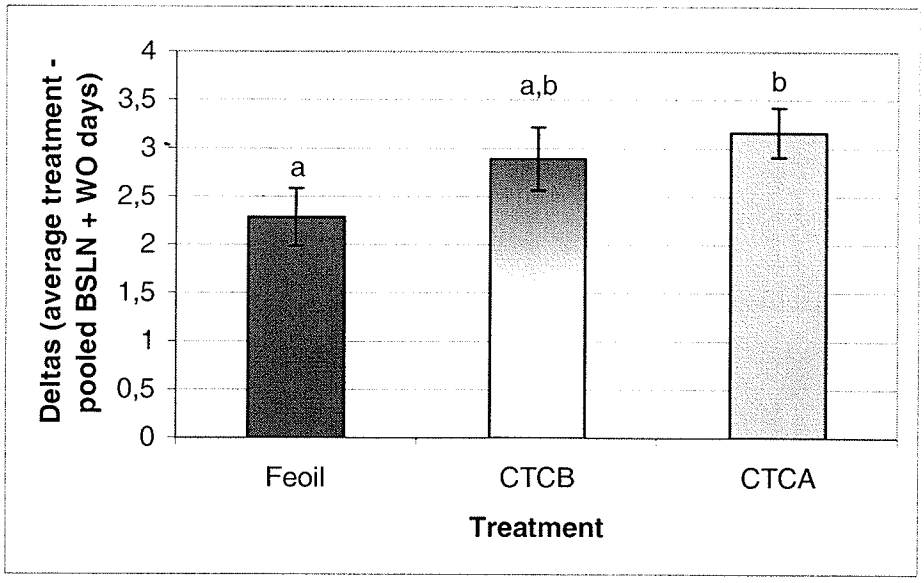

Figure 50. Difference (expressed as deltas) between residual non-heme iron in fecal samples collected during FEoil (120 mg Fe + vegetable oil), CTCA (0.4 g CTCMC) and CTCB (0.8 g CTCMC) treatments and the residual non-heme iron in fecal samples of a pooled average of baselines and washouts days for phase 2 (N = 12 subjects). Deltas for each FEoil, CTCA and CTCB treatment were calculated as the average of all treatment days minus the pooled average of the three baseline samples (BSLN1, 2 and 3) and the six washout samples (WO3, 4, 5, 8, 9, and 10). Average values sharing the same superscript letter are not statistically different by repeated measures ANOVA. Y-axis error bars represent SEM.

4.4.2 Interactions of Fecal Iron Concentration and Fecal Antioxidant Capacity

It was necessary to determine if the enhanced ROS production in fecal matter observed during days of supplementation with oral iron was due to the presence of higher concentrations of residual iron at the intestines. For this purpose, statistical associations using Cohen's kappa coefficient, Spearman's rank-order correlation and Pearson's correlations were calculated.

4.4.2.1 Findings from the pilot phase

The individual subject-by-subject agreement between non-heme iron and total hydroxylated products in fecal samples evaluated with Cohen's kappa coefficients ranges from 0.31 to 0.65 (Figures 51 through 55), indicating a fair to good agreement between both factors. On average (Figure 56), the interaction becomes poor (-0.03), mainly because the maximum concentrations of non-heme iron and ROS production are detected on different days for each individual. Fecal transit is determinant in this type of study and may explain the observed variations. It is well-known that colonic motility function varies broadly between normal subjects; some reports show variations in transit time of approximately 3-fold in healthy males (Wyman *et al*, 1978). This could explain why maximum cumulative iron concentrations are detected in different days for each subject.

Spearman's and Pearson's correlations (Figure 57) are significant though ($r = 0.422$ and $r = 0.334$), confirming individual interactions and demonstrating that there is a concrete overall association between fecal non-heme iron and fecal antioxidant capacity.

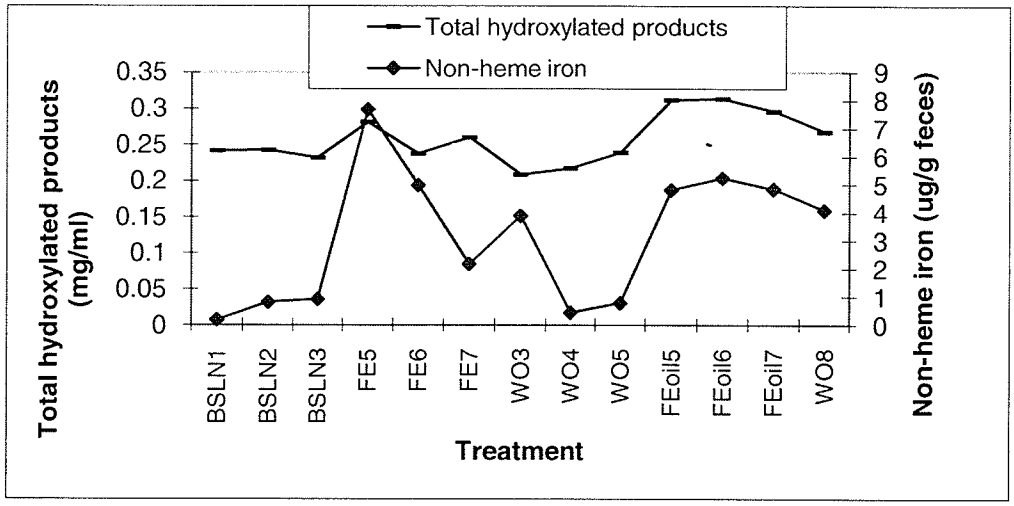

Figure 51. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 001 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships. Cohen´s kappa coefficient = 0.44 (fair agreement). BSLN = baseline, FE = 120 mg oil, FEoil = 120 mg Fe + vegetable oil, WO = washout.

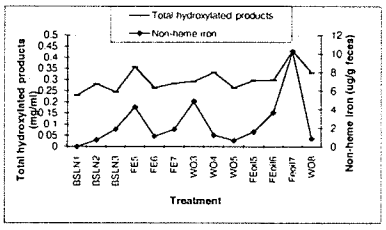

Figure 52. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 002 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Cohen´s kappa coefficient = 0.63 (good agreement). BSLN = baseline, FE = 120 mg oil, FEoil = 120 mg Fe + vegetable oil, WO = washout.

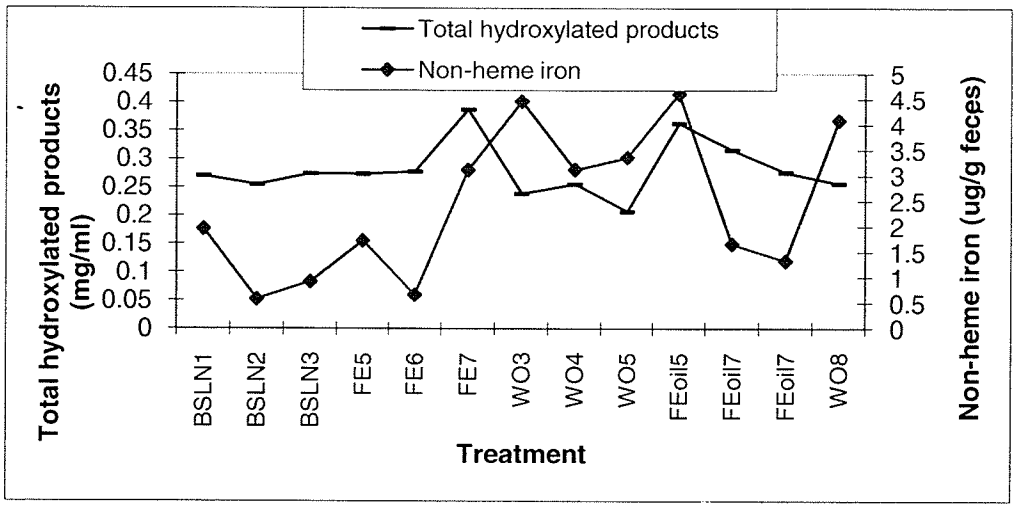

Figure 53. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 003 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Cohen´s kappa coefficient = 0.35 (fair agreement). Y-axis error bars represent SEM. (BSL = baseline, FE = 120 mg oil, FEoil = 120 mg Fe + vegetable oil, WO = washout).

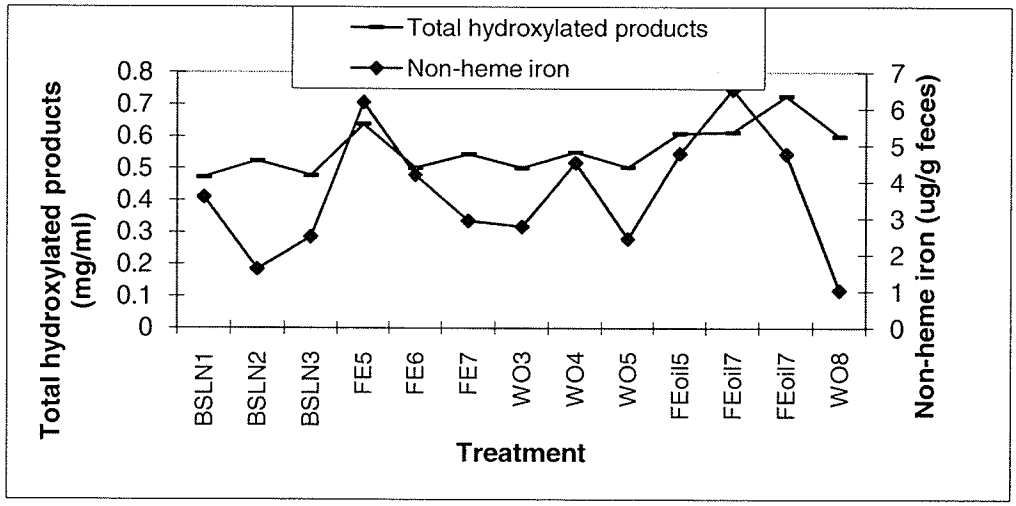

Figure 54. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 004 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Cohen´s kappa coefficient = 0.35 (fair agreement). (BSL = baseline, FE = 120 mg oil, FEoil = 120 mg Fe + vegetable oil, WO = washout).

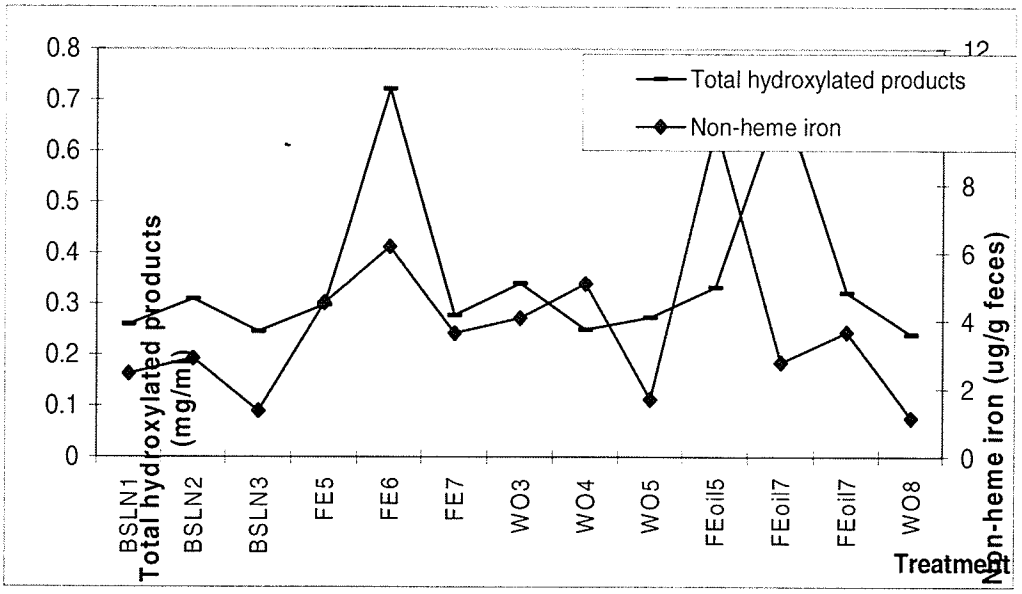

Figure 55. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 005 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Cohen's kappa coefficient = 0.31 (fair agreement). Y-axis error bars represent SEM. (BSL = baseline, FE = 120 mg oil, FEoil = 120 mg Fe + vegetable oil, WO = washout).

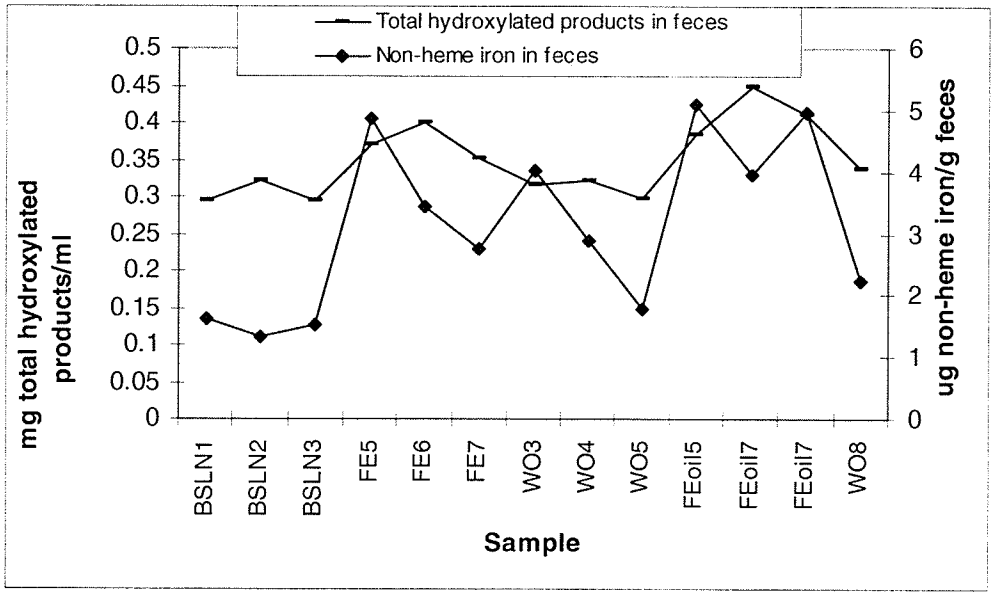

Figure 56. Comparison of average total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from all individuals participating in phase 1 (N = 5 subjects). Graph is not in real time scale; days where no samples were collected are not shown. Cohen's kappa coefficient = -0.03 (poor agreement). (BSL = baseline, FE = 120 mg oil, FEoil = 120 mg Fe + vegetable oil, WO = washout).

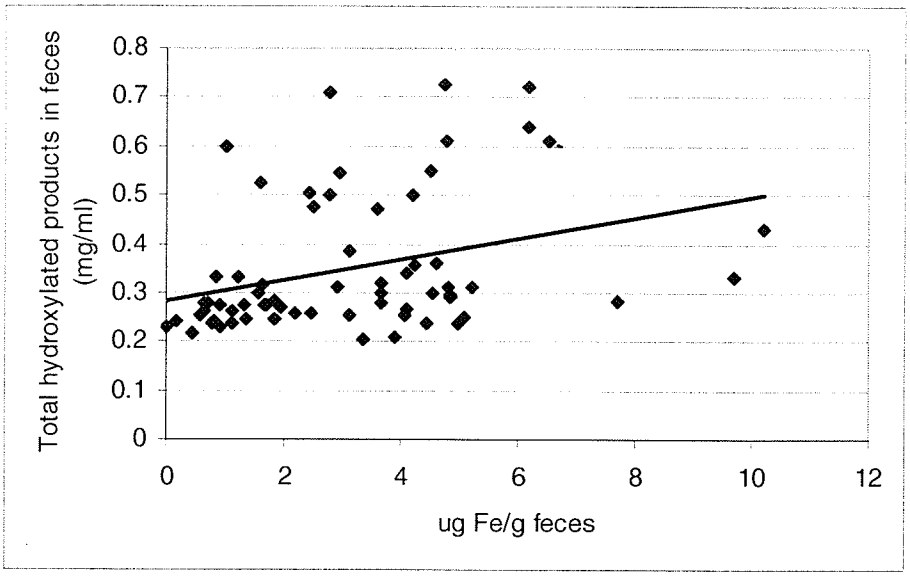

Figure 57. Correlation between free radical production in feces and residual non-heme iron in the same samples for phase 1 (N = 5, n = 65 plotted samples). Spearman's correlation = 0.422 (significant at 0.01 level), Pearson's correlation = 0.334 (significant at 0.01 level).

4.4.2.2 Findings from the definitive phase and combined data

The second phase enabled us to continue exploring the relationship between iron and free radical production in the gut. Kappa coefficient for the association between average hydroxylated products and non-heme iron concentrations is 0.64, good agreement. The correlation between these two factors, shown in Figure 58, is weaker but still significant (Pearson's = 0.199, p = 0.003 and Spearman's = 0.188, p= 0.006), compared to the results from the pilot study (Spearman's = 0.422 and Pearson's = 0.334) (Figure 57). In this case, the association between the two factors might be disrupted by the CTCMC since the antioxidants from the CTCMC suppress ROS production. Appendix 7 contains the individual associations between non-heme iron and total hydroxylated products for each individual participating in phase 2.

To confirm if the observed association persisted during the period when no CTCMC was administered and was affected by CTCMC supplementation, we calculated the correlation between free radical production in feces and residual non-heme iron under habitual conditions using the samples from FEoil, baseline and washout periods for both phases (Figure 59), during iron supplementation and washout periods (Figure 60), and without iron supplementation with oil (Figure 61). In absence of CTCMC, the correspondence is significant (Pearson = 0.279, p = 0.001 and Spearman = 0.321, p<0.001). When using exclusively samples from iron + CTCMC supplementation (Figure 62), the parallelisms disappear (Pearson = 0.049, p = 0.687 and Spearman = 0.089, p= 0.467).

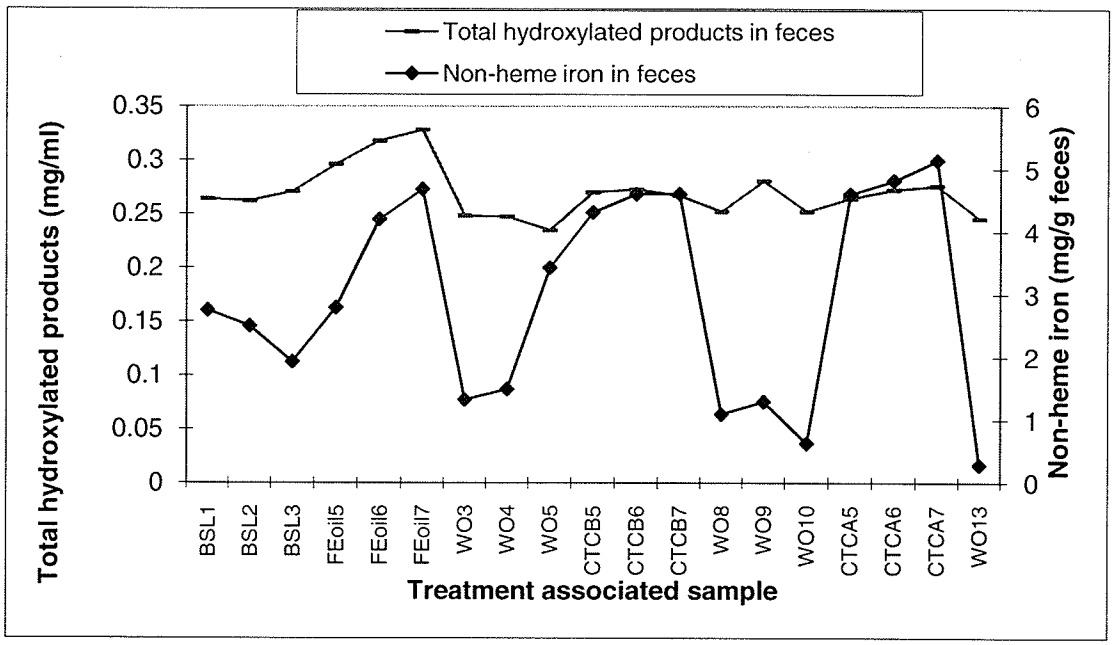

Figure 58. Comparison of average total hydroxylated products (mg/ml) and non-heme iron (µg iron/g feces) in feces samples from all individuals participating in phase 2 (N = 12 subjects). Graph is not in real time scale, days where no samples were collected are not shown. Cohen´s kappa coefficient = (0.64). (BSL = baseline, FEoil = 120 mg Fe + vegetable oil, WO = washout, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC).

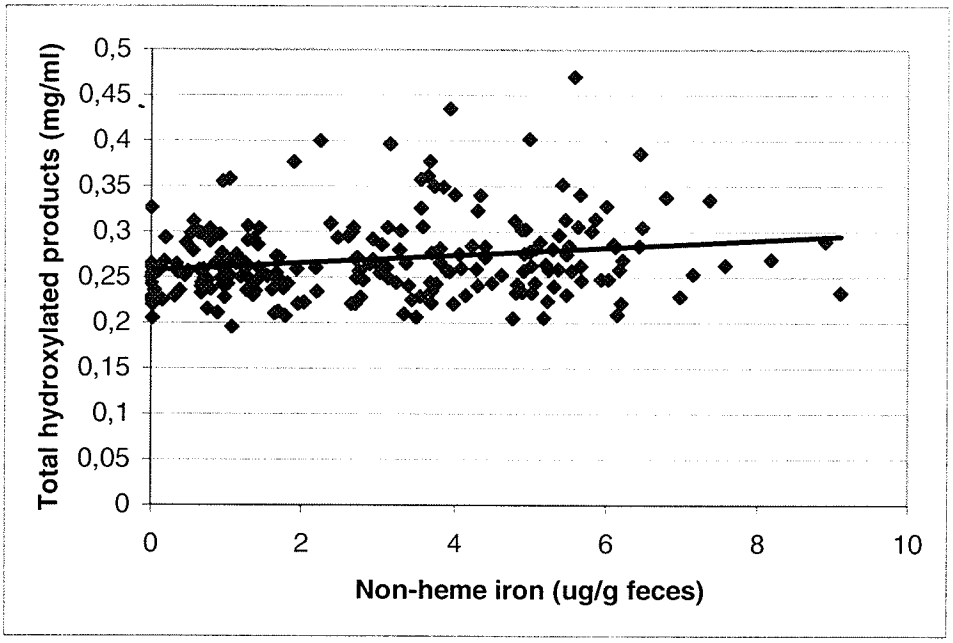

Figure 59. Correlation between free radical production in feces and residual non-heme iron in all the same samples of participants from phase 2 (N = 12 subjects, n = 216 plotted samples). Pearson's correlation = 0.199 (p = 0.003, significant at 0.001 level), Spearman's correlation = 0.188 (p= 0.006, significant at 0.001 level)

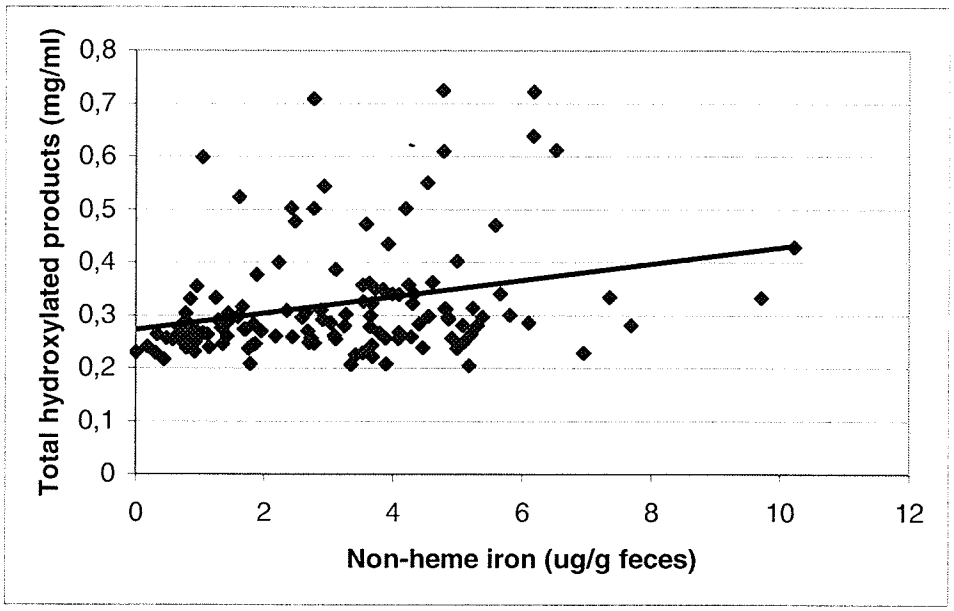

Figure 60. Correlation between free radical production in feces and residual non-heme iron in all treatment samples of participants from phase 1 (N = 5 subjects) and samples from FEoil (120 mg Fe + vegetable oil), BSLN (baseline) and washout treatments of participants from phase 2 (N = 12 subjects). (N = 17 subjects, n = 129 plotted samples). Pearson's correlation = 0.279 (p = 0.001, significant at 0.01 level), Spearman's correlation = 0.321 ($p<0.001$, significant at 0.01 level). These samples were selected to examine the correspondence between the two factors in absence of CTCMC.

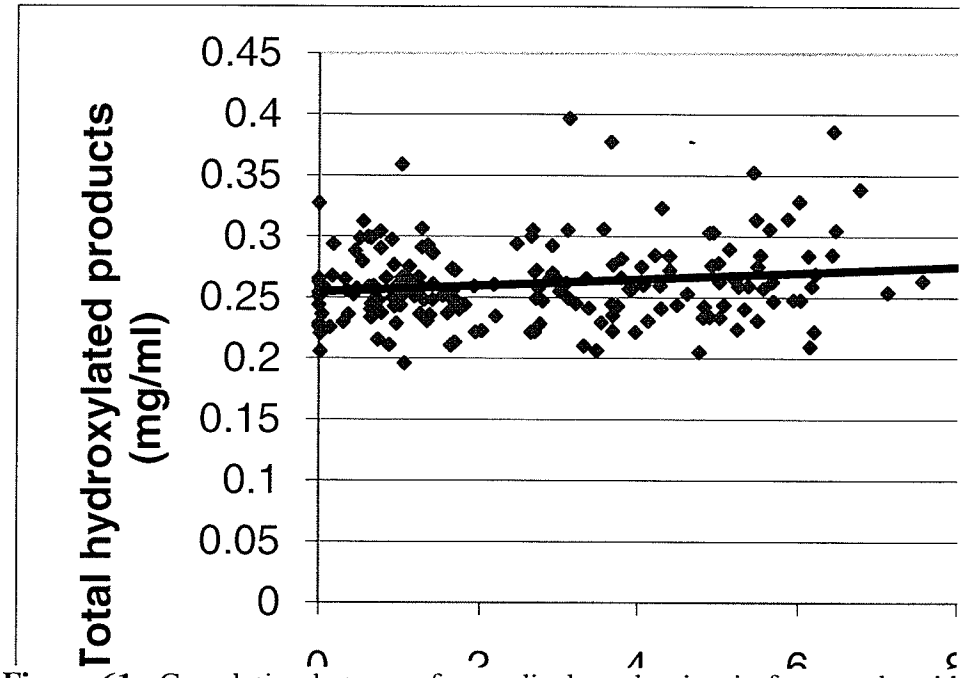

Figure 61. Correlation between free radical production in feces and residual non-heme iron in the samples from treatments CTCA (0.8 g CTCMC), CTCB (0.4 g CTCMC), BSLN (baseline) and WO (washout) samples for participants from phase 2 (N = 12 subjects, n = 182 plotted samples). Pearson correlation = 0.167 (p = 0.025), Spearman's correlation = 0.173 (p = 0.074). These samples were selected to examine the correspondence between the two factors in the situation without oral supplementation with iron and oil.

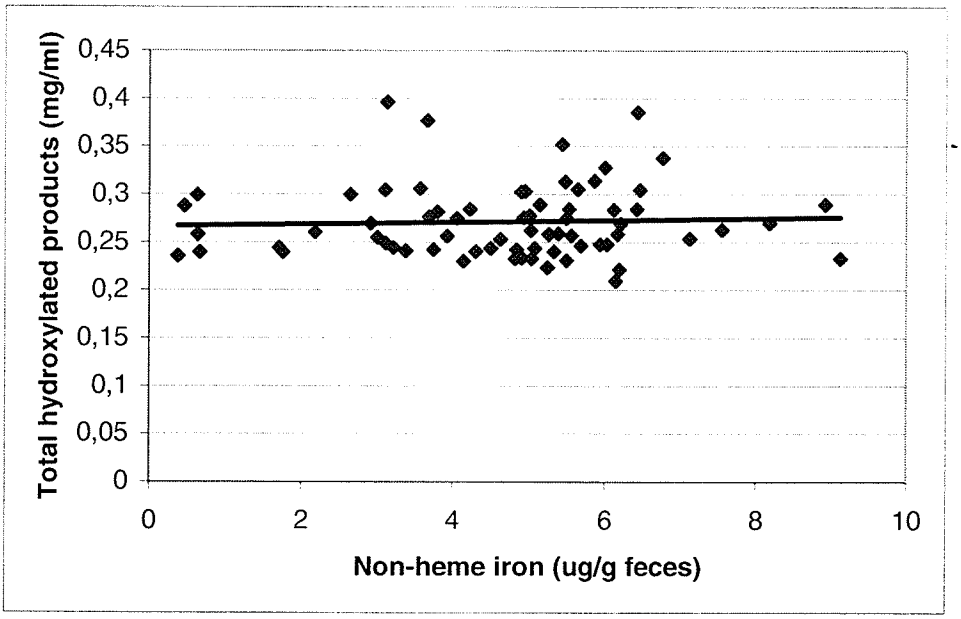

Figure 62. Correlation between free radical production in feces and residual non-heme iron in the samples from treatments CTCA (0.8 g CTCMC) and CTCB (0.4 g CTCMC) for participants from phase 2 (N = 12 subjects, n = 69 plotted samples). Pearson correlation = 0.049 (p = 0.687), Spearman's correlation = 0.089 (p= 0.467). These samples were selected to examine the correspondence between the two factors in the exclusive situation of oral supplementation with iron and CTCMC.

4.4.3 Summary Discussion

As reported elsewhere, high levels of iron promote oxidative stress in feces (Lund *et al*, 1998, 1999 and 2000; Stone *et al*, 2004). In this study we found significant associations between free radical production and residual non-heme iron in fecal matter, confirming the reports from other researchers. Despite these associations, we can not explain the differential ROS production by any influence of residual iron in the fecal samples because the quantified amount of residual non-heme iron may not reflect the net excretion of iron, since we did not perform 24-hour fecal collections due to logistical difficulties. We can further develop the consequences of the 40-fold disproportion between the residual non-heme iron passing through into the stools in concentrations of

5 µg/g (from supplemental and dietary sources) and the charge of ferric ions from the assay added at 200 µg/g of stool. Any amount of ROS that would be generated only from the residual iron in the stool sample with the addition of EDTA as a chelator would likely be well below the threshold of detection for the indicator species by HPLC. In no way, therefore, could the higher and lower amounts of fecal iron, oscillating through the 0.0 to 5 µg/g range of concentration, explain any differential production in the *in vitro* situation of the assay reaction. This exclusion, then, adds credibility to our underlying notion, expressed in Figures 38, 39 and 40, that the main effect of supplemental iron was to have eroded the fecal buffering capacity in stool sample during is residence time in the colon.

Finally, the associations between residual fecal iron and ROS production fade away with in the presence of the oral supplementation of CTCMC. This clearly is not through any effect of modifying iron absorption or transit times, since the concentrations of residual non-heme iron measured in stools from the iron-supplementation periods were identical across all three treatment periods (Figures 51 - 56). We postulate that the antioxidants must disrupt the relationships surrounding ROS generation in some way in the fecal samples. This can be visualized in Figures 58 and 59, which shows the correlation between free radical production in vitro in feces and residual non-heme iron is strongest excluding the influence of CTCMC exposure, and totally absent when focused exclusively on periods of oral antioxidant supplementation.

4.5. Findings related to the Biological Significance of Urinary Biomarkers Responses within the "Physiological Range"

All of the previous sections were related in one way or another to the questions surrounding the effect of oral iron supplementation. With regard to oxidation responses in the fecal environment (*in situ* in the colon) we were able to document powerful effects and their reversal by oral antioxidant supplementation. The residual non-heme iron passing through the colon set the stage for the fecal oxidation. However, the iron challenges proved to have no effect on urinary biomarkers (8-OHdG and TBARS) that reflect systemic oxidation of DNA and lipid membranes respectively. Nevertheless, two observations on the urinary biomarkers outside of the context of iron supplementation draw our attention. The first was an unexpected and interesting correspondence in day-to-day fluctuations of the two systemic oxidation biomarkers. The other was the effect that oral antioxidant supplementation had on this biomarkers independent of exposure to oral iron. We focus on the evidence for these biomarker-related findings in this section.

4.5.1 The nature of the urinary biomarkers responses in terms of what could be considered the "physiological range"

Insofar as our subjects were healthy young adults in Guatemala City without diagnosed pathologies, we would expect the underlying systemic oxidation to be normative for healthy subjects. We looked to develop a comparative perspective on biomarker responses in other samples of healthy individuals from manufacturer's norms and published literature.

The urinary biomarkers responses from the participants in both phases of this study were compared with those from other assessments to try determining what habitual levels could be considered "normal physiological values". The distributors of the 8-OHdG assay kits report a range of 18 – 80 ng 8-OHdG/ml for urine samples from normal subjects (Genox Corporation, personal communication). When using this parameter as a reference, 20.2% of the urine samples are above the upper limit, which is indicative of enhanced *in vivo* DNA oxidation (Figure 63). If the baseline creatinine-corrected values for normal adults of at least 35 y of age from a study by Chin and colleagues (2008) at the Universiti Kebangsaan in Malaysia, are applied instead (9.8 ± 3.4 ng/mg creatinine), a larger percentage of urine samples (72%) are above the reference range (defined as two times the standard deviation) (Figure 64). Average urinary 8-OHdG excretion for the Guatemalan sample (41.1 ± 40.3 ng/mg creatinine) is four-fold higher than the Malaysian study population.

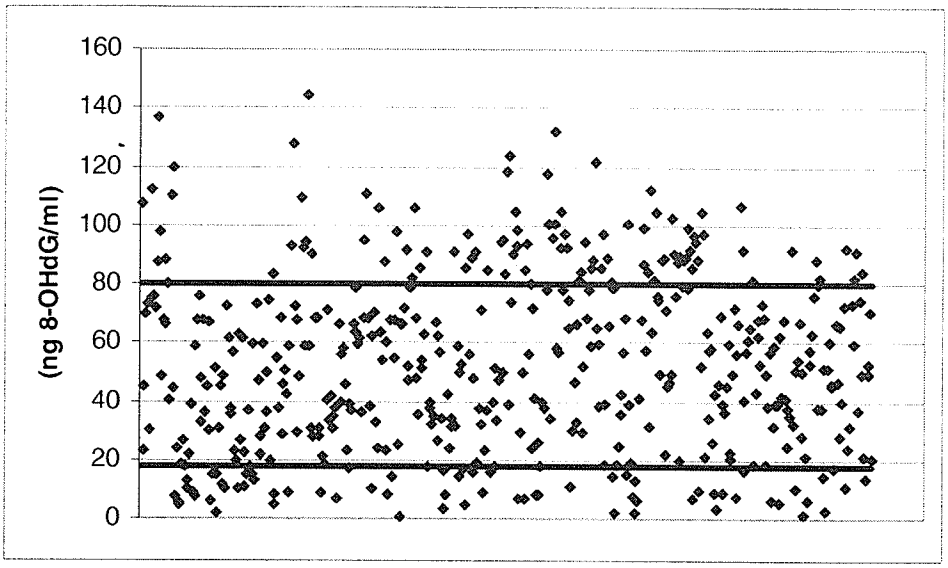

Figure 63. Total uncorrected 8-OHdG for urine samples from subjects participating in both phases (N = 17 subjects, n = 466 plotted samples) with reference to the normal range of 8-OHdG reported for normal adults (ng/ml) by the manufacturers. (Source: Genox Corporation, personal communication). The reference range is shown as thick black lines. A total of 94 (20.2%) analyzed had values above the reference range, indicative of *in vivo* inflammation.

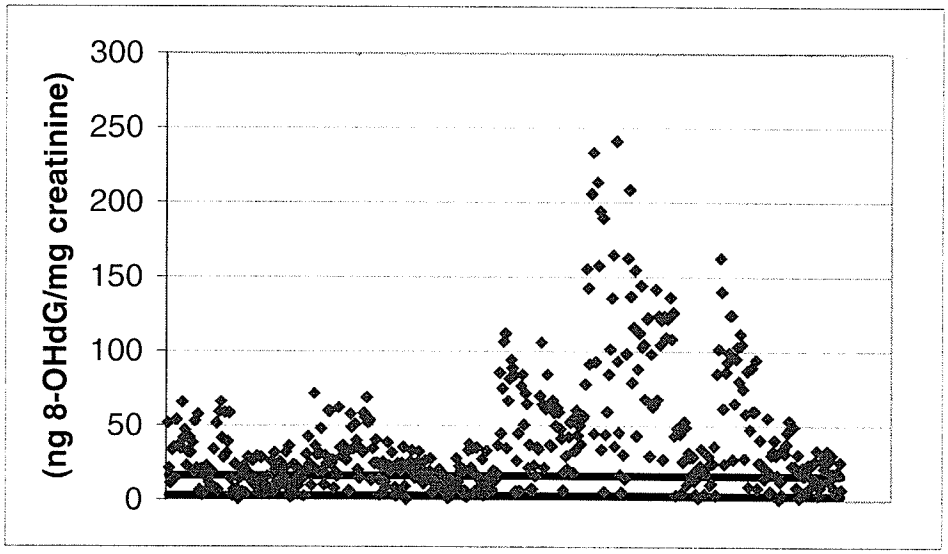

Figure 64. Total creatinine-corrected 8-OHdG values for urine samples from subjects participating in both phases (N = 17 subjects, n = 466 plotted samples) with reference to the average 8-OHdG (9.8 ± 3.39 ng/mg creatinine) reported for normal adults of at least 35 y of age by Chin *et al*, 2008. The reference range shown as thick black lines represents ± 2SD. A total of 338 of the 466 samples (72%) analyzed had values above the upper reference limit (+2SD).

A study by Actis-Goretta and colleagues (2004) was used to set a "reference range" for urinary TBARS excretion. In their study, TBARS were assessed on young healthy males and females aged 33 – 45 years, and reported values of 4.7 ± 0.2 nmol/ml. Comparing the uncorrected TBARS concentrations from the Guatemalan study with this reference range (defined as ± 2SD), it can be observed that only 27% of the urine samples from all the participants are above the upper reference limit (+ 2SD) (Figure 65). In average, the Guatemalan subjects have lower concentrations of urinary MDA (4.1 nmol MDA/ml) compared to the reference value (4.7 nmol MDA/ml), but the subject-to-subject variability measured as standard deviation (± 1.99) is almost ten-fold as the population from the Actis-Goretta study.

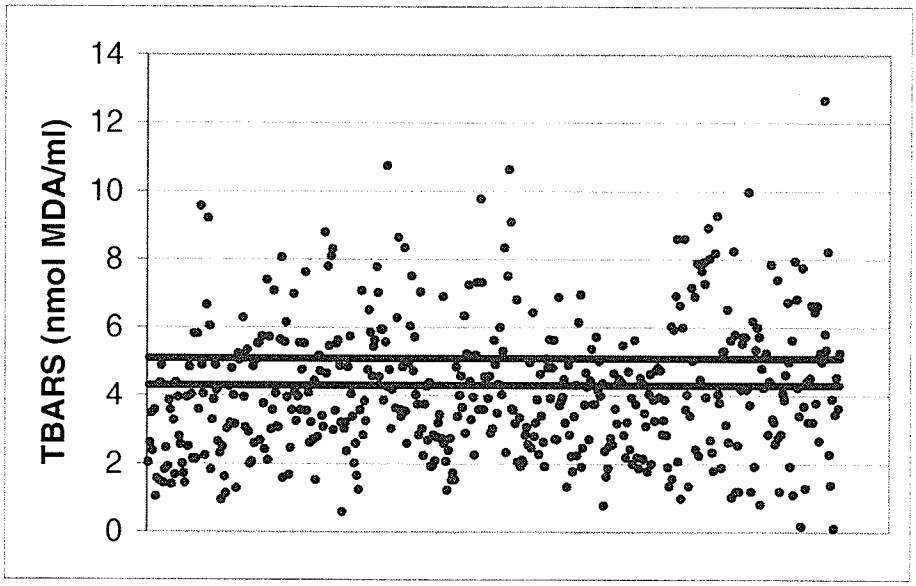

Figure 65. Total uncorrected TBARS values for urine samples from subjects participating in both phases (N = 17 subjects, n = 466 plotted samples) with reference to the normal range of TBARS reported for normal young adults aged 33- 45 y (4.7 ± 0.2 nmol/ml) by Actis-Goretta *et al*, 2004. The reference range shown as thick black lines represents ± 2SD. A total of 124 of the 466 samples (27 %) analyzed had values above the above the upper reference limit (+2SD).

4.5.2 Correspondence of Day-to-Day Fluctuation of TBARS and 8-OHdG within the Same Individual

Little is known about the dynamics of free radical damage under normal conditions. The previous section exemplified how difficult it is setting a normal physiological range that would allow categorizing individual oxidative damage and determine what could be considered detrimental to health. Even more, throughout this study we became aware of an interesting association between the two evaluated systemic oxidation biomarkers, suggesting that there are complex relationships and mechanisms that still need to be explored.

4.5.2.1 Findings from the pilot phase

Figures 66 to 70 illustrate individual correspondences between urinary excretion of 8-OHdG and TBARS for all the subjects in phase 1. To determine the strength of the association between the two factors, Cohen's kappa scores were estimated for each subject. These scores were calculated by counting how many relative increments and decrements occurred simultaneously between the 8-OHdG and the TBARS throughout the study. The number of correspondences and non-correspondences were introduced in a 2 x 2 matrix and analyzed with an online kappa score calculator (Department of Obstetrics and Gynaecology, University of Hong Kong). Resulting individual kappa scores ranged from 0.24 (fair agreement) to 0.71 (good agreement). The same analysis was performed for the average response of all the study population from phase 1 (Figure 71), confirming

the existence of a strong correspondence between both factors (kappa score = 0.81, very good agreement).

Spearman and Pearson's correlations were 0.465 and 0.293 respectively (both significant at 0.01 level) (Figure 72), providing more evidence about the parallelisms that exist during the normal fluctuations of these biomarkers.

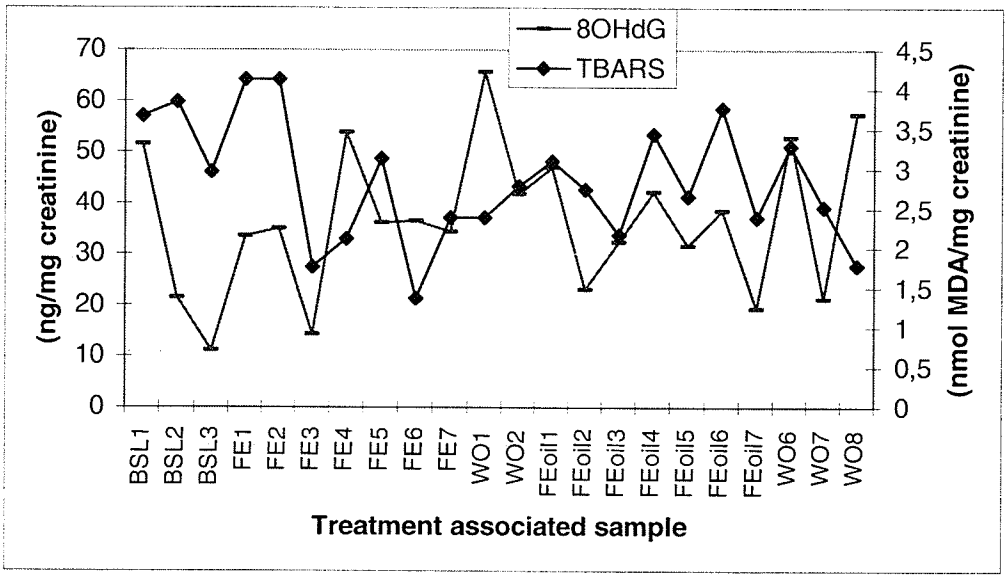

Figure 66. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 001 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Cohen's kappa coefficient = 0.51 (moderate agreement). (BSL = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout).

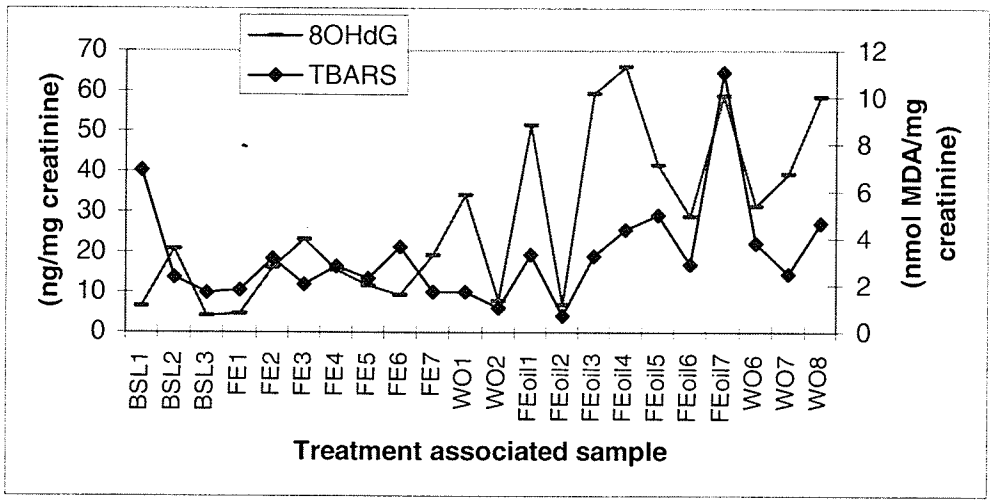

Figure 67. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 002 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Cohen´s kappa coefficient = 0.33 (fair agreement). (BSL = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout).

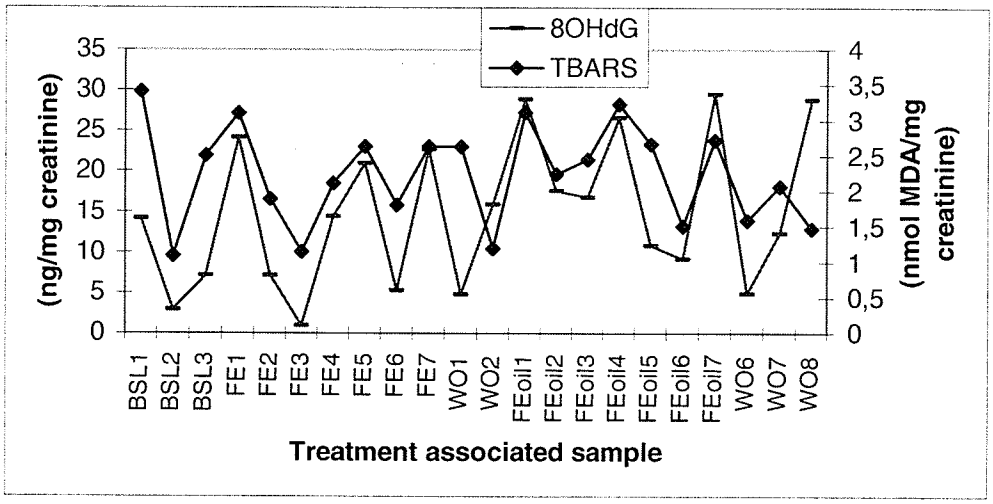

Figure 68. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 003 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Cohen´s kappa coefficient = 0.71 (good agreement). (BSL = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout).

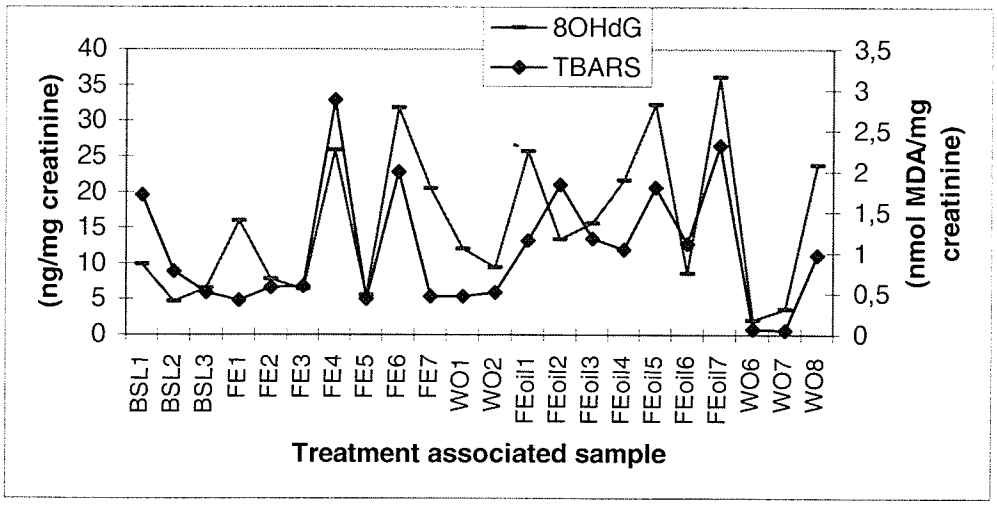

Figure 69. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 004 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Cohen´s kappa coefficient = 0.43 (moderate agreement). (BSL = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout).

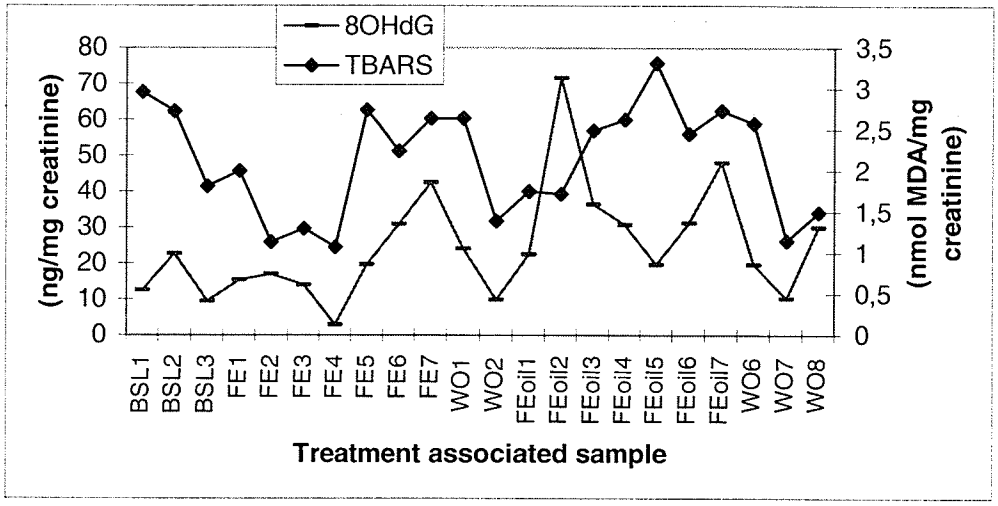

Figure 70. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 005 participating in phase 1. Graph is not in real time scale, days where no samples were collected are not shown. Cohen´s kappa coefficient = 0.24 (fair agreement). (BSL = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout).

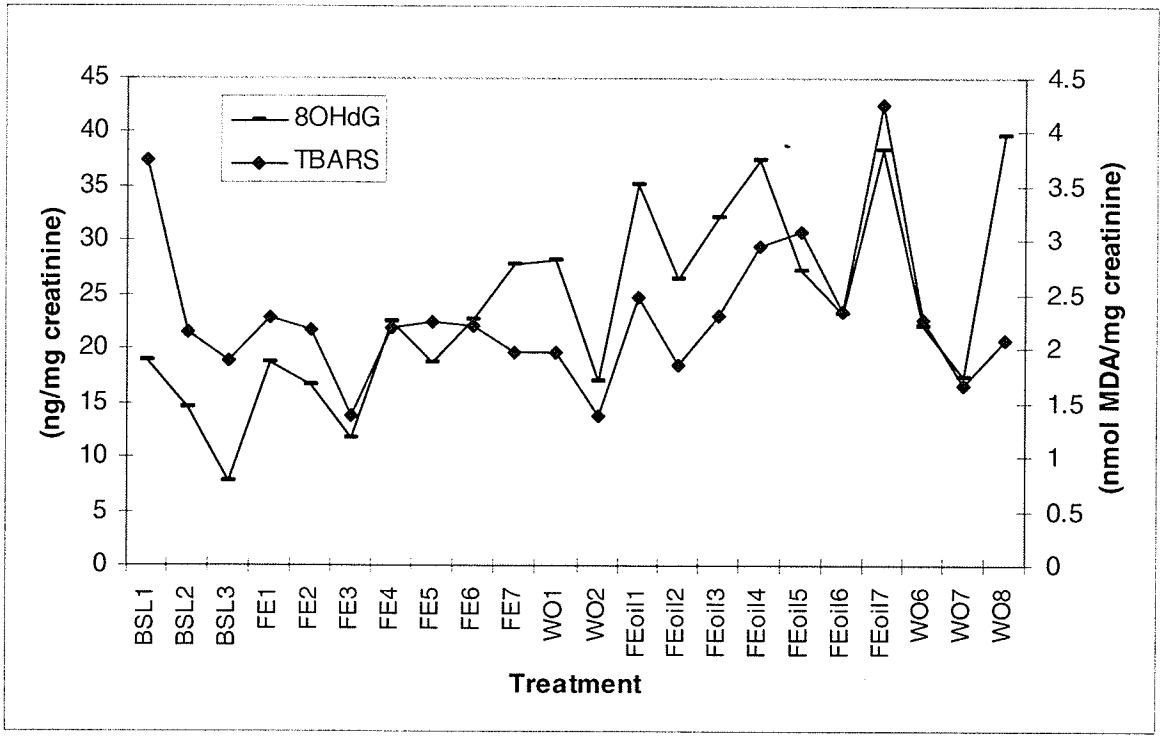

Figure 71. Comparison of average 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples for individuals in phase 1 (N = 5 subjects). Graph is not in real time scale, days were no samples where collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships. Cohen´s kappa coefficient = 0.81 (very good agreement). (BSL = baseline, FE = 120 mg Fe, FEoil = 120 mg Fe + vegetable oil, WO = washout).

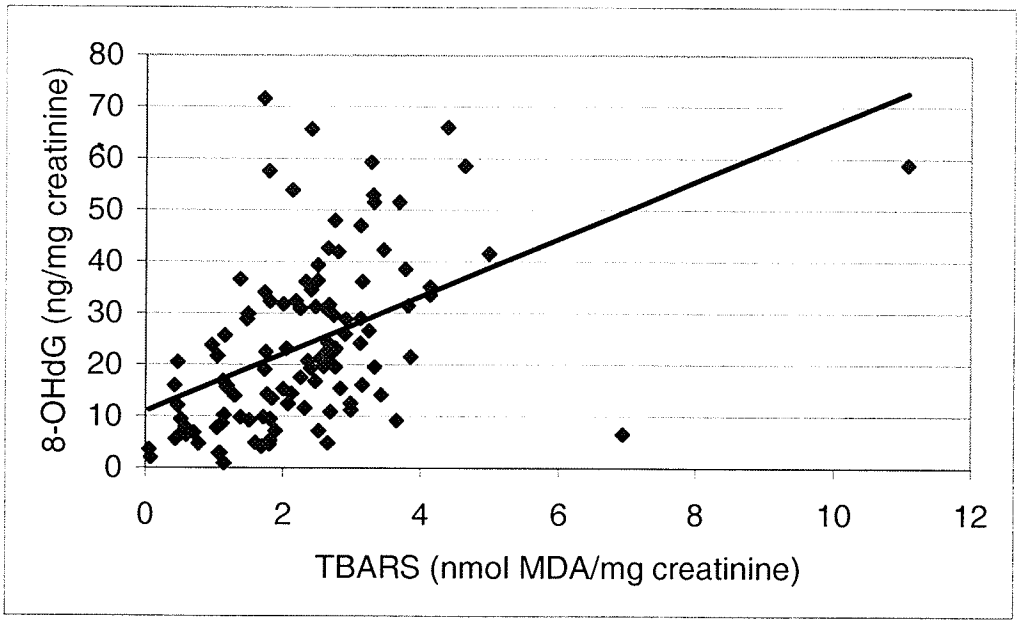

Figure 72. Correlation between 8-OHdG and TBARS in urine samples for phase 1 (N = 5 subjects, n = 110 plotted samples). Spearman correlation = 0.465 (significant at 0.01 level), Pearson correlation = 0.293 (significant at 0.01 level).

4.5.2.2 Findings from the definitive phase and combined data

We were interested in determining if the same disruptive effect of the antioxidants on the association between free radical production and non-heme iron in the feces would also be observed at a systemic level with these two biomarkers. Figure 73 shows a clear association between both factors in the first segment of the graph, which includes samples from baseline, FEoil treatment and the first washout period (WO1 and WO2) (kappa score = 0.46, moderate agreement). The association disappears when the CTCMC was supplied. To confirm the association between 8-OHdG and TBARS away from CTCMC supplementation, Spearman and Pearson's correlations were calculated using only data points from baseline, FEoil and washout treatment days, excluding CTCA and CTCB treatments. These analysis show very strong and significant correlations: Spearman's

correlation = 0.742 ($p < 0.001$, significant at 0.01 level), Pearson´s correlation = 0.817 ($p < 0.001$, significant at 0.01 level) (Figure 74).

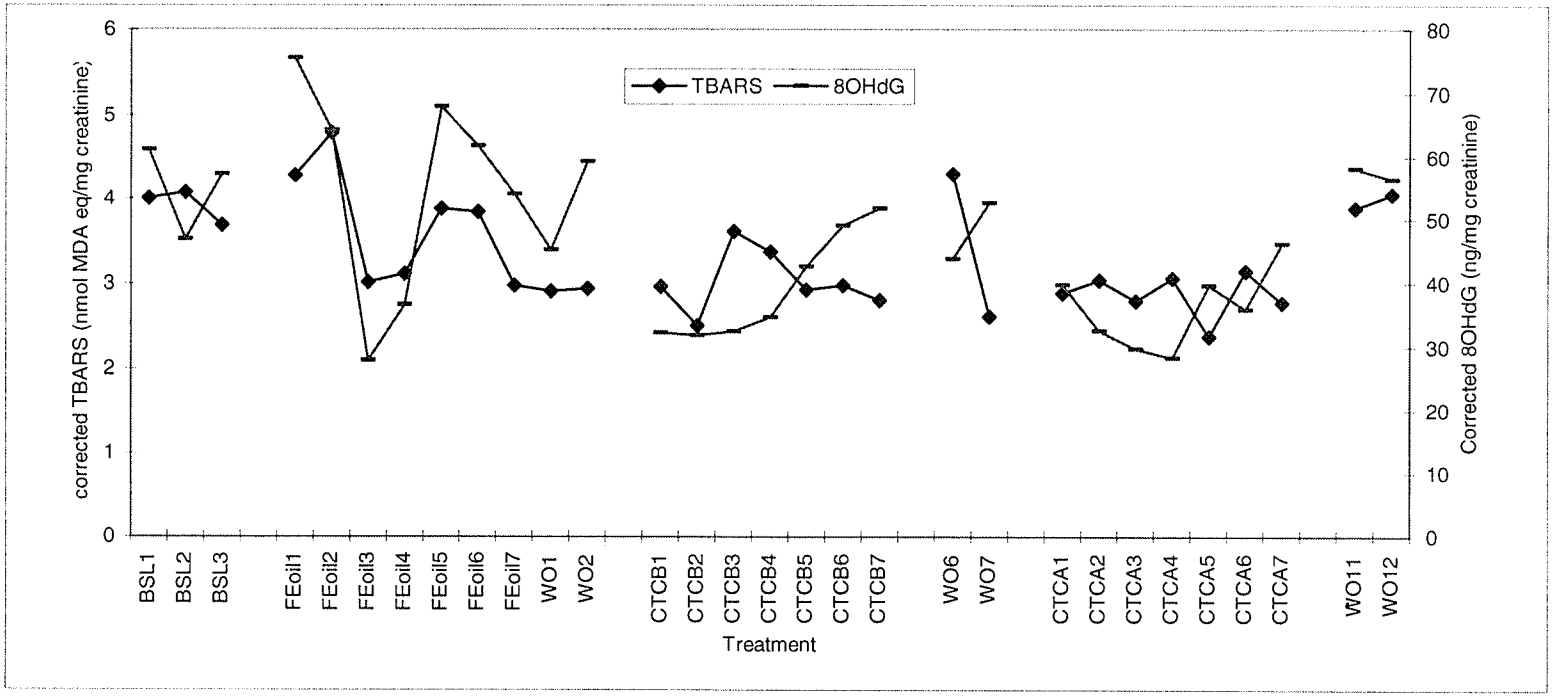

Figure 73. Comparison of average corrected TBARS and 8-OHdG in urine samples from all individuals participating in phase 2 (N = 12 subjects). Graph is not in real time scale, days were no samples were collected are not shown. Kappa coefficient for the treatment associated samples BSLN1 through WO2 = 0.46 (moderate agreement). Kappa coefficient for all the treatment days including days where CTCMC was administrated were not calculated since there is an effect of the CTCMC that smoothes the fluctuations of both biomarkers. (BSL = baseline, FEoil = 120 mg Fe + vegetable oil, WO = washout, CTCB = 0.4 g CTCMC, CTCA = 0.8 g CTCMC).

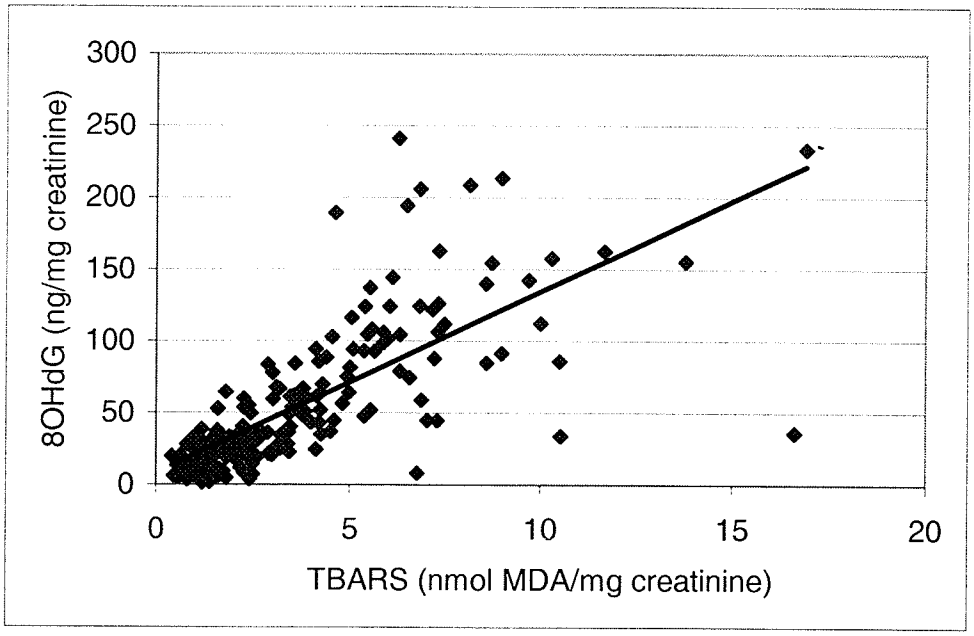

Figure 74. Correlation between 8-OHdG and TBARS in urine samples for phase 2 (N = 12 subjects, n = 189 plotted samples). Correlation does not include samples from CTCA (0.8 g CTCMC) and CTCB (0.4 g CTCMC) treatments. Spearman correlation = 0.742 ($p < 0.001$, significant at 0.01 level), Pearson´s correlation = 0.817 ($p < 0.001$, significant at 0.01 level)

Appendix 6 contains the individual comparisons between urinary TBARS and 8-OHdG for the 12 participants in the second phase.

4.5.3 Response to urinary TBARS and 8-OHdG to oral antioxidant supplementation

Even though the administration of iron had no effect on systemic oxidation in these subjects, the addition of oral supplementation with CTCMC appears to have a demonstrable effect. This effect seems to be, first, a dampening effect on the normal fluctuations of TBARS and 8-OHdG throughout time. The wide oscillations with FEoil or baseline conditions become narrower. More interestingly, it was also capable of

suppressing background excretion levels of 8-OHdG below habitual levels. This suggests a potentially potent antioxidant effect of carotenes and/or tocotrienols on normal DNA oxidation ($p<0.05$) (Figure 22). Statistical analysis showed that there are significant differences in the production of TBARS between the iron plus refined palm oil (FEoil) and CTCA (0.8 g CTCMC) treatments ($p<0.05$), as well as differences in 8-OHdG excretion between the FEoil and the CTCB (0.4 g CTCMC) treatments ($p<0.05$) (Figure 16).

4.5.4 Summary Discussion

Evidence collected from the assays carried out in this study illustrate how different environments, diets, experimental conditions and inter-individual variations does not allow us to make generalizations about highly variable and sensitive biomarkers such as TBARS and 8-OHdG. We were incapable of defining a normative physiological range with our data and the results from other researchers. We were able though, to get a general idea of the differences between oxidation levels for different populations.

In terms of DNA oxidation, the Guatemalan population presents higher levels of DNA damage compared to those from the population in the study by Chin and his group. Nevertheless, 8-OHdG values obtained from routine quality control assays with urines from normal men and women performed by the distributors of the assay kits (Genox Corporation) show a different picture. When we use Genox's parameters, the number of urine samples from the Guatemalan population above the upper limit is 3-fold lower than when we use the parameters from the study by Chin. For this reason, we can not construct a universal parameter that could describe "normal" levels of DNA damage.

During the course of this research, we made some unexpected findings that relate the fluctuations observed in both 8-OHdG and TBARS excretion within their habitual ranges. We were able to demonstrate that there is a strong and significant association between both biomarkers of *in vivo* oxidation within the 'non-pathological' range of oxidative stress in these healthy males, and that this correspondence is disrupted with the antioxidant supplementation. We can not conclude about the biological implications of these observations, but there would seem to be a biological meaning to the fluctuations in the urinary excretion of the oxidation biomarkers (TBARS and 8-OHdG). We ask ourselves if these associations are also observed under pathological states as well.

One thing that has become clear is that the antioxidants from the CTCMC have an authentic effect on the oxidative stress levels of these adults. Chin and colleagues (2008) found a significant reduction in urinary excretion of 8-OHdG after 6 months of supplementing healthy Malaysian adults over 50 years of age with 160 mg of a tocotrienol and tocopherol mix, but did not find any suppression on a younger group with subjects aged 35-49 years. The dose of tocopherols and tocotrienols they used in their study is twice as high (160 mg/day) as the one in ours (74 mg/day in the CTCA treatment), their participants are older in average, but most important, we used a mix of antioxidants that included carotenoids as well.

It is possible that in this trial we might have observed an early suppressing phenomenon that could extinguish with time, but it is also possible that the carotenoids present in the CTCMC boost antioxidant action to the extent that they are capable of

reducing habitual urinary 8-OHdG excretion in younger people who may have less oxidative damage than adults over 50.

In summary, a protective effect of the CTCMC on the oxidation response of individuals in their native environment is suggested by this unexpected observation period. More research is necessary to determine the minimum dose required to achieve a consistent suppressing effect on both DNA and membrane lipid oxidation, and whether any functional improvements or resistance to disease might result from consistent consumption.

5.0 SUMMARY/CONTRIBUTIONS TO KNOWLEDGE/ SUGGESTIONS FOR FUTURE WORK

The present work allowed us to explore the dynamics of the oxidation processes occurring during oral iron supplementation in non-anemic individuals. We could not create an *in vivo* oral-iron-challenge response at a systemic level in our study population with the proposed iron supplementation scheme, and thus, we were unable to test the effect of simultaneous supplementation with antioxidants from the CTCMC. We were able though, to demonstrate that the supplementation with 120 mg of iron per day for seven days is capable of inducing oxidative stress in the gut by depleting the antioxidant capacity state of the feces. Apparently, residual non-heme iron passing through the intestines lessens the antioxidants present in the fecal matter. The antioxidants (carotenoids, tocopherols and tocotrienols) from the Carotino® Tocotrienol Carotenoid Mixed Concentrate (CTCMC) restored the fecal capacity state depleted by supplemental iron and surprisingly, they also reduced DNA and membrane lipid oxidation below habitual levels.

We cannot draw definite conclusions about the hypothesis that tested the dose-dependent action of the CTCMC. At a systemic level, both concentrations of CTCMC caused different responses in the urinary biomarkers of oxidation, but in the intestines both 0.4 and 0.8 g CTCMC doses consistently reduced ROS production in the fecal samples. This opens the door to potential new research that will help determine the minimum effective concentration of CTCMC that will have an overall protective effect against systemic and *in situ* oxidation.

Contrary to our original premise, the vegetable oil used in the oral iron-in-oil-challenge did not have a protective effect against the theoretical oxidation produced by iron supplementation. Actually, the vegetable oil did not influence systemic oxidative stress but it did enhance ROS production in the gut. The mechanisms by which oil affects intestinal oxidation still need to confirmed and elucidated, assuming the tendency seen is a real oil-dependent effect.

Examining the findings from the urinary biomarkers, the question raised as to what could be considered "normal levels" of urinary TBARS and 8-OHdG excretion. For this purpose we tried, unsuccessfully, to create a parameter to define normative values of urinary biomarkers by comparing our results with those from other researchers in the literature. The problem was that there are major factors such as: diet, environmental and experimental conditions which influence these highly-sensitive oxidative stress markers and incapacitate us from setting such range. Since urinary excretion of TBARS and 8-OHdG remained unaffected by supplementation with ferrous sulphate, unlike the experience of Schümann *et al* with young German subjects, we considered the values observed during and away iron supplementation without antioxidants to be "habitual levels". Comparative research could clarify the issue as to why the German population responded to the iron-challenge and the Guatemalan sample did not, this might resolve if there are differences in the excretion of TBARS and 8-OHdG to oral iron or if the results published by Schümann *et al*, are irreproducible.

Fortuitously we made some interesting findings in which we discovered a very strong and significant association between the two biomarkers of oxidation -- TBARS and 8-OHdG -- under habitual, non-pathological conditions. The addition of antioxidants disrupts the correspondence significantly. The biological significance of these observations remains unknown, pointing out to potentially exciting *in vivo* investigation of systemic oxidation in healthy human subjects. It will also be interesting to evaluate if these associations are also observed during pathological states and what are the implications of such a phenomenon.

After evaluating the effects of supplemental iron on healthy, non-anemic men, the next step is to assess the implications of the ongoing iron supplementations practices on the oxidative status of women in reproductive age, especially in the anemic. These are the group of adults most subject to oral iron interventions. It was important to conduct this research with healthy men to avoid confounders that would interfere with the observations under normal metabolic and physiological circumstances. Now that it has been defined that these prophylactic doses of iron, used to treat iron deficiency anemia, have no effect on systemic oxidative stress in our population, it is important to determine what the consequences on the colonic oxidative milieu of women are. Other prenatal supplementation regimens use lower iron concentrations (30 mg, 60 mg) which calls for further research to test dose-response effects of supplemental iron on stool ROS production at lower concentrations than the one used in this study (120 mg).

Animal studies are necessary to complement these results and give us a better understanding of the processes taking place within the colonocytes during exposure to

high concentrations of iron. The question remains as to what extent the depleted antioxidant capacity state in the fecal stream caused by long-term iron supplementation induces DNA damage that could precipitate into carcinogenesis.

This has been an innovative research in the sense that we looked at iron-induced oxidation as a process occurring at both systemic and intestinal levels. Among the biggest contributions to knowledge was the application of an *in vitro* assay to assess fecal ROS production in an *in vivo* context. This is especially important given the difficulty to evaluate colonic oxidative damage in humans. The applications of this method are extensive. Colonic oxidative stress could be studied in parasitic intestinal diseases, Crohn's disease, and ulcerative colitis, among many others. Prospective long-term studies assessing the role of dietary fiber, antioxidants, or red meats on the intestinal oxidative status could be done in a non-invasive and comfortable way in human subjects.

6.0 REFERENCES

Actis-Goretta L, Carrasquedo F and Fraga CG. 2004. The regular supplementation with an antioxidant mixture decreases oxidative stress in healthy humans. Clin Chim Acta 349: 97–103.

Acworth IN and Bailey B. 1995. *The Handbook of Oxidative Metabolism.* ESA. USA.

Astley SB, Hughes DA, Wright AJ, Elliott RM and Southon S. 2004. DNA damage and susceptibility to oxidative damage in lymphocytes: effects of carotenoids in vitro and in vivo. Br J Nutr. 91:53-61.

Badcock NR, Zoanetti GD and Martin ES. 1997. Nonchromatographic assay for malondialdehyde–thiobarbituric acid adduct with HPLC equivalence. Clin Chem. 43:1655-1657.

Bayorh MA, Abukhalaf IK and Ganafa AA. 2005. Effect of palm oil on blood pressure, endothelial function and oxidative stress. Asia Pac J Clin Nutr. 14:325-339.

Benade AJ. 2003. A place for palm fruit oil to eliminate vitamin A deficiency. Asia Pac J Clin Nutr. 12:369-372.

Boateng J, Verghese M, Chawan CB, Shackelford L, Walker LT, Khatiwada J and Williams DS. 2006. Red palm oil suppresses the formation of azoxymethane (AOM) induced aberrant crypt foci (ACF) in Fisher 344 male rats. Food Chem Toxicol. 44:1667-1673.

Bullen JJ, Rogers HJ, Spalding PB and Ward CG. 2006. Natural resistance, iron and infection: a challenge for clinical medicine. J. Med Microbiol. 55:251–258.

Cairo G and Pietrangelo A. 2000. Iron regulatory proteins in pathobiology. Biochem. J. 352:241-250.

Casanueva E and Viteri FE. 2003. Iron and oxidative stress in pregnancy. J. Nutr. 133:1700S–1708S.

Chin SF, Hamid NA, Latiff AA, Zakaria Z, Mazlan M, Yusof YA, Karim AA, Ibahim J, Hamid Z and Ngah WZ. 2008. Reduction of DNA damage in older healthy adults by TriE® Tocotrienol supplementation. Nutrition 24:1-10.

Chung CJ, Huang CJ, Pu YS, Su CT, Huang YK, Chen YT and Hsueh YM. 2007. Urinary 8-hydroxydeoxyguanosine and urothelial carcinoma risk in low arsenic exposure area. Toxicol Appl Pharmacol. Article in press.

Clift E. *Food for the body, fuel for life.* Perspectives in Health.1997; 2(1). URL: http://www.paho.org/English/DPI/Number3_article2.htm. (July 8, 2005).

Conrad ME and Umbreit JM. 2000. Iron absorption and transport-an update. Am J Hematol. 64:287-298.

Darley-Usmar V and Starke-Reed PE. 2000. Antioxidants: strategies for interventions in aging and age-related diseases. A workshop sponsored by the National Institute on Aging and by the Office of Dietary Supplements. Antioxid Redox Signal. 2:375-377.

Department of Obstetrics and Gynaecology. The Chinese University of Hong Kong. *Cohen's kappa calculator.* URL: http://department.obg.cuhk.edu.hk/researchsupport/Cohen_Kappa_matrix.asp. (December, 2007 and January, 2008)

Department of Reproductive Health and Research (RHR), World Health Organization. 1998. *Postpartum care of the mother and newborn: a practical guide.* URL: http://www.who.int/reproductive-health/publications/msm_98_3/msm_98_3_5.html. (July 8, 2005).

Droge W. 2002. Free radicals in the physiological control of cell function. Physiol Rev. 82: 47–95.

Esterhuyse AJ, du Toit EF and van Rooyen J. 2005. Dietary red palm oil supplementation protects against the consequences of global ischemia in the isolated perfused rat heart. Asia Pac J Clin Nutr. 14:340-347.

Faulks M and Southon S. 2001. Carotenoids, Metabolism and Disease. Pages 1-12 in *Handbook of Nutraceuticals and Functional Foods.* CRC Press. Boca Raton, Florida.

Ferrari CK and Torres EA. 2003. Biochemical pharmacology of functional foods and prevention of chronic diseases of aging. Biomed Pharmacother. 57:251-260.

Fisher AEO and Naughton DP. 2004. Iron supplements: the quick fix with long-term consequences. Nutr J. 3:251-260.

Food and Nutrition Board. Institute of Medicine. National Academies. 1998. *Prevention of Micronutrient Deficiencies: Tools for Policymakers and Public Health Workers.* Washington, DC, National Academy Press. 224 pp.

Food and Nutrition Board. Institute of Medicine. National Academies. 2000. *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids.* Washington, DC, National Academy Press. 529 pp.

Food and Nutrition Board. Institute of Medicine. National Academies. 2001. *Dietary Reference Intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc.* Washington, DC, National Academy Press. 800 pp.

Food and Nutrition Board. Institute of Medicine. National Academies. 2004. *Dietary Referente Intakes (DRIs): Recommended Intakes for Individuals.* URL: http://www.iom.edu/Object.File/Master/21/372/0.pdf. (April 10, 2008)

Franzetti S, Mejia LA, Viteri FE and Alvarez E. Body iron reserves of rural and urban Guatemalan women of reproductive age. Arch Latinoam Nutr. 1984; 34(1): 69-82.

Fu S, Davies MJ, Stocker R and Dean RT. 1998. Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque. Biochem. J. 333: 519-525.

Galloway R and McGuire J. 1994. Determinants of compliance with iron supplementation: supplies, side effects or psychology? Soc Sci Med. 39:381-390.

Glei M, Latunde-Dada GO, Klinder A, Becker TW, Hermann U, Voigt K and Pool-Zobel BL. 2002. Iron overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A. Mutat Res. 519:151-161.

Global Micronutrient Project. 2001. *The Micronutrient Report: Current Progress and Trends in the Control of Vitamin A, Iodine, and Iron Deficiencies.* URL: http://www.tulane.edu/~internut/publications/MI%20REPORT.pdf. (July 8, 2005).

Gutteridge, JMC. 1995. Lipid Peroxidation and Antioxidants as Biomarkers of Tissue Damage. Clin Chem. 41:1819-1828.

Hallberg L, Hultén L and Gramatkovski E. 1997. Iron absorption from the whole diet in men: how effective is the regulation of iron absorption? Am J Clin Nutr. 66:347-356.

Horl W. 2007. "Iron therapy for renal anemia: how much needed, how much harmful?" Pediatr Nephrol. 22:480–489.

Huma N, Salim-Ur-Rehman, Anjum FM, Murtaza MA and Sheikh MA. 2007. Food fortification strategy—preventing iron deficiency anemia: a review. Crit Rev Food Sci Nutr. 47:259-65.

Huycke MM and Gaskins HR. 2004. Commensal Bacteria, Redox Stress, and Colorectal Cancer: Mechanisms and Models. Exp Biol Med. 229:586–597.

Jacobs DR Jr, Anderson LF and Blomhoff R. 2007. Whole-grain consumption is associated with a reduced risk of non-cardiovascular, non-cancer death attributed to inflammatory diseases in the Iowa Women's Health Study. Am J Clin Nutr. 85:1606-1614.

Jialal I and Devaraj S. 2003. Antioxidants and atherosclerosis: don't throw out the baby with the bath water. Circulation. 107:926-928.

Kabat GC and Rohan TE. 2007. Does excess iron play a role in breast carcinogenesis? An unresolved hypothesis. Cancer Causes Control. 18:1047-53.

Kamireddy R, Kavuri S, Devi S, Vemula H, Chandana D, Harinarayanan S, James R and Rao A. 2002. Oxidative stress in pediatric nephritic syndrome. Clinica Chimica Acta. 325:147-150.

Khanna S, Roy S, Ryu H, Bahadduri P, Swaan PW, Ratan RR and Sen CK. 2003. Molecular basis of vitamin E action: tocotrienol modulates 12-lipoxygenase, a key mediator of glutamate-induced neurodegeneration. J Biol Chem. 278:43508-43515.

Kiokias S and Gordon MH. 2003. Dietary supplementation with a natural carotenoid mixture decreases oxidative stress. Eur J Clin Nutr. 57:1135-1140.

Klassen P, Biesalski HK, Mazariegos M, Solomons NW and Furst P. 2004. Classic dengue fever affects levels of circulating antioxidants. Nutrition. 20:542-547.

Knöbel Y, Glei M, Osswald K and Pool-Zobel BL. 2006. Ferric iron increases ROS formation, modulates cell growth and enhances genotoxic damage by 4-hydroxynonenal in human colon tumor cells. Toxicol In Vitro. 20:793-800.

Knöbel Y, Weise A, Glei M, Sendt W, Claussen U and Pool-Zobel BL. 2007. Ferric iron is genotoxic in non-transformed and preneoplastic human colon cells. Food Chem Toxicol. 45:804-811.

Koorts AM and Viljoen M. 2007. Ferritin and ferritin isoforms II: protection against uncontrolled cellular proliferation, oxidative damage and inflammatory processes. Arch Physiol Biochem. 113:55-64.

Kritchevsky, D. 2000. Impact of red palm oil on human nutrition and health. Food and Nutrition Bulletin. 21:182-188.

Kruger MJ, Engelbrecht AM, Esterhuyse J, du Toit EF and van Rooyen J. 2007. Dietary red palm oil reduces ischaemia-reperfusion injury in rats fed a hypercholesterolaemic diet. Br J Nutr. 97: 653-660.

Lee BL, New AL and Ong CN. 2003. Simultaneous determination of tocotrienols, tocopherols, retinol, and major carotenoids in human plasma. Clin Chem. 2003 49:2056-2066.

Lindshield BL and Erdman JW. 2006. Carotenoids. Pages 184-197 in *Present Knowledge in Nutrition, Vol 1.* Bowman BA and Russell RM, ed. 9th edition. International Life Sciences Institute. Washington DC.

Lund EK, Wharf SG, Fairweather-Tait S and Johnson IT. 1998. Increases in the concentrations of available iron in response to dietary iron supplementation are associated with changes in crypt cell proliferation in rat large intestine. J Nutr. 128:175-179.

Lund EK, Wharf SG, Fairweather-Tait S and Johnson IT. 1999. Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. Am J Clin Nut. 69:250-255.

Lund EK, Wharf SG, Fairweather-Tait S and Johnson IT. 2001. Chronic exposure to high levels of dietary iron fortification increases lipid peroxidation in the mucosa of the rat large intestine. J Nutr. 131:2928-2931.

Mahapatra S and Manorama R. 1997. The protective effect of red palm oil in comparison with massive vitamin A dose in combating vitamin A deficiency in Orissa, India. Asia Pac J. Clin Nutr. 6:56-59.

Marotta F, Yoshida C, Barreto R, Naito Y and Packer L. 2007. Oxidative-inflammatory damage in cirrhosis: effect of vitamin E and a fermented papaya preparation. J Gastroenterol Hepatol. 22:697-703.

Mayes PA. 1994. Lípidos de importancia fisiológica. Pages 169-192 in *Bioquímica de Harper.* Murray RK, Granner DK, Mayes PA, and Rodwell VW. ed. Editorial El Manual Moderno, S.A. de C.V. México D.F.

Mayne S. 2003. Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. J Nutr. 133:933S-940S.

McLean E, Cogswell M, Egli I, Wojdyla D and de Benoist B. 2007. Worldwide prevalence of anemia in pre-school children, pregnant women and non-pregnant women

of reproductive age. Pages 1-12 in *Nutritional Anemia.* Kraemer K and Zimmermann MB, ed. Sight and Life Press. Basel, Switzerland.

Menchú MT, Mendez H, Barrera MA and Ortega L. 1996. Tabla de Composición de Alimentos de Centro América. INCAP/OPS, Guatemala.

Mínguez-Mosquera MI, Hornero-Mendez D and Pérez-Gálvez A. 2002. Carotenoids and provitamin A in functional foods. Pages 1201-157 in *Methods of Analysis for functional foods and nutraceuticals.* Hurst WJ ed. CRC Press. Boca Raton, Florida.

Ministerio de Salud Pública y Asistencia Social. Programa de Seguridad Alimentaria y Nutricional. Departamento de Regulación de los Programas de Atención a las Personas. Dirección de Regulación, Vigilancia y Control de la Salud. 2006. *Lineamientos para la suplementación con vitamina A, hierro, ácido fólico a niños y niñas de 6 a 59 meses, mujeres en edad fértil, mujeres embarazadas y 6 meses después del parto.* Guatemala, Guatemala.

Nagesundram B, Unnithan UR, Choo YM and Sundram K. 2000. Characteristics of red palm oil, alpha carotene- and vitamin E- rich refined oil for food uses. Food and Nutrition Bulletin. 21:189-194.

Nelson JL, Bernstein PS, Schmidt MC, Von Tress MS and 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.

Nelson RL, Yoo SJ, Tanure JC, Andrianopoulos G and Misumi A. 1989. The effect of iron on experimental colorectal carcinogenesis. Anticancer Res. 9:1477-1482.

Nur Azlina MF, Nafeeza MI and Khalid BAK. 2005. A comparison between tocopherol and tocotrienol effects on gastric parameters in rats exposed to stress. Asia Pac J Clin Nutr. 14: 358-365.

Owen, RW, Spiegelhalder B and Bartsch H. 2000. Generation of oxygen reactive species by faecal matrix. Gut. 46:225-232.

OXItek. *TBARS assay.* URL: www.zeptmetrix.com/0801192. (January, 2008)-.

Ozbay B and Dulger H. 2002. Lipid peroxidation and antioxidant enzymes in Turkish population: Relation to age, gender, exercise, and smoking. Tohoku J Exp Med. 197:119-124.

Pan American Health Organization. *Suministro de alimentos y estado nuticional: Guatemala.* URL: www.paho.org/english/sha/gutrstp.htm. (September 13, 2005).

Poredos P. *Systemic effects of intermittent claudication.* E-journal of Cardiology Practice. European Soc of Cardiology. Vol 4 No. 2. URL: http://www.escardio.org/knowledge/cardiology_practice/ejournal_vol4/vol4n2.htm. (January 4, 2006).

Proteggente AR, Rehman A, Halliwell B and Rice-Evans CA. 2000. Potential problems of ascorbate and iron supplementation: pro-oxidant effect in vivo? Biochem Biophys Res Commun. 277:535-540.

Schümann K, Kroll S, Weiss G, Frank J, Biesalski HK, Daniel H, Friel J and Solomons NW. 2005. Monitoring of hematological, inflammatory and oxidative reactions to acute oral iron exposure in human volunteers: Preliminary screening for selection of potentially-responsive biomarkers. Toxicology. 212:10-23.

Schümann K, Ettle T, Szegner B, Elsenhans B and Solomons NW. 2007. On risks and benefits of iron supplementation recommendations for iron intake revisited. J of Trace Elem in Med and Biol. 21:147-168.

Schwedhelm E, Maas R, Troost R and Boger RH. 2003. Clinical pharmacokinetics of antioxidants and their impact on systemic oxidative stress. Clin Pharmacokinet. 42: 437-459.

Scrimshaw N. 2000. Nutritional potential of red palm oil for combating vitamin A deficiency. Food Nutr Bull. 21:195-201.

Seril DN, Liao J, West AB and Yang GY. 2005. High-iron diet: foe or feat in ulcerative colitis and ulcerative colitis-associated carcinogenesis. J Clin Gastroenerol. 40:391-397.

Siemianowicz K, Gminski J, Francuz T, Wojcik A and Posielezna B. 2003. Activity of antioxidant enzymes in children from families at high risk of premature coronary heart disease. Scan J Clin Invest. 63:151-158.

Simic MG, Jovanovic SV and Niki E. 1992. Mechanisms of lipid oxidative processes and their inhibition. Pages 14-32 in *Lipid Oxidation in Food.* St. Angelo AJ. ed. American Chemical Society, Washington DC.

Simon S. *What is kappa coefficient?.* URL: http://www.childrens-mercy.org/stats/definitions/kappa.htm. (November 19, 2007).

Skoog DA, West DM, and Holler FJ. 1997. *Fundamentos de química analítica.* 4th. ed. Editorial Reverté, Barcelona, Spain.

Stoltzfus RJ and Dreyfuss ML. 1999. Guidelines for the Use of Iron Supplements to Prevent and Treat iron Deficiency. International Nutritional Anemia Consultative Group (INACG). Washington DC, International Life Science Institute.

Stone V, Johnston H and Clift MH. 2007. Air pollution, ultrafine and nanoparticle toxicology: cellular and molecular interactions. Trans Nanobioscience. 6:331-340.

Stone WL, Koyamangalath K, Campbell E, Qui M, Whaley SG and Yang H. 2004. Tocopherols and the treatment of colon cancer. Ann N.Y. Acad Sci. 1031:223-233.

Taketani, S. 2005. Acquisition, mobilization and utilization of cellular iron and heme: endless findings and growing evidence of tight regulation. Tohoko J. Exp Med. 205:297-318.

Tapia A and Araya M. 2006. Estrés oxidativo, prooxidantes y enfermedad de Crohn. Rev Med Chile. 134:95-100.

Tappel A. 2007. Heme of consumed red meat can act as a catalyst of oxidative damage and could initiate colon, breast and prostate cancers, heart disease and other diseases. Med Hypotheses. 68:562-564.

Tsukahara H, Sekine K, Uchiyama M, Kawakami H, Hata I, Todoroki Y, Kiraoka M, Kaji M, Yorifuji T, Momoi T, Yoshihara K, Beppu M and Mayumi M. 2003. Formation of advanced glycosilation end products and oxidative stress in young patients with Type 1 diabetes. Ped Res. 54: 419-424.

Tsukahara H, Shibata R, Ohshima Y, Todoroki Y, Sato S, Ohta N, Hiraoka M, Yoshida A, Nishima S and Mayumi M. 2003. Oxidative stress and altered antioxidant defenses in children with acute exacerbation of atopic dermatitis. Life Sci. 72: 2509-2516.

Tyssandier V, Reboul E, Dumas JF, Bouteloup-Demange C, Armand M, Marcand J, Sallas M and Borel P. 2003. Processing of vegetable-borne carotenoids in the human stomach and duodenum. Am J Physiol Gastrointest Liver Physiol. 284: G913–G923.

United States Department of Agriculture. *USDA National Nutrient Database for Standard Reference*. URL: http://www.nal.usda.gov/fnic/foodcomp/search/index.html. (January - March, 2008).

Upritchard JE, Schuurman C, Wiersma A, Tijburg L, Coolen S, Rijken PJ and Wiseman, SA. 2003. Spread supplemented with moderate doses of vitamin E and carotenoids reduce lipid peroxidation in healthy, nonsmoking adults. Am J Clin Nutr. 78:985-992.

van der Logt EMJ, Roelofs HMJ, Wobbes T, Nagengast FM and Peters WHM. 2005. High oxygen radical production in patients with sporadic colorectal cancer. Free Rad Biol and Med. 39:182-187.

van Stuijvenberg ME, Dhansay MA, Lombard CJ, Faber M and Benade AJS. 2001. The effect of a biscuit with red palm oil as a source of β-carotene on the vitamin A status of primary school children: a comparison with β-carotene from a synthetic source in a randomized controlled trial. Eur. J. Clin Nutr. 55:657-662.

Varvarovska J, Racek J, Stozicky F, Soucek J, Trefil L and Pomahacova R. 2003. Parameters of oxidative stress in children with Type 1 diabetes mellitus and their relatives. Diabetes Complications. 17:7-10.

Vertuani S, Angusti A and Manfredini S. 2004. The antioxidants and pro-antioxidants network: an overview. Curr Pharm Des. 10:1677-1694.

Walter PB, Knutson MD, Paler-Martinez A, Lee S, Xu Y, Viteri FE and Ames BN. 2002. Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. PNAS. 99:2264-2269.

Wildman EC. 2001. Nutraceuticals: A brief review of Historical and Teleological Aspects. Pages 1-12 in *Handbook of Nutraceuticals and Functional Foods*. Wildman EC. Ed. CRC Press. Boca Raton, Florida.

Winklhofer-Roob BM, Rock E, Ribalta J, Shmerling, DH and Roob JM. 2003. Effects of vitamin E and carotenoid status on oxidative stress in health and disease. Evidence obtained from human intervention studies. Mol Aspects Med. 24:391-402.

Wish JB. 2006. Assessing iron status: beyond serum ferritin and transferrin saturation. Clin J Am Soc Nephrol. 1:S4–S8.

Wong RH, Kuo CY, Hsu ML, Wang TY, Chang PI, Wu TH and Huang S. 2005. Increased levels of 8-hydroxy-2 -deoxyguanosine attributable to carcinogenic metal exposure among schoolchildren. Environ Health Perspect. 113:1386-1390.

World Health Organization and Food and Agriculture Organization of the United Nations. 2004. *Vitamin and mineral requirements in human nutrition.* URL: http://whqlibdoc.who.int/publications/2004/9241546123.pdf. (July 8, 2005).

Wyman JB, Heaton KW, Manning AP and Wicks CB. 1978. Variability of colonic function in healthy adults. Gut. 19:146-150.

Yao D, Shi W, Gou Y, Zhou X, Yee Aw T, Zhou Y and Liu Z. 2005. Fatty acid-mediated intracellular iron translocation: a synergistic mechanism of oxidative injury. Free Radic Biol Med. 39:1385-1398.

Yeum KJ, Aldini G and Russell R. 2007. Antioxidant nutrients and oxidative DNA damage in humans. Sight and Life Magazine Issue 1/2007.

Yip, R. 2001. Iron. Pages 311 – 328 in *Present Knowledge in Nutrition, Vol 1.* Bowman BA and Russell RM, ed. 8th edition. International Life Sciences Institute. Washington DC.

Yoshida Y, Niki E and Noguchi N. 2003. Comparative study on the action of tocopherols and tocotrienols as antioxidant: chemical and physical effects. Chem Phys Lipids. 123:63-75.

Zagre NM, Delpeuch F, Traissac P and Delisle H. 2003. Red palm oil as a source of vitamin A for mothers and children: impact of a pilot project in Burkina Faso. Public Health Nutr. 6:733-742.

Zeba AN, Prével YM, Somé IT and Delisle HF. 2006. The positive impact of red palm oil in school meals on vitamin A status: study in Burkina Faso. Nutr J. 5:17.

Zingg JM and Azzi A. 2004. Non-antioxidant activities of vitamin E. Curr Med Chem. 11:1113-1133.

APPENDIX 1: General information questionnaire used for recruiting participants

GENERAL INFORMATION QUESTIONNAIRE

Date: _____

Personal Data:

Name of participant	
Age	
Date of birth	
Address	
Phone	
E-mail address	

Medical History:

- Smokes? Yes ____ No ____
- Suffers from gastrointestinal disorders? Yes ____ No ____
  If so, which ones: _____
- Suffers from hematological disorders? Yes ____ No ____
  If so, which ones: _____
- Suffers anemia? Yes ____ No ____
- Takes nutritional supplements? Yes ____ No ____
  If so, which ones: _____
- Suffers an inflammatory condition (for example malaria)? Yes ____ No ____
  If so, which one: _____
- Has intolerance to iron supplements? Yes ____ No ____

Consent form:

Signed consent form: Yes ____ No ____

APPENDIX 2: Consent form used in first phase (pilot study)

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: PILOT STUDY: *In situ* and *in vivo* modification by palm oil on the oxidative response to commonly-prescribed doses of oral iron

Protocol Number: B2006:076

Principal Investigator:

Monica Orozco
Doctoral Student
Department of Human Nutritional Sciences
Faculty of Human Ecology
University of Manitoba
Fort Garry Campus
204-474-7030

Co-Investigators:

Dr. James K. Friel
Department Head
Department of Human Nutritional Sciences
Faculty of Human Ecology
University of Manitoba
Fort Garry Campus
204-474-7030

Dr. Michael Eskin
Professor
Department of Foods and Nutrition
Faculty of Human Ecology
University of Manitoba
Fort Garry Campus
204-474-8078

Dr. Trust Beta
Assistant Professor
Department of Food Science
University of Manitoba
Fort Garry Campus
204-474-8214

Dr. Noel Solomons
Scientific Director

Center for the Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM)
Guatemala City, Guatemala
(502) 24733942

Sponsor: Malaysian Palm Oil Board (MPOB)

You are being asked to participate in a human research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

The main researcher is receiving financial support to conduct this study.

Purpose and nature of this study:

Iron is an essential nutrient, necessary for oxygen transport on red blood cells and in tissues. It is also involved as a constituent of enzymes. When there is iron deficiency, a condition called anemia occurs. Children, pregnant women and women in reproductive age are the most vulnerable groups.

Oral iron supplementation is the most commonly used therapy to treat iron deficiency anemia. Guatemala's Health and Public Assistance Ministry has recommended pregnant women to take a dose of 120 mg per week while the international standards recommend from 30 to 120 mg/day to prevent iron deficiency in vulnerable populations. Recently it has been demonstrated that chronic, daily dosing of iron may be associated with chemical effects on the substance of cells that make up your body.

You are being asked to participate in a human research study. This study is a pilot study conducted to evaluate the effect of a palm oil on your ability to resist oxidation caused by normal doses of supplemental iron prescribed to pregnant women in case of anemia and learn more about the mechanisms on how the oil might work in order to provide efficient protection. We also want to standardize lab tests and procedures to be used in a future study with the same methodologies.

Methods:

Five participants will take part of this. If you participate, you will have the following tests and procedures: will be asked to take daily doses of iron alone and iron mixed with oil during short periods of time (7 days each) and to give us several urine and feces

samples, as well as two blood samples. We will give you a calendar of the days in which you have to take the iron and when you have to give us your blood, urine and feces samples. The participation involves being studied for an overall period of 43 days, during which 25 days will be considered sample-collection days. The 43 days are divided in 2 cycles of 21 days each. On the days of collection you will be asked to collect the first urine you excrete after you wake up, a feces sample collected according to your normal bowel habit and bring them to the laboratory at the Universidad del Valle for storage and further processing. You should not do any vigorous exercise on the days of sample collection. A trained technician, nurse or physician will take two blood samples at the beginning and at the end of the study. After we take your blood sample, you will have a small breakfast.

After you have provided us with your samples, we will ask you to take either of the following treatments:

TREATMENT A: 120 mg of iron in form of a syrup plus water
TREATMENT B: 120 mg of iron in form of syrup with 5 ml of commercial refined palm oil.

You will have to take all of the treatments mentioned above during 7 days each. There will be periods of time in between each treatment in which you just take water, but you will have to give us your urine and feces samples.

You will also have to record in a "Food Diary" everything you eat during two complete weeks during the study. This will allow us to know if there are other major sources of iron in your diet that may act as confounders in the biochemical analysis.

The researcher may decide to take you off this study if you have or develop a persistent inflammatory or infectious condition during the study.

You can withdraw from the study at any time. But if you decided to do so, we encourage you to talk to any member of our research staff.

Who can participate in this study:

Male healthy adult subjects who sign an informed written consent and are willing to provide all the blood, feces and urine samples required for this study.

Who can NOT participate in this study:

People who smoke, suffer from anemia, have a history of gastrointestinal disorders, intolerance to iron supplements, hematological disorders, or consume other nutritional supplements.

Risks and discomforts:

Your participation in this study involves fasting during the blood collection days, consumption of medicinal doses of iron and the provision of urine and feces samples. Fasting is associated with low levels of blood sugar which can produce lightheadedness.

The amounts of iron to be administrated are less than the ones used to treat therapeutically iron deficiency anemia (standard therapeutic dose for iron deficiency anemia is 300 mg or iron per day for 30 days). The iron will be mixed with a flavored syrup that may have a strong metallic taste that can be unpleasant and in some cases it can produce nausea and vomiting. Stools will often be black as a consequence of the iron supplementation.

We will take a 10 ml blood sample (equivalent to 2 teaspoons) at the beginning and end of the study; the collection may be uncomfortable due to the insertion of the hypodermic needle. A small bruise may appear on the puncture site.

Benefits:

By participating in this study, you will provide us with important information of benefit to science and society. You will help us understand and find ways to reduce the effects of iron exposure due to supplementation in public health and clinical applications. You will also get information about your health at the moment of the study. As a compensation for your collaboration you will receive USD100.00 after the first cycle of supplementation and USD 150.00 at the end of the last cycle of supplementation, making a total of USD 250.00.

Costs:

All clinic and professional fees, diagnostic and laboratory tests which will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Confidentiality:

The information obtained in this research may be published or presented in conferences and/or public forums. Your name will not be used or revealed, we will use code numbers to refer to your records. The data obtained from your exams will be stored on a computer and on written records. Despite the efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. If the law requires it, your personal information may be disclosed.

The University of Manitoba Biomedical Research Ethics Board may review the records related to the research, for quality assurance purposes.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your medical/research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the offices of CeSSIAM.

Voluntary participation/Withdrawal from the study:

Your participation in this study is voluntary. You may refuse to participate or withdraw from the study at any time.

You are not waiving any of your legal rights by signing this consent form or releasing the investigator or sponsor from their legal and professional responsibilities.

Medical Care for Injury Related to the Study

If you should become physically injured as a result of any research activity, the study doctor will provide any necessary treatment, at no charge, to help you promptly recover from the injury.

If you should become physically injured as a result of any research activity, the study doctor will provide treatment, at no charge, to help you recover from the injury, but we will not cover any expenses in case of hospitalization.

Questions:

You are free to ask any questions about your participation in the study and your rights. If you have any questions during or after the study, or if you have a research-related injury, please contact Ms. Monica Orozco at (502) 24733942 or Dr. Noel Solomons at the same phone number.

For questions related to your rights as a participant in a research, you may contact the Dr. Fernando Beltranena at (502) 2362-2429.

Do not sign this consent form until all the information is clear and your questions have been answered.

Statement of Consent:

I have read this consent form. I have had the opportunity to discuss this research study with Monica Orozco and or the other researchers and staff. All my questions were

answered by them in language I understand. The risks and benefits haven been explained to me and are clear. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I can withdraw at any time. I freely agree to participate in this research study.

I understand that the information regarding my personal identity will be kept confidential, but that confidentiality is not fully guaranteed. I authorize the inspection of my records by the Food and Drug Administration, the Health Protection Branch, government agencies and institutions in other countries, The University of Manitoba Biomedical Research Ethics Board and CeSSIAM's Human Research Ethics Board.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

Date: _____

Printed name: _____

Signature: _____

Witness signature: _____

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent

Date: _____

Printed name: _____

Signature: _____

APPENDIX 3: Consent form used in second phase (Intervention trial)

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: *In situ* and *in vivo* modification by palm fruit-derived carotenes and tocotrienols on the oxidative response to commonly-prescribed doses of oral iron

Protocol Number: B2006:076

Principal Investigator: **Monica Orozco**
Doctoral Student
Department of Human Nutritional Sciences
Faculty of Human Ecology
University of Manitoba
Fort Garry Campus
204-474-7030

Co-Investigators: **Dr. James K. Friel**
Department Head
Department of Human Nutritional Sciences
Faculty of Human Ecology
University of Manitoba
Fort Garry Campus
204-474-7030

Dr. Michael Eskin
Professor
Department of Foods and Nutrition
Faculty of Human Ecology
University of Manitoba
Fort Garry Campus
204-474-8078

Dr. Trust Beta
Assistant Professor
Department of Food Science
University of Manitoba
Fort Garry Campus
204-474-8214

Dr. Noel Solomons
Scientific Director
Center for the Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM)

Guatemala City, Guatemala
(502) 24733942

Sponsor: Malaysian Palm Oil Board (MPOB)

You are being asked to participate in a human research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

The main researcher is receiving financial support to conduct this study.

Purpose and nature of this study:

Iron is an essential nutrient, necessary for oxygen transport on red blood cells and in tissues. It is also involved as a constituent of enzymes. When there is iron deficiency, a condition called anemia occurs. Children, pregnant women and women in reproductive age are the most vulnerable groups.

Oral iron supplementation is the most commonly used therapy to treat iron deficiency anemia. Guatemala's Health and Public Assistance Ministry has recommended pregnant women to take a dose of 120 mg per week while the international standards recommend from 30 to 120 mg/day to prevent iron deficiency in vulnerable populations. Recently it has been demonstrated that chronic, daily dosing of iron may be associated with chemical effects on the substance of cells that make up your body.

You are being asked to participate in a human research study. The purpose of this study is to find out what effects (good and bad) a palm oil-derived concentrate, rich in antioxidants (vitamin E and carotenoids), has on your ability to resist oxidation caused by normal doses of supplemental iron prescribed to pregnant women in case of anemia. We also want to learn more about the mechanisms on how this concentrate might work in order to provide efficient protection.

Methods:

Twelve participants will take part of this study. If you take part in this study, you will have the following tests and procedures: you will be asked to take daily doses of iron alone and iron mixed with oil during short periods of time (7 days each) and to give us several urine and feces samples, as well as two blood samples. We will give you a calendar of the days in which you have to take the iron and when you have to give us

your blood, urine and feces samples. The participation involves being studied for an overall period of 65 days, during which 37 days will be considered sample-collection days. On the days of collection you will be asked to collect the first urine you excrete after you wake up, a feces sample collected according to your normal bowel habit and bring them to the laboratory at the Universidad del Valle for storage and further processing. You should not do any vigorous exercise on the days of sample collection. A trained technician, nurse or physician will take two blood samples at the beginning and at the end of the study. After we take your blood sample, you will have a small breakfast.

After you have provided us with your samples, we will ask you to take either of the following treatments:

TREATMENT A: 120 mg of iron in form of syrup with 5 ml of refined palm oil
TREATMENT B: 120 mg of iron in form of syrup with 5 ml of refined palm oil plus 0.5 g CTCMC
TREATMENT C: 120 mg of iron in form of syrup with 5 ml of refined palm oil plus 1 g CTCMC

You will have to take all of the treatments mentioned above during 7 days each. There will be periods of time in between each treatment in which you just take water, but you will have to give us your urine and feces samples.

You will also have to record in a "Food Diary" everything you eat during three complete weeks during the study. This will allow us to know if there are other major sources of iron in your diet that may act as confounders in the biochemical analysis.

The researcher may decide to take you off this study if you have or develop a persistent inflammatory or infectious condition during the study.

You can withdraw from the study at any time. But if you decided to do so, we encourage you to talk to any member of our research staff.

Who can participate in this study:

Male healthy adult subjects who sign an informed written consent and are willing to provide all the blood, feces and urine samples required for this study.

Who can NOT participate in this study:

People who smoke, suffer from anemia, have a history of gastrointestinal disorders, intolerance to iron supplements, hematological disorders, or consume other nutritional supplements.

Risks and discomforts:

Your participation in this study involves fasting during the blood collection days, consumption of medicinal doses of iron and the provision of urine and feces samples. Fasting is associated with low levels of blood sugar, which can produce lightheadedness.

The amounts of iron to be administrated are less than the ones used to treat therapeutically iron deficiency anemia (standard therapeutic dose for iron deficiency anemia is 300 mg or iron per day for 30 days). The iron will be mixed with a flavored syrup that may have a strong metallic taste that can be unpleasant and in some cases it can produce nausea and vomiting. Stools will often be black as a consequence of the iron supplementation. There are no known risks of consuming vitamin E, vitamin A and carotenoids in the amounts we are supplementing them.

We will take a 10 ml blood sample (equivalent to 2 teaspoons) at the beginning and end of the study; the collection may be uncomfortable due to the insertion of the hypodermic needle. A small bruise may appear on the puncture site.

Benefits:

By participating in this study, you will provide us with important information of benefit to science and society. You will help us understand and find ways to reduce the effects of iron exposure due to supplementation in public health and clinical applications. You will also get information about your health at the moment of the study. As a compensation for your collaboration you will receive USD117.00 per month for the duration of the study, approximately USD 350.00 in total.

Costs:

All clinic and professional fees, diagnostic and laboratory tests which will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Confidentiality:

The information obtained in this research may be published or presented in conferences and/or public forums. Your name will not be used or revealed, we will use code numbers to refer to your records. The data obtained from your exams will be stored on a computer and on written records. Despite the efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. If the law requires it, your personal information may be disclosed.

The local Human Subjects Committee and the University of Manitoba Biomedical Research Ethics Board may review the records related to the research, for quality assurance purposes.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your medical/research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the offices of CeSSIAM.

Voluntary participation/Withdrawal from the study:

Your participation in this study is voluntary. You may refuse to participate or withdraw from the study at any time.

You are not waiving any of your legal rights by signing this consent form or releasing the investigator or sponsor from their legal and professional responsibilities.

Medical Care for Injury Related to the Study

If you should become physically injured as a result of any research activity, the study doctor will provide any necessary treatment, at no charge, to help you promptly recover from the injury.

If you should become physically injured as a result of any research activity, the study doctor will provide treatment, at no charge, to help you recover from the injury, but we will not cover any expenses in case of hospitalization.

Questions:

You are free to ask any questions about your participation in the study and your rights. If you have any questions during or after the study, or if you have a research-related injury, please contact Ms. Monica Orozco at 24733942 or Dr. Noel Solomons at the same phone number.

For questions related to your rights as a participant in a research, you may contact the Dr. Fernando Beltranena at (502) 2362-2429.

Do not sign this consent form until all the information is clear and your questions have been answered.

Statement of Consent:

I have read this consent form. I have had the opportunity to discuss this research study with Monica Orozco and or the other researchers and staff. All my questions were answered by them in language I understand. The risks and benefits haven been explained to me and are clear. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I can withdraw at any time. I freely agree to participate in this research study.

I understand that the information regarding my personal identity will be kept confidential, but that confidentiality is not fully guaranteed. I authorize the inspection of my records by the Food and Drug Administration, the Health Protection Branch, government agencies and institutions in other countries, The University of Manitoba Biomedical Research Ethics Board and CeSSIAM's Human Research Ethics Board.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

Date: _____

Printed name: _____

Signature: _____

Witness signature: _____

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent

Date: _____

Printed name: _____

Signature: _____

APPENDIX 4: Ethical approval letters from Bannatyne Campus Research Ethics Board and CeSSIAM´s IRB

UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. J. Friel
Sponsor: Malaysian Palm Oil Board (MPOB)

Protocol Reference Number: B2006:076
Date of REB Meeting: May 29, 2006
Date of Approval: July 13, 2006
Date of Expiry: May 29, 2007

Protocol Title: "In situ" and "in vivo" Modification by Palm Fruit-Derived Carotenes and Tocotrienols on the Oxidative Response to Commonly-Prescribed Doses of Oral Iron"

The following is/are approved for use:

- Protocol Version 1 dated March 27, 2006
- Research Participant Information and Consent Form (English and Spanish) Version 2 dated July 13, 2006
- Research Participant Information and Consent Form for Group 1 – Pilot Study (English and Spanish) Version 2 dated July 13, 2006
- Advertisements submitted July 13, 2006
- General Information Questionnaire Version 1 dated March 27, 2006
- Food Diary submitted April 16, 2006

The above was approved by Dr. Ian Maclean, Acting Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your letter dated July 5, 2006 and electronic mails dated July 13, 2006. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations*.

This approval is valid for one year from the date of the meeting at which it was reviewed. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

Ian Maclean, Ph.D
Acting Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to the REB Secretary
Telephone: (204) 789-3255/ **Fax:** (204) 789-3414

Comité Prociegos y Sordos de Guatemala
Hospital de Ojos y Oídos "Dr. Rodolfo Robles V."
Diagonal 21, 19-19 zona 11
Tels: 502 - 24730258, 24730375
FAX 502 - 24733906
Guatemala Centro América

Guatemala 20 de abril de 2006

Doctor
Noel W. Solomons
Coordinador del Centro de Estudios
De Sensoriopatìas, Senectud e Impedimentos y
Alteraciones Metabòlicas (CESSIAM)
Presente

Estimado Dr. Solomons:

De la manera màs atenta me permito informar a usted, que la Comisiòn de Estudios en Humanos, ha considerado que no tiene ningún inconveniente en cuanto a que el trabajo: "***Modificación in situ e in vivo de la respuesta oxidativa a dosis de hierro comúnmente prescritas por carotenos y tocotrienoles derivados de la fruta palma africana***" se lleve a cabo, ya que no lesiona los derechos de las personas.

Por lo anterior, no se tiene inconveniente en que se proceda a su ejecución.

Atentamente,

Dr. Fernando Beltranena Valladares
Jefe Comisión de Estudios en Humanos
CESSIAM

APPENDIX 5: Additional results

Table 13. Hemoglobin values at baseline and end of the intervention for phases 1 and 2 for each participating subject

	PHASE 1: Pilot study		
Subject	Baseline (mg/dL)	Final (mg/dL)	Delta (mg/dL)
001	15.8	15.1	-0.7
002	15.4	15.8	0.4
003	15.3	15.8	0.5
004	16.2	16.7	0.5
005	16.5	17.4	0.9
Average	**15.84**	**16.16**	**0.32**
	PHASE 2: Intervention trial		
006	15.5	14.9	-0.6
007	16.5	16.3	-0.2
008	16.4	17.1	0.7
009	15.7	15.4	-0.3
010	15.1	15.5	0.4
011	14.8	14.9	0.1
012	15.5	15.6	0.1
013	16.4	17.5	1.1
014	15.4	14.7	-0.7
015	16.2	15.9	-0.3
016	15.7	16.3	0.6
017	15.9	16.1	0.2
Average	**15.8**	**15.8**	**0.09**

Table 14. Creatinine values at the end of the study for phases 1 and 2

Subject	Urinary creatinine (mg/dL)
	PHASE 1: pilot study
001	208.4
002	114.5
003	213.8
004	230.7
005	300.8
Average (SD)	**213.6 ± 66.6**
	PHASE 2: Intervention trial
006	114.1
007	274.4
008	322
009	256.5
010	110.8
011	156.2
012	41.6
013	72.5
014	198.4
015	65.6
016	165.6
017	275.1
Average (SD)	**171.1 ± 93.9**

APPENDIX 6: Individual correlations between TBARS and 8-OHdG for final intervention trial (N = 12)

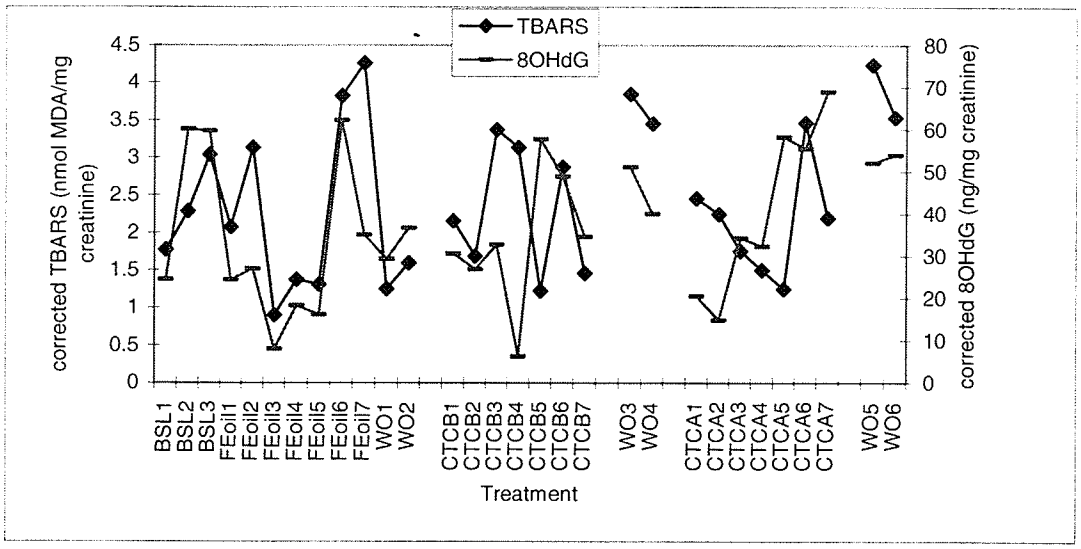

Figure 75. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 006 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

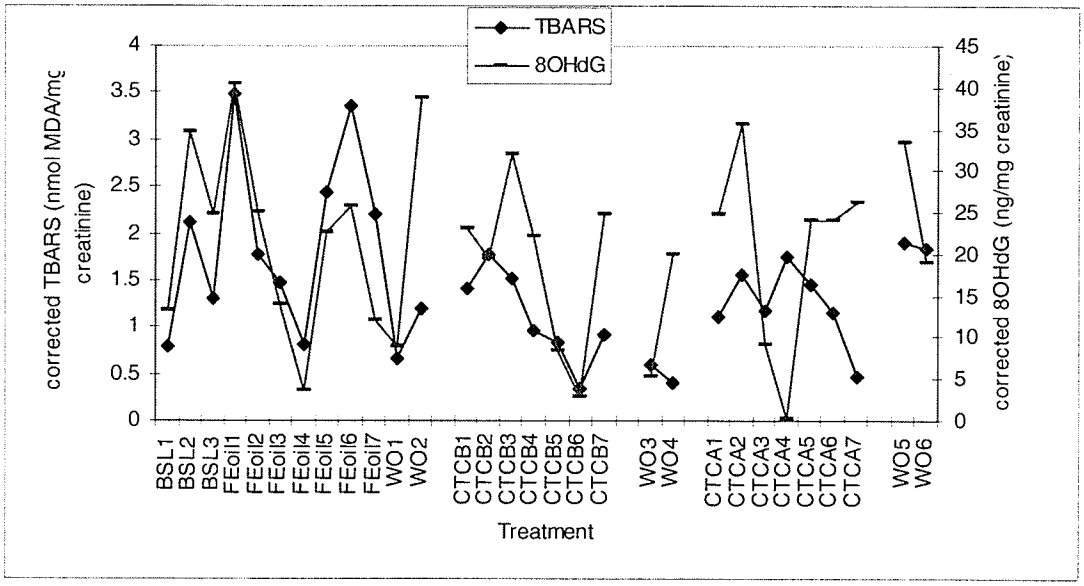

Figure 76. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 007 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

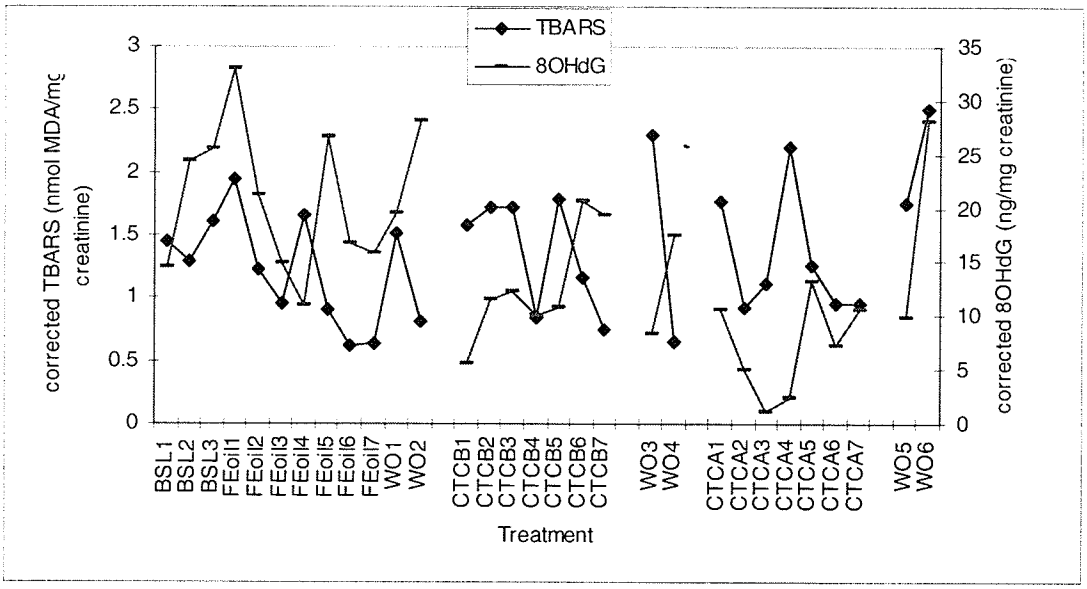

Figure 77. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 008 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

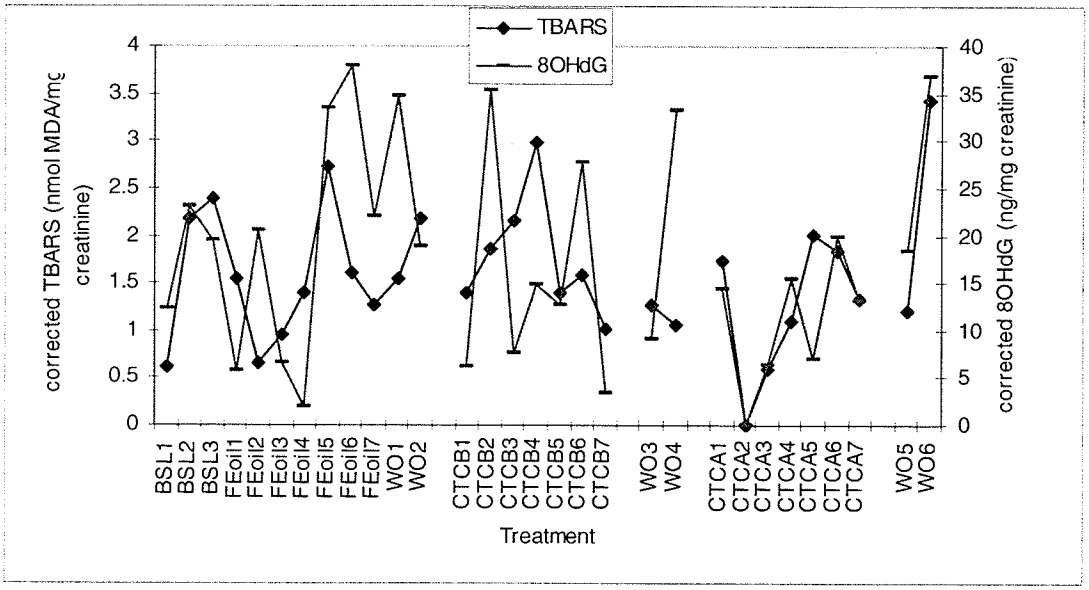

Figure 78. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 009 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

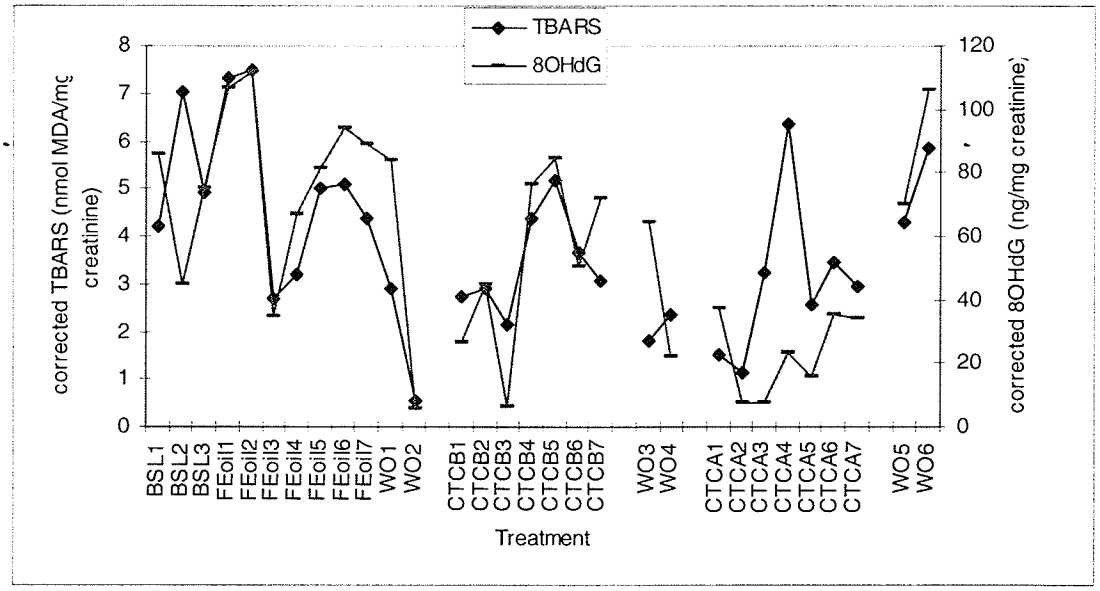

Figure 79. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 010 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

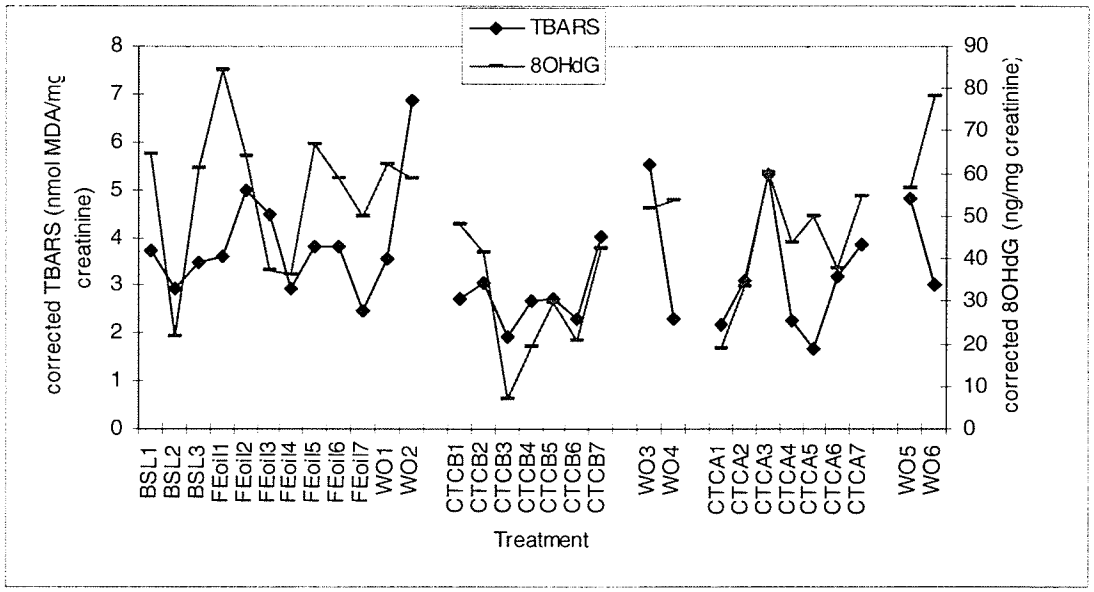

Figure 80. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 011 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

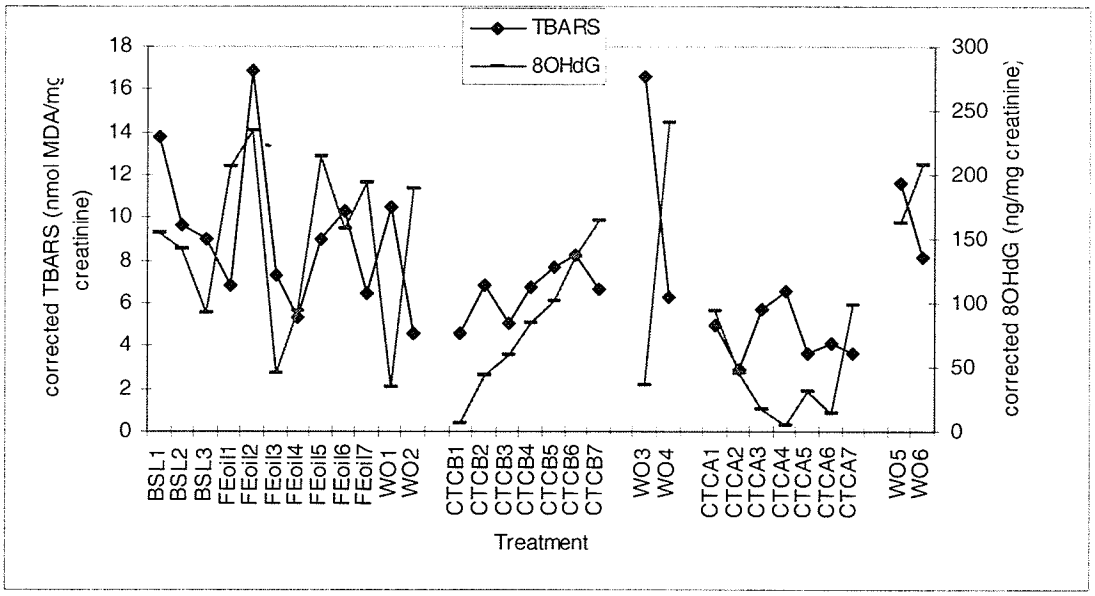

Figure 81. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 012 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

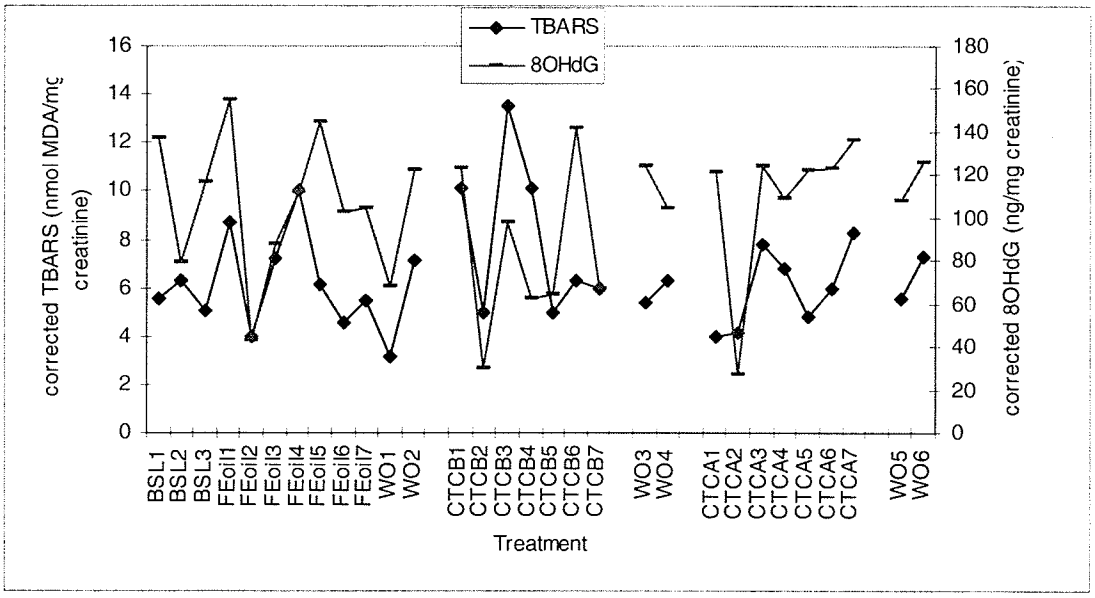

Figure 82. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 013 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

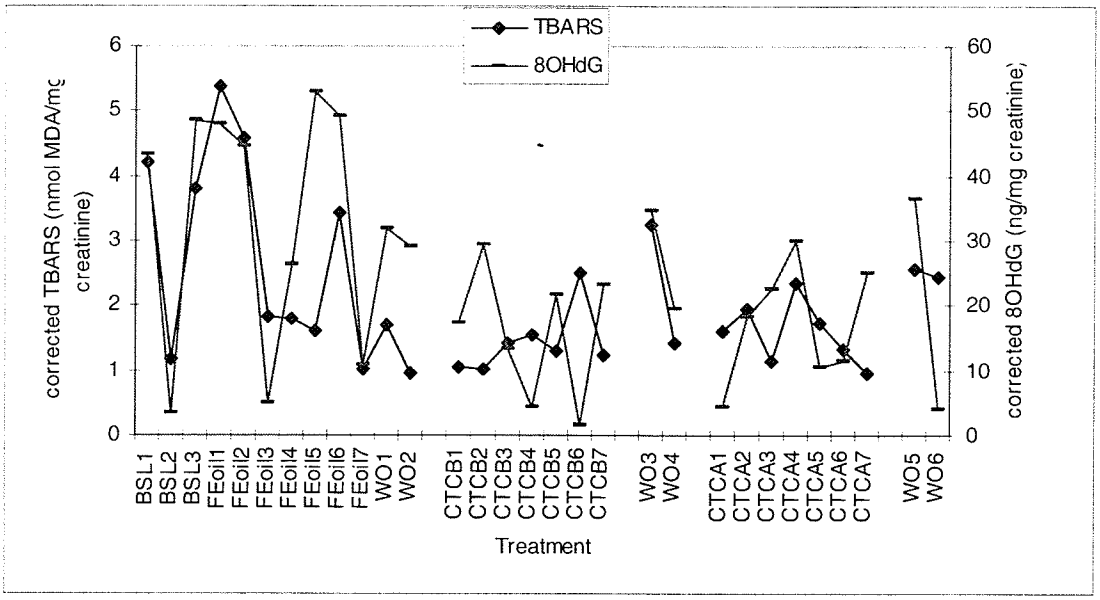

Figure 83. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 014 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

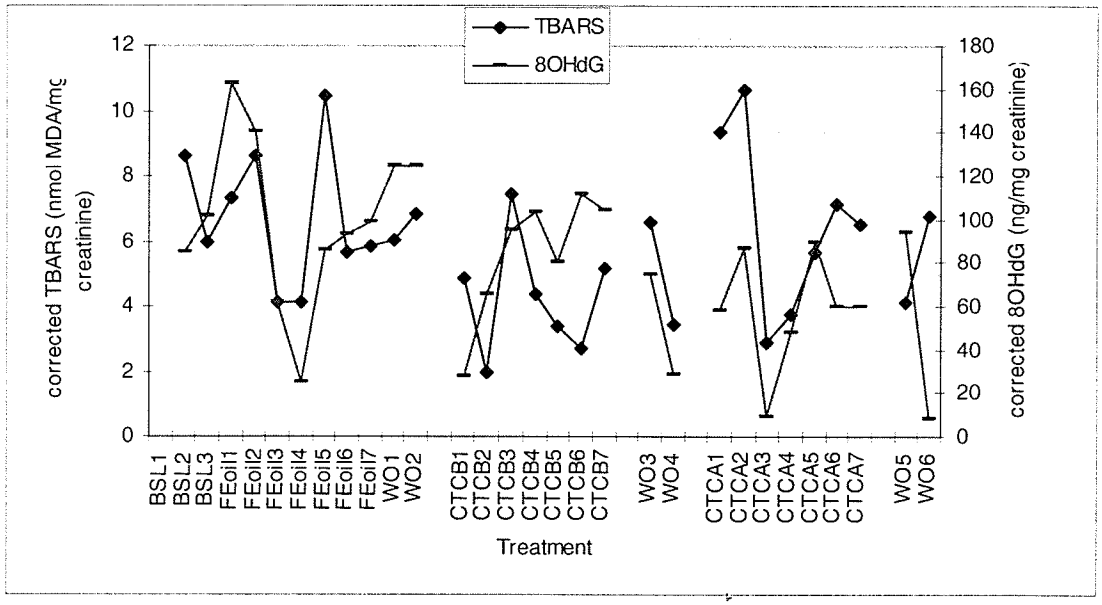

Figure 84. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 015 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

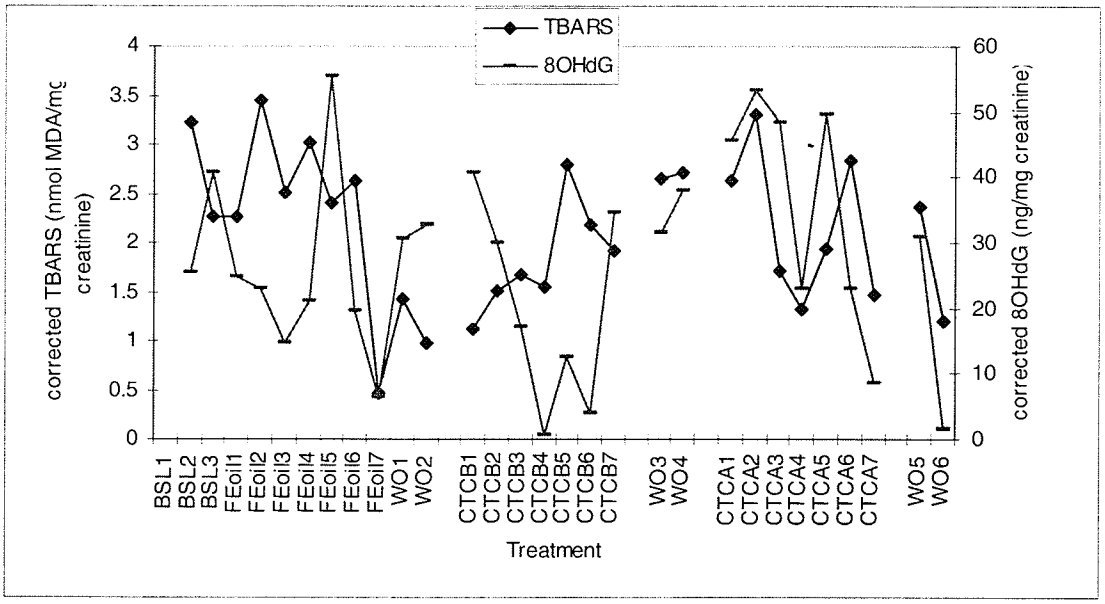

Figure 85. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 016 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

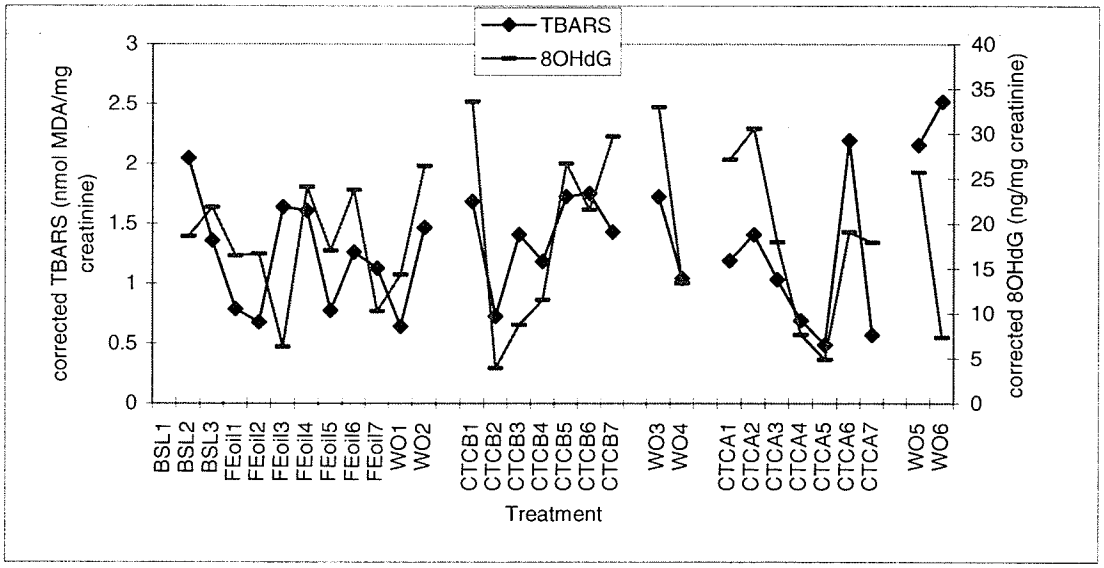

Figure 86. Comparison of production of 8-OHdG (ng/mg creatinine) and TBARS (nmol MDA/mg creatinine) in urine samples from subject 017 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown.

APPENDIX 7: Individual correlations between non-heme iron and total hydroxylated products for the final intervention trial (N = 12)

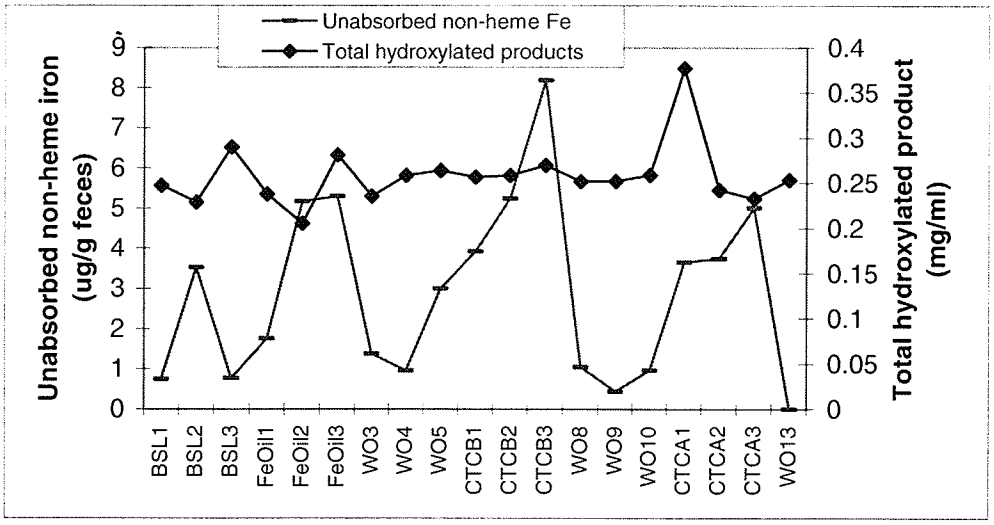

Figure 87. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 006 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

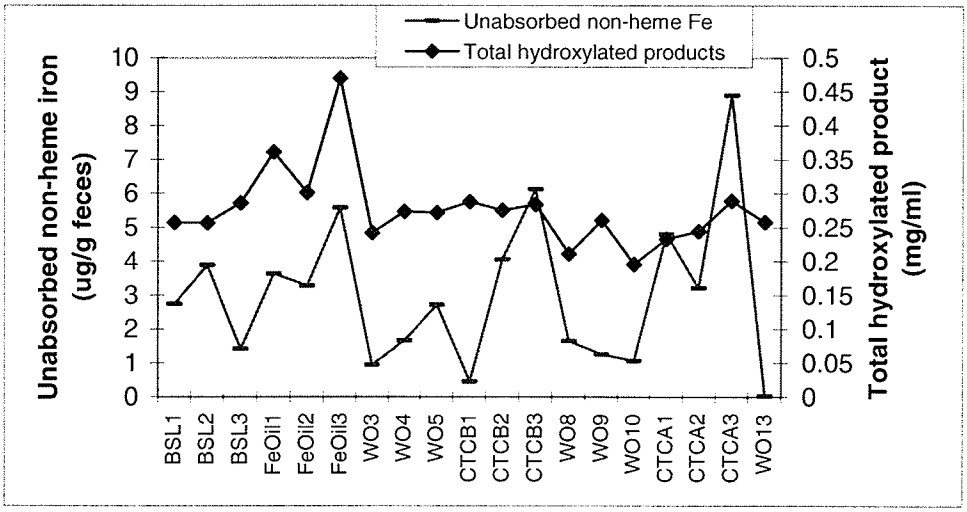

Figure 88. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 007 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

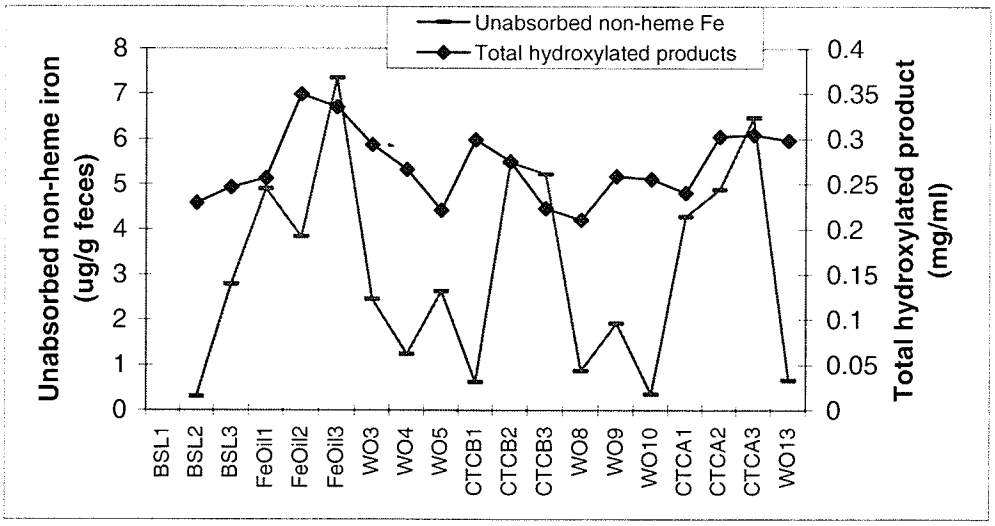

Figure 89. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 008 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

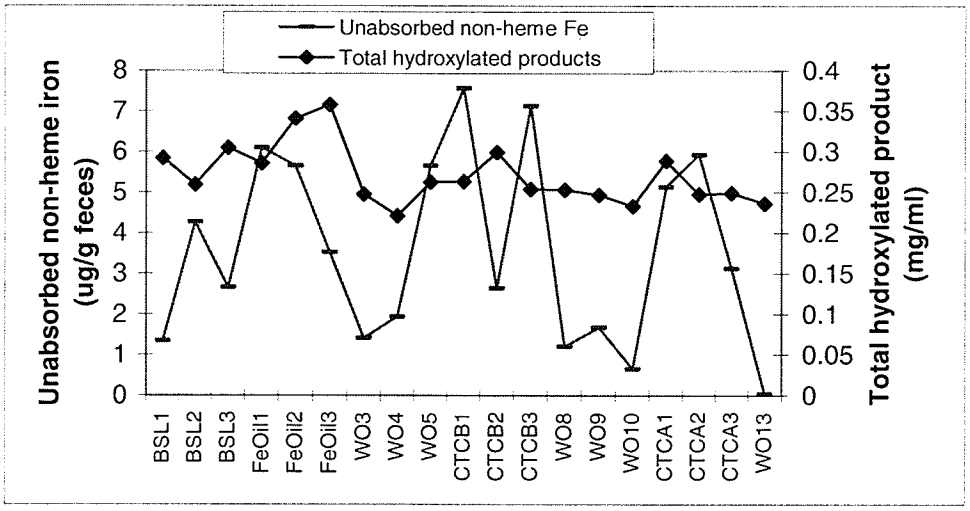

Figure 90. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 009 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

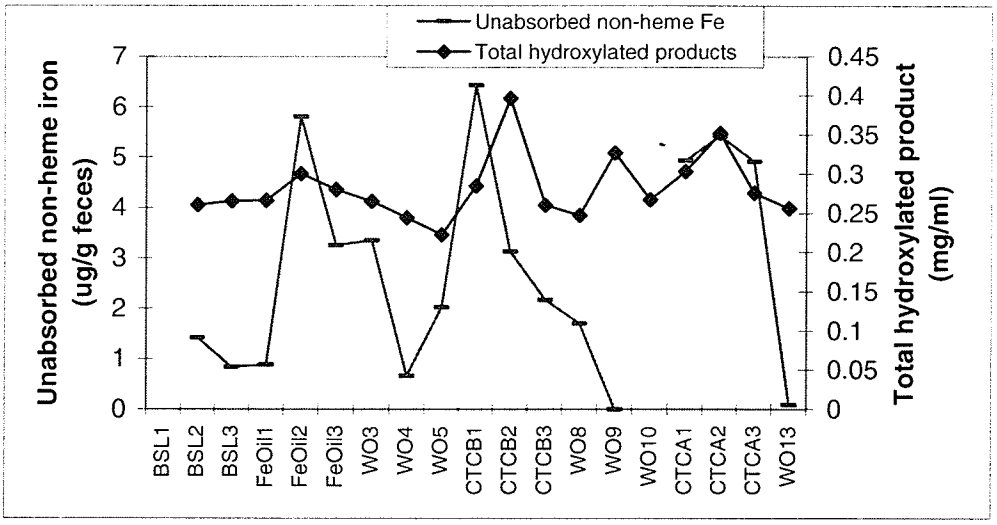

Figure 91. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 010 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

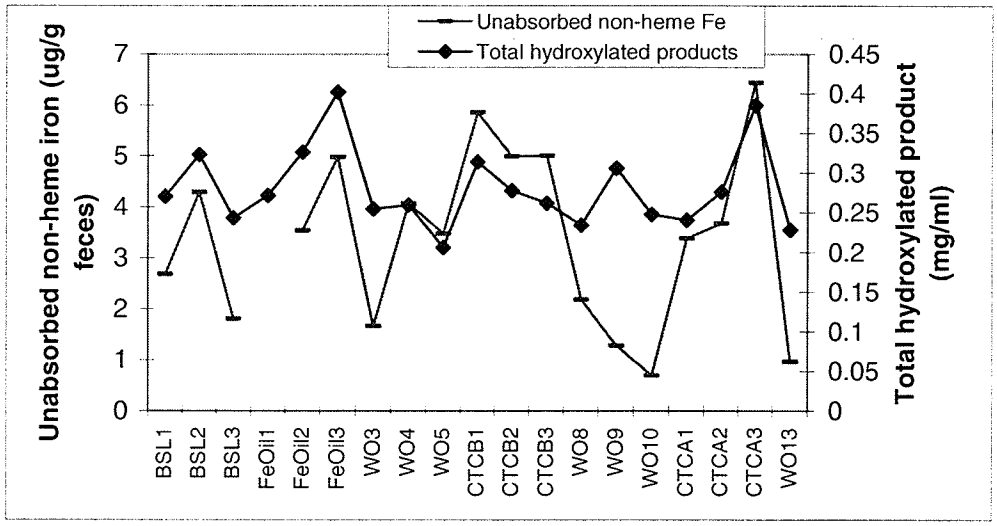

Figure 92. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 011 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

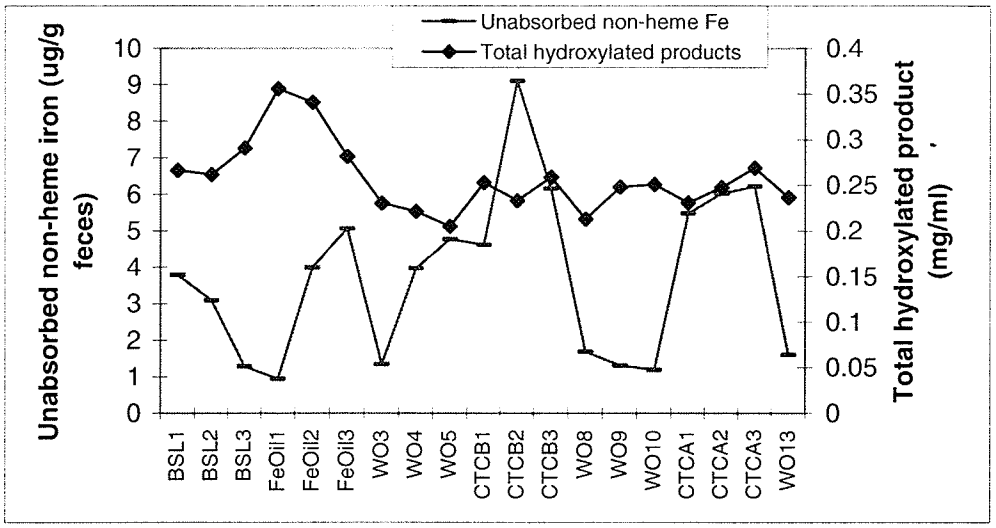

Figure 93. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 012 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

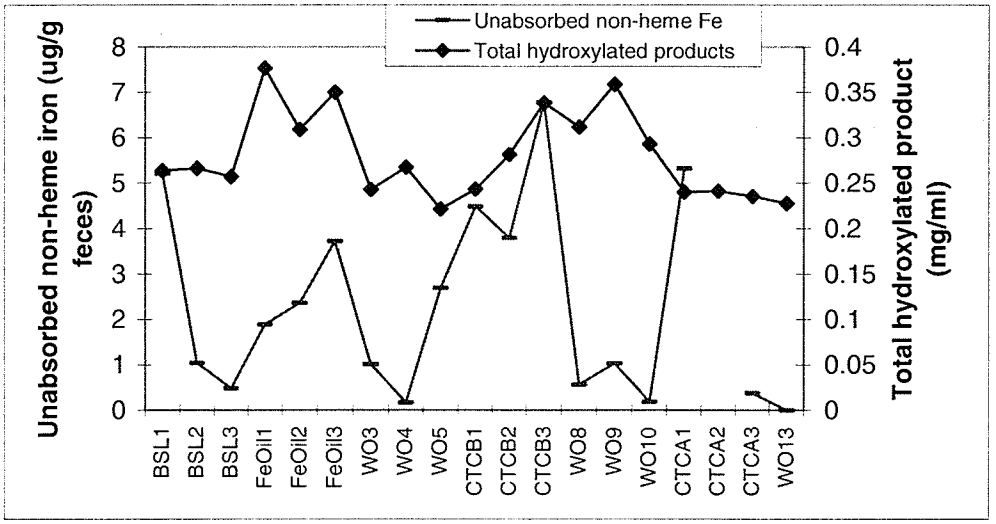

Figure 94. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 013 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

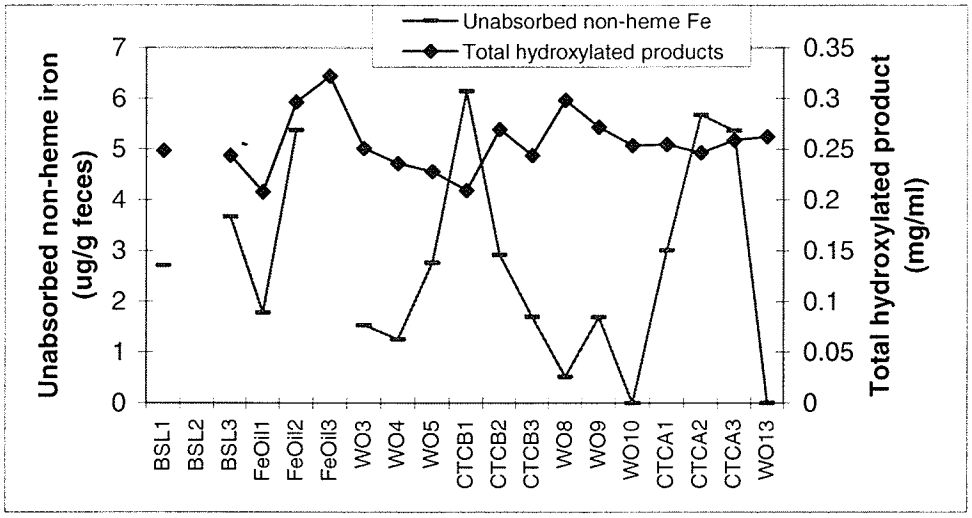

Figure 95. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 014 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

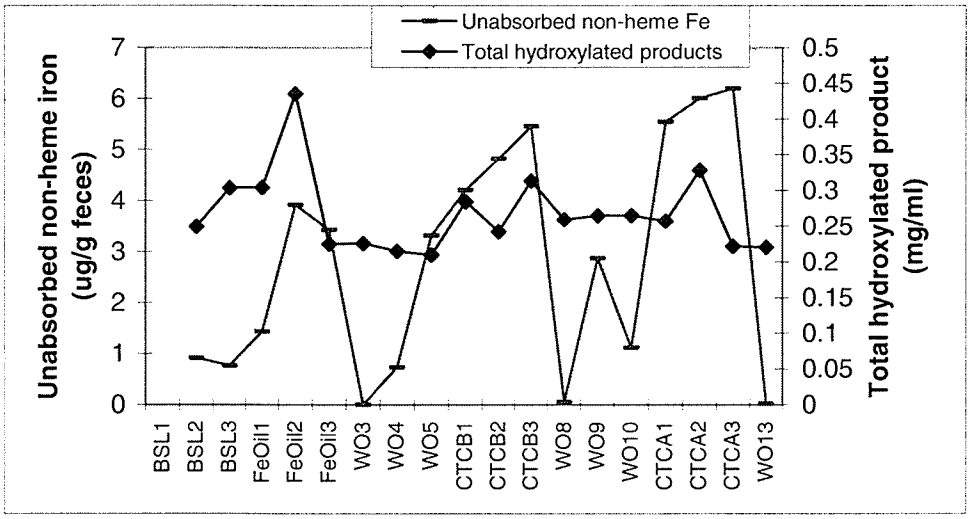

Figure 96. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 015 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

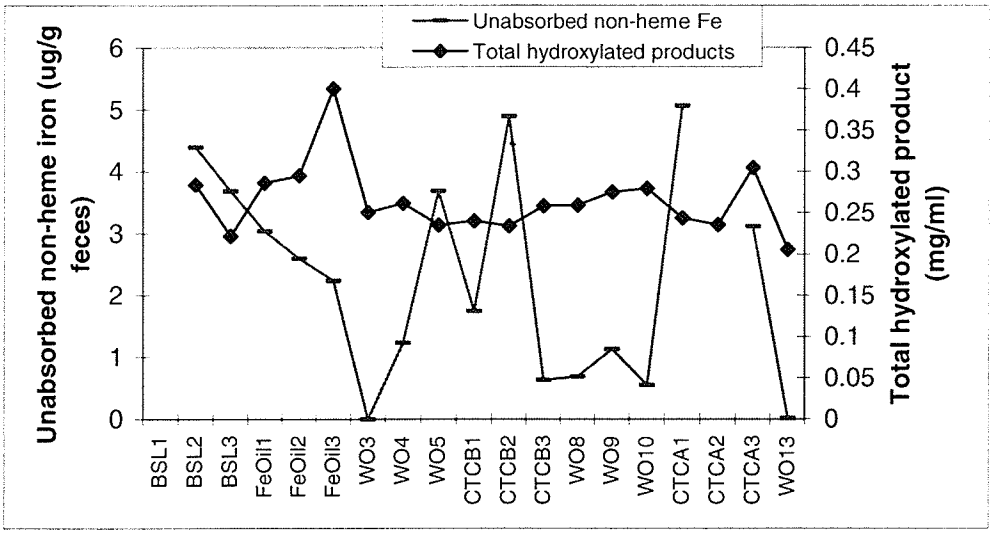

Figure 97. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 016 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.

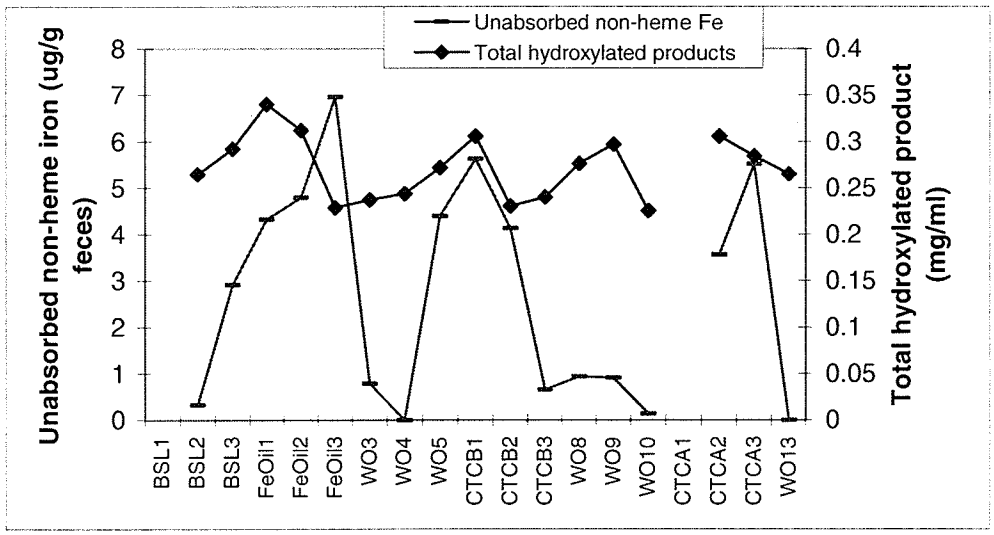

Figure 98. Comparison of total hydroxylated products (mg/ml) and non-heme iron (μg iron/g feces) in feces samples from subject 017 participating in phase 2. Graph is not in real time scale, days were no samples were collected are not shown. Variance bars (SEM) have been omitted on purpose so as not to obscure the inter-variable relationships.