Studies of genetic, gastrointestinal, renal and dietary factors in white and black South African subjects as a possible key to understanding the relative absence of calcium oxalate kidney stone disease in the black population

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Summary

The incidence of urolithiasis in South Africa's black population is extremely rare (<1%) while in the white population it is similar to that of western countries (~15%). The present thesis was aimed at shedding more light on the complex nature of the physicochemical, biochemical and physiological mechanisms in black South Africans which provide this group with a natural protection against urolithiasis in contrast to their white compatriots. Four studies comprise this thesis.

In the first study, the frequency of alanine:glyoxylate aminotransferase (*AGT*) Proline11Leucine (*Pro11Leu*) polymorphism was investigated to ascertain whether this polymorphism was a contributory factor in the differences in stone incidences between the two race groups. This study was prompted by the lack of any genetic studies attempting to explain this anomaly in stone incidence in the two race groups. Three groups of healthy male subjects *('rural' black (n=10), 'semi-urban' black (n=10) and white subjects (n=20)* participated in the study. Each subject collected a spot morning urine sample and provided a blood sample which was drawn by a qualified phlebotomist on an EDTA-tube. In addition, 24hr food dietary records as well as a questionnaire which assessed the subjects' food frequency diet, medical history, medications, lifestyle and other practices of interest as well as history of kidney stone disease were completed. Determination of AGT Pro11Leu polymorphism through DNA sample analysis was achieved using the polymerase chain reaction-restriction fragment length polymorphism method. Data revealed that the frequency of this polymorphism was similar between the subjects from the two race groups who participated in this study. It seems that this polymorphism may not play a key role in the low incidence of stone formation in the black group, but further studies involving much larger cohorts of participants are needed before the role of genetics can be fully resolved.

In the second study, the role of gut permeability as a key to identifying possible differences in the handling of dietary oxalate in healthy black (*n=10*) and white (*n=10*) South African male subjects following various dietary challenges was investigated. The study was divided into three protocols - (*regular diet (P1), standardized diet (P2) and high-oxalate standardized diet (P3*). Each protocol was conducted over a 3-day period with a one week wash-out period between protocols. Subjects followed the respective protocol's diets on day 1 and 2. On day 3 of each protocol, subjects provided a baseline

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sample (1hr) following an overnight fast, to test for the presence of endogenous sugars, and then ingested 200 ml of a dual-sugar isotonic test solution containing 5g lactulose (*LA*) and 2g mannitol (*MA*) dissolved in tapwater. Subjects then collected a 5hr test sample followed by an 18hr post-test sample. The ratios of the percentage recoveries of two sugar probes (*LA%/MA%*), an accurate index of gut permeability, were determined on the 5hr test samples using high performance anion exchange chromatography coupled with pulsed amperometric detection. The 24hr samples were reconstituted for measurements of urine parameters including urinary oxalate excretions. Physicochemical risk factors such as the relative supersaturation of calcium oxalate and Tiselius risk index were computed from the urinary parameters.

The results from this study showed that administration of the high-oxalate standardized diet in protocol 3 reduced MA excretions by more than 50% in the two race groups indicating a defect in the mucosal defense system in both groups. Based on this observation, it is tentatively concluded that there was an induced general malabsorption of nutrients following this protocol. The gut permeability index was slightly higher in blacks than in whites (*0.007 vs 0.003, respectively*) while the Tiselius risk index was unfavourably and significantly elevated in the black group (*p=0.0003*). Although the latter parameter was also elevated in white subjects it did not change significantly in this group (*p=0.1016*). These observations were surprising considering the low incidence of stone formation in the black population. Equally surprising was the fact that data showed that black subjects handled oxalate more transiently than whites as demonstrated by the significantly elevated levels of mean urinary excretion of oxalate in the black group (*p=0.0021*) but not in the white group (*p=0.8491*). Further research is warranted in this area.

In the third study, the effects of vitamin E (*dl-α-tocopheryl acetate*) ingestion on lipid peroxidation and urinary kidney stone risk factors were investigated. In this study, healthy black (*n=5*) and white (*n=5*) male subjects from the two race groups were required to ingest one vitamin E capsule (*400IU*) every day immediately after supper for 60 days. Blood samples (5ml) were collected in EDTA tubes by a qualified phlebotomist from each subject at baseline (*day 0*) and postsupplementaion (*day 60*) following an overnight fast. 24hr urine samples were also collected on the days in which blood samples were drawn. Subjects were instructed to continue taking their free and

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unrestricted regular diets for the duration of the study period and to complete 24hr dietary food records on the days in which urine and blood samples were collected. The subjects' dietary food intakes were assessed using a *Foodfinder 2* computer software programme. Plasma vitamin E (*α-tocopherol)* levels were determined using high performance liquid chromatography (*HPLC*) while urinary and plasma thiobarbituric reactive substances (*TBARS*) levels were measured using OXI-TEK TBARS assay kits. 24hr urine samples were rigorously analyzed for biochemical risk factors using routine standard biochemical techniques.

The results from this study appear to show a dual-role for vitamin E ingestion in the two race groups. On one hand, plasma α-tocopherol and urinary citrate were favourably and significantly elevated in both groups while on the other hand, urinary calcium, oxalate, relative supersaturation and Tiselius risk index as well as urinary and plasma TBARS (*MDA equivalents*) were not altered. Subtle differences in handling mechanisms of vitamin E in black and white subjects were apparent. Overall, vitamin E appeared to have minimal beneficial effects in the small group of black and white South African subjects, which supports the findings of previous studies on vitamin E ingestion in healthy subjects. However, the findings of favourable and significant increase in mean urinary excretion of citrate coupled with an increase of plasma α-tocopherol levels in both groups supports the protective role of vitamin E ingestion in calcium oxalate urolithiasis albeit it does not explain the differences in stone incidences in the two race groups.

In the final study, the effects of omega-3 fish oil ingestion alone (*protocol 1*) and in tandem with vitamin E (*dl-α-tocopheryl acetate*) (*protocol 2*) on urinary kidney stone risk factors was investigated. 20 healthy male subjects participated in this study, one group of black (*n=5*) and white (*n=5*) subjects participated in protocol 1 and ingested a fish oil capsule (*salmon oil, 1000mg*) providing 164 mg eicosapentaenoic acid (*EPA*) and 110 mg docosahexaenoic acid (*DHA*) every day immediately after supper for 30 days while a second group of black (*n=5*) and white (*n=5*) healthy male subjects participated in protocol 2 and ingested the aforementioned fish oil capsule in tandem with vitamin E (*dl-α-tocopheryl acetate, 400IU*) capsule for a similar period. Following an overnight fast, two venous blood samples (5ml) were collected (*EDTA tube and lithium heparin tube*) by a qualified phlebotomist from each subject at baseline (*day 0*) and postsupplementation

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(*day 30*). Blood samples collected in lithium heparin tubes were used for analysis of plasma uric acid using Synchron LX systems kits and for analysis of ferric reducing antioxidant power (*FRAP*) using colorimetric FRAP assay kits. Blood samples collected in EDTA tubes were analysed for plasma vitamin E (*α-tocopherol*) using HPLC and for plasma malondialdehyde (*MDA*) using BIOXYTECHMDA-586 kits. 24hr urine samples were also collected on the days in which blood samples were drawn and were analysed as described above.

The results for protocol 1 showed no significant changes in any of the urinary or plasma risk factors and biomarkers for calcium oxalate stone formation in the two race groups. However, protocol 2 favourably and significantly raised plasma α-tocopherol levels in both groups but did not have any effect on the other parameters. Between-group comparisons revealed that there were no statistically significant differences at baseline for this parameter in the two race groups ($p=0.0828$). However, significantly higher plasma α-tocopherol levels were observed post-supplementation (*day 30*) in whites than in blacks in protocol 1 ($p=0.0372$) and protocol 2 ($p=0.0577$). These observations are counter-intuitive considering the lower incidence of stone formation in the black group.

Although no profound insights were gained regarding explanations for the rarity of stone incidences in the black population as compared to their white compatriots, the present thesis has made significant contributions to the body of scientific knowledge in this area. The results of this thesis have demonstrated that explanation of the phenomenon of stone rarity in blacks is extremely complex and serve to motivate further studies to elucidate this intriguing phenomenon.

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Appendix CD: Windows XP

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α	alpha
β	beta
$(NH_4)_2Ox$	ammonium oxalate
μ	micro
μg	microgram
μl	microlitre(s)
μM	micromolar
4-AAP	4-amino antipyrine
Ag	silver
AgCl	silver chloride
AGT	alanine:glyoxylate aminotransferase
all-rac	racemic mixture
ANOVA	analysis of variance
B	black subject(s)
BMI	body mass index
BRI	BONN-Risk index
CaOx	calcium oxalate
Co.	company
COD	calcium oxalate dihydrate
COM	calcium oxalate monohydrate
Conc	concentration
COT	calcium oxalate trihydrate
CSSR	centre for social science research
DCHBS	dichloro-2-hydroxybenzene sulfonate
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
DTT	dithiothreitol
EDP	equity development programme
EDTA	ethylene diamine tetraacetic acid
EPA	eicosapentaenoic acid
FA	fatty acids
FO	fish oil

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FRAP	ferric reducing antioxidant power
g	gravitational centrifugal force
g	gram(s)
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HPAEC	high performance anion exchange chromatography
HPLC	high performance liquid chromatography
hr	hour(s)
I.D.	internal diameter
IU	international units
KJ	kilojoule(s)
L	litre(s)
LA	lactulose
LPO	lipid peroxidation
Ltd	limited
MA	mannitol
MDA	malondialdehyde
mg	miligram(s)
$MgCl_2$	magnesium chloride
mg/L	milligram per liter
min	minute(s)
ml	mililitre(s)
mM	milimolar
mmol	milimole
mol	mole
MSL	metastable limit
MUFA	monounsaturated fatty acids
n-3	omega 3
Na_2Ox	sodium oxalate
NAG	N-acetyl-β-glucosaminidase
NaOH	sodium hydroxide
ng	nanogram
ng/µl	nanogram per microlitre

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nm	nanometer
nmol	nanomole
NSAIDs	nonsteroidal anti-inflammatory drugs
O. formigenes	oxalobacter formigenes
°C	degree celcius
PAD	pulsed amperometric detection
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction – restriction fragment length polymorphism
PGE	prostaglandin E
PH1	primary hyperoxaluria 1
PolyU	Hong Kong Polytechnic University
Pro11Leu	proline11leucine
PUFA	polyunsaturated fatty acids
RB	'rural' black subject(s)
RE	retinol equivalents
REC REF	research ethics committee reference
rpm	revolutions per minute
RS	relative supersaturation
SB	'semi-urban' black subject(s)
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
TBA	thiobarbituric acid
TBARS	thiobarbituric reactive substances
TOMP	tetramethoxypropane
TRI	Tiselius risk index
UCT	university of Cape Town
UV-Vis	ultraviolet-visible region
UWC	university of Western Cape
W	white subject(s)

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Chapter One: General Introduction

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1.1 Introduction

Urolithiasis (kidney stone disease) is the third most common pathological disease afflicting the urinary tract, next to infection and prostatic pathology. It is a multi-factorial disease, the on-set and severity of which is influenced by both genetic and environmental factors (Blacklock 1982, Li *et al.* 1985, Ljunghall *et al.* 1985, Kohri *et al.* 1988, Smith 1989, Curhan *et al.* 2004, Holmes and Assimos 2004, Goldfarb *et al.* 2005, Moe and Bonny 2005, Stoller and Rubenstein 2005). It is likely due to a variety of etiological factors, many of which currently remain unknown.

A number of well recognized risk factors favour the development and progression of stone disease. These include biochemical/metabolic perturbations, anatomical anomalies within the kidney and genetic susceptibility (Friedman 2000, Jonassen *et al.* 2004). Although, it is difficult to determine how these factors converge to promote stone disease, it is an accepted factor that stones can form anywhere in the urinary tract, from kidneys to the bladder (Jonassen *et al.* 2004, Khan 2006). There is interest in the revival of Randall's plaque as the possible first step in calcium stone formation (Evan *et al.* 2003). These apatite plaques begin at the tip of Henle's loop, and as yet little is known regarding the factors that cause them, or the factors that lead to calcium oxalate stone growth on Randall's plaques.

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It is also worth noting that in the industrialized and affluent countries, stone formation is generally restricted to the kidneys, where the most common type is calcium oxalate. In fact, approximately 80% of all kidney stones are composed of calcium oxalate and calcium phosphate, while the remaining percentage is shared among struvite (10%) and uric acid (9%), with only 1% of stones composed of cystine or ammonium acid urate or are diagnosed as drug-related stones (Ramello *et al.* 2000, Coe *et al.* 2005, Khan 2006).

It is of interest that in South Africa, calcium oxalate stones occurs in the white population (~15%) to the same extent as in other western countries, but that the incidence of stones in the black population is extremely rare (<1%) (Muskat 1951, Wise and Kark 1961, Modlin 1967, Whalley *et al.* 1998, Whalley *et al.* 1999). Studies described in the present thesis are aimed at shedding more light on the complex nature of the physicochemical, biochemical and physiological mechanisms in black South Africans which provide this group with a natural protection against urolithiasis in contrast to their white compatriots.

1.2 Epidemiology and risk factors in urolithiasis

The incidence, prevalence and recurrence of stone formation is a worldwide disease sparing no age, gender, geographical or cultural groups (Moe 2006).

Age and gender

Although the aetiology of urolithiasis in children is different to that of adults, kidney stones can develop at any age, from as early as the first 2-3 months of life (van't Hoff 2004). However, stone disease varies according to gender. For males (*who form the basis of investigations of this thesis*) the incidence of stone formation has been reported to be most prevalent between the ages of 30 and 60 years while in females the risk is said to peak between the ages of 20 and 30 (Johnson *et al.* 1979, Iliatt *et al.* 1982, Hesse *et al.* 1986, Curhan *et al.* 1993, Curhan *et al.* 2004). In addition, the risk slowly falls with age in both groups. A study by Hesse and co-workers (1986) has shown that certain urinary parameters active in urolithiasis are influenced by age-dependent metabolism. This is in agreement with findings of earlier studies that reported similar observations (Ljunghall *et al.* 1977a, Iliatt *et al.* 1982, Robertson *et al.* 1984). For example, magnesium excretion was shown to decline continually after the age of 30. This was associated with absorption of magnesium in the bowel, which declines with increasing age (Hesse *et al.* 1986).

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The prevalence of stone formation in males is estimated to be between 7 and 15 % (Chandoke 2002) while it is reported to be only between 3 and 6 % in females (Fetter *et al.* 1963, Ljunghall 1977b, Scott *et al.* 1977, Hesse *et al.* 2003, Stamatelou *et al.* 2003, Dall'Era *et al.* 2005). A recent study by Scales and co-workers (2007) has reported a change in the prevalence of gender of patients treated for stone disease from 1.7:1 to 1.3:1 male-to-female ratio. Nevertheless, an explanation for gender disparity is based on several scientific observations. Firstly, it has been suggested that in males, high testosterone levels may cause an increased endogenous oxalate production by the liver, resulting in an increased urinary oxalate excretion (Baker *et al.* 1996, Lee *et al.* 1996, Yoshihara *et al.* 1999). Secondly, the low stone incidence in females may be accounted for by the presence of estrogens, which are known to decrease urinary oxalate excretion, plasma oxalate levels and kidney oxalate deposition (Liao and Richardson

1972, Finlayson 1974, Ferrari *et al.* 2007). Thirdly, another possible explanation is that females have been shown to excrete a high level of urinary citrate, which may protect them from calcium urolithiasis (Welshman and McGeown 1975).

Occupation

A number of studies have found an interesting correlation between stone disease and occupation. It has been shown that occupation and social class are related to a higher urinary stone risk. For example, in a study by Ekane and co-workers (1997), manual workers presented a much lower frequency of urinary stones than professional and managerial groups. Although other factors might be involved, such as infrequent voiding that may increase stone forming salts, it is possible that this may be due to the manual workers' active lifestyle. Nonetheless, this finding has been corroborated by other studies that have consistently shown that the more sedentary the work, the higher is the prevalence of stone formation (Borghi *et al.* 1993, Zheng *et al.* 2002).

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Economics

The health care costs associated with treatment of stone disease has risen over recent years. These have been estimated in the USA at $ 2-5.3 billion/year and about € 54.38 million in Germany (Strohmaier and Hormann 2000, Chandoke 2005, Porena *et al.* 2007). Moreover, it has also been reported in the UK, that every stone episode costs the local health authority almost £2000 (Robertson 2006). Notwithstanding that the prevalence and incidence of nephrolithiasis are estimated at 5-10% and 100-300/100000/year respectively, and that the relapses occur in 50-70% of all cases (Saita *et al.* 2007), the rising medical cost of treatment of stone disease, is another stark reminder that prevention of stone formation is of great importance.

Geography and climate

The overall probability of forming stones has been shown to vary in different parts of the world. The risk of developing stone disease in normal adults appears to be lower in Asia (*1-5%)* than Europe (*5-9%*) and North America (*12% in Canada and 13% in USA*) (Ramello *et al.* 2000). These discrepancies could be attributable to various reasons

related to different lifestyle, diet, climate, daily water intake, physical activity and corporeal overweight (Yoshida and Okada 1990, Curhan *et al.* 1993, Trinchieri 1996, Hesse *et al.* 2003). In addition, other authors have attributed these differences in stone incidences to comorbidity (*diabetes, overweight, hypertension, surgery for obesity, etc*) (Hall *et al.* 2001, Timio *et al.* 2003, Daudon *et al.* 2006, Lieske *et al.* 2006, Cupisti *et al.* 2007).

According to Rodgers (2006), urolithiasis in Africa has not been extensively investigated, due to a general lack of research facilities in the continent. However, there are regions (*Ethiopia, Cameroon, Niger, Algeria, Egypt*) in which endemic paediatric bladder stones are known to occur, regions (*South Africa, Tanzania, Northern Sudan*) in which calcium oxalate upper urinary tract stones have been reported and regions (*Nigeria, Southern Sudan and South Africa*) where the occurrence of stones has been found to be extremely rare in some population groups. It is further stated that, the endemic stones in Africa maybe due to infection, hyperuricosuria (*malnutrition*) and poor diet. The stone rarity in Southern Sudan has been attributed to the relatively low temperature, high relative humidity, low urinary calcium and high urinary volume (Kambal *et al.* 1981) while Esho (1978) attributed the stone rarity in Nigeria to low calcium content of the drinking water, the low consumption of dairy products and the labour intensive lifestyle.

Of particular concern, is the highest risk of stone formation that has been reported in Saudi Arabia, with 20.1% of the population afflicted with the disease (Robertson and Hughes 1994) and the notorious South Eastern United States 'stone belt' (Blacklock 1982). These geographic areas are well-known for their hot climate. It is plausible that increased vitamin D levels that may arise, possibly due to exposure to ultra-violet portion of sunlight, which may activate the conversion of 7-dehydrocholesterol to vitamin D_3, which (*together with its hydroxylated derivate, 1.25-dihydroxyvitamin D_3*) has been reported to act on the intestine and bone to increase plasma calcium and phosphate ions levels (Broadus *et al.* 1984). As a result of this activation, an increase in intestinal calcium absorption and urinary excretion may occur, which might be responsible for the reported stone propensity (Robertson and Peacock 1981, Broadus *et al.* 1984). In addition, it is also possible that, high temperatures may increase perspiration, which may lead to increased incidence of stone formation as a result of urines being more

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concentrated with stone forming salts (Al-Dabbagh and Fahadi 1977, Schwille and Hermann 1992).

Furthermore, seasonal stone recurrence has also been observed, with incidences reported to be higher in spring and summer than in autumn and winter (Parry and Lister 1975, Bartoletti *et al.* 2007). It seems very likely that this can be ascribed to low fluid intake and low urinary output during the cooler seasons. This has been confirmed by studies showing the highest incidence of ureteral stones during the summer months (Prince *et al.* 1956, Alhadramy 1997).

Race

It is interesting that certain races or groups of people appear to be immune to urinary stones. These include the Indians of Mexico, Peru, Ecuador and Bolivia, South African blacks, inhabitants of Greenland and Aborigines (Muskat 1951, Wise and Kark 1961, Modlin 1967, Widdowson and McCance 1970, Finlayson 1974, Scott *et al.* 1977, Whalley *et al.* 1998, Whalley *et al.* 1999).

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Stone rarity in the inhabitants of Greenland has been attributed to their high consumption of dietary essential fatty acids (*oily fish*), which are rich in eicosapentaenoic acid. The latter has been shown to reduce urinary calcium, one of the major risk factors for stone formation (Dyerberg 1981, Dyerberg and Bang 1982, Buck *et al.* 1991, Yasui *et al.* 2001).

Of further interest in the context of this thesis, is that whites have been consistently shown to have a higher incidence of stone formation than blacks, irrespective of geographical and environmental area (Robertson and Peacock 1981, Soucie *et al.* 1994, Ramello *et al.* 2000, Maloney *et al.* 2005). Despite numerous research studies on this anomaly, there is currently no definitive conclusive explanation to this apparent natural immunity of the black population to stone disease (Muskat 1951, Wise and Kark 1961, Soucie *et al.* 1994, Whalley *et al.* 1998, Whalley *et al.* 1999, Rodgers 2006). It is clear that further studies are warranted in this regard. Indeed, this phenomenon forms the basis of the present thesis with special reference to the South African black and white population.

1.3 Urinary risk factors

Urolithiasis is associated with a variety of abnormalities in urinary composition, which are due to dietary indiscretions, physiological-metabolic disturbances or both (Pak 2004b, Taylor and Curhan 2004). These urinary risk factors have been identified as those urinary characteristics that are widely accepted to influence the likelihood of calcium stone formation or recurrence and are routinely measured as part of the metabolic investigations of both calcium stone formers and non-stone formers (Rodgers 2006, Sutton 2006).

Urinary risk factors for stone formation include pH, volume, calcium, oxalate, citrate, magnesium, phosphate and uric acid. A number of risk models of stone formation have been developed over the years (Robertson *et al.* 1978, Khan 1997, Robertson 2003, Jaeger and Robertson 2004). Fortunately, the correction of abnormal risk factors by dietary modification and pharmacologic intervention has been shown in several studies to reduce the risk of stone formation as well as prevent recurrent stone formation (Massey *et al.* 1993, Anbazhagan *et al.* 1999, Baggio *et al.* 2002, Rodgers and Lewandowski 2002, Massey 2003, Jaeger and Robertson 2004, Pak 2004b, Taylor and Curhan 2004, Goldfarb *et al.* 2005, Siener and Hesse 2005, Taylor *et al.* 2005, Hesse and Straub 2006, Thomas *et al.* 2008).

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Urinary calcium

The ultimate driving force for stone formation is supersaturation with respect to stone-forming salts, with urinary calcium being a major risk factor (*along with urinary oxalate in the case of calcium oxalate stones*). As stated earlier, about 80% of all kidney stones contain calcium. Most of these occur as calcium oxalate (Robertson *et al.* 1978, Wilson 1989, Hess *et al.* 1998). Moreover, between 30-60% of all patients with calcium oxalate kidney stones have increased urinary calcium excretion in the absence of elevated serum calcium. This condition is generally referred to as *idiopathic hypercalciuria* (Balaji and Menon 1997, Worcester and Coe 2008).

In 1974, Pak and co-workers suggested that idiopathic hypercalciuria is of heterogenous origin (*absorptive hypercalciuria, renal hypercalciuria and resorptive hypercalciuria*). The

primary defect in absorptive hypercalciuria, which is inherited as an autosomal dominant trait, is increased passive mucosal absorption of calcium and oxalate in the jejunum. Renal hypercalciuria, which is characterized by primary renal leak of calcium and resorptive hypercalciuria, which is secondary to increased bone demineralization, are relatively uncommon (Pak *et al.* 1974, Coe *et al.* 1992, Pak 2004a, Moe 2006).

Hypercalciuria is often a predisposing factor for nephrolithiasis (Coe and Kavalich 1974, Coe 1978, Coe *et al.* 1979, Licata *et al.* 1979, Curhan *et al.* 1993, Hess 1996, Messa *et al.* 1997, Heller 1999). In fact, early studies regarded raised urinary calcium to be the most important urinary risk factor for calcium oxalate urolithiasis. As a result, patients were advised to restrict calcium intake (Marshall *et al.* 1972, Bleich *et al.* 1979, Rao *et al.* 1982, Vahlensieck 1986, Rose 1987, Goldfarb 1994). However recent studies have shown that a reduction in calcium intake *increases* the risk of calcium stone formation and also causes a negative calcium balance and further loss of bone tissue (Coe *et al.* 1992, Curhan *et al.* 1993, Lemann 2002). This is probably because a low intake of calcium leads to an increase in the amount of oxalate available for absorption in the gut and a consequent increase in urinary oxalate (Marshall *et al.* 1972). Conversely, some evidence suggests that a high intake of dietary calcium inhibits the absorption of oxalate and in this way, is a means of reducing urinary oxalate excretion and therefore for reducing calcium oxalate stone forming risk (Zarembski and Hodgkinson 1969, Barilla *et al.* 1978, Hess *et al.* 1998, Holmes *et al.* 2001).

Urinary oxalate

Urinary oxalate was originally considered merely as a major component of calcium oxalate stones, forming crystals in the lumen of the renal tubules. However, there is now much evidence to suggest that oxalate is the limiting or determinant factor in urinary calcium oxalate crystallization (Robertson *et al.* 1978, Robertson and Peacock 1980, Borsatti 1991, Bensatal and Ouabrani 2008, Tsujihata 2008). In fact, even a slight increase in urinary oxalate has been shown to have adverse effects on calcium oxalate stone formation (Robertson *et al.* 1981, Taylor and Curhan 2004). Oxalate also affects renal tubular epithelial cells, influencing the pathogenesis of nephrolithiasis (Robertson *et al.* 1978, Kim *et al.* 2002).

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Many studies in normal individuals, have reported increases in oxaluria of approximately 8-289% following oxalate loads (Marshall *et al.* 1972, Barilla *et al.* 1978, Finch *et al.* 1981, Balcke *et al.* 1989, Nguyen *et al.* 1994, Hess *et al.* 1998, Liebman and Costa 2000, Holmes *et al.* 2001). As might be expected, other studies have shown that stone formers present a higher mean oxalate level in the urine than healthy controls (Schwille *et al.* 1989, Wilson *et al.* 1989). This has prompted Jaeger and Robertson (2004) to posit that being able to control the urinary excretion of oxalate would undoubtedly constitute a major break-through in the prevention of calcium oxalate stone recurrence.

In normal individuals, the majority of urinary oxalate is derived from the endogenous metabolism of glycine, glyoxylate and ascorbic acid, while 10 to 20% is derived from oral ingestion (Zimmerman *et al.* 2004). However, in patients, hyperoxaluria may be due to various factors. Firstly, primary hyperoxaluria type I (*described in more details in chapter two of this thesis*) is caused by deficiency of the enzyme alanine:glyoxylate aminotransferase in the liver, which causes glycolate to be oxidized to oxalate thus increasing urinary oxalate and the risk of calcium oxalate stone formation (Danpure *et al.* 2003, Danpure *et al.* 2005). Secondly, enteric hyperoxaluria can also occur in patients with short bowel syndrome, as malabsorption of fats may cause calcium to bind to the unabsorbed fats rather than oxalates (Dobbins and Binder 1976, Dobbins 1985, Laminiski *et al.* 1991, Thomas *et al.* 2008, Cirillo *et al.* 2008). Thirdly, it has been postulated that apparent inconsistencies between dietary oxalate and oxaluria can be explained by oxalate-degrading gastrointestinal bacteria (*e.g Oxalobacter formigenes)* (Holmes *et al.* 1995, Sidhu *et al.* 1998, Sidhu *et al.* 1999, Sidhu *et al.* 2001, Siener *et al.* 2001, Kwak *et al.* 2003, Massey 2003, Jaeger and Robertson 2004, Hoppe *et al.* 2005).
These bacteria may be important for controlling oxaluria and may be a significant factor contributing towards stone rarity. There is some compelling evidence to support this latter assertion. In studies in stone-formers, the absence of *O. formigenes* has been associated with an increase in urinary oxalate (Kwak *et al.* 2003). Furthermore, several studies have also shown that stone-formers generally have lower levels of colonisation with *O. formigenes* than non-stone-formers (Sidhu *et al.* 1998, Sidhu *et al.* 1999, Sidhu *et al.* 2001, Kumar *et al.* 2002).

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Urinary citrate

Urinary citrate is widely regarded as an extremely important naturally-occurring inhibitor of calcium stone formation (Fleisch 1978, Pak 1994, Ryall 1997, Chow *et al.* 2004, Rodgers *et al.* 2005). Indeed, several studies have reported hypocitraturia in calcium stone formers (Pak *et al.* 1978, Rudman *et al.* 1982, Nicar *et al.* 1983, Pak *et al.* 1985, Nicar *et al.* 1987, Cupisti *et al.* 1992).

The most important cause of this condition in calcium stone formers is probably metabolic acidosis, which causes increased proximal tubular reabsorption of citrate and is seen in 15% to 63% of patients with urolithiasis (Nicar *et al.* 1983, Balaji and Menon 1997). In addition to these clinical scenarios, *in vitro* crystallization studies have also shown that citrate is able to complex urinary calcium thereby reducing its ionic concentration and concomitantly reducing the risk of calcium oxalate stone formation (Meyer and Smith 1975, Ryall *et al.* 1981, Hallson *et al.* 1983, Kok *et al.* 1986, Tiselius *et al.* 1993).

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Urinary magnesium

Magnesium is known for its ability to complex oxalate, potentially reducing oxalate absorption in the gastrointestinal tract, and decreasing the risk of stone formation (Desmars and Tawashi 1973, Hallson *et al.* 1982, Rushton and Spencer 1982, Li *et al.* 1985, Trinchieri *et al.* 1992). For example, magnesium has been shown to lower the urinary supersaturation of calcium oxalate and increase urinary citrate in two separate studies by Lindberg (1990a, 1990b). This finding is in agreement with other studies that have also observed a decrease in calcium oxalate supersaturation in the urine following magnesium therapy (Liebman and Costa 2000, Taylor and Curhan 2004). Consequentially, this makes it a potent inhibitor of calcium oxalate stone formation.

However, several studies have reported contradictory findings on urinary magnesium levels. Some studies have shown lower urinary magnesium excretion in stone formers compared to controls (Faragalla and Gershoff 1963, Yendt 1970, Tiselius *et al.* 1978, Trinchieri *et al.* 1991, Trinchieri *et al.* 1992), while in other studies it was found to be equal to or higher (Johansson *et al.* 1980, Drach *et al.* 1985). To compound matters, one

randomized clinical trial demonstrated no effect of magnesium supplementation on recurrent calcium oxalate stone formation (Ettinger *et al.* 1988). More studies are required to elucidate the actions of magnesium on relative levels of urinary magnesium in stone formers and controls.

Urinary uric acid

Hyperuricosuria, which may occur as a result of an excessive dietary intake of purine, is the major cause of uric acid stones along with low urinary pH (Coe and Kavalich 1974, Moe 2006). Several other conditions are also known to induce supersaturation of uric acid and/or lower urinary pH. These include chronic dehydration, severe diarrhea and hyperuricemia (Vella *et al.* 2007).

In a study by Coe (1978), a third of calcium oxalate patients were shown to have a high urinary uric acid excretion, from which 70% was attributed to excessive intake of dietary protein while the remaining 30% was attributed to endogenous overproduction of uric acid. Previous studies have shown that allupurinol therapy can reduce stone recurrence in calcium oxalate stone patients with hyperuricosuria by blocking uric acid production and reducing purine absorption (Pak *et al.* 1978, Ettinger 1991).

Urinary phosphate

An increase in urinary phosphate causes an increase in calcium phosphate complexation thereby reducing the risk of calcium oxalate crystallization (Schwille *et al.* 1989, Baumann *et al.* 2001). However, it concomitantly increases the risk of calcium phosphate stone formation. Calcium phosphate occurs in stones as either apatite (*the principal constituent of bones and teeth*) or brushite (*calcium monohydrogen phosphate*) (Mandel and Mandel 1989, Coe *et al.* 2005). It has been reported that calcium phosphate is present as a constituent of kidney stones in amounts ranging from 1-10% (Mandel and Mandel 1989) although Evan and co-workers (2005) estimate the more recent figure at 15%.

In a study comparing calcium phosphate and calcium oxalate stone formers, it was observed that calcium phosphate stone formers had high urine volume and calcium

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excretion with low citrate excretion relative to calcium oxalate stone formers (Evan *et al.* 2005). This was attributed to the morphological changes of each stone forming group which correlated with their metabolic profiles.

Urinary pH

Urine is a complex chemical solution containing dissociated and non-dissociated solutes in equilibrium. These equilibria are sensitive to changes in pH. Thus, the latter is a critical risk factor for urolithiasis.

As alluded to above, low urinary pH is a risk factor for uric acid stone formation. In fact, excessively low urine pH is much more common than hyperuricosuria as a cause of uric acid stone, as it can convert urinary urate into sparingly soluble uric acid, resulting in an increased risk of stone formation (Finlayson and Smith 1974, Moe *et al.* 2002, Tiselius 2003, Moe 2006). On the other hand, higher pH values pose a risk for calcium oxalate stone formation, while still higher values increase the risk of calcium phosphate (brushite) stone formation (Tiselius 1981, Tiselius and Larsson 1993, Højgaard *et al.* 1999).

This may seem like a gloomy situation, however it is not entirely so, as it has also been suggested that therapeutic alkalization for treatment of uric acid stones should be combined with calcium reducing measures so as to avoid brushite formation (Tiselius 1981). In addition, a high pH has been shown to have inhibitory activity towards calcium oxalate crystallization, as it causes more phosphate and citrate ions to be dissociated, which results in complexation with calcium, thereby reducing the urinary saturation of calcium oxalate (Kohri *et al.* 1993, Pak 1994, Rodgers *et al.* 2005).

The risk of calcium phosphate stone formation increases at pH above 6.2 (Højgaard *et al.* 1999, Jaeger and Robertson 2004). In fact, according to Jaeger and Robertson (2004), a small increase in pH above this value produces a logarithmic increase in the urinary concentration of phosphate ions. At a pH 5.75 half of the uric acid is ionized as urate salts and is soluble, while the other half exists as free insoluble uric acid. However, as pH falls below 5.5, uric acid stones can form as its concentration would exceed that of urate (Asplin 1996, Moe 2006, Vella *et al.* 2007). The pH range in which there is a high

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risk of calcium oxalate stones is not lucid. However, it has been reported that an alkaline pH seems to reduce the risk of calcium oxalate crystallization (Kohri *et al.* 1993, Pak 1994).

It has been suggested that the best case scenario with regard to urinary pH levels, is to maintain pH between 6-7, as the inhibitory activity of citrate and pyrophosphate is enhanced as a result of more of these species being in the active ionic form and the undissociated concentration of uric acid is low in this range (Pak 1994, Messa *et al.* 1997, Lewandowski *et al.* 2005).

Urinary volume

The single most common factor that initiates stone formation is inadequate fluid intake, which results in low volume of concentrated urine and relative urinary supersaturation of stone forming salts (Hall 1995, Moe 2006).

The positive effect of fluid intake on stone formation has been proven in epidemiological and prospective intervention studies (Pak *et al.* 1980, Borghi *et al.* 1996, Curhan *et al.* 1998, Moe 2006). For example, it is generally accepted that stone patients with a urine volume less than 2L/day can often avoid further events by increasing their fluid intake as shown in a randomized clinical trial by Borghi and co-workers (1996), who reported a significant and favourable decrease in urinary calcium and urinary oxalate thereby reducing the stone recurrent rate from 27% to 12%.

However, in attempts to increase fluid intake, caution should be exercised as some beverages have been reported to increase the risk of stone formation. Although the mechanism for the increased risk observed is unknown, two independent studies by Curhan and co-workers (1996,1998), on the effect of apple and grapefruit juices on stone formation risk, have shown that the risk of stone formation is increased by 35% and 37% respectively. Other workers have cautioned against the use of cola and hot chocolate, which because of their high oxalate content, probably contribute to their adverse effects on urolithiasis (Hesse *et al.* 1993, Rodgers 1999). Moreover, an increased calcium/creatinine ratio and elevated Tiselius Risk Index was observed after acute load of caffeine in a group of 39 normocalcaemic patients with calcium stones.

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Thus caffeinated beverages should also be consumed in moderation (Massey and Sutton 2004, Siener 2006). On the other hand, other fruit juices (*notably lemon*) have been shown to decrease the risk of calcium stone formation (Seltzer *et al.* 1996, Oussama *et al.* 2005, Kang *et al.* 2007).

Relative supersaturation and risk indices

Considerable effort has been expended in trying to develop robust indicators of the risk of stone formation. Risk indices include quotients calculated from the above urinary risk factors such as relative supersaturation (RS) of calcium oxalate (COM, COD and COT), brushite and uric acid (Werness *et al.* 1985, Ackermann *et al.* 1989, Brown *et al.* 1994). RS is the ratio between the activity product of the stone forming ions in urine and the corresponding solubility product obtained from artificial solutions. It gives an estimate of the driving forces favouring crystallization of common urinary salts and is readily computed using special software called EQUIL 1.5 (Werness *et al.* 1985).

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The BONN-Risk index (BRI), is based on *in vitro* studies of crystallization in urine (Laube *et al.* 2004, Laube *et al.* 2005). It combines the direct measurement of urinary free ionised calcium with an index of oxalate tolerance and calcium oxalate crystal formation. It can be calculated using the formula:

$$BRI = \frac{[Ca^{2+}]}{(Ox^{2-})} = \frac{\text{free ionised calcium } (mmol/L)}{\text{conc } (NH_4)_2Ox \text{ required to initiate spontaneous crystallization } (mmol/200\ ml\ urine)}$$

According to this formula, the risk for stone formation is low at BRI values less than 0.50, moderate at less than 1.00 and elevated at 2 and higher values (Laube *et al.* 2004).

The Tiselius risk index expresses the biochemical risk of calcium oxalate stone formation (Tiselius 1982). The mathematical expression is given by

$$\frac{(Ca/Cr)^{0.71} \times (Ox/Cr)}{(Mg/Cr)^{0.14} \times (Cit/Cr)^{0.10}}$$

Where Ca, Ox, Mg, Cit (*mmol/24hr*) and Cr (*mol/24hr*) are the urinary excretions of calcium, oxalate, magnesium, citrate and creatinine, respectively. Using the Tiselius risk index formula, normal values correspond to about 366±14, while a higher risk will correspond to a value about 527±17 (Tiselius 1982).

1.4 Diet

Diet plays an important role in the pathogenesis of calcium-containing kidney stones (Lewandowski *et al.* 2001, Massey 2003, Lewandowski and Rodgers 2004a, Taylor and Curhan 2004, Goldfarb *et al.* 2005, Massey *et al.* 2005, Siener and Hesse 2005, Taylor *et al.* 2005, Hesse and Straub 2006, Kynast-Gales and Massey 2007, Thomas *et al.* 2008). While most physicians agree that dietary modifications should be encouraged after the development of a kidney stone, consensus on the specifics of these modifications, with the exception of increasing fluid intake, has been lacking (Harvey *et al.* 1985, Curhan *et al.* 1993, Curhan *et al.* 1997a, Messa *et al.* 1997, Lemann 2002, Heller *et al.* 2003).

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Dietary oxalate

Since urinary oxalate concentration affects calcium oxalate relative supersaturation, any conditions that increase oxalate absorption from food or lead to increased oxalate production can cause calcium oxalate stone formation (Pak *et al.* 2004a, Coe *et al.* 2005).

About 85-90% of urinary oxalate comes from endogenous production in the liver, which is derived from three principal metabolic ways: ascorbic acid (30-50%), tryptophane (<10%) and glyoxylate (40%). Because oxidation of glyoxylate to oxalate is an irreversible process, accumulated glyoxylate consequently leads to hyperoxaluria, a major risk factor for calcium oxalate stone formation (Balaji and Menon 1997, Danpure *et al.* 2003, Danpure *et al.* 2005, Vella *et al.* 2007). The contribution of dietary oxalate to urinary oxalate is much higher than previously estimated and has recently been reported to be in the range 24.4±15.5% to 41.5±9.1% (Holmes *et al.* 2001).

It has been recognized for several years that the bioavailability of oxalate from different foods may be an important factor in determining how much oxalate is absorbed from a given diet (Brinkley *et al.* 1981). It has been shown in a rat model study that oxalate may be absorbed passively at all segments of the intestinal tract (Hatch and Freel 1995). In normal individuals, only small amounts of ingested oxalate are said to be absorbed into the body, with most estimates suggesting this to be 6-14%. On the other hand, stone formers may absorb ~50% more oxalate than normal individuals (Holmes *et al.* 1995, Hesse *et al.* 1999, Holmes and Assimos 2004). In addition, hyperoxaluric stone formers have been reported to absorb more oxalate than normo-oxaluric stone formers (Krishnamurthy *et al.* 2003). Holmes and Assimos (2004) posit that the reasons for these differences and their physiological, biochemical and genetic underpinnings require further research.

Nevertheless, studies have shown that oxalate-rich foods (*spinach, rhubarb, beetroot, peanuts, chocolate, parsley, strawberries, wheat bran and tea*), cause a significant increase in urinary oxalate excretion, increasing the risk of calcium oxalate stone formation (Finch *et al.* 1981, Brinkley *et al.* 1990, Hesse *et al.* 1993, Massey *et al.* 1993, Holmes and Kennedy 2000). Therefore calcium oxalate stone patients should reduce their intake of such foods.

Dietary calcium

According to Moe (2006), the recommendation for the optimum dietary calcium intake has changed from the traditional wisdom of calcium restriction, to high dietary calcium being protective, to a position in between, although some clarification is needed.

This may have been prompted by studies that have shown that a higher dietary calcium intake was associated with a reduced risk of incident stone formation (Bataille *et al.* 1983, Coe *et al.* 1992, Curhan *et al.* 1993, Curhan *et al.* 1997a, Messa *et al.* 1997, Lemann 2002, Heller *et al.* 2003). The mechanism by which dietary calcium reduces the risk of stone formation is unknown, but may be related to the impact on dietary oxalate absorption, as it has been shown that a higher calcium intake reduce dietary oxalate absorption in the gut, thereby lowering urinary oxalate excretion (Barilla *et al.* 1978, Hess *et al.* 1998, Curhan 2004).

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Furthermore, metabolic studies in healthy people have shown that when daily dietary calcium intake is reduced from high (*45 mmol/L per day, 1800 mg*) to intermediate (*30 mmol/L per day, 1200 mg*), there is a small increase of oxalate absorption, whereas stringent reduction (*5 mol/L per day, 200 mg*) greatly raises the oxalate absorption. Therefore it is recommended that a calcium intake between 800 mg and 1200 mg per day should be maintained rather than restricting or exceeding its input (Jaeger *et al.* 1985, Brinkley *et al.* 1990, Wahl and Hess 2000, Holmes *et al.* 2001, von Unruh *et al.* 2004, Moe 2006).

Interestingly, although a high dietary calcium intake has recently been shown to decrease the risk for calcium oxalate stone formation, studies on supplemental calcium have produced inconclusive evidence. A previous study by Curhan (1993), has also reported an increase in the risk of stone formation in females. This was attributed to the timing of ingestion of the supplement relative to meals, as it is postulated that when taken between meals rather than with them, supplemental calcium may increase gut intestinal tract calcium absorption, thereby increasing urinary calcium (Curhan *et al.* 1997a, Harvey *et al.* 1985).

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Dietary sodium

A high dietary sodium intake is a risk factor for stone formation, as it has been shown to increase urinary calcium excretion independent of calcium intake (Kok *et al.* 1990, Sakhaee *et al.* 1993, Pak 2004b). This is probably due to the inhibition of sodium and calcium reabsorption in the proximal tubule and along the loop of Henle (Muldowney *et al.* 1982, Curhan 2004).

The major effect of dietary sodium restriction is to reduce urinary calcium, although it has additional effects, such as raising urinary citrate and reducing sodium urate saturation, all of which can reduce calcium oxalate precipitation. Moreover, reducing dietary salt intake has a positive effect on both urinary calcium excretion and bone turnover, the latter aspect assuming clinical relevance in recurrent idiopathic calcium nephrolithiasis and postmenopausal women (Devine *et al.* 1995, Ferrari *et al.* 2007).

On the other hand, it has been shown that combined dietary protein with salt restriction is effective in reducing stone recurrence (Borghi *et al.* 2002). However, observational studies with high sodium intake have found an increased risk of stone formation in women but not in men (Curhan *et al.* 1993, Curhan *et al.* 1997a, Curhan *et al.* 2004). It is postulated that the differences may be due to true differences in the risk factors or an incomplete assessment of sodium intake. Nonetheless, the findings in men have been supported in a recent study, which showed that sodium salts such as sodium citrate and sodium bicarcabonate have no significant effect on urinary calcium in white South African males (Allie-Hamdulay and Rodgers 2005).

Dietary animal protein

Data from epidemiological studies provide evidence for a strong correlation between consumption of animal protein and the incidence of stone disease (Robertson *et al.* 1979, Yoshida and Okada 1990, Curhan *et al.* 1993, Hesse *et al.* 2003, Siener 2006).

Animal protein intake may increase the risk of stone formation as a result of increased excretion of calcium and uric acid and a decreased excretion of citrate (Robertson *et al.* 1979, Breslau *et al.* 1995, Curhan 2004). These findings have been supported by studies that have reported similar observations after a high purine intake (Brockis *et al.* 1982, Fellström *et al.* 1984, Nguyen *et al.* 2001, Reddy *et al.* 2002). It has been suggested that this may occur through an increased endogenous acid production with consequent metabolic acidosis, which may elicit buffering from bone, increasing calcium reabsorption (Brockis *et al.* 1982, Ferrari *et al.* 2007). Moreover, a high protein intake seems to affect the glomerular filtration rate by decreasing distal tubular cell calcium reabsorption. This mechanism may even be amplified in hypercalciuric patients (Schuette *et al.* 1981, Brandle *et al.* 1995).

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1.5 Genetics and urolithiasis

Kidney stones develop more frequently in individuals with a family history of stone formation than in those without. However, little information is available regarding whether the increased risk is attributable to genetic factors, environmental exposures or some combination thereof (Curhan *et al.* 1997b). The first clues that idiopathic nephrolithiasis might have a large genetic component were reported in 1894 when familial aggregations of urinary calculi were recorded (Griffin *et al.* 2004).

A number of studies (Coe *et al.* 1979, Marya *et al.* 1981, Goodman *et al.* 1995, Jaeger 1996, Goodman *et al.* 1997, Saborio and Scheinman 1998, Baggio 1999, Thomas and Stapleton 2000, Milosevic *et al.* 2002, Goldfarb *et al.* 2005, van Woerden *et al.* 2006, Gambaro and Abaterusso 2007) have focussed their investigations on the genetic aspects of kidney stone disease. It is very clear from these studies that a comparison of risks attributed to dietary factors and those attributed to genetic factors, indicates that the genetic risks exceed the risks associated with dietary factors. For example, the latter study by Gambaro and Abaterusso (2007) has observed that, genetic background accounts for 56% of the risk of stone formation. Indeed, this is larger than a combination of both dietary and environmental influences. It is probable that there is considerable genetic heterogeneity in renal stone disease.

Although an environmental effect cannot be excluded fully, findings in a study by Mente and co-workers (2006) have suggested that the disturbance in calcium metabolism in hypertension and kidney stone disease has a genetic basis. Another independent study has also shown that half of the variance of calciuria is due to genetic factors (Bianchi *et al.* 1988). This conclusion has been recently confirmed in families studied for nephrolithiasis (Loredo-Osti *et al. 2005*). In the context of the present thesis, a study investigating the influence of a genetic aspect (*frequency of alanine:glyoxylate aminotransferase Proline11Leucine polymorphism*) on the risk of kidney stone formation in black and white South African subjects will be described in much more detail in Chapter 2 of this thesis.

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1.6 Gut permeability and oxalate handling

Gut permeability describes a situation where the lining of the gut mucosa is damaged allowing toxic materials (*undigested foods, bacteria, toxins, parasites, etc.)* to pass into the body that would normally be eliminated. It is well recognized that the primary function of the gut mucosa is the digestion and absorption of nutrients, in addition to exhibiting a barrier function and to facilitate active transport (*through the cell themselves*) or paracellular pathway (*through 'tight junctions' between the cells*) (Allison *et al.* 1986, O' Boyle *et al.* 1998, Robertson 1999). According to Verdu and Collins (2004) infection, drugs, certain foods and psychological stress can cause the paracellular pathway to become more permeable or leaky, thus resulting in the so called 'leaky gut syndrome'.

One of the most commonly used non-invasive, highly sensitive tests for screening of diseases that affect gut permeability is the '*Lactulose-mannitol absorption test*' (Judy *et al.* 1989, Andre *et al.* 1991, Laudat *et al.* 1994, Paroni *et al.* 2006). A full description of this test including the relevance of abnormal gut permeability to renal stone formation is described in detail in Chapter 3 of this thesis.

1.7 Supplemental vitamin E

As stated earlier, it is well known that hyperoxaluria is a major risk factor for calcium oxalate urolithiasis and that stone formation is augmented and promoted when combined with cellular degradation products derived from renal tubular injury (Thamilsevan *et al.* 2003). This injury may be due to the production of free radicals in patients with calcium oxalate stones. The latter can be caused by several mechanisms, one of which is lipid peroxidation (LPO), a process in which tissue is damaged by superoxide, hydroxyl radicals or hydrogen peroxide. Exposure of renal cells to oxalate itself can induce peroxidative injury. However cells are endowed with several antioxidant systems including ascorbic acid, vitamin E and thiols which provide protection against oxidative damage (Scheid *et al.* 1996, Thamilselvan *et al.* 2000, Thamilselvan *et al.* 2003).It has been suggested in the latter study that antioxidant administration may prevent oxalate-mediated peroxide injury and hence may prevent calcium oxalate nucleation and retention in the renal tubules. A study of the effects of vitamin E ingestion on kidney stone risk factors as well as a full description of the relevance of lipid peroxidation to renal stone formation will be given in detail in Chapter 4 of this thesis.

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1.8 Supplemental fish oil (omega 3)

It has been well documented that ingestion of omega 3 (n-3) polyunsaturated fatty acids (PUFA) has positive effects on human health, especially the very long chain (n-3) molecules, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Kris-Etherton *et al.* 2002, Din *et al.* 2004, Schram *et al.* 2007). Several cohort, dietary intervention and case control studies have shown that fish and n-3 PUFAs confer significant health benefits (Kromhout *et al.* 1985, Burr *et al.* 1989, Dolecek *et al.* 1991, de Lorgeril *et al.* 1994, Siscovick *et al.* 1995, Daviglus *et al.* 1997, Albert *et al.* 1998, GISSI investigators 1999, Albert *et al.* 2002, Hu *et al.* 2002, Moore *et al.* 2006). For example, the low incidence of cardiovascular disease, kidney stone disease and other degenerative diseases including atherosclerosis in the inhabitants of Greenland has been attributed to their high consumption of oily fish with its high concentration of EPA (Dyerberg 1981, Dyerberg and Bang 1982, Buck *et al.* 1991, Yasui *et al.* 2001).

It has been proposed that greater intake of n-3 fatty acids such as EPA and DHA (*through dietary sources or fish oil supplementation*) may reduce the risk of kidney stone formation (Buck *et al.* 1991, Yasui *et al.* 2001, Naya *et al.* 2002, Taylor *et al.* 2005). Indeed, studies by Gambaro and co-workers (1995, 1997, 2000) have shown that dietary manipulation by long chain polyunsaturated fatty acids play an important role in the pathogenesis of calcium nephrolithiasis by *inter alia* reducing urinary parameters (*notably calcium and oxalate*). In fact, clinical and experimental investigations have underlined the important role of fatty acids in the control of hypercalciuria, a well-known risk factor for lithogenesis (Rothwell *et al.* 1993, Baggio *et al.* 1996, Baggio and Budakovic 2005). Many treatment modalities in clinical practice are directed towards reducing urinary calcium excretion and dietary modifications with long chain n-3 fatty acids might be a useful adjunct in the treatment of idiopathic hypercalciuric urolithiasis (Tulloch *et al.* 1994).

A study investigating the effects of fish oil (n-3) on urinary calcium, oxalate and other kidney stone risk factors will be described in Chapter 5 of this thesis.

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1.9 Fish oil and concomitant vitamin E requirement

Concurrent with the aforementioned proposed beneficial effects of fish consumption, several workers have cautioned that potential side effects are increased oxidative stress *in vivo,* as shown by studies that have reported increased lipid peroxidation observed following consumption of fish or fish oil (Yamamoto *et al.* 1985, Bartoli *et al.* 1988, Meydani *et al.* 1991, Wander *et al.* 1996, Wood *et al.* 2003).

It is well recognized that the major fat soluble antioxidant responsible for preventing oxidative damage to PUFA in membranes is vitamin E (Vatassery 1994, Turley *et al.* 1998, Wood *et al.* 2003). A study investigating the effects of n-3 fish oil ingestion on its own and in tandem with vitamin E in South Africa's relatively stone-free and stone prone groups will be fully described in Chapter 5 of this thesis.

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1.10 Urolithiasis in South Africa

As stated earlier, in South Africa, the incidence of urolithiasis in the black population is extremely rare (<1%) while in the white population it occurs to the same extent as in many western societies (~15%) (Muskat 1951, Wise and Kark 1961, Modlin 1967, Whalley *et al.* 1998, Whalley *et al.* 1999). Although the explanation for stone rarity in the black group is not apparent at this time, several studies have addressed this intriguing phenomenon during the past 40 years.

In 1967, Modlin suggested that it is reasonable to assume that the answer to this problem may be found in the differences between the compositions of urine in the black and white groups (Modlin 1967). However, those studies pursued in this regard have reported unexpected and perplexing results.

Urinary calcium and phosphate excretions have been found to be significantly lower in black subjects than in whites, but these values all lie within the normal range (Muskat *et al.* 1951, Modlin 1967, Whalley *et al.* 1998, Lewandowski *et al.* 2001, Rodgers and Lewandowski 2002). Thus, although the relatively lower excretion of these two parameters might explain a relatively lower incidence of stones in the black group, it cannot account for its near absence.

More intriguing are the results which have been reported for urinary oxalate, citrate and pH. Some studies have shown that there is no difference in oxalate excretion between the groups (Whalley *et al.* 1998, Lewandowski *et al.* 2001, Rodgers and Lewandowski 2002) which is surprising, given that this parameter is a critical determinant of calcium oxalate stone formation. Even more surprising is that one study involving a small group of subjects found higher urinary oxalate levels in black subjects (Lewandowski and Rodgers 2004a). Urinary citrate has been found to be lower in blacks (Modlin 1967, Whalley *et al.* 1998, Lewandowski *et al.* 2001). This too is surprising because this would suggest an increased predisposition towards stone formation. The same inference can be drawn about urinary pH which has been found to be lower in blacks (Modlin 1967, Lewandowski *et al.* 2001, Rodgers *et al.* 2005). Thus, urine composition values have proven to be counter-intuitive and are unable to adequately account for the difference in stone incidence in the two population groups.

Some authors believe that dietary differences can account for the difference in stone incidence between the two race groups (Wise and Kark 1961, Modlin 1967). Black South Africans have been reported to ingest significantly greater quantities of sodium in the form of seasoning or table salt than their white counterparts (Modlin 1967, Whalley *et al.* 1998). Modlin has attributed the low stone incidence in blacks to their relatively higher urinary ratio of sodium/calcium, which might play a protective role against urolithiasis in this group. In addition, hyperoxalurogenic eating habits (*high dietary intake of oxalate, low intake of calcium, and a low intake of magnesium*) have been reported among the black population (Viljoen and Gericke 2001). Despite this, studies have shown that urinary oxalate in this group is unaffected and lies within the normal range (Whalley *et al.* 1998, Rodgers and Lewandowski 2002, Lewandowski and Rodgers 2004a, Lewandowski and Rodgers 2004b, Lewandowski *et al.* 2005). There are difficulties in reliably measuring urinary oxalate, plasma oxalate and food oxalate (Holmes and Assimos 2004), which may in part explain the differences in the presented data that were obtained from literature.

Of particular interest is that studies in the Kidney Stone Research Laboratory at the University of Cape Town in South Africa, have found empirical evidence to suggest that different oxalate handling mechanisms exist in this group compared to the white population in which the stone incidence is comparable to the rest of the world (Lewandowski *et al.* 2001). For example, in the latter study, it was shown that when a

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high oxalate/low calcium diet was administered to white and black subjects, the urinary oxalate increased in the former as expected, but remained unchanged in the latter. In addition, other studies in the Kidney Stone Research Laboratory have shown that black subjects have significantly more oxalate degrading bacteria relative to healthy and stone-forming white subjects and that the oxalate-degrading bacteria capability of the bacteria isolated from black subjects is also significantly greater than that of the other two groups (Lewandowski *et al.* 2004a, Lewandowski *et al.* 2004b, Lewandowski *et al.* 2005). It was concluded that these results confirm that the South African black population is able to handle dietary oxalate in a more efficacious way than the white population, which might be a contributory factor to their low stone incidence.

Intriguingly, in another study by the same authors in which the [$^{13}C_2$] oxalate absorption test was applied on a standardized diet with normal oxalate intake it was observed that there is no significant difference between the two race groups with regard to gastrointestinal oxalate absorption (Lewandowski *et al.* 2005). These findings have prompted us to evaluate the role of gut permeability as a possible key to understanding the different oxalate handling in black and white South African subjects and therefore allude to the disparity of prevalence in the black group.

The handling of various lithogenic and anti-lithogenic dietary supplements in black and white subjects has also been investigated in the Kidney Stone Research Laboratory. Here too interesting results have been obtained. For example, in a study by Lewandowski and Rodgers (2004a), in which the renal response to 5 different dietary and supplemental challenges (*high dietary calcium, calcium, vitamin B6, L-glutamine and L-cysteine supplements*) in the two race groups was studied, it was reported that none of the challenges had an effect in black subjects while in white subjects, the calcium diet significantly increased urinary potassium (*p=0.0001*) and decreased the relative supersaturation of brushite (*p=0.035*), the calcium supplement significantly decreased the Tiselius risk index (*p=0.014*), vitamin B6 supplement significantly decreased urinary calcium (*p=0.016*), urinary phosphate (*p=0.027*) and the relative supersaturation of brushite (*p=0.004*), L-glutamine supplement significantly decreased relative supersaturation of calcium oxalate (*p=0.01*), L-cysteine supplement significantly decreased urinary calcium (*p=0.031*) and the Tiselius risk index (*p=0.013*). The authors speculated that a renal or gastrointestinal homeostatic adjustment occurs in the black group keeping urinary concentration of substances in balance.

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In another approach aimed at understanding the occurrence of calcium oxalate kidney stones in the white population but its rarity in the black population, the role of several urinary proteins (*urinary prothrombin fragment 1, Tamm Horsfall mucoprotein, bikunin, osteopontin and albumin*) with regard to their inhibitory or promotory properties in the urines from the two race groups has also been probed (Craig *et al.* 2000, Craig *et al.* 2001, Mabizela *et al.* 2004, Webber *et al.* 2004, Deppa *et al.* 2005, Rodgers *et al.* 2006). For example, in a study investigating *N*-linked glycans on urinary prothrombin fragment 1, it was shown that the *N*-glycans from black subjects had a significantly greater proportion of disialylated structures than those from white subjects (Webber *et al.* 2004) while Tamm Horsfall mucoprotein, which is a known inhibitor of calcium oxalate crystallization, has been reported to have different molecular structures in the two groups, with those isolated from the black group having superior inhibitory properties (Craig *et al.* 2000, Craig *et al.* 2001).

In addition, osteopontin derived from urine of black subjects has been shown to inhibit calcium oxalate aggregation and deposition to a greater extent and to promote nucleation to a lesser extent than that derived from white subjects (Deppa *et al.* 2005). In yet another study, Bikunin, a protease inhibitor inter-α-inhibitor (IαI), isolated from matrix extract proteins was found to be in greater amounts in black subjects' matrix when quantitatively compared to that from white subjects (Mabizela *et al.* 2004), indicating that it may play a contributory role in the extremely low incidence of urolithiasis in the black population. More recently, evidence of molecular and structural differences between albumin isolated from urines of black and white subjects were also reported (Rodgers *et al.* 2006). The aforementioned studies demonstrated that these urinary proteins may be key role players in protecting the South African black population against stone disease.

1.11 Objectives of this thesis

Interrogation of the available literature on studies of the South African black and white kidney stone phenomenon indicates that further investigations are warranted in many areas. The present thesis will address some of these.

Firstly, the absence of any genetic studies attempting to explain the differences of kidney stone disease incidence in South Africa's black and white population groups is notable.

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Thus, the recent speculation by Danpure (2005) that the presence, absence or frequency of the alanine:glyoxylate aminotransferase Proline11Leucine polymorphism might be a contributory factor in calcium oxalate kidney stone disease, provides a meaningful motivation for exploring whether there are differences in the frequency of this polymorphism in South Africa's black and white populations which might contribute towards explaining the difference in stone occurrence.

Secondly, as stated earlier, urinary oxalate is the most critical determinant of calcium oxalate stone formation relative to other risk factors like hypercalcuria, hyperuricosuria and hypocitraturia. As such, it is of fundamental interest and importance to investigate the role of gut permeability in South Africa' s black and white groups, as a possible key to providing insights into the alleged different renal handling mechanisms for oxalate in the two race groups.

Thirdly, the favourable results in the context of kidney stone risk factors, which have been reported following the ingestion of vitamin E and fish oil supplements, coupled with the observations of different handling by South African black and white subjects of supplements in general, provide the basis of the final set of objectives.

The objectives of this thesis are therefore:

(i) To investigate the absence, presence or frequency of the alanine:glyoxylate aminotransferase Pro11Leu polymorphism in the South African black and white population groups with respect to calcium oxalate kidney stone disease.
(ii) To investigate the role of gut permeability as a possible key to providing insights into the alleged renal handling mechanisms for oxalate in the two race groups.
(iii) To investigate the effects of supplemental vitamin E ingestion on kidney stone risk factors in the two race groups.
(iv) To investigate the effects of supplemental omega 3 fish oil ingestion on kidney stone risk factors in the two race groups.
(v) To investigate the additive effects of the simultaneous ingestion of vitamin E and omega 3 fish oil supplements on kidney stone risk factors in the two race groups.

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1.12 References

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Chapter Two

Alanine:glyoxylate aminotransferase Pro11Leu polymorphism in South Africa's black and white subjects with respect to calcium oxalate kidney stone formation

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2.1 Introduction

As described in the previous chapter, the incidence of urolithiasis in South Africa differs between different populations with urolithiasis being extremely rare in the black or indigenous populations (<*1*%) and more prevalent (~15%) in populations of European descent. The reasons for this anomaly are unknown in spite of many previous physicochemical and biochemical investigations (Muskat 1951, Wise and Kark 1961, Modlin 1967, Whalley *et al.* 1998, Whalley *et al.* 1999).

It is well recognized that calcium oxalate stone formation is influenced by the patient's genetic make-up (Goodman *et al.* 1995, Baggio 1999, Shah *et al.* 2005, Watts 2005, Wolf *et al.* 2005, Mittal *et al.* 2006, Gambaro and Abaterusso 2007). Although calcium oxalate nephrolithiasis is considered as being a polygenic disease (Curhan *et al.* 1997, Holmes *et al.* 1998), any genes influencing the excretion of urinary calcium, oxalate and citrate must be considered prime candidates for screening as genetic components in its pathogenesis (Goodman *et al.* 1995, Baggio 1999).

More than 50 mutations have been identified in the AGXT gene, half of which are point missense mutations. Many of these are predicted to decrease the stability of alanine:glyoxylate aminotransferase (AGT), a pyridoxal-phosphate dependent enzyme that metabolizes glyoxylate by converting it into glycine (Danpure 2005, Danpure 2006). Moreover, mutations of the AGXT gene lead to deficient hepatic AGT activity which causes primary hyperoxaluria 1 (PH1) (Langman 2004, Yuen *et al.* 2004). Although it has long been recognized that hyperoxaluria plays an important aetiological role in nephrolithiasis, until recently little consideration has been given to the possibility of a common genetic defect being responsible (Griffin 2004).

PH1 is an autosomal recessive disorder that leads to recurrent calcium oxalate nephrolithiasis, nephrocalcinosis, progressive renal failure and systemic oxalosis (Milosevic *et al.* 2002, Coulter-Mackie *et al.* 2005, Watts 2005, Milliner 2006, Kemper *et al.* 2007). When AGT is deficient, glyoxylate is either oxidized to oxalate or reduced to glycolate, leading to hyperoxaluria and hyperglycolic aciduria, respectively, which then culminate in PH1 (Yuen *et al.* 2004, Danpure 2006).

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AGT is localized exclusively in the peroxisomes in healthy people, but, it is instead localized mainly in the mitochondria in PH1 patients. When in the mitochondria, AGT is unable to perform its function because glyoxylate is synthesized in the peroxisomes (Lumb *et al.* 2000). It has been hypothesized that AGT mistargeting is the combined effects of the Pro11Leu polymorphism and the PH1-specific G170R mutations (Purdue *et al.* 1990). The presence of this polymorphism alone reduces the specific activity of AGT by up to 75% and it has at least 3 significant effects (Danpure *et al.* 2003). Firstly, it reduces the specific catalytic activity of purified recombinant AGT by a factor of three. Secondly, it redirects a small proportion (~5%) of AGT from the peroxisomes to mitochondria. Thirdly, it sensitises AGT and the untoward effects of many of the most common mutations found in PH1.

Buoyed by the speculation that the presence, absence or frequency of the alanine:glyoxylate aminotransferase (AGT) Pro11Leu polymorphism might be a contributory factor in an individual's susceptibility to idiopathic calcium oxalate kidney stones (Danpure 2005), this thesis investigates whether there are differences in the frequency of this polymorphism in South Africa's black and white populations which might be a contributory factor in explaining their difference in stone occurrence.

2.2 Subjects and methods

Study population

Fourty healthy South African male subjects (*20 whites and 20 blacks*) were recruited for the study. The white subjects were recruited from the student cohort of the University of Cape Town via advertisement on the university notice boards. The black subjects comprised two experimental groups: 10 '*semi-urban*' black subjects were recruited from the University of Cape Town student cohort in the same way as white subjects while 10 '*rural*' subjects were recruited from '*Khayelitsha Township*' near the city of Cape Town via referrals by volunteers familiar with the area.

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Inclusion criteria

Healthy black and white South African male subjects (*age 18-30 years*) were allowed to participate in this study. Black subjects were recruited from the same ethnic group '*Xhosa*'.

Exclusion criteria

Subjects were excluded if:

- they or their first-degree relatives had a history of urolithiasis or any renal disorder
- they had chronic digestive disease and/or gut disease
- they were taking any supplements and/or minerals

Questionnaire for subjects

Each subject was required to complete a questionnaire assessing their diet, medical history, medications, lifestyle and other practices of interest as well as history of kidney stone disease (*appendix CD/Chapter2/questionnaire*). In addition, a 24-hour dietary food record (*appendix CD/Chapter2/24hr dietary food record*) was also completed on the day of blood and spot urine collection and analysed using the *Foodfinder 2* software package (Langenhoven *et al.* 1991).

Blood sample collection

Five ml of venous blood was collected into EDTA-tubes and was assigned an unambiguous unique identity number. One ml blood sample was aliquoted into a clearly labelled 1.5 mL eppendorf tube and stored at -20 °C until analysis. The remainder of the sample was stored at -80 °C. Blood samples were used for isolating DNA, which was typed for Pro11Leu polymorphism.

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Detection of Pro11Leu polymorphism

The Pro11Leu polymorphism was detected by a PCR-RFLP (Polymerase chain reaction - restriction fragment length polymorphism) method (Caldwell *et al.* 2004).

DNA was extracted from 100µl whole venous blood using a DNeasy® Blood and Tissue Kit (*QIAGEN, Hilden, Germany*). DNA concentrations were estimated for all samples using a NanoDrop™ 1000 Spectrophotometer (*Thermo Scientific, Cheshire, United Kingdom*). DNA samples were individually amplified by polymerase chain reaction (*PCR*) using a GeneAmp® 2700 thermocycler (*Applied Biosystems, California, USA*). PCR amplification was performed in thin-walled 0.2ml PCR tubes containing the following reagents in a 20µl reaction volume: 10 to 50 ng genomic DNA, 0.25 units of Super-Therm DNA polymerase (*Hoffman-La-Roche, New Jersey, USA*), 1× reaction buffer (final concentration: 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol and stabilisers), 0.4 µM of primers MIT 2 (5'-GCACAGATAAGCTTCAGGGA-3') and EX-2R (5'-CTTGAAGGA TGGATCCAGGG-3'), 1 mM $MgCl_2$ and 0.2 mM dNTPs.

The PCR cycling parameters consisted of one denaturing step at 95°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and a final incubation step of 72°C for 10 minutes. For all samples, 15µl of the PCR product was digested overnight at 37°C using 10 units of Eco130I restriction enzyme (*StyI; Fermentas, Helsingborg, Sweden*) in 10× Buffer orange (*Fermentas, Helsingborg, Sweden*). Restriction enzyme digest products were electrophoresed on a 2% agarose gel and visualised with ethidium bromide, and the product size estimated according to a BenchTop 100bp DNA Ladder (*Promega, Southampton Hampshire, United Kingdom*).

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Spot urine collection and treatment

Subjects also provided a sample of a freshly voided morning spot urine on the day the blood sample was drawn. These were tested for haematuria and nitrite using urinalysis test strips (*Medi Test Combi 5N, Macherey-Nagel; Düren*). All urines tested negative. Urine pH (*pH 211 microprocessor pH meter, Hanna Instruments, Cape Town, South Africa*) and volume were routinely measured. Urine aliquots were filtered through a 0.74

µm filter to remove cellular debris and proteinaceous material. The samples were then analysed for calcium, potassium, magnesium and sodium (Trudeau and Freier 1967, Willis *et al.* 1967, Fernandez *et al.* 1971) using a Varian 1275 Model flame atomic absorption spectrometer. Oxalate was determined using oxalate decarboxylase (Chiriboga *et al.* 1963), while citrate was determined by conversion to oxaloacetate using citrate lyase (Gruber and Moellering 1966). Inorganic phosphorus was determined using ammonium molybdate (Dryer and Routh 1963), creatinine using picric acid (Rock *et al.* 1986) and uric acid using uricase (Fossati *et al.* 1980).

Statistical analysis

Data were analysed statistically using one-way analysis of variance (ANOVA), Pearson's chi-squared risk test method, and the student's *t-test*. Data were considered significant if $p \leq 0.05$.

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2.3 Results

Pro11Leu polymorphism

DNA concentration of the samples ranged between 2.4-22.8 ng/µl (*appendix CD/Chapter/ DNA concentrations*. The genotypes of each individual and genotype frequencies are shown in tables 2.1 to 2.4. Comparisons for the frequency distribution of the AGXT gene alleles for '*rural*' blacks, '*semi-urban*' blacks, combined blacks (*both 'rural and semi-urban'*) and white subjects are given in table 2.5.

Table 2.1 Distribution of the AGXT gene alleles in healthy 'rural' black subjects

Subjects	Bands	Sizes of bands		Alleles*	
GRB01	1	512		C	C
GRB02	1	512		C	C
GRB03	1	512		C	C
GRB04	1	512		C	C
GRB05	1	512		C	C
GRB06	1	512		C	C
GRB07	2	512	619	C	T
GRB08	1	512		C	C
GRB09	1	512		C	C
GRB10	2	512	619	C	T

Table 2.2 Distribution of the AGXT gene alleles in healthy 'semi-urban' blacks

Subjects	Bands	Sizes of bands		Alleles*	
GSB01	1	512		C	C
GSB02	1	512		C	C
GSB03	1	512		C	C
GSB04	1	512		C	C
GSB05	1	512		C	C
GSB06	2	512	619	C	T
GSB07	2	512	619	C	T
GSB08	2	512	619	C	T
GSB09	1	512		C	C
GSB10	1	512		C	C

* **Note**: Allele 'C' corresponds to the AGXT major allele encoding Pro11 and allele 'T' corresponds to the AGXT minor allele encoding Leu11.

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There were no notable differences in the AGXT gene allelic distribution between 'rural' black subjects and 'semi-urban' black subjects. Therefore data from the two black groups (*table 2.1 and 2.2*) were combined (*table 2.3*) for comparison with their white compatriots (*table 2.4*) and the results of the comparisons of the frequency distribution of the major and minor alleles of the AGXT gene in the two race groups are given in table 2.5.

Table 2.3 Distribution of the major and minor alleles of the AGXT gene in healthy combined black subjects (*both 'rural' and 'semi-urban' subjects*)

Subjects	Bands	Sizes of bands		Alleles	
GRB01	1	512		C	C
GRB02	1	512		C	C
GRB03	1	512		C	C
GRB04	1	512		C	C
GRB05	1	512		C	C
GRB06	1	512		C	C
GRB07	2	512	619	C	T
GRB08	1	512		C	C
GRB09	1	512		C	C
GRB10	2	512	619	C	T
GSB01	1	512		C	C
GSB02	1	512		C	C
GSB03	1	512		C	C
GSB04	1	512		C	C
GSB05	1	512		C	C
GSB06	2	512	619	C	T
GSB07	2	512	619	C	T
GSB08	2	512	619	C	T
GSB09	1	512		C	C
GSB10	1	512		C	C

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* **Note**: Allele 'C' corresponds to the AGXT major allele encoding Pro11 and allele 'T' corresponds to the AGXT minor allele encoding Leu11.

Table 2.4 Distribution of the major and minor alleles of the AGXT gene in healthy white subjects

Sample	Bands	Size of Bands		Alleles*	
GSW01	1	512		C	C
GSW02	1	512		C	C
GSW03	2	512	619	C	T
GSW04	1	512		C	C
GSW05	1	512		C	C
GSW06	2	512	619	C	T
GSW07	1	512		C	C
GSW08	1	512		C	C
GSW09	1	512		C	C
GSW10	1	512		C	C
GSW11	2	512	619	C	T
GSW12	1	512		C	C
GSW13	1	512		C	C
GSW14	1	512		C	C
GSW15	1	512		C	C
GSW16	1	512		C	C
GSW17	1	512		C	C
GSW18	2	512	619	C	T
GSW19	2	512	619	C	T
GSW20	1	512		C	C

Table 2.5 Frequency distribution of the major and minor alleles of AGXT gene in healthy black and white subjects

Subjects	Genotypes	
	Frequency CC	Frequency CT
'Rural' blacks	0.80	0.20
'Semi-urban' blacks	0.70	0.30
Combined blacks	0.75	0.25
Whites	0.75	0.25

* **Note**: Allele 'C' corresponds to the AGXT major allele encoding Pro11 and allele 'T' corresponds to the AGXT minor allele encoding Leu11.

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Comment

As shown in table 2.5, the frequency distribution of the AGXT gene allelic variants was 80% for the major allele 'C' and 20% for the minor allele 'T' in 'rural' black subjects while for 'semi-urban' black subjects it was 70% and 30% for the major and minor alleles, respectively. When black subjects were treated as one group, the frequency of the alanine:glyoxylate aminotransferase (AGT) Pro11Leu polymorphism in the black and white subjects was the same given the small sample sizes. The association of variables (*such as daily caloric intake and urinary parameters*) with the presence or absence of this polymorphism was statistically investigated for all subjects irrespective of race group.

Dietary analysis

Comparison of the mean nutrient intakes derived from the dietary records from 'rural' black subjects (*n=10*), 'semi-urban' black subjects (*n=10*) and white subjects (*n=20*) as well as those of the combined black subjects (*n=20*) are given in table 2.6 while the summary is given in table 2.7. The association of daily caloric intake with the distribution of genotypes 'CC' and 'CT' of the AGXT gene encoding the Pro11Leu polymorphism is given in table 2.8. The raw data are presented in appendix CD/Chapter 2/Dietary analysis.

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Table 2.6 Comparisons of the mean dietary intakes (SE) of black ('rural', 'semi-urban' and combined) and white subjects

Nutrients	**'rural' blacks (RB)**	**'semi-urban' blacks (SB)**	**Combined blacks (RB+SB)**	**Whites (W)**	**Statistical comparisons (*p-values*)**			
					RB vs SB	**RB vs W**	**SB vs W**	**(RB+SB) vs W**
BMI (*kg/m2*)	24.19(0.58)	24.64(0.58)	24.14(0.37)	24.68(0.36)	0.5885	0.4342	0.9574	0.6227
Energy (*kJ*)	10485 (1350)	11479 (1349)	10983 (935)	14910 (808)	0.6088	0.0059*	0.0285*	0.0030*
Moisture (*g/day*)	1262 (197)	1613 (500)	1437 (148)	3301 (200)	0.2471	<0.0001*	<0.0001*	<0.0001*
Total Protein (*g/day*)	87.3 (10.13)	88.6 (8.21)	88.0 (6.35)	123.7 (6.35)	0.9229	0.0037*	0.0028*	0.0003*
Total Fat (*g/day*)	60.5 (14.27)	93.3 (11.22)	76.9 (9.60)	131.9 (7.57)	0.0880	<0.0001*	0.0072*	<0.0001*
Carbohydrate (*g/day*)	369 (49.36)	339 (46.32)	354 (33.12)	370 (28.32)	0.6690	0.9762	0.5528	0.7103
Fibre (*g/day*)	29.9(3.86)	30.6(3.44)	30.3(2.52)	29.9(2.45)	0.0741	0.9847	0.8633	0.9076
Total Sugar (*g/day*)	53.6 (13.36)	26.8 (4.61)	40.2 (7.53)	69.7 (5.02)	0.9014	0.1809	<0.0001*	0.0024*
Oxalate(*mg)*	0.8(0.36)	317(46.30)	158(42.56)	152(15.30)	<0.0001*	<0.0001*	0.0002*	0.8856
Ca (*mg/day*)	421.6(65)	644.9(96)	533.3(62)	1142.7(65)	0.0690	<0.0001*	<0.0001*	<0.0001*
Mg (*mg/day*)	406(46)	396(35)	401(28)	503(25.59)	0.8652	0.0568	0.0222*	0.0114*
Phosphate (*mg/day*)	1326 (168)	1407 (139)	1366 (107)	2133 (138)	0.7135	0.0015*	0.0027*	<0.0001*
K (*mg/day*)	2470(540)	2864(378)	2667(324)	4321(278)	0.5577	0.0021*	0.0048*	<0.0001*
Na (*mg/day*)	2286(362)	3399(4538)	2793(318)	4302(264)	0.0710	0.0001*	0.0763	<0.0001*
Vitamin A (*RE/day*)	335 (174)	1284 (98)	809 (146)	1418 (137)	0.0002*	<0.0001*	0.5232	0.0043*
Vitamin B6 (*mg*)	1.56 (0.32)	2.31 (0.29)	1.94 (0.22)	3.06 (0.26)	0.1014	0.0017*	0.0860	0.0024*
Vitamin C(*mg*)	70(36)	86(24)	78(21)	108(11)	0.7127	0.1948	0.3318	0.2027
Vitamin D *µg)*	7.62(2.66)	4.31(0.87)	5.97(1.41)	5.07(0.58)	0.2515	0.2178	0.4603	0.5622
Vitamin E(*mg*)	12.13(2.43)	11.38(1.09)	11.75(1.23)	11.40(1.26)	0.8298	0.7405	0.9871	0.8397

*(*Significance at $p \leq 0.05$).*

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Comment

Consideration of the results in table 2.6 and the summary thereof in table 2.7 shows that for most of the nutrients (*labelled* *) there is no difference between the RB and SB groups but that in all cases, they are significantly lower than in the W group. Other nutrients (*labelled ϕ*) show subsets of the aforementioned trends with black subjects again having lower values. Oxalate is somewhat unique with SB surprisingly having the highest value. These trends will be fully interpreted in the discussion section of this chapter. Since in most cases there were no differences between RB and SB, consolidation of these groups into one is feasible. Statistical comparisons between combined blacks (*RB+SB*) and whites showed that in all of the * and ϕ nutrients, these nutrients were significantly lower for the combined blacks group than in W group. This trend will also be fully interpreted in the discussion section of this chapter.

Table 2.7 Summary of comparisons of mean dietary intakes of black (*'rural', 'semi-urban' and combined*) and white subjects

Nutrient intake	Summary
Energy *	B (combined) < W, (RB=SB)<W
Moisture *	B (combined) < W, (RB=SB)<W
Total Protein *	B (combined) < W, (RB=SB)<W
Total Fat *	B (combined) < W, (RB=SB)<W
Carbohydrate	No differences
Fibre	No differences
Total Sugar ϕ	B (combined) < W, SB<W
Oxalate	B (combined) =W, RB<W<SB
Calcium *	B (combined) <W, RB<SB<W
Magnesium *	B (combined) <W, (RB=SB)<W
Phosphate *	B (combined) <W, (RB=SB)<W
Potassium *	B (combined) <W, (RB=SB)<W
Sodium *	B (combined) <W, (RB=SB)<W
Vitamin A ϕ	B (combined) <W, RB<(SB=W)
Vitamin B6 ϕ	B (combined) <W, RB=SB, RB<W
Vitamin C	No differences
Vitamin D	No differences
Vitamin E	No differences

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Table 2.8 The associations of daily caloric intake with the distribution of genotypes 'CC' and 'CT' of the AGXT gene encoding the Pro11Leu polymorphism

Nutrient intake	**Genotype**		**P-values**
	CC (Average)	**CT (Average)**	
Energy (*kJ*)	13010.83	12752.80	0.8731
Moisture (*g/day*)	2461.00	2096.86	0.4221
Total Protein (*g/day*)	105.25	107.51	0.8731
Total Fat (*g/day*)	107.38	95.43	0.4868
Carbohydrate (*g/day*)	378.89	311.90	0.0830
Fibre (*g/day*)	29.39	32.02	0.5222
Total Sugar (*g/day*)	55.31	53.83	0.9008
Oxalate (*mg/day*)	156.80	149.30	0.8866
Calcium (*mg/day*)	804.31	938.90	0.3838
Magnesium (*mg/day*)	453.20	447.40	0.9044
Phosphate (*mg/day*)	1695.87	1911.30	0.3843
Potassium (*mg/day*)	3456.17	3607.80	0.7959
Sodium (*mg/day*)	3764.40	2896.60	0.1135
Vitamin A (*RE/day*)	1118.53	1097.80	0.9363
Vitamin B6 (*mg/day*)	2.60	2.18	0.3482
Vitamin C (*mg/day*)	93.17	92.90	0.9924
Vitamin D (*µg/day*)	5.21	6.44	0.4910
Vitamin E (*mg/day*)	11.58	11.57	0.9985

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Comment

There was no significant association between daily nutrient intakes and the presence or absence of the Pro11Leu polymorphism as shown in table 2.8.

Urinary analysis

Comparison of urine data from the freshly voided morning urine samples of black and white subjects is given in table 2.9 while the summary is given in table 2.10. The association of urinary composition of spot morning urine sample with the distribution of

genotypes 'CC' and 'CT' of the AGXT gene encoding the Pro11Leu polymorphism is given in table 2.11. The spot urine composition data are standardized and expressed per mg creatinine (*e.g. mg calcium/mg creatinine*) as the values were not obtained from 24hr urine samples (Ilich *et al.* 2009). The raw data from each subject are presented in appendix CD/Chapter 2/urinary analysis.

Table 2.9 Comparisons of mean spot urine parameters (SE) from urines of black ('rural', 'semi-urban' and combined) and white subjects

Parameter	**'rural' blacks (RB)**	**'semi-urban' blacks (SB)**	**Combined blacks (RB+SB)**	**Whites (W)**	**Statistical comparisons (*p-values*)**			
					RB vs SB	**RB vs W**	**SB vs W**	**(RB+SB) vs W**
pH	5.745 (0.122)	5.703 (0.192)	5.724 (0.111)	5.732 (0.067)	0.8554	0.9225	0.8582	0.9479
Volume (*ml*)	231.80 (45.22)	290.50 (30.17)	261.15 (29.27)	260.50 (36.03)	0.3292	0.6375	0.6061	0.9889
Citrate (*mg/mg creatinine*)	0.2985 (0.12)	0.3198 (0.12)	0.3073 (0.06)	0.1634 (0.06)	0.8977	0.1276	0.1588	0.1136
Oxalate (*mg/mg creatinine*)	0.0086 (0.002)	0.0105 (0.002)	0.0095 (0.003)	0.0116 (0.003)	0.5870	0.5740	0.8370	0.5936
Calcium (*mg/mg creatinine*)	0.0431 (0.02)	0.0650 (0.02)	0.0537 (0.013)	0.0412 (0.008)	0.5042	0.8955	0.3454	0.4945
Magnesium (*mg/mg creatinine*)	0.0338 (0.010)	0.0119 (0.010)	0.0227 (0.006)	0.0230 (0.007)	0.1283	0.3700	0.1590	0.9722
Sodium (*mg/mg creatinine*)	2.368 (0.98)	2.0187 (0.98)	2.1771 (0.50)	0.7949 (0.42)	0.8030	0.0405*	0.1065	0.0566**
Potassium (*mg/mg creatinine*)	1.2399 (0.22)	0.3639 (0.22)	0.7932 (0.13)	0.1832 (0.12)	0.0107*	<0.0001*	0.1116	0.0022*
Uric acid (*mg/mg creatinine*)	0.2330 (0.04)	0.2263 (0.04)	0.2297 (0.03)	0.2801 (0.02)	0.8966	0.2773	0.3099	0.1890
Phosphate (*mg/mg creatinine*)	1.0361 (0.42)	2.1023 (0.42)	1.5260 (0.27)	1.8135 (0.20)	0.0910	0.0355*	0.5762	0.4637

**Significance at $p \leq 0.05$, **approaching significance*

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Table 2.10 Summary of the comparisons of mean spot urine parameters

Parameter	Summary
pH	No differences
Volume	No differences
Citrate	No differences
Oxalate	No differences
Calcium	No differences
Magnesium	No differences
Sodium	B(combined)>W, (RB=SB)>W
Potassium	B(combined)>W, RB>SB>W
Uric acid	No differences
Phosphate	RB<W

Comment

Only two urinary parameters – sodium and potassium – demonstrated consistently significant intergroup differences between 'rural' black and combined black subjects in comparisons with white subjects. In all two cases, values were individually and collectively higher for the indicated black groups than for white subjects. However, this two parameters were not significantly different between 'semi-urban' black subjects and the white subjects.

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Table 2.11 The associations of mean spot urine parameters with the distribution of genotypes 'CC' and 'CT' of the AGXT gene encoding the Pro11Leu polymorphism

Parameter	Genotype (average)		P-values
	CC	CT	
pH	5.74	5.70	0.8088
Volume (*ml*)	282.10	197.00	0.1087
Citrate (*mg/mg creatinine*)	0.2669	0.1407	0.2329
Oxalate (*mg/mg creatinine*)	0.0123	0.0052	0.1122
Calcium (*mg/mg creatinine*)	0.0534	0.0296	0.2556
Magnesium (*mg/mg creatinine*)	0.0255	0.0149	0.2993
Sodium (*mg/mg creatinine*)	1.7159	0.7964	0.2798
Potassium (*mg/mg creatinine*)	0.5308	0.3601	0.4840
Uric acid (*mg/mg creatinine*)	0.2675	0.2170	0.2562
Phosphate (*mg/mg creatinine*)	1.8327	1.1811	0.1462

**Significance at* $p \leq 0.05$

Comment

Statistical analysis showed that there was no association between mean urinary composition (*mg*) of spot morning urine samples expressed per mg creatinine with the presence or absence of the Pro11Leu polymorphism as shown in table 2.11.

Questionnaire analysis

As previously described, all subjects completed questionnaires in which they provided details of their self-defined ethnic identity and place of birth (Caldwell *et al.* 2004). Similar information was also sought about their parents. An attempt was made to extend the enquiry to grandparents but efforts in this regard were unsuccessful as information was mostly unknown, with most respondents indicating that their grandparents had passed away. Subjects were also asked if any of their siblings, parents or grandparents had ever had a kidney stone or renal disease (Curhan *et al.* 1997). All respondents reported no family history of kidney stone formation and identified themselves as South Africans born in the country. Relatives of subjects were not contacted. Responses to the questionnaire were statistically analysed for the association of selected factors with the frequency of AGXT gene allelic variants (*genotype 'CC' and 'CT'*) using the Pearson's chi-squared test method and the student's *t-test*. The results are given in tables 2.12 and 2.13 below.

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Table 2.12 The associations of dietary habits and lifestyle with the distributions of genotypes 'CC' and 'CT' of the AGXT gene encoding the Pro11Leu polymorphism

Dietary habits	Genotype		p-values
	CC (%)	CT (%)	
Childhood diet			
Personal			0.4494
Vegetarian	0	0	
Mainly vegetarian but with some meat	2.5	2.5	
Mixed	47.5	12.5	
Mainly meat but with some vegetables	17.5	10	
Lots of meat	7.5	0	
Mother			0.1132
Vegetarian	2.5	0	
Mainly vegetarian but with some meat	10	5	
Mixed	50	12.5	
Mainly meat but with some vegetables	10	7.5	
Lots of meat	2.5	0	
Father			0.6603
Vegetarian	0	0	
Mainly vegetarian but with some meat	10	2.5	
Mixed	47.5	15	
Mainly meat but with some vegetables	12.5	7.5	
Lots of meat	5	0	
Current diet			
Personal			0.5817
Vegetarian	0	2.5	
Mainly vegetarian but with some meat	5	0	
Mixed	37.5	7.5	
Mainly meat but with some vegetables	22.5	15	
Lots of meat	10	0	
Mother			0.7024
Vegetarian	0	0	
Mainly vegetarian but with some meat	7.5	0	
Mixed	52.5	22.5	
Mainly meat but with some vegetables	12.5	2.5	
Lots of meat	2.5	0	

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Father			0.2487
Vegetarian	0	0	
Mainly vegetarian but with some meat	2.5	0	
Mixed	20	12.5	
Mainly meat but with some vegetables	15	12.5	
Lots of meat	7.5	0	
Other personal dietary habits			
Salty foods			0.8219
Not salted	10	2.5	
Lightly salted	52.5	20	
Very salted	12.5	2.5	
Salty snacks			0.5796
No	45	12.5	
Yes	30	12.5	
Add pepper_cooking			0.5094
No	27.5	12.5	
Lightly	40	12.5	
A lot	7.5	0	
Add pepper_eating			0.7601
No	45	17.5	
Lightly	27.5	7.5	
A lot	2.5	0	
Add sugar_tea/coffee			0.1705
No	12.5	0	
<2 teaspoons	35	12.5	
3 teaspoons	10	10	
<5 teaspoons	17.5	2.5	
>5 teaspoons	0	0	
Add sugar_pudding			0.3492
No	60	20	
<2 teaspoons	5	2.5	
3 teaspoons	0	2.5	
<5 teaspoons	7.5	0	
> 5 teaspoons	2.5	0	
Add sugar_cereal			0.8841
No	20	7.5	
<2 teaspoons	27.5	10	
3 teaspoons	10	5	
<5 teaspoons	12.5	2.5	
>5 teaspoons	5	0	

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Other (affluence)			
Personal_birthplace			0.4357
Village	22.5	2.5	
Township	7.5	2.5	
City	45	20	
Mother_birthplace			0.9036
Village	35	10	
Township	5	2.5	
City	35	12.5	
Father_birthplace			0.5587
Village	35	7.5	
Township	7.5	5	
City	32.5	12.5	
Mother_Job			0.5828
Farmer	0	0	
Farm labour	0	2.5	
Shop worker	10	5	
Street Cleaner	0	0	
Office worker	10	5	
Professional (e.g doctor, teacher)	30	7.5	
Policeman	0	0	
Seasonal or occasional employment	5	0	
Housewife	7.5	5	
Self-employed	7.5	0	
Hotel worker	0	0	
Politician	0	0	
Taxi/bus driver	0	0	
Domestic worker	2.5	0	
Unemployed	2.5	0	
Retired (Indicate field)	0	0	
Other specify	0	0	
Father_Job			0.4564
Farmer	0	2.5	
Farm labour	0	0	
Shop worker	7.5	0	
Street Cleaner	0	2.5	

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Office worker	7.5	0	
Professional (e.g doctor, teacher)	27.5	10	
Policeman	2.5	0	
Seasonal or occasional employment	0	0	
Househusband	2.5	0	
Self-employed	2.5	0	
Hotel worker	2.5	0	
Politician	2.5	0	
Taxi/bus driver	10	5	
Domestic worker	7.5	5	
Unemployed	0	0	
Retired (Indicate field)	5	0	
Other (specify)	0	0	
Exercise			0.8101
No	12.5	5	
Yes	62.5	20	
Exercise example			
Jogging			0.3613
No	57.5	22.5	
Yes	17.5	2.5	
Hiking			0.2985
No	67.5	7.5	
Yes	25	0	
Tennis			0.1251
No	67.5	17.5	
Yes	7.5	7.5	
Squash			1.1000
No	67.5	22.5	
Yes	7.5	2.5	
Soccer			0.6733
No	55	20	
Yes	20	5	
Gym (cardio)			0.1250
No	67.5	17.5	
Yes	7.5	7.5	
Gym (weights)			0.8380
No	75	22.5	
Yes	0	2.5	
Gym (both)			0.4076
No	67.5	20	

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Yes	7.5	5	
Rugby			0.1492
No	72.5	17.5	
Yes	2.5	7.5	
Golf			0.0794
No	60	22.5	
Yes	17.5	0	
Other			0.5321
No	60	22.5	
Yes	17.5	0	
Level exercise activity			0.7076
Not at all	5	2.5	
Some, but < average	17.5	5	
Average	17.5	10	
Fairly active, > average	12.5	5	
Very active	22.5	2.5	

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Table 2.13 The associations of food and beverage intakes with the distributions of genotypes 'CC' and 'CT' of the AGXT gene encoding the Pro11Leu polymorphism

Food and Beverage intakes	Genotype		p-values
	CC(%)	CT(%)	
Cereal			0.5819
No	27.5	10	
Everyday or two	40	15	
Every few weeks	7.5	0	
Once or twice a year	0	0	
Milk			0.4295
No	10	7.5	
Everyday or two	62.5	17.5	
Every few weeks	2.5	0	
Once or twice a year	0	0	
Yogurt			0.3084
No	60	25	
Everyday or two	5	0	
Every few weeks	10	0	
Once or twice a year	0	0	
Chocolate			0.0044
No	65	10	
Everyday or two	7.5	5	
Every few weeks	2.5	10	
Once or twice a year	0	0	
Cheese			0.5109
No	32.5	15	
Everyday or two	30	5	
Every few weeks	12.5	5	
Once or twice a year	0	0	
Eggs			0.2807
No	17.5	12.5	
Everyday or two	35	7.5	
Every few weeks	22.5	5	
Once or twice a year	0	0	
Porridge			0.7491
No	32.5	15	
Everyday or two	25	5	

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Every few weeks	15	5	
Once or twice a year	2.5	0	
Pasta			0.9397
No	47.5	15	
Everyday or two	22.5	7.5	
Every few weeks	5	2.5	
Once or twice a year	0	0	
Rice			0.8170
No	20	7.5	
Everyday or two	40	12.5	
Every few weeks	15	5	
Once or twice a year	0	0	
Fruits			0.0975
No	20	2.5	
Everyday or two	42.5	10	
Every few weeks	12.5	12.5	
Once or twice a year	0	0	
Vegetables/Salad			0.2413
No	25	15	
Everyday or two	25	2.5	
Every few weeks	25	7.5	
Once or twice a year	0	0	
Potatoes			0.4020
No	32.5	17.5	
Everyday or two	32.5	5	
Every few weeks	10	2.5	
Once or twice a year	0	0	
Fried Chips			0.4594
No	47.5	12.5	
Everyday or two	5	5	
Every few weeks	22.5	7.5	
Once or twice a year	0	0	
Chicken			0.2588
No	7.5	5	
Everyday or two	52.5	20	
Every few weeks	15	0	
Once or twice a year	0	0	
Beef/Pork/Mutton			0.8532
No	10	5	
Everyday or two	37.5	12.5	

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Every few weeks	27.5	7.5	
Once or twice a year	0	0	
Fish			0.60943
No	50	0	
Everyday or two	20	5	
Every few weeks	5	0	
Once or twice a year	0	0	
Cool drink (eg Coke)			0.0204
No	50	5	
Everyday or two	10	12.5	
Every few weeks	15	7.5	
Once or twice a year	0	0	
Juice			0.0031
No	30	7.5	
Everyday or two	37.5	2.5	
Every few weeks	7.5	15	
Once or twice a year	0	0	
Tea/Coffee			0.1444
No	12.5	0	
Everyday or two	52.5	25	
Every few weeks	10	0	
Once or twice a year	0	0	
Alcohol intake			
Example alcohol			
Beer			0.0794
No	0	2.5	
Yes	75	22.5	
Hard liquor			1.0000
No	30	10	
Yes	45	15	
Wine			0.3558
No	35	7.5	
Yes	40	17.5	
Homebrew			0.5119
No	60	17.5	
Yes	15	7.5	
Fermented cider			0.3458
No	50	12.5	
Yes	25	12.5	

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Frequency alcohol intake			0.3996
1-4 days per week	0	2.5	
5/more days per week	27.5	10	
1-3 days a month	7.5	0	
<once a month	25	7.5	
Amount alcohol per week			0.1808
(no. units x volume)			
No (O ml)	0	2.5	
<1000ml	17.5	7.5	
< 5000ml	57.5	15	
> 5000ml	0	0	

Comment

When comparing the usual patterns of food consumption, the only variables that showed an association with the distribution of the major AGXT allelic variants 'T' encoding the presence of Pro11Leu polymorphism was dietary intake of juice (*p=0.0031*), cool drink (*e.g coke*) (*p=0.0204*) and chocolate (*p=0.0044*). The association with alcohol intake (*beer*) tended towards significance (*p=0.0794*) while there was no association with the intake of other types of alcoholic beverages (*p>0.05*). There was no significant association of the frequency of this polymorphism with fish (*p=0.6094*) and meat intake in the studied group (*p=0.8532 for red meat and p=0.2588 for chicken*). All other food items investigated did not show a significant association with the polymorphism. Analysis of vitamin and supplement use is not included as the respondents were not taking any supplements and/or minerals.

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2.4 Discussion

It is well recognized that nephrolithiasis is a complex, multifactorial disease resulting from an interaction between environmental and genetic factors (Goodman *et al.* 1995, Baggio 1999, Watts 2005, Wolf *et al.* 2005, Mittal *et al.* 2006, Gambaro and Abaterusso 2007). According to Baggio (1999), the lack of convincing findings on genetic factors and genes responsible for nephrolithiasis is due to an inadequate understanding of the pathogenesis of this disease.

Although urolithiasis is a polygenic disease, there is some evidence for a monogenic basis for a small number of cases of hyperoxaluria, which predisposes the patient to nephrolithiasis (Holmes *et al.* 1998, Caldwell *et al.* 2004, Griffin 2004, Gambaro and Abaterusso 2007). It is widely accepted that primary hyperoxaluria 1 is due to a functional defect of the liver-specific peroxisomal enzyme AGT (Danpure 1998, Milosevic *et al.* 2002, Coulter-Mackie *et al.* 2003, Coulter-Mackie and Rumsby 2004, Yuen *et al.* 2004). While the liver-specific nature of AGT expression in humans precludes a comprehensive analysis of the relationship between the presence of the minor and major AGXT alleles and AGT activity *in situ* (Lumb *et al.* 2000), AGT itself has been shown to have a number of polymorphisms (including Pro11Leu polymorphism), which can change the amino acid sequence but have no apparent pathological effect (Rumsby *et al.* 2004).

In the present study, the potential influence of the frequency of this polymorphism in black and white South African subjects was examined with respect to the differences in stone incidence in the two groups. Firstly, the association of urinary parameters with the frequency of the above-mentioned polymorphism was investigated. No association was demonstrated. However, as stated previously this may have been due to the nature of the urine collections (spot) and the treatment of the data. For example, different results might be anticipated according to the time of day of the spot urine is collected. In fact, a "fasting" spot urine might miss peaks of urinary excretion following ingestion of food. It is uncertain whether measurements from 24hr urine samples would have shown any associations with this polymorphism. However, such collections were generally not feasible for this study. On the other hand, according to Ilich and co-workers (2009), although replacing burdensome 24hr urine collection with spot urine sampling might not provide associations in all cases, results show spot urine samples could be useful.

The two urinary parameters (*potassium and sodium*) that were significantly higher in 'rural' black (*$p<0.0001$ for potassium and $p=0.0405$ for sodium*) and combined black (*$p=0.0022$ for potassium and $p=0.0566$ for sodium*) subjects when compared with white subjects are recognized risk factors for kidney stone formation. It has been reported that low urinary potassium excretion may potentially increase the risk because of its associated calciuria (Parivar *et al.* 1996). Thus, the higher urinary excretion of potassium in blacks as opposed to whites may be a contributory factor in their low incidence of

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stone formation. In addition, it has also been reported that sodium intake of blacks is higher than that of whites (Modlin 1967, Whalley *et al.* 1998). Although this commensurate with high urinary sodium excretion, it seems to have no effect on urinary calcium excretion as it has also been reported that urinary calcium is lower in blacks than in whites (Modlin 1967, Whalley *et al.* 1998, Rodgers and Lewandowski 2002).

Secondly, association of dietary intake with polymorphism frequency was investigated. According to Curhan and co-workers (1997) environmental exposures, such as diet, are thought to play an important role in stone formation. In support, there is a wealth of data suggesting that this assertion might indeed be accurate (Curhan *et al.* 1993, Parivar *et al.* 1996, Lewandowski *et al.* 2001, Goldfarb *et al.* 2005). Interest in dietary analysis in the present study arose mainly because the qualitative relationship between AGT distribution and diet is compelling (Birdsey *et al.* 2005).

Analysis of dietary records supplied by all subjects, revealed a number of significant differences in dietary intakes between 'rural' black subjects, 'semi-urban' black subjects and white subjects. The lower nutrients intake in blacks of nutrients which are important in pathogenesis of kidney stones (*calcium, magnesium, phosphate, potassium, sodium, vitamin A and vitamin B6*) have been previously reported (Muskat 1951, Wise and Kark 1961, Modlin 1967, Whalley *et al.* 1998, Lewandowski *et al.* 2001). The low intake of calcium has been attributed to lactose intolerance in blacks (Viljoen and Gericke 2001) while low magnesium has been attributed to a low intake of vegetables (Whalley *et al.* 1998, Charlton *et al.* 2005).

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It is noted that in the present study, blacks had lower sodium intake than whites, which contradicts the findings of Modlin (1967). The reasons for this anomaly are unclear. According to Massey and Whiting (1996), dietary assessment of salt is very inaccurate. However, this does not seem to diminish its importance as it has been indicated that dietary salt is a much stronger predictor of urinary calcium than dietary calcium and protein (Brutis *et al.* 1994). In fact, a high sodium intake leads to increased urinary calcium excretion independent of calcium intake due to the inhibition of sodium and calcium reabsorption in the proximal tubule and along the loop of Henle (Muldowney *et al.* 1982, Curhan 2004). A positive association between sodium intake and new kidney

stone formation has been demonstrated in women but not in men (Curhan *et al.* 1993, Curhan *et al.* 1997, Taylor and Curhan 2004).

In the present study, there were no significant differences in the intake of vitamin B6 between 'rural' blacks and 'semi-urban' blacks ($p=0.1014$) as well as in 'semi-urban' blacks and whites ($p=0.0860$). Statistically significant differences in the intake of this vitamin were observed between 'rural' blacks and whites ($p=0.0017$) as well as between combined blacks and whites ($p=0.0024$). There seem to be controversy with regard to the role of vitamin B6 intake in stone formation. According to Lewandowski and Rodgers (2004), its role has not been clearly defined. In support, studies have shown that prolonged vitamin B6 deficiency can result in an increase in urinary oxalate excretion leading to a potentially higher incidence of calcium oxalate stones (Faber *et al.* 1963, Tommaso *et al.* 2002) while in contrast, a study by Curhan and co-workers (1999) has reported that a high intake of vitamin B6 is inversely associated with the risk of stone formation.

There were no significant differences in the intake of vitamin A between 'semi-urban' blacks and whites ($p=0.5232$). However, statistically significant differences were observed between 'rural' blacks and 'semi-urban' blacks ($p=0.0002$, $RB<SB$), 'rural' blacks and whites ($p<0.0001$) as well as combined blacks and whites ($p=0.0043$), with blacks having lower intakes of this vitamin than whites. The intake of vitamin A has been shown to be significantly higher in idiopathic calcium stone formers than normal control subjects (Al Zahrani *et al.* 2000). This is surprising as the deficiency of this vitamin has been reported to potentially aggravate tubular cell necrosis, an initial promoter of renal lithiasis, which can be caused by vitamin E deficiency and overproduction of free radicals (Sakly *et al.* 2003). However, it is worth mentioning that vitamin A might not be a potent free radical scavenger (Schunemann *et al.* 2001, Alfonso *et al.* 2005) as it has been shown to have a limited capacity to act as a scavenging antioxidant *in vivo* (Lesgards *et al.* 2005, Dragsted 2008).

The intake of dietary phosphate was similar between 'rural' blacks and 'semi-urban' blacks ($p=0.7135$) while it was lower in 'rural' blacks ($p=0.0021$), 'semi-urban' blacks ($p=0.0048$) and combined blacks ($p<0.0001$) when compared to their white compatriots. Previous studies have shown that dietary phosphate intakes of stone formers and

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healthy subjects are similar (Fellström *et al.* 1989, Trinchieri *et al.* 1991). Although dietary phosphate restriction has been reported to increase calcium excretion (Roberts and Knox 1990), neutral phosphate intake has been shown to decrease renal calcium excretion (Lau *et al.* 1979).

The intake of dietary potassium is also worthy of attention. Although it was similar between 'rural' blacks and 'semi-urban' blacks (*p=0.5577*) in the present study, it was lower in 'rural' blacks (*p=0.0021*), 'semi-urban' blacks (*p=0.0048*) and combined blacks (*p<0.0001*) when compared to their white compatriots. This supports the findings of a recent study which has also shown racial differences in potassium intakes, with blacks typically consuming less dietary potassium than whites (Turban *et al.* 2008). This is surprising as it has been shown that potassium administration reduces and potassium deprivation increases urinary calcium excretion in healthy adults (Lemann *et al.* 1991, Rafferty *et al.* 2005). In fact, a prospective study of dietary calcium and other nutrients and the risk of symptomatic kidney stones has also shown that potassium intake is inversely related to the risk of stone formation (Curhan *et al.* 1993). It has also been shown that the mean daily intakes of potassium were greater in patients with hyperoxaluria than in stone formers with normal oxalate excretion (Siener 2003) while no differences were found in the potassium intake of stone formers compared to healthy subjects (Fellström *et al.* 1989).

It is noted that since most food sodium and potassium are absorbed, there is an obvious discrepancy in that the sodium and potassium diets of whites are higher than those of blacks, but their urinary sodium and potassium values are lower. The possible explanation for this anomaly is that urinary solutes were determined in spot urine samples (standardized with creatinine values) rather than in 24hr urines.

The intake of oxalate is interesting. As mentioned in the introduction, there does not seem to be any consistent trends. The relatively high intake of oxalate in blacks has been previously attributed to a high intake of spinach (Viljoen and Gericke 2001). Whether this is peculiar to 'semi -urban' blacks (SB) or not remains to be established in future studies. However, attention has certainly been drawn in previous studies that the dietary features identified in the present project (and others) for black subjects – lower calcium, relatively high oxalate, low magnesium and low vitamin B6 – are prescriptive

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features for hyperoxaluria which, as has been noted, was not observed. Although the one day diet diary is an imperfect way to assess long-term dietary intakes, the high oxalate intakes in semi-urban blacks and extraordinarily low dietary intake in rural blacks is notable. According to Kennedy *et al.* 2004, 24hr dietary recall tends to underestimate and the food frequency questionnaire tends to overestimate nutrient intakes. It is uncertain whether the extraordinarily low oxalate intakes are a manifestation of this type of questionnaire or are peculiar to rural blacks or not. Perhaps this remains to be established in future studies.

There were no significant associations between daily caloric intakes and the presence or absence of the Pro11Leu polymorphism. Unfortunately this study could not be extended to grandparents. It has been suggested that the frequency of the Pro11Leu polymorphism is much higher in populations in which the ancestral diet is extremely meat-rich than it is in those in which it is more mixed or more vegetarian (Caldwell *et al.* 2004). It would have been interesting to explore this relationship. Thus it was not surprising that there were no significant association between fish (*p=0.6094*) and meat-intake and the frequency of this polymorphism (*p=0.8532 for red meat and p=0.2588 for chicken*). A previous study investigating genetic and dietary influences on nephrolithiasis, also observed that the consumption of the amount of meat and fish were not significantly related to kidney stones (Goldfarb *et al.* 2005). Reasons for this are unclear. Moreover, in another study by Curhan and co-workers (1997) investigating family history and risk of kidney stones, it was also found that the magnitude of the risks for animal protein appeared to be higher in men with a family history, but these were not statistically different from the risks in men without.

Thirdly, the association of the frequency of this polymorphism with alcohol intake (*beer*) tended towards significance (*p=0.0794*) but there was no association with the intake of other types of alcoholic beverages (*p>0.05*). Goldfarb and co-workers (2005) found that the consumption of alcohol was marginally associated with a decreased report of kidney stones compared to no recent intake of alcohol ($P_{trend}=0.06$). Other studies have also demonstrated protective effects for wine and beer (Curhan *et al.* 1996, Krieger *et al.* 1996, Hirvonen *et al.* 1999, Goldfarb *et al.* 2005). Interestingly, in the latter study, there was an increased prevalence of stones among current nondrinkers of alcohol.

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Fourthly, in the present study, the only observed significant associations of dietary intake with the frequency of the polymorphism in both groups were that of juice (*p=0.0031*), cool drink (e.g coke) (*p=0.0204*) and chocolate (*p=0.0044*). Thus, in the present analysis, routine consumers of these items are more likely to have the polymorphism than non-users.

In summary, the differences in urinary compositions of spot morning urine samples and dietary intakes in 'rural' and 'semi-urban' black subjects and white subjects do not appear to have led to any notable variation in the frequency of the alanine:glyoxylate aminotransferase (AGT) Pro11Leu polymorphism in the studied groups, as experimental data revealed that the frequency of this polymorphism is the same in the white and black subjects who participated in this study (i.e *75% and 25% of the AGXT major allele 'C' and minor allele 'T', respectively*).

It has been stated earlier that in healthy individuals AGT is localised exclusively in the peroxisomes in liver parenchymal cells but in PH1 patients it is mistargeted to mitochondria (Cooper *et al.* 1988, Danpure *et al.* 2003).

It is well recognized that pro11Leu polymorphism redirects a small proportion of AGT from the peroxisome to mitochondria (Danpure *et al.* 1994, Danpure 1998). This effect is predicted to be detrimental due to the fact that the peroxisome, not the mitochondria, is the major site of synthesis of its substrate (*glyoxylate*). Although AGT is still catalytically active after the import, it is metabolically ineffective. Thus, it would seem reasonable to expect that this polymorphism should occur at a low frequency in normal individual. Therefore, at first sight, it is difficult to understand why the minor AGXT allele 'T' encoding the Pro11Leu polymorphism should occur at such a high frequency in the healthy Caucasians, Sami, European and North American populations (Danpure *et al.* 1994, Danpure 1998, Caldwell *et al.* 2004, Coulter-Mackie *et al.* 2005, Ross *et al.* 2006, Kozlov *et al.* 2008) as well as in the present study.

A plausible explanation is provided by Danpure (2006), who has proposed that the evolutionary history of mammals suggests that this mistargeting in humans, which might lead to a subcellular distribution of AGT, is more compatible with an individual's lifestyle (*e.g. omnivorus dietary habits rather than herbivorous*). In support, previous studies have also shown an adaptive dietary lifestyle for the high prevalence of the AGXT allele

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'T' encoding this polymorphism in healthy populations (Caldwell *et al.* 2004, Kozlov *et al.* 2008). This might explain the observed frequency for white subjects in the present study, which is similar to that previously reported for healthy Caucasians as described below (Danpure *et al.* 1994, Danpure 1998, Coulter-Mackie *et al.* 2005). Although scant data exists in literature on the frequency of Pro11Leu polymorphism, the latter studies have shown that in Caucasian populations, the so-called major AGXT allele 'C' has a frequency of about 80% while the minor AGXT allele 'T' has a frequency of about 20%. This is in very good agreement with data in the present study.

According to Lumb and co-workers (2000), the explanation of the high frequency of the minor AGXT allele in European and North American population (~20%) is unclear. Nevertheless, the frequency of this polymorphism seems to vary between human populations, with the highest frequency reported in the Sami (27.9%) and the lowest in the Japanese (2%) and Chinese (2.3%), who have a more mixed ancestral diet (Ross *et al.* 2006).

Furthermore, it has been reported that the frequency of this polymorphism is low (2.3-6.9%) in eastern and southern Asia, intermediate (8.9-10.9%) in Africa and high (14.6-27.9%) in Europe and the Middle East (Caldwell *et al.* 2004). Interestingly, the frequency of the AGXT minor allele 'T' in the Kola Sami is very close to the frequency of this allele in the Swedish Sami at 27.9%, although it is lower in Norwergians (19.7%) and Russians (17.6%) (Kozlov *et al.* 2008). This has been attributed to the traditional diet of the Sami, which has been reported to be high in meat, based on circumpolar hunting, fishing and reindeer herding.

As the reasons for the high frequency observed especially in black subjects are vague, it is tempting to look at other possible explanations such as family history of stone formation. However, this seems unlikely, as all subjects reported an absence of family history of stone formation albeit that the present study did not extend to grandparents and great-great grandparents. Perhaps the answer might be provided by recent reports that have indicated that true lack of stones in subjects reporting no family history of stones is less easily documented, as renal stones may remain asymptomatic for years since detection of such subclinical disease would require radiological imaging (Griffin 2004, Goldfarb *et al.* 2005). In fact, according to Griffin (2004), many individuals with renal stones may never have symptomatic disease, and their stones may go unnoticed

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throughout life or be diagnosed as an incidental radiological finding during investigation of unrelated symptoms. In support, radiological studies have shown a prevalence of 3.5% for renal stone disease among a random population (Scott 1987).

The present study has some limitations. These limitations include the small sample size, the lack of control of diets, the use of a spot urine rather than a 24hr collection, and the inherent errors in estimating dietary intakes. It is noted that large-cohort studies are needed for much deeper and rigorous investigations. In addition, the present results are only applicable to the black ethnic group which participated in the study, namely '*Xhosas*'. However, the selection of only one ethnic group was necessary as it eliminated confounding factors due to possible genetic diversity among the ethnic groups from the black population. It might be interesting to investigate this polymorphism in black and white calcium oxalate stone formers and PH1 patients, but this is likely to be extremely difficult and probably unfeasible given the dire paucity of both types of subjects and the near absence of stone formation in South African blacks.

The strong evidence of the importance of environmental factors in renal stone disease seems to diminish the role of genetic factors, but these are clearly involved (Baggio 1999). According to Goldfarb and co-workers (2005), despite suggestive data favouring a heritable component of stone disease in the general population, the genetic basis for calcium stones in general remains unknown.

Notwithstanding that the present study had limitations, the question of whether the reported differences in stone incidence between the two groups has a genetic basis could not be answered from the present data, as the results have clearly indicated that there is no significant differences in the frequency of the alanine:glyoxylate aminotransferase (AGT) Proline11Leucine polymorphism in the black and white subjects who participated. Nonetheless, in view of the fact that the frequency of this polymorphism (*a genetic factor*) did not vary, it might seem reasonable to tentatively assume that environmental factors might help explain the high proportion of stone disease variation between the two groups and that other genetic and non-genetic factors (*that remain to be identified*) rather than this polymorphism might be responsible for the reported differences in stone incidence. Further research is undoubtedly warranted in this area.

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Chapter Three

Investigation of the role of gut permeability as a key to identifying possible differences in the handling of dietary oxalate in black and white South African subjects

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3.1 Introduction

In the study described in the previous chapter, an attempt was made to evaluate and investigate whether the reported differences in the incidence of stone formation between black (<1%) and white (~15%) South African subjects have a genetic basis. It was shown through DNA sample analysis that the frequency of alanine:glyoxylate aminotransferase Pro11Leu polymorphism does not appear to be a contributory factor towards the prevalence of stone formation in the white group. As such, it is able to explain stone rarity in the black group. Further studies are deemed necessary to investigate other avenues that might explain this phenomenon. One such area might be to investigate the role of gut permeability to establish whether different oxalate handling mechanisms occur in the two race groups and therefore allude to their disparity in stone prevalence. This Chapter describes such a study.

It was pointed out in Chapter 1 (*section 1.6*), that previous studies have found empirical evidence to suggest that different oxalate handling mechanisms exist in the black group compared to the white group and that it proceeds via mechanisms other than dietary and renal (Lewandowski *et al.* 2001, Rodgers and Lewandowski 2002, Lewandowski *et al.* 2004a, Lewandowski *et al.* 2004b). Moreover, it has also been shown that there is no significant difference between the two race groups with respect to gastrointestinal oxalate absorption (Lewandowski *et al.* 2005).

Four mechanisms have been identified that could account for elevated urinary oxalate. Firstly, increased dietary intake of oxalate allows for more oxalate to reach the colon, increasing its availability for absorption (Brinkley *et al.* 1981, Massey *et al.* 1993, Pak *et al.* 2004, Coe *et al.* 2005). Secondly, intestinal hyperabsorption of oxalate might occur (Stauffer 1977, Dobbins 1985, Cirillo *et al.* 2008, Thomas *et al.* 2008). Thirdly, a deficiency of oxalate-degrading bacteria (*in particular O. formigenes*) has been suggested as causing an increase in the risk of hyperoxaluria and subsequently calcium oxalate urolithiasis (Sidhu *et al.* 1998, Sidhu *et al.* 1999, Sidhu *et al.* 2001, Holmes and Assimos 2004, Lewandowski and Rodgers 2004a). Finally, increased endogenous production of oxalate is yet another mechanism (Finch *et al.* 1981, Jaeger and Robertson 2004, Lewandowski *et al.* 2004b).

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The hypothesis in the present study is that blacks have a better intestinal integrity and therefore better intestinal control mechanisms which allow for less oxalate to be absorbed when challenged by a high dose of oxalate. On the other hand, it is hypothesized that the integrity of the gut wall might be lowered (*made more permeable*) in whites and thus passive intestinal absorption of oxalate could occur when challenged by a high dose of oxalate.

This hypothesis seems plausible, since a previous study has reported that when a high oxalate/low calcium diet was administered to white and black subjects, the urinary oxalate increased in the former as expected, but remain unchanged in the latter (Lewandowski *et al.* 2001). Moreover, black subjects have been shown to have hyperoxalurogenic eating habits (*high dietary intake of oxalate, low intake of calcium, low magnesium intake*), while their urinary oxalate lies within the normal range (Whalley *et al.* 1998, Viljoen and Gericke 2001, Rodgers and Lewandowski 2002, Lewandowski *et al.* 2005).

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In 2005, Coe stated that as a clinician, he would counsel patients to avoid boluses of oxalate. The effects of oral oxalate loads in both calcium oxalate stone formers and normal individuals have been investigated by several workers with many studies reporting increases in oxaluria of approximately 8-289% (Marshall *et al.* 1972, Barilla *et al.* 1978, Finch *et al.* 1981, Balcke *et al.* 1989, Nguyen *et al.* 1994, Hess *et al.* 1998, Liebman and Costa 2000, Holmes *et al.* 2001). In addition, in a study by Krishnamurthy and co-workers (2003), investigating urinary response to an oral oxalate load (*5 mM sodium oxalate in 250 ml distilled water*), recurrent calcium stone formers with mild hyperoxaluria were reported to have higher fasting urinary oxalate and an exaggerated urinary response to an oral oxalate load compared with recurrent calcium stone formers with normal urinary oxalate excretion. It was also shown that stone formers without hyperoxaluria excrete similar fractions of an oral oxalate load. Renal oxalate excretion following an oral oxalate load (*500 mg*), was also investigated in patients with urinary calculus disease (*15 calcium oxalate stone formers, 7 non-oxalate stone formers and 10 healthy controls*), it was shown that the mean increments of urinary oxalate were significantly higher in calcium oxalate stone formers than in both healthy controls and non-oxalate stone formers (Ebisuno *et al.* 1986).

On the other hand, in a study by Holmes and co-workers (2005), the authors concluded (*after administering oral oxalate loads of up to 8 mmol sodium oxalate*) that no evidence of acute renal injury or oxidative stress was observed under these experimental conditions.

According to Cirillo and co-workers (2008), calcium oxalate stones may be caused by colonic oxalate hyperabsorption (*secondary to gut dysfunction*) or by parenteral nutrition. Although relationships between malabsorptive intestinal diseases and urinary stones are less well defined, it is known that increased gut permeability can cause hyperabsorption of nutrients which can lead to the passive diffusion of oxalate (Stauffer 1977, Dobbins 1985). Patients with ileal resection and jejuno-ileal bypass are at an increased risk of forming calcium oxalate renal calculi because of enhanced absorption of dietary oxalate (Stauffer 1977). Thus, gut diseases may cause the formation of urinary stones through changes in the metabolism of oxalate, calcium and uric acid.

The most widely accepted method for the evaluation of changes in gut permeability and monitoring the intestinal mucosa integrity is the measurement of permeation through the gut mucosa of two or more sugar probes of different molecular weight (Hamilton *et al.* 1987, Lifschitz and Shulman 1990, Lostia *et al.* 2008). As alluded to in the first chapter, one of the most commonly used non-invasive, highly sensitive tests for screening of diseases that affect gut permeability is the '*Lactulose-Mannitol absorption test*' (Juby *et al.* 1989, Andre *et al.* 1991, Laudat *et al.* 1994, Paroni *et al.* 2006). This test is based on coupled oral ingestion of dual-sugar probes, lactulose (LA) (*Figure 3.1*) and a low molecular weight polyol, mannitol (MA) (*Figure 3.2*) and determination of the ratio of their excretion rate in urine (Liu *et al.* 2004).

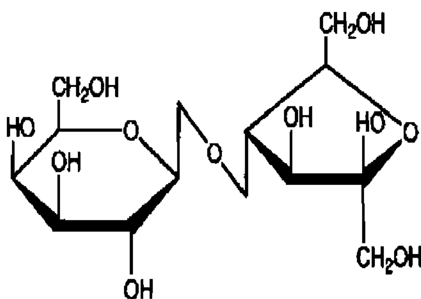

Figure 3.1 LA (*a synthetic disaccharide*)

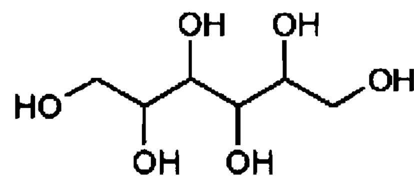

Figure 3.2 MA (*a monosaccharide*)

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The test uses LA and MA for measuring differential sugar absorption mainly because they have a negligible affinity for the monosaccharide transport system and are passively absorbed and not metabolized before urine excretion, therefore intraindividual differences in gastric emptying, small gut transit, and urinary excretion are eliminated (Juby *et al.* 1989, Marsillo *et al.* 1989, Barboza *et al.* 1999, Liu *et al.* 2004). Moreover, LA and MA excretion are thought to be good markers of permeation via transcellular and paracellular pathways, respectively and as shown in figure 3.3, MA theoretically enters the cell through the hydrophilic portion of the cell membrane, while LA goes through the tight junctions and extrusion zones of the intervillous spaces (Barboza *et al.* 1999, Farhadi *et al.* 2003). Consequently, the loss of mucosal integrity should cause increased LA absorption, while the loss of absorptive areas decreases the absorption of MA.

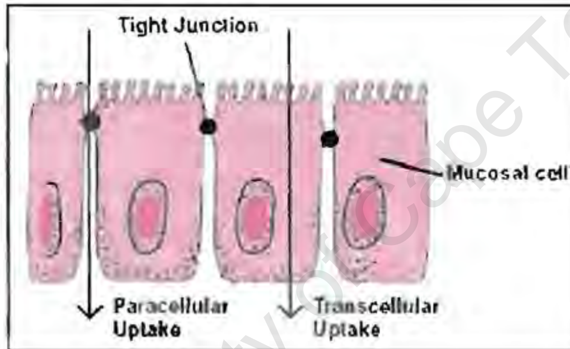

Figure 3.3 Permeation through the intestinal mucosa can be via transcellular uptake (MA) or paracellular uptake (LA) *(http://www life- enthusiast. com/index/articles/ cabot/leaky_gut_syndrome)*

Mucosal damage or loss of villus surface area (*as shown in figure 3.4*) alters the rate of permeation of the sugars across the mucosal surface, with decreased MA and increased LA recoveries, resulting in high LA%/MA% ratios (Kukuruzovic *et al.* 1999). Thus, MA recoveries are a measure of mucosal absorptive capacity, whereas LA permeability reflects barrier function.

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Figure 3.4 Diagram showing mucosal damage or loss of villus surface (*damaged intestinal microvilli*) (*http://test.gsd/.com/home/assessments/ip/*)

If the mucosal barrier is damaged, the gut is more permeable to intact sugars and proteins, therefore an abnormal intestinal permeability with an imbalance in the di/mono saccharide excretion in urine will indicate either an abnormal passive uptake of larger molecules (*e.g LA*) through the paracellular route across the damaged mucosal barrier or a reduction of the integral mucosa with a reduced passage of the smaller molecules (*e.g MA*) (Sullivan *et al.* 1992, Peters *et al.* 1994, Catassi *et al.* 1997, Marsilio *et al.* 1998, Kukuruzovic *et al.* 1999, Haase *et al.* 2000, Generoso *et al.* 2003, Liu *et al.* 2004, Paroni *et al.* 2006).

It is widely accepted in literature that for an oral dose of 5g LA and 2g MA, the reference intervals determined for the percentage recoveries of sugars excreted during the 5hr period are %LA (*0.069-0.393%*) mean (*0.231%*) and %MA (*5.0-22.0%*) mean (*13.6%*), the ratio of LA%/MA% (*0.006-0.030*) mean (*0.018*) (Menzies *et al.* 1979, Ford *et al.* 1985, Elia *et al.* 1987, Juby *et al.* 1989, Kynaston *et al.* 1993, Dastych *et al.* 2008). These ranges are very similar to published data in other studies by Pearson and co-workers (1982) and Fleming and co-workers (1990,1993), who report a mean %LA excretion of 0.25% (*range 0.065-0.45%*), a mean % MA excretion of 14% (*range 8.5-24.5%*) and a mean LA%/MA% ratio of 0.018 (*range 0.005-0.028*) (Pearson *et al.* 1982, Fleming *et al.* 1990). Although they used a different method of analysis (*spectrophotometery vs chromatographic methods in above studies*), Benjamin and co-workers (2008), have also recently reported a similar range, LA% 0.293% (*range 0.0089-0.665*), MA% 14.2% (*range 4.95-30.8*) and LA%/MA% ratio of 0.0164 (*range 0.0018-0.0548*).

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For example, in an effort to determine gut permeability in patients with coeliac disease, a previous study has found that the median % urinary recoveries of LA was significantly higher in patients with the disease than in controls (*0.63 vs 0.18,* $p<0.001$). Although the difference was not statistically significant, the mean MA% recovery was lower in patients with the disease than in controls (*17.6 vs 18.5*). Moreover, the median urinary LA%/MA% ratio was significantly higher in patients with the disease than in controls (*0.038 vs 0.014,* $p<0.001$). It is worth noting that 16 of the 29 patients with coeliac disease showed a LA%/MA% ratio within the normal limits (<0.044) (Catassi *et al.* 1997).

In addition, the LA/MA gut permeability test has also been evaluated in 100 healthy controls and 47 patients with Crohn's disease, on a whole, patients with the disease were shown to have lower mean MA and higher mean LA and mean LA/MA ratio than controls (Andre *et al.* 1988). These findings have recently been confirmed by Generoso and co-workers (2003) who evaluated gut permeability in 25 healthy volunteers and reported a LA/MA ratio (*gut permeability index*) of 0.018 ± 0.014.

Several methods (*paper/thin layer chromatography, enzymatic method, gas liquid chromatography and high performance liquid chromatography*) have been developed to assay sugar probes for determination of gut permeability, however, they have been shown to be time-consuming, increase the possibility of analytical errors, limit the number of samples that can be processed and require derivatization (Menzies 1973, Menzies *et al.* 1978, Laker 1979, Laker and Mount 1980, Behrens *et al.* 1984, Petchey and Crabbe 1984, Engelhardt and Ohs 1987, Fleming *et al.* 1993, Kynaston *et al.* 1993, Willems *et al.* 1993). Fortunately, high performance anion-exchange chromatography coupled with pulsed amperometric detection (*HPAEC-PAD*) using an alkaline elution protocol has proven to be a method well-suited for the separation of neutral, acidic, and reduced sugars (*e.g LA and MA*) and as such was selected to be used in the present study (Fleming *et al.* 1990, Ricci *et al.* 2001, Liu *et al.* 2004, Bruggink *et al.* 2005). Moreover, this technique has the advantage of greatly increased sensitivity, specificity, minimal sample preparation and derivatization is not required (Rocklin 1983, Fleming *et al.* 1990).

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The present Chapter describes an investigation of the effect of a dietary oxalate load (*with normal calcium intake*) on urinary oxalate excretion and gut permeability in subjects from South Africa's two population groups.

3.2 Subjects and Methods

Study population

10 healthy black and 10 white South African age-matched male volunteers were recruited from the student cohort of the University of Cape Town via advertisement on the university notice boards. After verbal and written information had been provided, the subjects signed a declaration of informed consent form in order to participate in the tests (*see appendix CD/Chapter 3/informed consent form*).

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Biographical data

For each subject, brief information about their social and medical history was collected. (*see appendix CD/Chapter 3/Biographical data*).

Inclusion criteria

Healthy black and white South African male subjects in the age range 18-30 years without any family history of renal or kidney stone disease were recruited for the study. In addition, standard tests with urinary dip sticks (*Medi Test Combi 5N, Macherey-Nagel; Düren*) confirmed their health status.

Exclusion criteria

Subjects were excluded from the study if they:

- had a history of gastrointestinal disease (e.g celiac disease, inflammatory bowel disease, irritable bowel disease, food allergies) or any history of digestive, gastrointestinal disorders or complains
- had galactosaemia.
- were diabetic.

- were on any medication or taking supplements that might affect LA and MA metabolism e.g. neomycin, ciprofloxacin, sodium polystyrene sulfonate, oral tetracyclines, potassium supplements, antiacids and antibiotics.
- were on nonsteroidal anti-inflammatory drugs (*NSAIDs*)
- had a family history of renal or kidney stone disease
- had a history of excess alcohol consumption

Study design

The study was divided into three protocols conducted over a three week period:

Week 1. **Protocol 1**: Regular diet (*3 days*)
Week 2. **Protocol 2**: Standardized diet (*3 days*)
Week 3. **Protocol 3**: High-oxalate standardized diet (*3 days*)

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On day 3 of each protocol, the gut permeability test was performed and urinary oxalate as well as other urinary parameters were measured. Risk indices of stone formation were computed. The three protocols were separated by a wash-out period of four days. All subjects followed and completed all three protocols and no adverse effects were reported.

Protocol 1 (Week 1, Day 1 – Day 3)

Subjects were requested to take their regular diet for three consecutive days and keep 3x 24hr dietary food records (*see appendix CD/Chapter 3/24hr dietary food record*). The subjects' regular home-diet food intake was assessed using the *FoodFinder 2* computer software programme (Langenhoven *et al.* 1991) (*see appendix CD/Chapter 3/dietary analysis*).

On day 3, following an overnight fast (19h45-7h45), subjects were required to discard the first morning urine sample and then collect a pre-test (1hr) sample in order to confirm that no endogenous sugars were present (Andre *et al.* 1988, Ventura *et al.* 2006). At 08H45, while on an empty stomach, subjects ingested a dual-sugar isotonic test solution consisting of 5g LA and 2g MA dissolved in 200 ml of tapwater (Ventura *et al.* 2006). After 1hr (09h45), an intake of fluids was encouraged to maintain an

adequate urine output (Goren *et al.* 1995). Caledon mineral water was provided for this purpose: 700 ml until 14h00, 600 ml until 19h00 and a further 700 ml until 22h00. Caledon water was used as it has a low content of all minerals, especially calcium (*6.1 mg/L*). The content of each mineral in Caledon 'Still" water is given in table 3.3. Subjects were allowed to ingest food 0of the respective diets after the second hour (10h45) (Kynaston *et al.* 1993, Goren *et al.* 1995, Duerksen *et al.* 2005). The timing of the meals was as follows: Breakfast (10h45), Lunch (12h45), Snack (16h00), Supper (18h00) and late meal (19h45).

The 5hr test sample was then collected (09h00-14h00), followed by the 18hr post-test sample (14h00-08h00). Urine volume was recorded for each fraction. After thorough mixing of individual fractions, a 10 ml aliquot was stored without preservatives (van Nieuwenhoven *et al.* 1999) at -20 °C until determination of gut permeability (Fleming *et al.* 1990, Generoso *et al.* 2003, Bosi *et al.* 2006, Paroni *et al.* 2006, Ventura *et al.* 2006). In addition, 24hr urine samples were reconstituted from the urine fractions (pre-test, 5hr test and 18hr post-test) from each subject for measurement of urinary parameters.

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Protocol 2 (Week 2, Day 1 – Day 3)

In the second week, the same subjects followed a standardized diet for three consecutive days. The standardized diet was based on the 2001 South African food-based dietary guidelines (Vorster *et al.* 2001). The meals and liquid intakes were prescribed with regard to type, quantity and timing. The actual food ingested and nutrient contents of the diet are listed in table 3.1 and 3.2, respectively. On day 3, urine fractions were collected and analysed as described in protocol 1.

Table 3.1 Constituents of the strict and controlled standardized diet for protocol 2

Meals (Week 2)	**Day 1-Day 3**
Breakfast (*10h45*)	100g bread rolls, 8g butter 15g apricot jam 18g melrose cheese 55g cornflakes 250ml coffee (2 sachet sugar (10g) + 1 sachet creamer (4g) 1 muesli bar (Kellog's all-bran bar,27 g), 150 g banana
Lunch (*12h45*)	60 g turkey ragout, 60 g creamy sauce 120 g mixed vegetables 120 g pasta
Snack (*16h00*)	150 g apple, 175 g low fat fruit yoghurt
Supper (*18h00)*	3 slices of whole wheat bread 10 g margarine 30 g cheddar cheese, 17 g cream cheese 50 g tomato 150 g apple
Late night snack (*19h45*)	1 slice of whole wheat bread 5 g margarine, 17 g cream cheese 50 g tomato

Beverages: (2 l/day): *700 ml Caledon water until 14h00, 600 ml Caledon water until 19h00 700 ml Caledon water until 22h00*

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Table 3.2 Nutrients contents of the standardized diet (protocol 2)

Nutrients	**Standardized diet**
Energy (*kJ*)	11060
Moisture (*g/day*)	1204.0
Total Protein (*g/day*)	82.9
Total Fat (*g/day*)	79.6
Carbohydrate (*g/day*)	357.7
Fibre (*g/day*)	36.8
Total sugars (*g/day*)	104.6
Oxalate (*mg/day*)	55
Calcium (*mg/day*)	923
Magnesium (*mg/day*)	391
Phosphate (*mg/day*)	1377
Potassium (*mg/day*)	2687
Sodium (*mg/day*)	2773
Vitamin A (*μg/day*)	1065
Vitamin B6 (*mg/day*)	2.58
Vitamin C (*mg/day*)	99
Vitamin D (*μg/day*)	3.23
Vitamin E (*mg/day*)	7.04

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Table 3.3 The mineral contents of Caledon 'still' water used in this study

Mineral	**Quantity** (*mg/l*)
Sodium	17
Potassium	5
Calcium	6.1
Magnesium	1.0
Iron	0.01
Manganese	0
Chloride	23
Sulphate	1.7
Bicarbonate	32

Protocol 3 (Week 3, Day 1 – Day 3)

In the third week, the same subjects followed a high-oxalate standardized diet for three consecutive days. The meals and liquid intakes were the same as for the standardized diet protocol (*protocol 2*), except that 10 mmol sodium oxalate dissolved in 200ml tapwater was also ingested at breakfast on each day (Holmes *et al.* 2005). On day 3, urine fractions were again collected and analysed as in protocol 1 and 2.

3.3 Determination of gut permeability index using high performance anion exchange chromatography (HPAEC-PAD)

Reagents and standard solutions

Standard carbohydrates were used for the preparation of the calibrator and test solutions: D-MA (99+%) (*Sigma-Aldrich, Germany*), and LA (*crystalline powder*) (*Medpro Pharmaceutica (Pty, Ltd, Bellville, South Africa*). Low carbonate sodium hydroxide 50% (v/v) was obtained from Fluka, Deisenhofen, Germany and sodium oxalate *(assay min 99.0%)* was purchased from Sigma-Aldrich, Germany. MilliQ water was obtained from MilliQ water purification system (*Millipore, Berdford, USA*). Stock analyte standard solution was prepared by dissolving the appropriate amount of each carbohydrate in MilliQ water to give a final concentration of 157 ppm for MA and 132 ppm for LA. This stock solution was stored at -20 °C until analysis, when it was diluted to produce working standard solutions at different concentrations as shown in appendix CD/Chapter 3/concentrations of working standards.

HPAEC-PAD sample analysis

Stored samples were defrosted and pooled before analysis (Monteleone *et al.* 2004). Briefly, 1ml aliquot was taken from each pre-test sample from all subjects and pooled according to race group. The 5hr test samples of protocol 1 as well as samples from protocol 2 and protocol 3 were also pooled in a similar manner.

The pooled samples were then diluted (5x) with MilliQ water. Diluted samples were then filtered through 0.45 µm membranes (*Target, Cape Town, South Africa*) and C18

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reverse phase solid phase extraction cartridges *(Isolute, Biotage, Virginia, USA)* to remove proteins. Samples were then analysed by HPAEC-PAD for determination of the recoveries percentages of LA and MA as described below.

Carbohydrate profiling was performed on a Dionex BioLC ion chromatograph system (*Dionex Sunnyvale, CA, USA*) equipped with an AS50 autosampler (*Rheodyne rotary injection valve with a 25 µl PEEK sample loop*), ED50 pulsed-amperometric electrochemical detector, LC30 oven and GS50 pump (*Dionex*). The detector cell incorporated a gold working electrode and a pH Ag/AgCl reference electrode. To control the complete system and to realize acquisition and processing of chromatographic data, a personal computer equipped with the Chromeleon® chromatography management system was used.

Profiling of MA and LA was achieved using a CarboPac MA1 column (*250 mm X 4 mm, I.D., Dionex*) coupled with a CarboPac MA1 guard column (*50 mm X 4 mm, I.D., Dionex*). Isocratic elution with 300 mM NaOH at a flow rate of 0.4ml/min was employed for separation of the analytes. This mobile phase was degassed with nitrogen and then continuously purged with helium during elution. Quantification was achieved from peak areas calculated from LA and MA standard curves (figures 3.5a and 3.5b). The use of an available internal standard (*arabinose*) was investigated but unfortunately it overlapped with the MA peak. Each pooled urine fraction was analysed in triplicate.

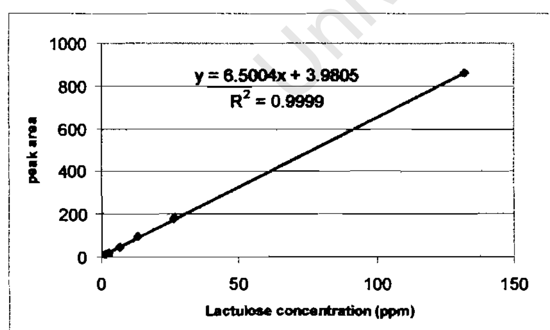

Figure 3.5a Standard curve for LA determinations

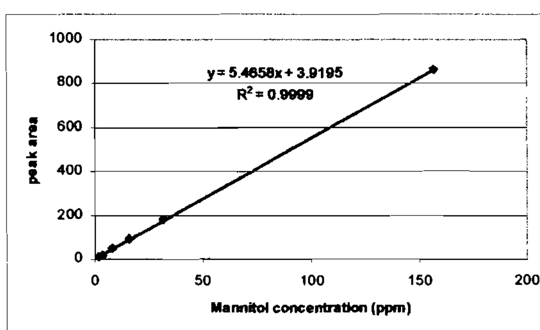

Figure 3.5b Standard curve for MA determinations

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Urine reconstitution, treatment and physicochemical properties

As stated earlier, in all three protocols, 24hr urine samples were reconstituted from the urine fractions (pre-test, *5hr test and 18 hr post-test*) from each subject. Each 24hr urine sample was tested for haematuria and nitrite using urinalysis test strips (*Medi Test Combi 5N, Macherey-Nagel; Düren, Germany*). None of the samples tested positive for either blood or infection. Urine pH (*pH 211 microprocessor pH meter, Hanna Instruments, Cape Town, South Africa*) and volume were routinely measured. Aliquots were filtered through a 0.75 µm filter (*Macherey-Nagel, Düren, Germany*) to remove cellular debris and proteinaceous material. The urine samples were analysed as described in Chapter 2 (*section 2.2*). Relative supersaturation (RS) values of calcium oxalate (COM) were computed using EQUIL 1.5 computer software programme (Ackermann *et al.* 1989) while the Tiselius risk index (Tiselius 1982) was calculated for each urine sample as described in Chapter 1 (*section 1.3*).

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Statistical analysis

Statistical comparison of mean dietary intake was achieved using one-way analysis of variance (ANOVA) while data from 24hr urine samples were analyzed using repeated measures ANOVA. Both analyses were computed using STATISTICA version 8.0. Data were considered statistically significant if $p \leq 0.05$.

3.4 Results

Dietary analysis

In week 1 (*protocol 1*), subjects were instructed to consume their regular diet for three consecutive days and keep 3x 24hr dietary food records. The subjects' regular home-diet food intake was assessed using a *FoodFinder 2* computer software programme (Langenhoven *et al.* 1991). Comparison of mean nutrient intakes for black and white subjects when they were on their regular diet is given in table 3.4 as well as those of the standardized diet.

Table 3.4 Comparisons of the mean dietary intakes (SE) of regular diets of black and white subjects and standardized diet

Nutrients	Blacks (B)	Whites (W)	Standard diet	B vs W p-values
BMI (*kg/m²*)	24.28(0.89)	25.74(0.89)	——	0.2627
Energy (*kJ*)	10729 (1144)	15733(699)	11060	0.0015*
Moisture (*g/day*)	2122(207)	3137(296)	1204	0.0095*
Total protein (*g/day*)	82.20(5.59)	123.33(8.63)	82.9	0.0008*
Total fat (*g/day*)	102.36(9.75)	124.80(6.92)	79.6	0.0770
Carbohydrate (*g/day*)	320(34.11)	394(39.60)	357.7	0.1724
Fibre (*g/day*)	26.95(3.39)	31.48(3.61)	36.8	0.3725
Total sugars (*g/day*)	32.06(3.64)	66.53(53)	104.6	0.0008*
Oxalate (*mg/day*)	328(46.77)	142(12.36)	55	0.0012*
Calcium (*mg/day*)	708(83)	1182(70)	923	0.0004*
Magnesium *mg/day*)	348(8)	513(33)	391	0.0001*
Phosphate (*mg/day*)	1360(117)	2187(175)	1377	0.0010*
Potassium *mg/day*)	2764(269)	4407(386)	2687	0.0026*
Sodium (*mg/day*)	3011(387)	4242(452)	2773	0.0820
Vitamin A (*µg/day*)	1263(175)	1575(118)	1065	0.1550
Vitamin B6 *mg/day*)	2.27(0.30)	3.14(0.36)	2.58	0.0794
Vitamin C (*mg/day*)	135(29)	106(13)	99	0.3728
Vitamin D (*µg/day*)	6.24(0.72)	5.78(0.54)	3.23	0.6320
Vitamin E (mg/day)	12.29(1.16)	13.64(1.08)	7.04	0.4204

**Significance at $p \leq 0.05$*

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Comment

Dietary analysis of mean intake of nutrients of black and white subjects on their regular diet revealed that dietary oxalate was significantly higher in black subjects ($p=0.0012$) while total protein ($p=0.0008$), total sugar ($p=0.0008$) as well as calcium ($p=0.0004$), magnesium ($p=0.0001$), phosphate ($p=0.0010$) and potassium ($p<0.0026$) were significantly lower.

Analysis of gut permeability index

Data obtained from HPAEC-PAD chromatograms for determination of gut permeability indices in black and white subjects following the three protocols are given below.

HPAEC-PAD analysis of standard solution

A standard mixture of LA (*132 ppm*) and MA (*157 ppm*) was eluted isocratically with an alkaline mobile phase (*300 mM NaOH*), the resultant HPAEC-PAD chromatogram is shown in figure 3.6.

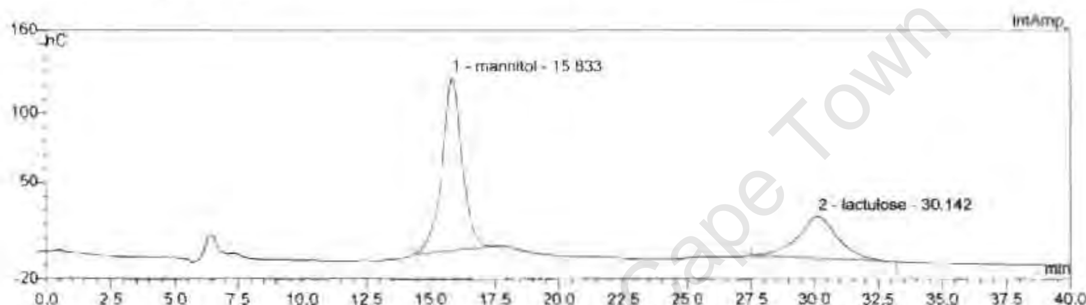

Figure 3.6 *Typical HPAEC-PAD chromatogram of a standard mixture of MA (157 ppm) and LA (132 ppm) (The chromatographic conditions for this and all samples analysed are as given in Section 3.3 of this Chapter).*

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Comment

As revealed in this representative chromatogram of the standard solution, the two carbohydrate sugars LA and MA are well-resolved, with their respective peaks and retention times being displayed. It is noted that there appears to be a much lower electrochemical detector response for LA than MA (Fleming *et al.* 1990, Kynaston *et al.* 1993, Barboza *et al.* 1999) as also evident in the relative small peak of LA in the chromatogram. As expected, the monosaccharide MA was eluted first (~*16min*), while the disaccharide LA exhibited a longer retention time (~*30min*). This maximum separation of the peaks under the chromatographic conditions described was deemed to have been achieved in a reasonable time. As such, this isocratic elution (*with the indicated alkaline mobile phase*) was employed for analysis of all samples. The concentrations of the working standard solutions used for preparation of the calibration

curve are indicated in the appendix (*see appendix CD/Chapter 3/concentrations of working standard solutions*).

Typical HPAEC-PAD chromatograms obtained from urines of black and white subjects for protocol 1 (*regular diet*) are given below while the chromatograms obtained from analysis of the pooled urine fractions (*pre-test and 5hr test samples*) for all three protocols from black and white subjects are given in the appendix (*see appendix CD/ Chapter 3/HPAEC-PAD chromatograms*). The samples were pooled as done by Monteleone *et al.* 2004, due to financial constraints.

Typical HPAEC-PAD chromatograms from samples of black subjects

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Typical chromatograms obtained from pre-test and 5hr pooled urine fractions from black subjects for Protocol 1 (*regular diet*) are shown in figures 3.7 and 3.8, respectively. The remaining chromatograms from urine fractions from this group for protocol 2 (*standardized diet*) and protocol 3 (*high-oxalate standardized diet*) are given in the appendix as indicated above.

P1B1

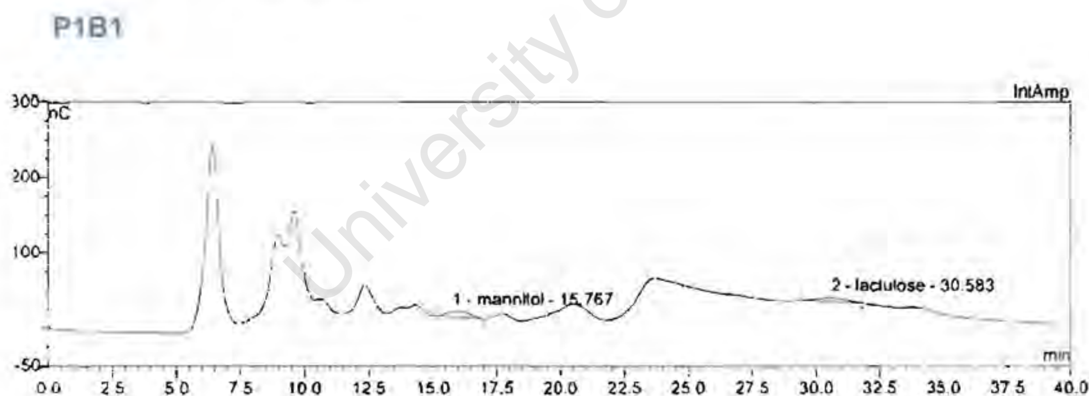

Figure 3.7 *HPAEC-PAD chromatogram from pooled pre-test samples from black subjects before LA and MA oral load while on protocol 1 (P1B1=protocol 1, black subjects, pooled pre-test sample)*

P1B5

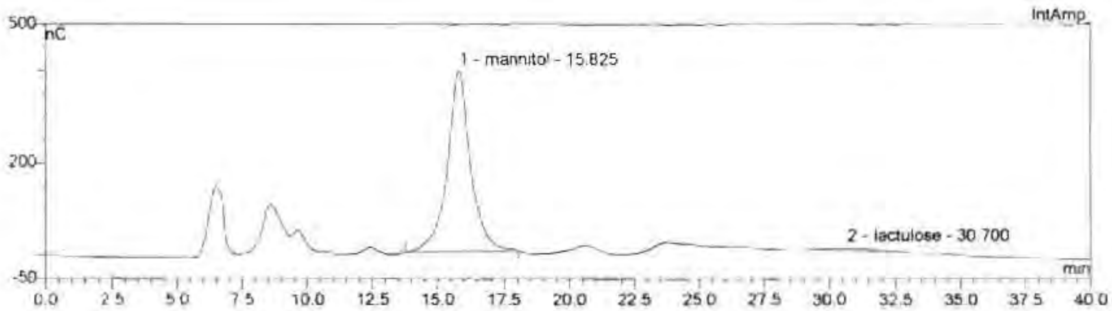

Figure 3.8 *HPAEC-PAD chromatogram from pooled 5hr test samples from black subjects after 5g LA and 2g MA oral load while on protocol 1 (P1B5=protocol 1, black subjects, pooled 5hr test sample)*

Typical HPAEC-PAD chromatograms of samples from white subjects

Typical chromatograms obtained from pre-test and 5hr pooled urine fractions from white subjects for protocol 1 are shown in figures 3.9 and 3.10, respectively. All other chromatograms from pooled urine fractions from this race group for protocol 2 and 3 are also indicated in the appendix (*see appendix CD/ Chapter 3/HPAEC-PAD chromatograms*).

P1W1

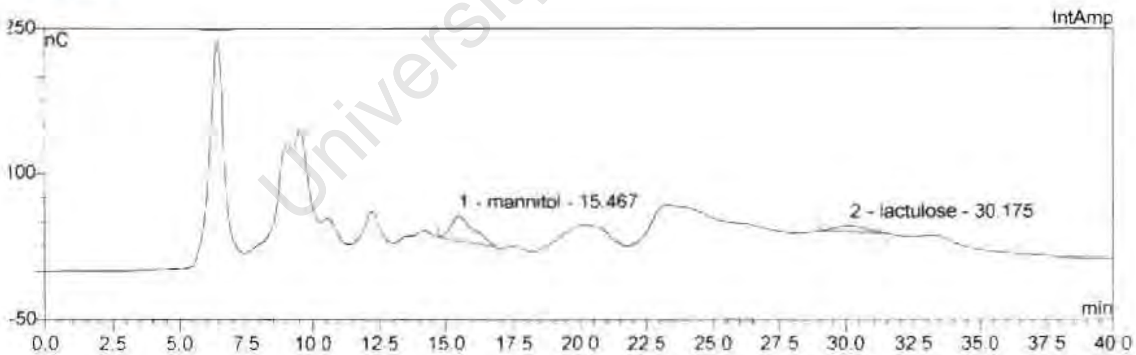

Figure 3.9 *HPAEC-PAD chromatogram from pooled pre-test samples from white subjects before LA and MA oral load while on protocol 1(P1W1=protocol 1, white subjects, pooled pre-test sample)*

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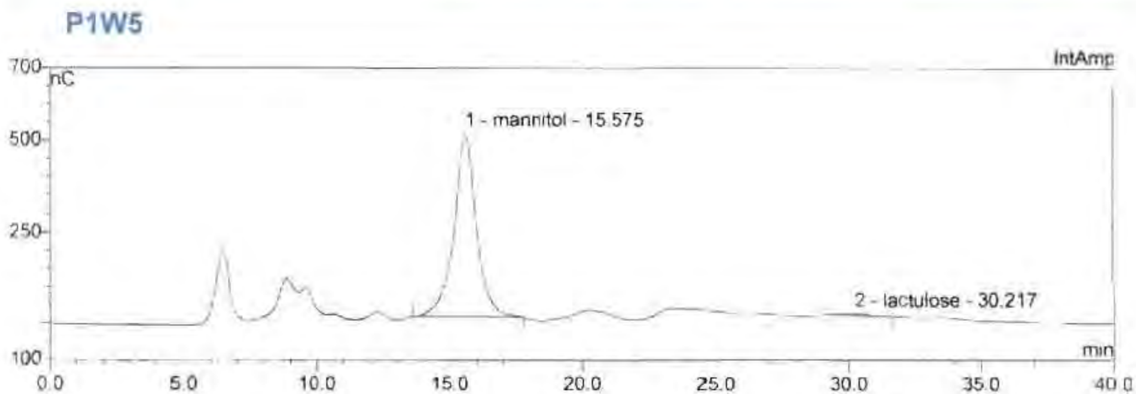

Figure 3.10 *HPAEC-PAD chromatogram from 5hr pooled test samples from white subjects after 5g LA and 2g MA oral load while on protocol 1(P1W5=protocol 1, white subjects, 5hr pooled test sample)*

Comment

The general HPAEC-PAD chromatograms for each sample from the pooled urine fractions from black and white subjects indicate a large error for LA as small values were obtained. This might introduce an error in the ratio (*small number/big number*). It appears that the pulsed-amperometric electrochemical detector used might e insensitive to LA since other studies have also shown a lower pulsed-amperometric electrochemical detector response for LA (Fleming *et al.* 1990, Kynaston *et al.* 1993, Barboza *et al.* 1999). Perhaps a more sensitive analytical technique is required to detect urinary sugar excretions in individual subjects. It is also worth pointing out that each analysis was performed in triplicate. The chromatogams from both race groups show few peaks and the labelled analyte peaks (*LA and MA*) are clearly recognizable. Similar patterns are apparent. For example, in protocol 1, at first glance the pre-test pooled urine fractions from both groups show less MA excretion than the 5hr test samples obtained after the oral load (*MA and LA test solution*). It also appears as if LA excretion for this protocol was not affected by the oral load as it does not seem to be different when comparing the pre-test and 5hr excretions in the two groups.

Percentage recoveries of LA, MA and the LA%/MA% ratios

The actual recoveries of LA and MA computed from all the pooled urine fractions using peak areas on Chromeleon® software and urine volumes are given in table 3.5. Volumes for individual samples for all three protocols are given in appendix CD/Chapter 3/urine volumes.

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Table 3.5 Urine volumes and concentrations of LA and MA from pooled 5hr test samples from urines of black and white subjects collected on day 3 of each protocol

Protocol	Blacks			Whites		
	volume (*ml*)	Concentration (µg/ml)		volume (*ml*)	Concentration (µg/ml)	
		LA	MA		LA	MA
P1 (*regular diet*)	4180	4.00	399.50	4455	3.00	503.00
P2 (standardized diet)	6401	9.00	358.00	5740	7.50	358.00
P3 (high-oxalate standardized diet)	6085	2.10	137.05	6280	1.00	123.00

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Comment

The LA and MA concentration values (*table 3.5*) obtained from peak areas were used to calculate the recoveries of LA and MA after taking into consideration the total combined amount of ingested analytes and the total combined volumes (*table 3.5*). The combined total volume of the pooled urine fractions was also determined in the same way. The urinary recoveries (µg) of LA and MA were determined as µg/ml X volume (ml) (Duerksen *et al.* 2005, Lostia *et al.* 2008). The respective percentage recoveries of the ingested dose of LA and MA were then calculated.

For calculating percentage recoveries of LA and MA in the pooled 5hr test urine fractions from the two race groups, the combined total amount of test solution ingested by subjects from each race group was taken into account. For example, for MA, 2g MA was ingested by each of the 10 subjects per group contributing to the pooled urine fractions, therefore 20g MA was taken as the ingested amount for the calculation of %MA recoveries.

Results are expressed in two ways. Firstly, they are expressed as the percentage recoveries of the ingested dose of LA and MA present in the pooled 5hr test urine fractions. Secondly, they are expressed as the percentage excretion LA%/MA% ratio (*an accurate and reliable indicator of gut permeability*) (Catassi *et al.* 1997, Generoso *et al.* 2003, Bosi *et al.* 2006) and are given in table 3.6 to table 3.10. No attempt was made to

correct for the small amount of endogenously produced MA appearing in the urine as in the studies by Northop *et al.* 1990 and Goren *et al.* 1995. Hypothesis tests (*p-values*) are not possible in this case because urine samples were pooled for each race group so the data consist of a single observation in each group (Underhill and Bradfield 2001).

Table 3.6 Percentage recoveries of LA, MA and LA/MA ratios from pooled *5hr test* urine fractions from urines of black subjects for all three protocols (*P1, P2 and P3*)

	% LA	**% MA**	**LA% /MA%**
Protocol 1	0.03	8.3	0.004
Protocol 2	0.12	11.4	0.010
Protocol 3	0.03	4.2	0.007

Table 3.7 Percentage recoveries of LA, MA and LA/MA ratio from pooled *5hr test* urine fractions from urines of white subjects for all three protocols (*P1, P2 and P3*)

	% LA	**% MA**	**LA% /MA%**
Protocol 1	0.03	11.2	0.003
Protocol 2	0.09	10.3	0.009
Protocol 3	0.01	3.9	0.003

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Table 3.8 Comparisons of the percentage recoveries of LA, MA and LA/MA ratios from pooled *5hr test* urine fractions from urines of black and white subjects for protocol 1(*regular diet*)

% Recoveries of probes	**Blacks**	**Whites**
% LA	0.03	0.03
% MA	8.3	11.2
LA%/MA%	0.004	0.003

Table 3.9 Comparisons of the percentage recoveries of LA, MA and LA/MA ratios from pooled *5hr test* urine fractions from urines of black and white subjects for protocol 2 (*standardized diet*)

% Recoveries of probes	**Blacks**	**Whites**
% LA	0.12	0.09
% MA	11.4	10.3
LA%/MA%	0.010	0.009

Table 3.10 Comparisons of the percentage recoveries of LA, MA and LA/MA ratios from pooled *5hr test* urine fractions from urines of black and white subjects for protocol 3 (*high-oxalate standardized diet*)

% Recoveries of probes	**Blacks**	**Whites**
% LA	0.03	0.01
% MA	4.2	3.9
LA%/MA%	0.007	0.003

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Comment

The percentage recoveries of urinary MA excretion in all three protocols ranged from 4.2-11.4 % of the ingested dose in black subjects while in white subjects the range was similar (3.9-11.2%). Low percent recovery ranges of urinary LA were observed in the two race groups, with 0.03-0.12% of the ingested dose recovered for black subjects while in white subjects the recovery range was 0.01-0.09 %.

Data analysis of gut permeability index in the two race groups reveal that in all three protocols, the LA%/MA% ratio (*an index of gut permeability*) was within normal reference intervals (*range of present study 0.003-0.010 vs range from literature values 0.006-0.030*). In protocol 1, following regular diet, the ratio was similar between the two groups (*0.004 vs 0.003, for blacks and whites, respectively*). Administration of a standardized diet did not have a noticeable effect on the gut permeability as it remained similar in both groups (*0.010 vs 0.009*) for blacks and whites respectively, albeit there was a slight

increase in MA excretion in the black subjects, it was accompanied by a slight increase in LA excretion, hence the ratio did not vary. On the other hand, administration of a high-oxalate standardized diet in protocol 3, led to a reduction of more than 50% in MA excretion in black and white subjects. However, the gut permeability index was still within the normal range in both groups, due to the very low recoveries of LA. In addition, the gut permeability index for this protocol was slightly higher in black subjects than white subjects (*0.007 vs 0.003, respectively*).

Analysis of reconstituted 24hr urines

Comparisons of urinary parameters and computed risk indices for black and white subjects following protocol 1 (*regular diet*), protocol 2 (*standardized diet*) and protocol 3 (*high-oxalate standardized diet*) are given in figures 3.11 to 3.21.

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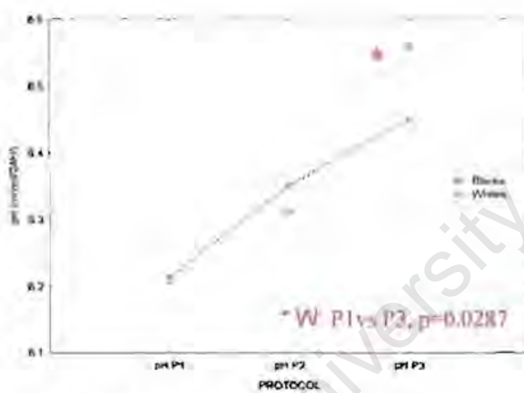

Figure 3.11 Comparisons of mean pH

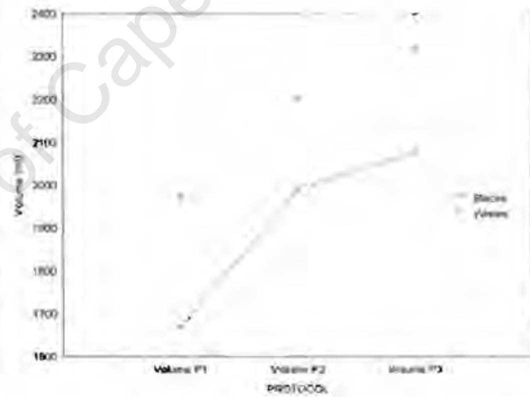

Figure 3.12 Comparisons of mean volumes

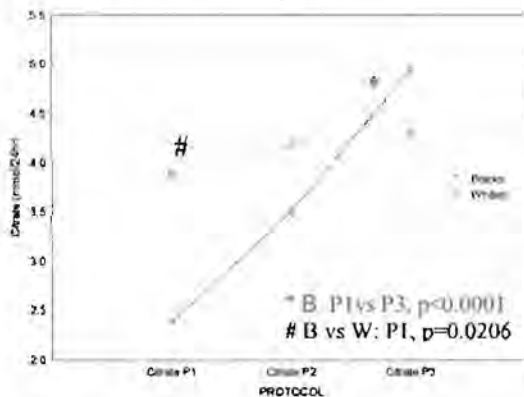

Figure 3.13 Comparisons of mean citrate

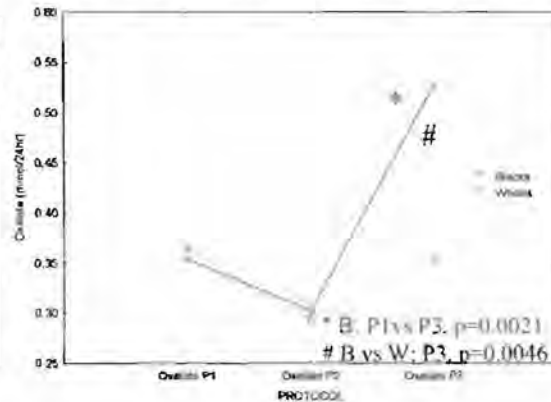

Figure 3.14 Comparisons of mean oxalate

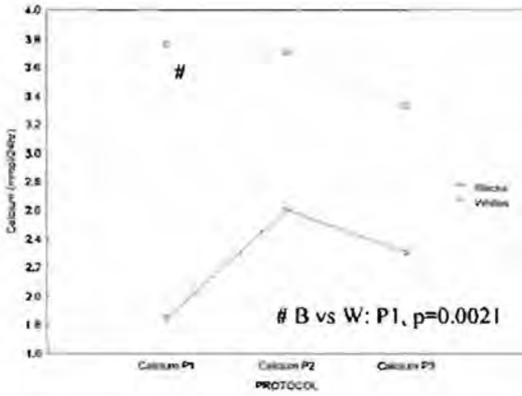

Figure 3.15 Comparisons of mean calcium

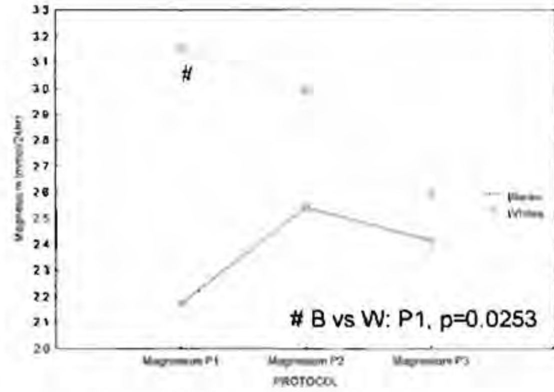

Figure 3.16 Comparisons of mean magnesium

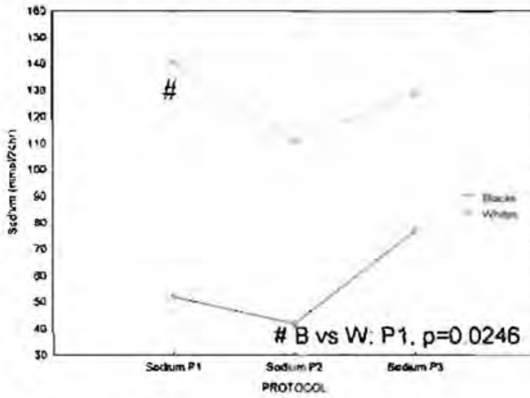

Figure 3.17 Comparisons of mean sodium

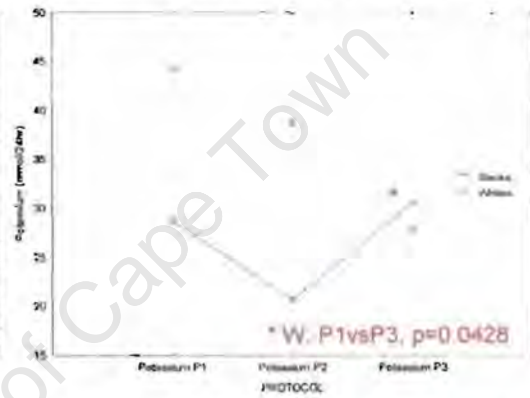

Figure 3.18 Comparisons of mean potassium

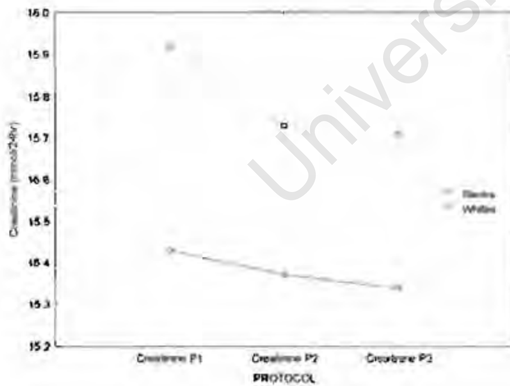

Figure 3.19 Comparisons of mean creatinine

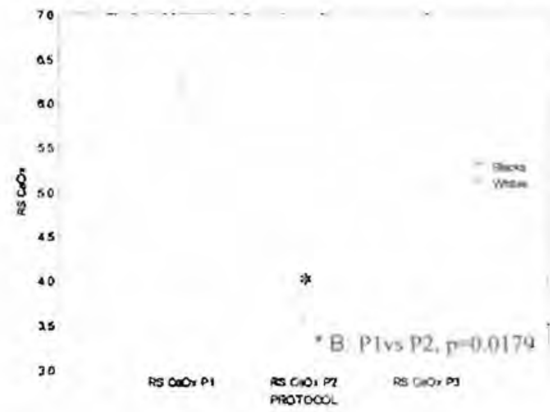

Figure 3.20 Comparisons of mean RS CaOx (COM)

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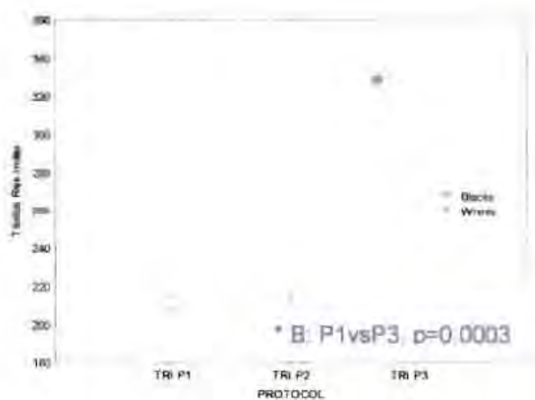

Figure 3.21 Comparisons of mean Tiselius risk index

Comment

It has been stated earlier in chapter one that diet plays a crucial role in the pathogenesis of calcium-containing kidney stones and that it has been shown to have a key role in determining urine chemistry and as a consequence, can influence the risk of stone formation (Rodgers and Lewandowski 2002, Taylor and Curhan 2004). Indeed, the comparisons of the mean 24hr urinary composition data obtained from black and white subjects in present study, indicate as shown in the figures 3.11 to 3.21, that the administration of the three protocols P1 (*regular diet*), P2 (*standardized diet*) and P3 (*high-oxalate standardized diet*) induced remarkable changes (*both favourable and unfavourable*) to the most important urinary risk factors for stone formation in both groups (*although to different extents in some instances*).

Inter alia, in black subjects, the urinary parameters that increased significantly is oxalate in P3 ($p=0.0021$) while an increase in citrate was approaching significance in P2 and reached significance in P3 ($p=0.0607$ *and* $p<0.0001$, *respectively*). In white subjects, although potassium showed a decreasing trend from P1 to P3, the decrease only reached statistical significance in P3 ($p=0.0428$).

When making comparisons of the urine parameters in black and white subjects for each protocol, experimental data further showed that, citrate and oxalate were significantly higher in blacks than in whites in P1 and P3 ($p=0.0206$ *and* 0.0046, *respectively*). Urinary calcium, sodium and magnesium were significantly higher in whites than in

blacks for protocol 1 (*p=0.0021, p=0.0246 and p=0.0253, respectively*). It is also noted that although the urinary pH showed increasing trends from P1 to P3 in both groups, it only reached statistical significance in the white group, in P3 (*p=0.0287*).

With regard to the relative supersaturation of calcium oxalate (COM) values (*computed from all the urinary parameters shown in the figures above*), although there were no significant differences when comparing values obtained from black and white subjects for this risk index, there was a significant decrease from P1 to P2 for black subjects (*p=0.0179*). The Tiselius risk index (*computed from urinary calcium, magnesium, citrate, oxalate and creatinine only*) was significantly higher in black subjects for P3 (*p=0.0003*) while it did not change significantly in white subjects (*p=0.2281*), although it showed a similar trend (*an increase in P3).*

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3.5 Discussion

In this chapter, determination of the gut permeability index in black and white South African subjects using the LA-MA absorption test, has been described. Discussion of the study described in this Chapter will commence with a critical assessment of the technique used for evaluating permeability. The choice of analysis of permeability of the two sugars using the HPAEC-PAD technique, proved to be a relatively good option as it allowed both analytes (*LA and MA*) to be determined even in relatively small amounts *(for example, 1.0 µg/ml obtained for the recoveries of LA from the pooled 5hr test sample from white subjects in protocol 3*). Moreover, all the peaks were well-resolved, with elution of both analytes achieved in a reasonable time (*~30min*).

The use of the alkaline mobile phase (e.g *NaOH*) is a critical factor in analysis of carbohydrates by HPAEC-PAD. This is mainly because most carbohydrates are weak acids (*with pKa values in the range 12-14*), and at high pH values, their hydroxyl groups are consequently partially or totally transformed into oxyanions, which enable this class of compounds to be chromatographed as anions (Corradini *et al.* 1997, Andersen and Sørensen 2000). Conversely, the use of such high pH eluents, might also serve as a drawback as it may cause carbonate, a divalent anion at pH 12, to bind strongly to the columns and interfere with carbohydrates binding, causing a drastic decrease in column selectivity and a loss of resolution and efficiency (Cataldi *et al.* 1998). Nevertheless, the

eluent used in the present study was relatively carbonate-free as it was first purged with nitrogen and then continually degassed with helium during elution to prevent absorption of atmospheric carbon dioxide and subsequent production of carbonate, which would act as a displacing ion and shorten retention times.

In addition, the pulsed-amperometry detector used in HPAEC-PAD technique, allows for detection of only those compounds containing functional groups oxidizable at the detection voltage employed (Generoso *et al.* 2003, Casabuono *et al.* 2005). Thus, neutral and cationic samples were eluted in the void volume of the column. However, noting that urine is a complex matrix sample, it is comforting that, even if such species were oxidizable, they did not interfere with the analysis of the carbohydrate components of interest (*LA and MA*) as seen in all the chromatograms which were obtained (*see appendix CD/Chapter 3/HPAEC-PAD chromatograms*).

The interference of the available internal standard (*arabinose*) with the MA peak (*MA*) (*chromatogram not shown*) culminated in it being discarded. However, in order to overcome this limitation, each pooled sample was analysed in triplicate under similar conditions. As a result, analysis of results can be regarded with some degree of confidence.

Much of the published work on permeability testing has been done on diseases *(e.g pediatrics, coeliac disease, Crohn's disease, chronic diarrhoea and malnutrition)* that affect gut permeability. Although early studies have linked intestinal diseases to renal colic (Dowling *et al.* 1971, Admirand 1972, Smith *et al.* 1972, Stauffer 1977, Dobbins 1985), laboratory data on gut permeability tests with respect to kidney stone disease appear to be lacking. Thus, findings in the present study could not be compared with any published reference values.

However, as stated earlier, the results compare favourably with other studies investigating gut permeability in other diseases (*e.g pediatrics, coeliac disease, Crohn's disease, chronic diarrhoea and malnutrition*) as well as in healthy subjects as described in this chapter *(introduction)* (Wheeler *et al.* 1978, Menzies *et al.* 1979, Ford *et al.* 1985, Elia *et al.* 1987, Juby *et al.* 1989, Sullivan *et al.* 1992, Peters *et al.* 1994, Iqbal *et al.*

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1996, Catassi *et al.* 1997, Marsilio *et al.* 1998, Kukuruzovic *et al.* 1999, Haase *et al.* 2000, Generoso *et al.* 2003, Liu *et al.* 2004, Dastych *et al.* 2008).

Although analysis of percentage recoveries of the analytes in the present study was performed in pooled test samples as done by Monteleone and co-workers (2004), it is conceded that individual variability was lost. For example, in a study by Iqbal and co-workers (1996), it was also reported that 33% Indians and 45% Afro-Caribbeans had values outside the normal white range while four otherwise healthy patients (*three Afro-Caribbens and one Indian*) fell within the coeliac patients' range.

In the present study, the levels of percentage recoveries of urinary LA and MA have been reported and gut permeability index (*LA%/MA% ratio*) was determined in a stone-prone population and a relatively stone-free population following administration of 3 protocols, protocol 1 (*regular diet),* protocol 2 *(standardized diet),* and protocol 3 *(high-oxalate standardized diet*). In addition, urinary parameters have also been measured including urinary oxalate excretion, a major risk factor for kidney stone disease in the two race groups and risk indices for stone formation were computed.

As stated earlier, the hypothesis in the present study is that blacks have a better intestinal integrity and therefore better intestinal control mechanisms that allows for less oxalate to be absorbed when challenged by a high dose of oxalate. Whereas, in whites, the integrity of the gut wall could be lowered (*made more permeable*) and thus passive intestinal absorption of oxalate could occur when challenged by a high dose of oxalate.

The results obtained in the present study are intriguing. In all three protocols consistently low percentage recoveries of LA were observed in both groups (0.01-0.15%) although this was within the aforementioned normal reference interval range, the reason for this observation is unclear. It appears that the pulsed-amperometric electrochemical detector used might be insensitive to LA as analysis was done in triplicate. It is also possible that both premucosal, mucosal and postmucosal factors could be responsible for the low recoveries of LA. In a study by Monteleone and co-workers (2004), in which intestinal permeability in patients with aneroxia nervosa was investigated, reduced recoveries of LA was ascribed to changes in the anatomophysiology of intestinal mucosa leading to a decreased passage of sugar into systemic circulation.

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For apparently healthy subjects, it is not far-fetched to speculate that this could also occur through bacterial degradation of LA (*colonic or in urine*) (Goren *et al.* 1995, Söderholm *et al.* 1999, Farhadi *et al.* 2008, Vilela *et al.* 2008). However, this seems unlikely as errors such as bacterial degradation of the sugars should affect both of them equally (Vilela *et al.* 2008), casting doubt about this possibility. Although there are no data available on renal clearance of LA, it appears as if this clearance is dependent on the quantity of LA present in the circulation as shown in a study by van Nieuwenhoven and co-workers (2000), who observed a lower recovery of LA at 5hr after a higher quantity of LA ingested in a dose-dependency study.

Furthermore, according to Fasano (2001), the paracellular route, through which LA is thought to permeate, is the dominant pathway for passive solute flow across the intestinal epithelial barrier, and its permeability depends on the regulation of intercellular tight junctions, also known as the zonula occludens. In recent years, much has been discovered about the structure, function and regulation of tight junctions although the precise mechanism(s) through which they operate is still incompletely understood. Nevertheless, most authors agree that LA permeates the mucosa via the paracellular pathway *(through the tight junctions)* (Catassi *et al.* 1997, Marsilio *et al.* 1998, Kukuruzovic *et al.* 1999, Haase *et al.* 2000, Generoso *et al.* 2003, Liu *et al.* 2004, Dastych *et al.* 2008). Thus, it is conceivable that the low absorption of LA observed could be indicative of an indication of the integrity of the gut mucosa in both groups, which might have led to the reduced passage of this sugar probe through the tight junctions.

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Data from HPAEC-PAD chromatograms revealed interesting trends with regard to MA recoveries in the 5hr test samples from the two race groups. In fact, the most prominent abnormality observed in this study, along with the augmented urinary oxalate excretion in black subjects, was the reduction of mannitol permeability in both groups following the administration of a high-oxalate standardized diet. In addition, the trend of this reduction is rather closely similar in both groups. For example, in black subjects, the percentage recoveries of MA was reduced from 11.4% in the standardized diet protocol to 4.2% in the high-oxalate standardized diet, while in white subjects the reduction was analogous from 10.3% to 3.9%. Although the reduction of MA recoveries was extreme (*more than 50%)* in both groups, it was still within the aforementioned normal reference intervals.

It has been theorized that MA diffuses through small water-filled pores in the enterocyte cell membrane, reflecting total transcellular transport of basal surface area. (Bao *et al.* 1996). It is commonly assumed that reduced MA percentage reflects the reduction in the total surface area of the small intestine caused by villous atrophy. Moreover, the present findings of reduced MA recoveries in both groups in protocol 3 seem to be in agreement with studies that have shown reduced MA permeability in patients with active celiac disease and other diseases that cause villous hypotrophy as a result of reduced absorptive area (Catassi *et al.* 1997, Vilela *et al.* 2007). According to Andre and co-workers (1988), permeation of MA is generally low except in patients free from localisation of Crohn's disease in the small bowel. This is in line with the concept that MA absorption is mainly transcellular and located in the small bowel. It is further posited that the low permeability in Crohn's disease could be a consequence of either intestinal hurry or of a smaller surface of absorption possibly related to a reduction in the mean cellular pore size.

With respect to the influence of diet on permeability between the two race groups, it was observed that in week 1, protocol 1 while following their respective regular diet, there were no differences in the gut permeability index in the two groups as shown by similar LA%/MA% ratios (*0.004 vs 0.003, for blacks and whites respectively*), in addition, there was also no variation in the gut permeability index in week 2, protocol 2, when both subjects were on a similar, strict and controlled standardized diet (*0.010 vs 0.009, for blacks and whites respectively*). However, after the administration of the high-oxalate standardized diet in week 3, protocol 3, there was a marked reduction of MA (*more than 50%*) in both groups and the gut permeability index was slightly higher in black subjects than in white subjects *(0.007 vs 0.003, respectively*). Statistical significance could not be determined for these observations (*see page 107*). Nonetheless, the LA%/MA% ratios were still within the normal range due to the very low LA excretion values obtained. Thus, the present results are noteworthy in this additional respect that data analysis clearly revealed that the gut permeability index in both South African black and white race groups was within the normal range in all three protocols (*range 0.003-0.010 vs 0.006-0.030, range of present study vs range from literature values respectively*).

Nonetheless, taken together, the cited data obtained in the present study in protocol 3 (*i.e reduced MA and low LA percent recoveries*) imply an indication of a defect in the

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observation in protocol 3, the present author might tentatively conclude that there was an induced general malabsorption of nutrients after the high-oxalate standardized diet in this regard.

It is feasible that the differences observed after the high-oxalate standardized diet can be attributable to the different renal handling mechanisms of oxalate in the two race groups. This is in consideration of the fact that, in protocol 3 of this study, experimental data showed that after oral oxalate loads urinary oxalate was significantly higher in black subjects compared to white subjects ($p=0.0046$), indicating an increased risk of stone formation in the black subjects. This was further confirmed and manifested in the observed significantly higher Tiselius risk index for this group which reached statistical significance in P3 ($p=0.0003$) as opposed to white subjects who did not show a significant increase in this risk index ($p=0.2281$).

This is rather surprising considering the reported low incidence of stone formation in the black population. Although in one hand it confirms the findings by Lewandowski and co-workers (2001) who observed higher urinary oxalate levels in black subjects compared to white subjects after an oxalate-rich meal. On the other hand, it not only contradicts the findings in recent studies (Rodgers and Lewandowski 2002, Lewandowski *et al.* 2004a, Lewandowski *et al.* 2004b) but it also disproves the hypothesis mentioned earlier in this chapter.

It is noteworthy that in Lewandowski study (2001), observations were based on oxalate intake from spinach, it is uncertain whether the results observed with a spinach-derived oxalate load would compare with those observed with sodium oxalate due to poorly defined bioavailability of oxalate in spinach. It is also possible that the oxalate contained in food may be handled differently from that of a crystalline salt (Holmes *et al.* 2005).

Factors that may modulate the absorption of oxalate include co-ingestion of calcium and magnesium, the presence of oxalate degrading bacteria and inherent absorption characteristics (Holmes and Assimos 2004). It has been postulated that lower dietary calcium (*in blacks*) could cause reduced complexation with oxalate in the gastrointestinal tract leading to increased absorption and hence higher urinary oxalate (Messa *et al.* 1997, Lewandowski *et al.* 2005) while a previous study had demonstrated that blacks

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1997, Lewandowski *et al.* 2005) while a previous study had demonstrated that blacks have significantly more oxalate-degrading bacteria including *O. formigenes* and greater oxalate-degrading capability than white subjects (Lewandowski and Rodgers 2004a). However, in the present study, when both groups were following high-oxalate standardized diet with normal calcium and similar magnesium intake, it may be speculated that the data show that blacks handled oxalate more transiently than white subjects. This striking and rapid increase (transient/temporary) in the urinary oxalate excretion after oral oxalate load, proves the absorption of oxalate in the upper gastrointestinal tract and agrees with the investigation of Balcke *et al.* 1989.

Although the reasons for this observation are not clear, transient hyperoxaluria has been observed in a number of studies after oxalate loads (Marshall *et al.* 1972, Barilla *et al.* 1978, Finch *et al.* 1981, Balcke *et al.* 1989, Nguyen *et al.* 1994, Hess *et al.* 1998, Liebman and Costa 2000, Holmes *et al.* 2001). In most studies, the transient hyperoxaluria that occurred (*increases of about 8-289%*) was equivalent to that observed chronically in individuals with primary hyperoxaluria where such conditions can cause pathological changes (Jaeger and Robertson 2004). Nonetheless, since Holmes and co-workers (2005) did not observe renal injury (*urinary N-acetyl-β-glucosaminidase (NAG)* and *γ-glutamyl transpeptide (GGT)* or oxidative stress (*urinary isoprostanes*) after oxalate loads of up to 8mmol sodium oxalate in normal individuals, these markers were not investigated in this study.

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Apart from excessive intakes of oxalate, increased urinary oxalate excretion could originate from its precursors (*ascorbic acid and glycolate*) or from increased endogenous synthesis as in primary hyperoxaluria (Finch *et al.* 1981, Jaeger and Robertson 2004). It is generally accepted that oxalate absorbed from the diet is almost all excreted from the body by the kidney. In 2005, Holmes and co-workers concluded that oxalate is rapidly absorbed and cleared by the kidney by filtration and secretion following an oral oxalate load. If this is indeed correct as the present author believe, then it seems credible that this renal oxalate secretion process, which has a significant role in the renal handling of an oral oxalate load, occurs differently in the two race groups.

Nevertheless, in addition to the aforementioned differences in urinary oxalate excretion in the two race groups, an increase in urinary citrate excretion reached significance only

in black subjects (*p<0.0001*) in protocol 3. Although such an increase in urinary citrate after oral oxalate loads has been reported before in the two groups, its mechanism is still unexplained in literature (Lewandowski *et al.* 2001).

The available evidence argues against the hypothesis which motivated this study, namely that blacks have a better intestinal integrity and therefore better intestinal control mechanisms which allow for less oxalate to be absorbed when challenged by a high dose of oxalate while in whites the integrity of the gut wall might be lowered (*made more permeable*) and thus passive intestinal absorption of oxalate could occur when challenged by a high dose of oxalate. Nonetheless, of particular interest is that despite both race groups following similar and strictly controlled protocols, some important differences with regard to gut permeability index and urinary stone risk factors were observed in the two race groups. This lends auxiliary credence to the notion that different renal handling mechanisms of lithogenic (*and antilithogenic*) substances occur in these groups and that these might contribute to the differences in stone incidences in the two groups.

To explore this idea further, anti-lithogenic substances (*vitamin E and fish oil*) were administered to both groups singly and in combination. These studies are described in Chapters 4 and 5 of this thesis.

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3.6 References

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Chapter Four

Investigation of the effects of vitamin E ingestion on lipid peroxidation and urinary kidney stone risk factors in black and white South African subjects

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4.1 Introduction

Vitamin E is a mixture of α-, β-, δ-, and γ-tocopherol and tocotrienols, differing in the methyl substitutions on the chromanoxyl ring, and the saturation of the phytyl tail. The most extensively studied of these is α- tocopherol (*figure 4.1*) because it has the highest biological activity and possesses potent antioxidant activity (Lodge *et al.* 2000).

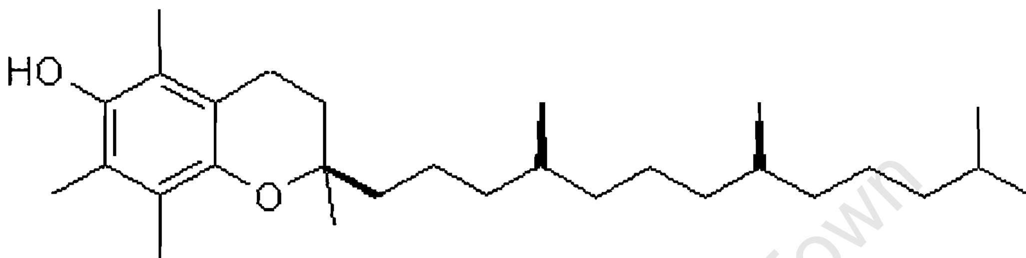

Figure 4.1 Chemical structure of vitamin E *(α-tocopherol) (*Brigelius-Flohé and Traber 1999*)*

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A well-established chemical property of vitamin E is that it is an antioxidant, which has been shown to protect important compounds, including vitamin A, from degradation in the laboratory. It probably also serves this function in organisms (Campbell 1991, Sakly *et al.* 2003). It has been reported that the interaction of vitamin E with membranes enhances its effectiveness as an antioxidant (Brigelius-Flohé and Traber 1999, Brigelius-Flohé 2003, Frank 2005, Weinberg 2005, Sen *et al.* 2006).

As with other fat-soluble vitamins, the absorption of tocopherols is dependent on the ability to digest and absorb fat. Previous studies have centred on the physical form in which it reaches the intestine, its location within the mucosal cells, the efficiency of absorption, and the effect of dietary fat unsaturation on absorption or intestinal destruction (Marks 1975, Munson and Falusy 1976, Blatt *et al.* 2001).

It is widely accepted that free radicals are highly reactive molecules that damage cells throughout the body and that free radical-mediated oxidative stress is implicated in the genesis and exacerbation of degenerative diseases including urolithiasis (Brown *et al.* 1994, Seven *et al.* 1996, Selvam and Kalaiselvi 2001, Zhao *et al.* 2004).

Many researchers believe that supplementing with antioxidants (*e.g. vitamin E*) can dramatically reduce free radical damage, prevent and delay the onset of chronic degenerative diseases and possibly extend lifespan (Zhao *et al.* 2004). In fact, vitamin E has been shown to be involved in free radical physiology and antioxidant defense mechanisms (van Acker *et al.* 1993, Pope *et al.* 2000). Vitamin E is able to prevent free radical formation or terminate their reaction and is therefore, essential to protect molecules and biological systems from oxidative damage (Selvam 2002).

Lipid peroxidation (LPO) products such as thiobarbituric reactive agents (TBARS) and malondialdehyde (MDA) and antioxidant enzymes can be measured in urine and blood as markers of cellular injury (Huang *et al.* 2003, Khan 2005). Previous studies have shown that CaOx stone formers have elevated plasma TBARS (Anuradha *et al.* 1988, Ravichandran and Selvam 1990, Anbazhagan *et al.* 1999) and elevated urinary N-acetyl-β-glucosaminidase (NAG) (Huang *et al.* 2003). This is a tubular enzyme found primarily in the proximal tubular cells of the kidney, which is one of the most sensitive marker enzymes for renal tubular injury (Green *et al.* 2005, Khan 2005, Tungsanga *et al.* 2005). In the study by Huang and co-workers (2003), it was shown that NAG of stone formers was positively and significantly correlated with their urinary oxalate and calcium. Measurement of these markers is therefore a useful indicator of stone risk profiles.

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Administration of vitamin E rapidly restored plasma antioxidant levels and reduced urinary excretion of oxalate and calcium in kidney stone patients (Anbazhagan *et al.* 1999). In another study involving hypertensive and hyperoxaluric patients, supplementation with vitamin E decreased LPO products and increased antioxidant enzymes and vitamin E in plasma (Sumitra *et al.* 2005). It also restored the biochemical and kinetic properties of urinary Tamm-Horsfall glycoprotein, which is a well known inhibitor of calcium oxalate crystallization. In addition, a recent study involving urogenital tuberculosis patients showed that administration of vitamin E enhanced the antioxidant status of plasma thereby preventing membrane injury and consequently, the risk of stone formation (Srinivasan *et al.* 2004a).

The potential role of free radicals, reactive oxygen species and antioxidants in the aetiology of chronic diseases has stimulated extensive research (Knight 1998, Santanam *et al.* 1998, Toyokuni 1999, Trevisan *et al.* 2001). According to Khan (2005),

recent studies have provided evidence for the development of oxidative stress in the kidneys of stone patients.

As part of the motivation and rationale of the present thesis, an investigation of effects of vitamin E ingestion on the biomarkers of oxidative stress in South Africa's stone-prone and relatively stone-free population groups was undertaken. Details of this study are described in this Chapter.

4.2 Subjects and Methods

Study population (Inclusion criteria)

Healthy South African black (*n=5*) and white (*n=5*) male subjects were recruited from the student cohort of the University of Cape Town via advertisement in the university notice boards. The number of subjects corresponds to that of similar studies (Burton *et al.* 1998, Couderc *et al.* 1998, Meagher *et al.* 2001, Traber *et al.* 2001, Rabovsky *et al.* 2006). The age range of subjects was 18-30 years in each group.

Exclusion criteria

Subjects were excluded if:

- they were on any medication or taking supplements that might affect vitamin E metabolism e.g. warfarin, dalteparin sodium, enoxaparin sodium
- they had high blood pressure and/or diabetes
- they had a family history of renal or kidney stone disease

Study design

Subjects were required to ingest one vitamin E capsule (*dl-α-tocopheryl acetate, 400 IU)* per day every day immediately after supper for 60 days (Meagher *et al.* 2001, Srinivasan *et al.* 2004a, Srinivasan *et al.* 2004b, Sivrioglu *et al.* 2007). The vitamin E capsules were manufactured by Vitalfarm (Pty) Ltd, Cape Town, South Africa.

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Venous blood samples (*5ml*) were collected in EDTA tubes by a qualified phlebotomist from each subject on day 0 (*baseline*) and on day 60 (*post-supplementation*), following an overnight fast (*~12hrs*). Blood samples were kept in the dark and on ice until centrifugation. Plasma samples were prepared within 1hr of blood collection by centrifugation (*3000 rpm for 10 min*) (Horoz *et al.* 2005, Rabovsky *et al.* 2006) using Labofuge 200, Heraeus Sepatech, Germany. Plasma vitamin E was determined using high performance liquid chromatography (HPLC) method. Urinary and plasma TBARS were measured using commercially available assay kits (*OXI-TEK TBARS Assay Kit, ZeptoMetrix, Buffalo, New York, USA).* Details of both analyses are provided below. 24hr urine samples were also collected on the day in which blood samples were drawn for measurement of urinary parameters as described in Chapter 2, Section 2.2.

Subjects were instructed to continue taking their free and unrestricted regular diet for the duration of the study (Turley *et al.* 1998, Wander and Du 2000, Higdon *et al.* 2001) so that their habitual intake of macronutrients was maintained throughout the study period. 24hr dietary food records were also collected on the days in which blood and urine samples were drawn (*see appendix CD/Chapter 4/24hr dietary food record*). The subjects' dietary food intake was assessed using a *FoodFinder 2* computer software programme (Langenhoven *et al.* 1991) (*see appendix CD/Chapter 4/dietary analysis*).

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4.3 Determination of plasma vitamin E (HPLC)

Plasma vitamin E (*α-tocopherol)* levels were analyzed by the African Micronutrient Research Group, Stellenbosch University, using the procedure based on that described by Catignani and Bieri (1983). The test procedure is aimed at the quantitation of Vitamin A and E levels in human plasma or serum samples. In this method, serum or plasma are first deproteinised by precipitation following which the fat soluble components, including vitamin A and vitamin E, are extracted with hexane. After evaporation of the hexane, the residue is dissolved in methanol and aliquots used for quantitative determination by high performance liquid chromatography (HPLC).

HPLC analysis was conducted on a Waters solvent delivery system consisting of two solvent pumps (model M45) and a gradient controller (model 680), Waters model 717 plus autosampler and a Linear Programmable UV-VIS Detector (Model SSI 525).

Analytes were separated on a Supelco LC-18, 250mm x 4,6mm, 5µm analytical column together with a Supelcosil™ LC-18 guard pack. Recording and analysis of data were done on EZChrom Elite/Client Server (ver 2.3) Chromatography Data System (Scientific Software Inc., USA) under Windows 95.

4.4 Determination of urinary and plasma TBARS

Urinary and plasma lipid peroxide thiobarbituric acid reactive substances (TBARS) were measured as MDA equivalents using commercially available kit (*OXI-TEK TBARS Assay Kit, ZeptoMetrix, Buffalo, New York, USA) (*Kwon and Watts 1964*).* In this assay, malondialdehyde (MDA) forms a 1:2 adduct with thiobarbituric acid (TBA) to form an MDA-TBA adduct as shown in the reaction scheme (*figure 4.2*), which can be measured by spectrometry at a wavelength of 532 nm (Armstrong and Browne 1994, Yagi 1998).

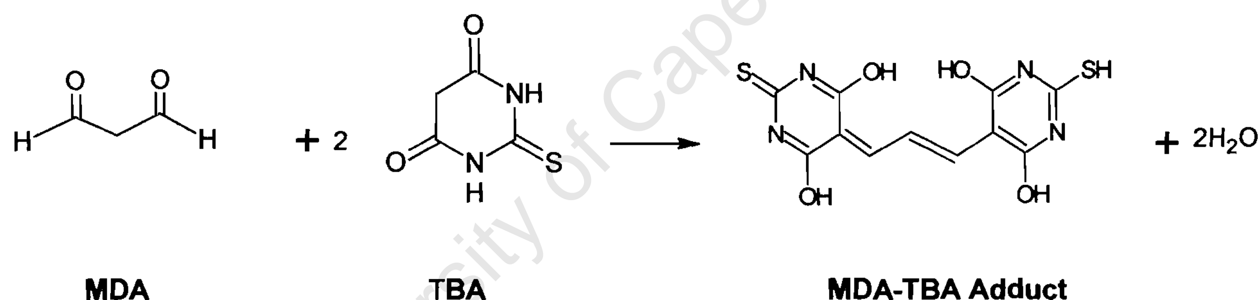

Figure 4.2 Reaction scheme of MDA with TBA

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Preparation of solutions

The following solutions were provided with the kit: TBA (4 vials) containing 0.53 grams TBA, TBARS diluent 1 (4x50 ml) containing acetic acid, TBARS diluent 2 (4x50 ml) containing sodium hydroxide, MDA standard (20 ml) containing 100 nmol/ml malondialdehyde Bis (dimethyl acetal), MDA diluent (100 ml) containing sterile deionized water and sodium dodecyl sulphate (SDS) solution (30 ml).

A TBA/Buffer reagent was prepared as follows: TBA was added to a mixing vessel containing half a bottle of TBARS diluent 1. The vial was mixed with the remaining half of

TBARS diluent 1 and added to the mixing vessel. A full bottle of TBARS diluent 2 was then added while mixing until the TBA was completely dissolved.

Assay procedure

A series of five standards in MDA standard diluent was prepared using the dilution scheme as shown in table 4.1. The MDA standard was used undiluted for the 100 nmol/ml concentration. All reagents were allowed to reach room temperature before use. Samples or standards (100 µl) were then pipetted into properly labeled tube. 100 µl SDS solution was added to each tube with mixing. 2.5 ml TBA/Buffer reagent was added forcefully down the side of each tube, which was then covered with glass marbles (*provided with the kit*) and incubated at 95 °C for 60 minutes. The samples or standards were then cooled to room temperature in an ice bath for 10 min. The mixtures were then centrifuged (*Labofuge 200, Heraeus Sepatech, Germany*) at 3000 rpm for 15 min. The supernatants were removed from samples for analysis and absorbances were read at 532 nm using a spectrophotometer (*Spectronic Unicam Helios, Cambridge, England*). All assays were performed in duplicate. Mean data obtained from absorbance readings of standard solutions (table 4.1) were used to prepare a calibration curve as shown in figure 4.3 and are expressed as MDA equivalents (*nmol/ml*) (Kwon and Watts 1964). The calibration curve was then used to calculate the respective concentrations of urine and plasma TBARS (*as MDA equivalents*) (*section 4.6*).

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Table 4.1 Dilution scheme for preparations of MDA standards

Standard	MDA (nmol/ml)	MDA (µl)	MDA diluent (µl)	Mean Absorbance (532 nm)
0	0	0	1000	0
1	12.5	125	875	0.0755
2	25	250	750	0.1525
3	50	500	500	0.2565
4	100	1000	0	0.5390

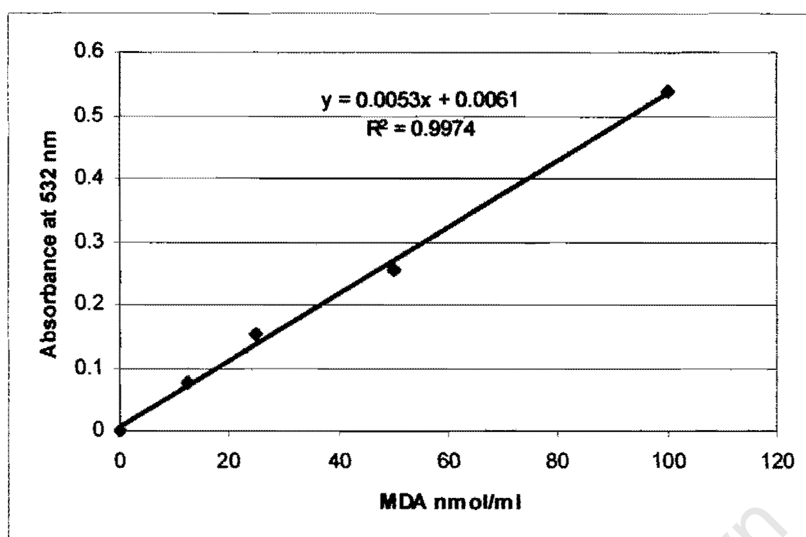

Figure 4.3 The standard curve for TBARS determinations (*MDA nmol/ml*)

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4.5 Urine analysis

24hr urine samples were collected on day 0 (baseline) and on day 60, following vitamin E supplementation. Urinary parameters were measured as described in Chapter 2, Section 2.2 of this thesis.

4.6 Statistical analysis

Data were statistically analysed using repeated measures ANOVA, STATISTICA version 8.0. Data were considered statistically significant if $p \leq 0.05$.

4.7 Results

Dietary analysis

Comparison of the mean nutrient intakes derived from the 24hr dietary records from black and white subjects are given in table 4.2 while the summary of changes is given in table 4.3. The raw data are presented in appendix CD/Chapter 4/Dietary analysis.

Table 4.2 Comparisons of mean dietary intakes (SE) of black and white subjects before and following vitamin E ingestion (*60 days*)

Nutrients	Black (B)		p-values	White (W)		p-values	B vs W, p-values	
	Day 0	Day 60		Day 0	Day 60		Day 0	Day 60
BMI (*kg/m²*)	25.30(0.98)	———	———	25.81(0.98)	———	———	0.7263	———
Energy (*kJ*)	8720 (1623)	8922 (1270)	0.7360	13815 (1622)	14133 (1270)	0.5970	0.0364*	0.0332*
Moisture (*g/day*)	1870 (392)	1729 (469)	0.3150	3287 (392)	3303 (469)	0.9072	0.0476*	0.0317*
Total Protein (*g/day*)	86(5.4)	87(6.2)	0.8190	121(5.4)	116(6.2)	0.2630	0.0018*	0.0057*
Total Fat (*g/day*)	104(15)	103(15)	0.7659	111(15)	109(15)	0.6598	0.7828	0.7975
Carbohydrate (*g/day*)	325(25)	311(21)	0.5141	339(25)	350(21)	0.6074	0.6858	0.2601
Fibre (*g/day*)	25.10 (3.45)	22.62 (4.03)	0.2444	27.00 (3.45)	29.37 (4.03)	0.2635	0.7285	0.2344
Total Sugar (*g/day*)	36.91 (7.89)	38.56 (8.33)	0.4453	60.44 (7.89)	60.75 (8.33)	0.8851	0.0733	0.0880
Oxalate (*mg/day*)	262(47)	251(41)	0.1399	119(47)	120(41)	0.7903	0.0492*	0.0646**
Calcium (*mg/day*)	659(69)	683(55)	0.6062	1151(69)	1088(55)	0.1907	0.0002*	0.0001*
Magnesium (*mg/day*)	274(20)	280(13)	0.6236	381(20)	372(13)	0.4809	0.0001*	0.0030*
Phosphate (*mg/day*)	1817 (286)	1670 (253)	0.1099	1999 (286)	1997 (253)	0.7941	0.6453	0.4435
Potassium (*mg/day*)	2835(491)	2846(493)	0.8447	3550(491)	3497(493)	0.3661	0.3342	0.3766
Sodium (*mg/day*)	2769(626)	2738(634)	0.6613	3641(626)	3609(634)	0.6518	0.3565	0.3569
Vitamin A (*RE/day*)	1031 (197)	1050 (198)	0.5381	1157 (197)	1140 (198)	0.5870	0.6627	0.7556
Vitamin B6 (*mg/day*)	2.56 (0.39)	2.44 (0.35)	0.4170	2.83 (0.39)	2.80 (0.35)	0.8650	0.6199	0.4965
Vitamin C (*mg/day*)	121(24)	122(23)	0.7629	105(24)	103(23)	0.6286	0.6580	0.5806
Vitamin D (*µg/day*)	3.32 (1.09)	3.30 (1.10)	0.4740	4.00 (1.09)	4.00 (1.10)	0.9454	0.6733	0.6643
Vitamin E (*µg/day*)	9.88 (1.20)	8.97 (0.95)	0.3024	11.88 (1.20)	10.92 (0.95)	0.2824	0.2205	0.2296

**Significance at $p \leq 0.05$, **approaching significance*

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Table 4.3 Summary of comparisons of mean dietary intakes between the two race groups

Nutrients	Comparison	
	Day 0	Day 60
Energy*	B < W	B < W
Moisture*	B < W	B < W
Total protein*	B < W	B < W
Oxalate	B > W**	B > W**
Calcium*	B < W	B < W
Magnesium*	B < W	B < W

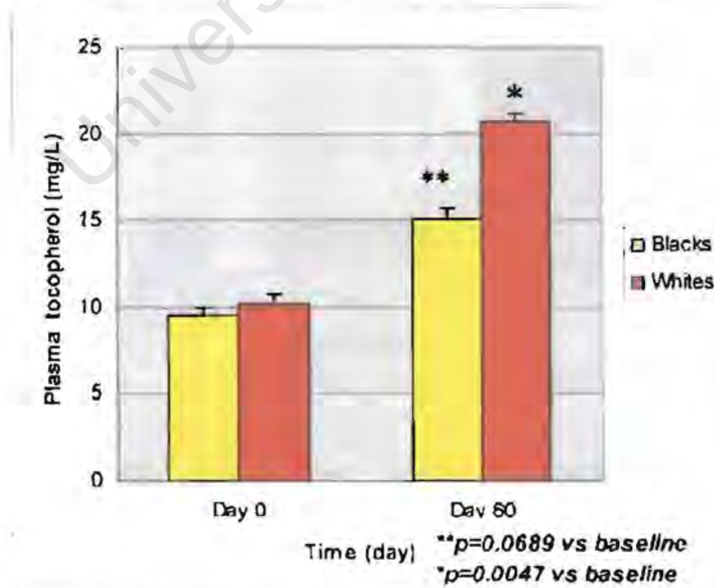
* *Significantly different $p \leq 0.05$, ** approaching significance*

Comment

Comparison of mean dietary intakes between the two race groups for moisture, energy, total protein, calcium and magnesium followed the sequence B<W, the only exceptions were mean intake of oxalate which followed the sequence B>W.

Plasma vitamin E

Plasma vitamin E was determined as described in section 4.3 of this Chapter and is expressed as its most active form α-tocopherol (Brigelius-Fohlé and Traber 1999). Comparisons of the relative levels of plasma α-tocopherol obtained in both race groups before and after vitamin E ingestion (*60 days*) are given in figure 4.4.



* *Significance at $p<0.05$, **approaching significance*

Figure 4.4 Comparisons of mean plasma α-tocopherol of black and white subjects

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Comment

At baseline, no significant differences were observed in mean plasma α-tocopherol values between black and white subjects (*p=0.8272*). Following supplementation, a favourable increase (*from 9.48 ± 1.09 to 15.14 ± 1.83 mg/L*) was observed for black subjects which approached significance (*p=0 0689)*. However, a significant and favourable increase (*from 10.22 ± 0.77 to 20.72 ± 4.13 mg/L*) was observed in white subjects (*p=0.0047*). No significant differences were observed when comparing plasma α-tocopherol levels in the two groups at day 60 (*p=0.1153*).

Urinary TBARS

Mean urinary TBARS expressed as MDA equivalents (*µmol/ g creatinine*) (Jacob *et al.* 2003, Goulart *et al.* 2005) were determined from the calibration curve shown in (*figure 4.4*). Comparisons of the relative levels in black and white subjects at baseline and after vitamin E ingestion (*60 days*) are given in figure 4.5 below.

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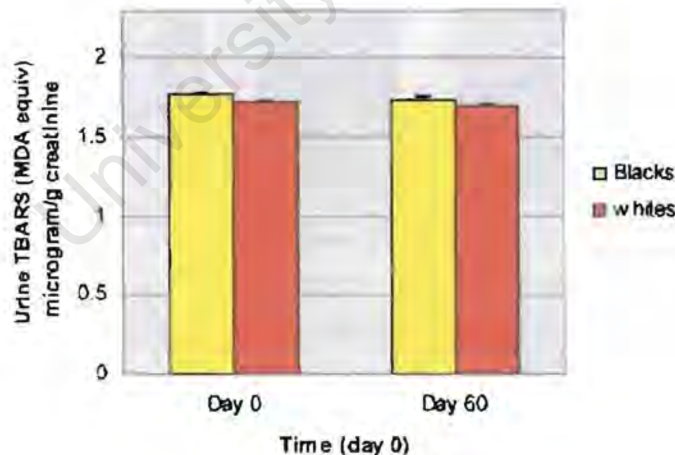

Figure 4.5 Comparisons of mean urinary TBARS of black and white subjects

Comment

No significant changes or differences were observed when making within-groups or inter-group comparisons for this parameter before or following vitamin E ingestion (*60 days*).

Plasma TBARS

Mean plasma TBARS expressed as MDA equivalents (*nmol/ml*) (Kwon and Watts 1964, Ide *et al.* 2002) were determined from the calibration curve shown in (*figure 4.3*). Comparisons of the relative levels in black and white subjects at baseline and after vitamin E ingestion (*60 days*) are given in figure 4.6 below.

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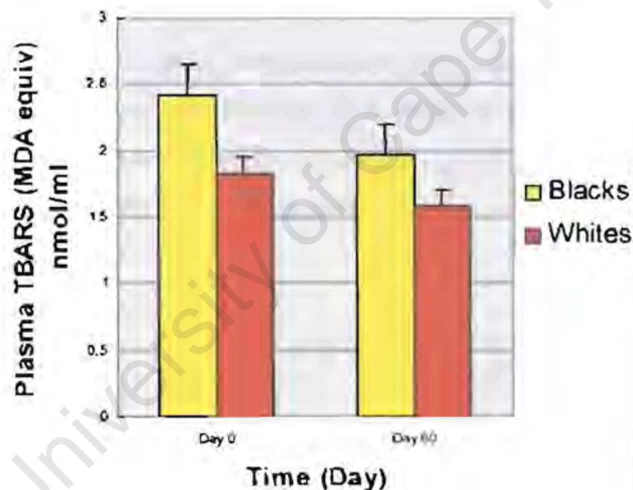

Figure 4.6 Comparisons of mean plasma TBARS of black and white subjects

Comment

At baseline, no significant differences were observed in mean plasma TBARS (*MDA equivalents*) between black and white subjects (*p=0.2782*). Following vitamin E ingestion for 60 days, data showed that relative plasma TBARS (*MDA equivalents*) values were slightly reduced in blacks (*from 2.42±0.48 to 1.96±0.23 nmol/ml*) as well as in whites (*from 1.82±0.48 to 1.57±0.23 nmol/ml*). These slight changes/reductions were not statistically significant in both groups (*p=0.1789 in blacks and 0.4449 in whites*). No

significant differences were observed when comparing the relative levels in the two groups at day 60 (*p=0.4685*). It appears as if plasma TBARS (*MDA equivalents*) values were slightly higher in blacks than whites on both days albeit the differences were not statistically significant (*p>0.05*).

Urine analysis

Comparison of mean 24hr urine data from the two race groups before and following vitamin E ingestion is given in table 4.4 while the summary of changes in parameters investigated in this study following vitamin E ingestion is given in table 4.5. The raw data from each subject are presented in appendix CD/Chapter 4/urinary analysis.

Comparison of mean urinary parameters between the two race groups showed that, urinary calcium was significantly higher in whites than in blacks at baseline (day 0) and at day 60 (*p=0.0170* and *p=0.0386, respectively*). Magnesium was slightly higher in whites at baseline, approaching significance (*p=0.0609*). Following vitamin E ingestion for 60 days, the only urinary parameter that changed significantly was citrate excretion, which was elevated in both groups (*p=0.0017 for blacks and p=0.0001 for whites*). No other changes were observed.

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Table 4.4 Comparisons of mean 24hr urine parameters (SE) from urines of black and white subjects before and following vitamin E ingestion (*60 days*)

Parameter	Black (B)		p-values	White (W)		p-values	B vs W, p-values	
	Day 0	Day 60		Day 0	Day 60		Day 0	Day 60
pH	6.70 (0.19)	6.77 (0.12)	0.5660	6.29 (0.19)	6.35 (0.12)	0.6374	0.1042	0.0957
Volume (*ml/24hr*)	1588 (123)	1719 (117)	0.2084	1453 (123)	1596 (117)	0.1736	0.4449	0.4854
Citrate (*mmol/24hr*)	2.71 (0.40)	4.94 (0.55)	0.0017*	1.96 (0.40)	5.27 (0.55)	0.0001*	0.2905	0.6369
Oxalate (*mmol/24hr*)	0.21 (0.03)	0.24 (0.03)	0.5608	0.23 (0.03)	0.28 (0.03)	0.2431	0.6384	0.2394
Calcium (*mmol/24hr*)	2.22 (0.62)	2.72 (0.47)	0.2506	4.43 (0.62)	4.56 (0.47)	0.7432	0.0170*	0.0386*
Magnesium (*mmol/24hr*)	2.02 (0.41)	2.01 (0.35)	0.9738	3.11 (0.41)	2.99 (0.35)	0.8030	0.0609*	0.0873
Sodium (*mmol/24hr*)	222.2 (60)	273.16 (41)	0.2256	208.52 (60)	213.86 (41)	0.8940	0.8548	0.4342
Potassium (*mmol/24hr*)	47.56 (16)	58.10 (15)	0.1617	55.06 (16)	68.76 (15)	0.0800	0.7406	0.6391
Urate (*mmol/24hr*)	2.68 (0.62)	3.32 (0.48)	0.2098	4.08 (0.62)	4.28 (0.48)	0.6813	0.1015	0.2464
Creatinine (*mmol/24hr*)	14.64 (1.34)	15.08 (1.14)	0.4869	16.90 (1.34)	17.08 (1.14)	0.7732	0.2306	0.2845
Phosphate (*mmol/24hr*)	20.98 (5.30)	27.88 (4.45)	0.3802	31.16 (5.30)	38.80 (4.45)	0.3338	0.1609	0.1344
RS CaOx (COM)	5.31 (0.46)	5.34 (0.38)	0.9233	6.88 (0.46)	6.76 (0.38)	0.6971	0.0243*	0.0375*
RS brushite	7.16E-9 (1.13E-9)	7.13E-9 (9.25E-10)	0.9669	3.37E-9 (1.13E-9)	3.72E-9 (9.25E-10)	0.6140	0.0270*	0.0422*
RS uric acid	1.60 (0.96)	1.13 (0.64)	0.3462	3.25 (0.96)	2.85 (0.64)	0.4134	0.1814	0.1665
Tiselius risk index	157(47)	176(17)	0.7240	227(47)	236(17)	0.8613	0.1772	0.2416

**Significance at $p \leq 0.05$*

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Table 4.5 Summary of changes in parameters following vitamin E ingestion (*60 days*)

Parameter	Blacks	Whites
Plasma α-tocopherol	↑ *	↑ *
Urinary TBARS	___	___
Plasma TBARS	___	___
Calcium	___	___
Citrate	↑ *	↑ *
Oxalate	___	___
RS CaOx (COM), brushite, uric acid	___	___
Tiselius Risk Index	___	___

**Significance at $p \leq 0.05$*

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4.8 Discussion

It is well-recognized that vitamin E is the major lipid soluble antioxidant in humans and thus plays the largest role in protecting cell membranes (Emmert and Kirchner 1999). Although the antioxidant defence system includes endogenous components and exogenous antioxidants which may be obtained by dietary intake, diet alone is not enough to provide the required amount of antioxidants such as vitamin E that minimize or prevent deleterious actions of free radicals that may induce oxidative stress (Jain *et al.* 1998, Panza *et al.* 2008). Therefore supplementation becomes necessary. In fact, it is the only feasible way to acquire large doses of vitamin E. For example, it would take 1000 almonds, containing 33472 J and 658 g of fat, to provide 400 IU of vitamin E (Butler 1997), which was readily provided in the present study through supplementation of one capsule per day.

It is recommended that 2 or more markers be used for accurate and consistent evaluation of oxidative stress in humans (Niki *et al.* 2005). In 2002, Block and co-workers, suggested that investigators of causal mechanisms or the association between oxidative stress and disease, should collect data on one or both lipid peroxidation markers (*eg TBARS and/or MDA or Isoprostanes*) while other studies have used plasma

vitamin E as an additional measure (Selvam and Ravichandran 1993, Srinivasan *et al.* 2004a, Srinivasan *et al.* 2004b).

In the present study, plasma vitamin E and lipid hydroperoxide TBARS (*as MDA equivalents*) in urine and in plasma (*indeces of oxidative stress*) as well as urinary risk factors were investigated in both race groups. At baseline, there were no significant differences between the two groups with respect to plasma α-tocopherol (*p=0.8272*). Supplementation with vitamin E for 60 days significantly elevated the relative levels of plasma α-tocopherol in both groups (*p=0.0689 for blacks and p=0.0047 for whites*) but to different degrees. Inter-group comparisons of postsupplementation levels showed that the effect was slightly higher in the white group albeit not significant (*p=0.1153*). Studies usually associate decreased plasma α-tocopherol with increased susceptibility to lipid peroxidation and increased plasma α-tocopherol levels with prevention of membrane injury and consequently, the risk of stone formation (Belcher *et al.* 1993, Srinivisan *et al.* 2004b). This significant and favourable increase in levels of plasma α-tocopherol in both groups is in good agreement with previous studies that have shown enhancement of antioxidant status of plasma following vitamin E ingestion (Selvam and Ravichandran 1993, Srinivasan *et al.* 2004a, Srinivasan *et al.* 2004b).

As mentioned earlier, the increasing evidence of the involvement of lipid peroxidation in various disorders and diseases has prompted most investigators to examine TBARS (*as MDA equivalents*) as it is a byproducts of lipid peroxidation (Clarkson and Thompson 2000, Niki *et al.* 2005). According to McCall and Frei (1999), lipid peroxidation studies with precise endpoints such as specific classes of lipid peroxides (*TBARS and/or MDA*) are likely to be of considerable value.

In the present analysis, data showed that vitamin E (400IU) ingestion for 60 days did not significantly change urinary TBARS (*MDA equivalents*) values in either race groups (*p=0.7772 in blacks and p=1.0000*). Although, this is in contrast with a study by Cadenas and co-workers (1996) who observed a 27% decrease in urinary TBARS after vitamin E ingestion (*100mg, 30 days*) in healthy subjects, it is in agreement with observations by Kosugi and co-workers (1995) who also reported no change in this parameter after administering vitamin E (*300 mg, 50 days*).

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With regard to plasma TBARS (*MDA equivalents*) values, there were no changes in this parameter in either race group (*p=0.1789 in blacks and p=0.4449 in whites*) following vitamin E ingestion (*400IU, 60 days*). Although a slight reduction occurred in both groups, it was not statistically significant. This is in agreement with a study by Mol and co-workers (1997) in which no consistent or significant change was observed in this parameter following vitamin E ingestion (*600IU, 4 weeks*) while it contradicts that of Sakuma and co-workers (1997) who observed a significant decrease in plasma TBARS following vitamin E ingestion (*300mg, 4 weeks*).

In general, in the present study, blacks tended towards having slightly higher plasma TBARS values than whites albeit not statistically significant (*p=0.2782 at baseline*) and (*p=0.4685 post-supplementation*). Although Block and co-workers (2002) has also reported higher values of this parameter in healthy African American as opposed to their white compatriots, this is surprising in view of the lower stone incidence in black subjects. On the other hand it might help explain the greater effect of vitamin E in raising plasma α-tocopherol levels in whites as opposed to their black compatriots as this biomarker also serve as a sensitive functional assessment of vitamin E status in biological fluids (Drury *et al.* 1997).

The results of other prospective controlled human trials of the effects of vitamin E on lipid peroxides such as TBARS (*MDA equivalents*) are contradictory. Studies show either a reduction (*normalization*) of TBARS and/or MDA in kidney stone patients with hyperoxaluria (Anbazhagan *et al.* 1999) as well as in healthy males (Kanter *et al.* 1993, Meydani *et al.* 1993, Rokitzki *et al.* 1994, Cadenas *et al.* 1996, Sakuma *et al.* 1997), an increase in healthy males (Sacheck *et al.* 2003) or no effect in healthy subjects (Kosugi *et al.* 1995, Mol *et al.* 1997, McBride *et al.* 1998, Huang *et al.* 2002) as well as in healthy smokers (Mol *et al.* 1997) in both urine and plasma samples.

Although the TBARS assay *(as MDA equivalents*) is the most widely used to determine lipid peroxidation (Armstrong and Browne 1994, Ide *et al.* 2002), its determinations may not fully reflect the real situation. In fact, the present author is aware that, when this technique is used on human fluids, false-positives may be produced as stated by Clarkson and Thompson (2000). For example, aldehydes other than MDA may also react with thiobarbituric acid to produce compounds such as iron (II), heme proteins and

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purines that absorb ultraviolet-visible light within the same wavelength (532 nm) as MDA (Mukai and Goldstein 1976, Esterbauer *et al.* 1989, Duthie 1991, Frankel 1991, Block *et al.* 2002). The decomposition of lipid peroxides during the test itself may also mask the actual MDA concentration before testing (Green *et al.* 2005, Dragsted 2008).This test is therefore considered to be a general indicator of oxidative stress only, rather than a specific marker of lipid peroxidation (Armstrong and Browne 1994, Trevisan *et al.* 2001).

Regarding urine chemistry contradictory reports exist about the effect of vitamin E ingestion. In rat model studies, one study reported that vitamin E prevented hyperoxaluria (Kumar and Selvam 2003) while in another study hyperoxaluria and tubular enzymuria were not completely prevented (Huang *et al.* 2006). It is worth mentioning that, in the former study, vitamin E was administered in tandem with selenium. Perhaps an explanation for this anomaly is provided by Green and co-workers (2005), who observed that lipid peroxidation is not the underlying cause of renal injury in hyperoxaluric rats.

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In human studies, a reduction of urinary calcium and oxalate in kidney stone patients with hyperoxaluria (Anbazhagan *et al.* 1999) and in urogenital tuberculosis patients (Srinivasan *et al.* 2004a, Srinivasan *et al.* 2004b) has been reported following vitamin E ingestion. In contrast, in the present study, no changes were observed in urinary calcium (*p=0.2506 in blacks and p=0.7432 in whites*) and oxalate (*p=0.5608 in blacks and p=2431 in whites*) following supplementation in both groups. It is noted that, although urinary calcium was significantly higher in whites than blacks on day 60 (*p=0.0386*), this difference was not attributed to vitamin E ingestion as it was also significantly higher at baseline (day 0) (*p=0.0170*).

The causes of differential effectiveness of vitamin E in epidemiological, clinical and experimental studies are not clearly defined in literature (Rimm *et al.* 1993, Stampfer *et al.* 1993, Sakly *et al.* 2003, Huang *et al.* 2006, Thabet and Chan 2006). Some authors have speculated that it might be related to many variables such as nature and dosage of vitamin E, timing of therapy, stage of various diseases including stone formation, age of patients and degree of renal insufficiency among other factors (McCall and Frei 1999, Sacheck *et al.* 2003).

Although no changes in urinary calcium and oxalate were observed in the present study, elevated urinary excretion of citrate following vitamin E ingestion was observed in both race groups (*p=0.0017 for blacks and p=0.0001 for whites*). Such an effect has been reported by other workers in kidney stone patients with hyperoxaluria (Anbazhagan *et al.* 1999) and urogenital tuberculosis patients (Srinivasan *et al.* 2004a, Srinivasan *et al.* 2004b). According to Khan (2005), urinary citrate is involved in maintaining endogenous antioxidants. However, of particular importance is that an increase in the excretion of urinary citrate is widely accepted as being highly favourable for reducing the risk of stone formation (Fleisch 1978, Pak 1994, Ryall 1997, Chow *et al.* 2004, Rodgers *et al.* 2005). It has also been suggested that increases in citraturia reduce the retention of calcium oxalate crystals by complexation with calcium ions, thereby lowering the rate of recurrence of stone formation (Anbazhagan *et al.* 1999). Further investigations as well as experiments examining the mechanism underlying the altered citrate excretion in the present study as well as in other studies (Anbazhagan *et al.* 1999, Srinivasan *et al.* 2004a, Srinivasan *et al.* 2004b) are needed as it is a beneficial effect of the treatment which might decrease stone risk.

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Because citraturia increased in both groups, it seemed reasonable to anticipate a lowering of RS CaOx and RS brushite. However, no changes were observed in these parameters following vitamin E ingestion in either group. The reasons for these observations are not very lucid. It is probable that it may be related to other subtle changes in urine composition values, which were statistically too small to detect individually, but which had a collective influence on the RS values. No significant changes were observed in the computed Tiselius risk index for calcium oxalate stone formation following vitamin E ingestion in both race groups.

In the present study, comparisons of mean dietary intakes between the two groups showed that moisture, energy, total protein, calcium and magnesium were significantly lower in blacks than in whites on both day 0 and day 60 ($p<0.05$) while oxalate was significantly higher in blacks than whites at day 0 ($p=0.0492$), but this difference only approached significance on day 60 ($p=0.0646$). It is noted that mean dietary intakes of vitamin E were not significantly different between the two groups at baseline (day 0) and at day 60. Thus the observed changes in the present study can be attributed to the

vitamin E supplement with some degree of confidence because the intake of all other nutrients did not change during the 60 day trial.

Although this study is comparable to other studies that have involved similar small numbers of subjects (Burton *et al.* 1998, Couderc *et al.* 1998, Meagher *et al.* 2001, Traber *et al.* 2001, Rabovsky *et al.* 2006), it is recognized that it is a preliminary report only and that it may have precluded detection of subtle effects of vitamin E. Since the study was designed to detect changes of at least 10% in any parameters (*as done by Meagher and co-workers 2001*), the subtle changes were not detected.

Studies have revealed that oxidative damage is more related to hyperoxaluria and crystalluria rather than to tissue crystal deposits and that the process of membrane damage is greatly increased with progressive crystal deposition (Thamilselvam *et al.* 1999, Selvam 2002). Urinary N-acetyl-β-glucosaminidae (NAG) a lysosomal enzyme found primarily in the proximal tubular cells of the kidney, is one of the most sensitive marker enzymes for renal tubular injury (Green *et al.* 2005, Khan 2005, Tungsanga *et al.* 2005). However, since no change or increase in urinary oxalate was observed in both race groups, NAG was not investigated in the present study as it has been reported that it is significantly and positively correlated with urinary oxalate (Khan *et al.* 1989, Winter *et al.* 1996, Huang *et al.* 2000, Perez-Blanco *et al.* 2000, Tungsanga *et al.* 2005).

There is evidence that vitamin E is transported in plasma lipoproteins and is incorporated into lipid bilayers of biological membranes where it determines oxidative susceptibility (Drevon 1991, Brigelius-Fohlé and Traber 1999). Thus, after its intestinal absorption, it is packaged into chylomicrons, which are secreted into the systemic circulation (Herrera and Barbas 2001, Wood *et al.* 2003). The literature abounds with basic science showing that vitamin E inhibits smooth muscle cell proliferation, platelet adhesion and aggregation and monocyte endothelial adhesion (Emmert and Kirchner 1999, Sen *et al.* 2006, Blumberg and Frei 2007, Traber 2007). Thus, it is difficult to argue that the mechanistic actions of vitamin E are unknown. However, these effects are unrelated to the antioxidant activity of vitamin E (Zingg and Azzi 2004).

As alluded to earlier in this Chapter (*Introduction*), the antioxidant activity of vitamin E is attributed to its ability to react with free radicals. Vitamin E as one of hydroxyl radical

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scavengers, is thought to abolish the accumulation of lipid peroxidation products such as TBARS and/or MDA in tissues under urolithic conditions (Selvam 2002).

There is a paradox between the dramatic responses to vitamin E in animal studies and the lack of efficacy in some human studies (McCall and Frei 1999, Thabet and Chan 2006). The currently available markers of oxidative damage seem to be unable to pick up antioxidant effects of supplementation with vitamin E after dietary intervention (Dragsted 2008). In fact, according to Huang and co-workers (2006), the use of antioxidant therapy to prevent renal stone formation is not yet established and its mechanism remains obscure.

In summary, the present study appears to show a dual-role for vitamin E ingestion in the two race groups. On one hand, plasma α-tocopherol and urinary citrate were favourably and significantly elevated in both groups while on the other hand, urinary calcium, oxalate, relative supersaturation and Tiselius risk index as well as urinary and plasma TBARS (*MDA equivalents*) were not altered. Subtle differences in handling mechanisms of vitamin E in black and white subjects were apparent. Overall, vitamin E appeared to have minimal beneficial effects in the small group of black and white South African subjects, which supports the findings of previous studies on vitamin E ingestion in healthy subjects (Patrignani *et al.* 2000, Meagher *et al.* 2001, Block *et al.* 2008). However, the findings of favourable and significant increase in urinary excretion of citrate coupled with an increase of plasma α-tocopherol levels in both groups supports the protective role of vitamin E ingestion in calcium oxalate urolithiasis albeit it does not fully explain the differences in stone incidence in the two South African race groups. The limitations of this study were small number of subjects and lack of dietary control. Further research is warranted in large cohort studies before firm conclusions can be drawn.

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4.9 References

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Chapter Five

Investigation of the effects of Omega 3 (*n-3*) fish oil ingestion alone and in tandem with vitamin E (*dl-α-tocopheryl acetate*) on urinary kidney stone risk factors in black and white South African subjects

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5.1 Introduction

As mentioned earlier in the first chapter (*General Introduction*), fish oil ingestion has been reported to play a role in many degenerative diseases including kidney stone formation (Rothwell *et al.* 1993, Tulloch *et al.* 1994, Claassen *et al.* 1995, Kruger *et al.* 1995, Kruger *et al.* 1998, Schlemmer *et al.* 1999, Kelly *et al.* 2003, Baggio and Budakovic 2005, Kruger and Schollumb 2005).

The possibility that dietary fish oil ingestion may benefit stone patients has been studied in rats (Buck *et al.* 1991, Ali and Bashir 1994, Daly 1996, Baggio *et al.* 2002, Fernandez *et al.* 2004) and humans (Rothwell *et al.* 1993, Claassen *et al.* 1995, Kruger *et al.* 1995, Kruger *et al.* 1998, Schlemmer *et al.* 1999, Yasui *et al.* 2001, Kelly *et al.* 2003, Kruger and Schollumb 2005). Most experts agree that amelioration of kidney stone risk factors following fish oil ingestion might occur through reduction of urinary calcium and oxalate and enhancing protective mechanisms. These favourable changes in urinary calcium and oxalate following fish oil ingestion have been attributable to the incorporation of EPA in the diet, which can provide a substitute metabolic pathway and a unique way of correcting the biochemical abnormalities of idiopathic urolithiasis (Yasui *et al.* 2001).

However, it is worth noting that, in the aforementioned study by Yasui and co-workers (2001), a significant reduction in urinary calcium was only observed in the hypercalciuric group but not in the normocalciuric group and that in another clinical study involving 18 hypercalciuric recurrent stone patients, the reduction in urinary calcium was accompanied by decreases in the excretion of magnesium and citrate while oxalate excretion was unchanged, thereby indicating that overall, fish oil had a limited impact on the risk profile for recurrent urolithiasis (Rothwell *et al.* 1993).

Of concern is that some investigators have observed increased lipid peroxidation after fish oil ingestion and have concluded that it may consequently increase the need for vitamin E supplementation (Harmon *et al.* 1966, Yamamoto *et al.* 1985, Meydani *et al.* 1987, Bartoli *et al.* 1988, Chautan *et al.* 1990, Meydani *et al.* 1991, Nair *et al.* 1993, Wander *et al.* 1996, Wood *et al.* 2003). On the other hand, other studies did not find any evidence of lipid peroxidation and have concluded that there is no basis for additional

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vitamin E after fish oil ingestion (Turley *et al.* 1998, Wander and Du 2000, Higdon *et al.* 2001).

In light of the aforementioned inconclusive studies, further research is deemed necessary to investigate the extent to which fish oil increases the necessity for antioxidant nutrients (Sanders and Hinds 1992). In addition, it is of special interest that vitamin E therapy may also be considered as a means of correcting deficient plasma antioxidant status in chronic kidney failure (Thabet and Chan 2006). In fact, it is noted that in the previous study of this thesis (*Chapter 4*), the present author observed a favourable and significant increase in plasma α-tocopherol in whites ($p=0.0044$) while in blacks, the favourable increase was approaching significance ($p=0.0689$) following vitamin E (*400IU, 60 days*) ingestion.

Thus, an investigation of the effects of n-3 fish oil ingestion alone and in tandem with vitamin E in South Africa's stone-prone and relatively stone-free population groups on kidney stone risk factors was undertaken. Details of this study are described in this chapter.

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5.2 Subjects and methods

Study population (Inclusion criteria)

20 healthy South African black ($n=10$) and white ($n=10$) male subjects (*age range 18-30 years*) were recruited from the student cohort of the University of Cape Town via advertisement in university notice boards.

Exclusion criteria

Subjects were excluded if they:

- were on any medication or taking supplements that might affect vitamin E metabolism e.g. warfarin, dalteparin sodium, enoxaparin sodium
- had high blood pressure and/or diabetes
- had a family history of renal or kidney stone disease

Study design

Protocol I: Fish oil and urolithiasis

Healthy black (*n=5*) and white (*n=5*) subjects were required to ingest an n-3 fish oil capsule (*salmon oil, 1000mg)* providing 164 mg eicosapentaenoic acid (*EPA*) and 110 mg docosahexaenoic acid (*DHA*) every day immediately after supper for 30 days (Baggio *et al.* 1996, Baggio *et al.* 2000, Sivrioglu *et al.* 2007). The fish oil capsules were obtained from Dis-chem Pharmacy (Pty) Ltd, Johannesburg, South Africa. They were provided in sealed opaque containers and did not contain any added vitamin E. Subjects were instructed to store the capsules in the refrigerator at 4 °C (Wander *et al.* 1996).

Protocol 2: Fish oil, vitamin E and urolithiasis

A second group of healthy black (*n=5*) and white (*n=5*) subjects were required to ingest the aforementioned n-3 fish oil capsule in tandem with a vitamin E capsule (*dl-α-tocopheryl acetate, 400 IU capsule/day*), every day immediately after supper for 30 days (Baggio *et al.* 1996, Baggio *et al.* 2000, Sivrioglu *et al.* 2007). The vitamin E capsules were manufactured by Vitalfarm (Pty) Ltd, Cape Town, South Africa.

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Study protocol

In both protocols I and 2, subjects were instructed to take their free and unrestricted regular diet for the duration of the study (Turley *et al.* 1998, Wander and Du 2000, Higdon *et al.* 2001) so that their habitual intake of macronutrients was maintained throughout the study period. 24hr dietary food records were collected on the days in which blood and urine samples were drawn (*see appendix CD/Chapter 5/24hr dietary food record*). The subjects' dietary food intake was assessed using a *FoodFinder 2* computer software programme (Langenhoven *et al.* 1991) (*see appendix CD/Chapter 5/dietary analysis*).

Following an overnight fast (*~12hrs*), two venous blood samples (*5ml*) were collected (*EDTA tube and lithium heparin-tube*) by a qualified phlebotomist from each subject on day 0 (*baseline*) and on day 30 (*postsupplementation*). Blood samples were kept in the

dark and on ice until centrifugation. Plasma samples were prepared within 1hr of blood collection by centrifugation (*3000 rpm for 10 min for EDTA samples and 3500 rpm for 10 min for lithium heparin samples*) (Horoz *et al.* 2005, Rabovsky *et al.* 2006) using Labofuge 200, Heraeus Sepatech, Germany.

Samples collected in lithium heparin-tubes were used for analysis of plasma uric acid (*described in section 5.5*) and ferric reducing ability of plasma (FRAP) (*described in section 5.6*) while samples collected in EDTA-tube samples were used for analysis of plasma α-tocopherol (*described in section 5.3*) and malondialdehyde (*MDA*) (*described in section 5.4*). Assays from the lithium heparin-tube samples were measured within 2hrs of sample collection while aliquots of EDTA-plasma were frozen at -80 °C for the subsequent assays (*batch-analysis*) of the remaining analytes. 24hr urine samples were also collected on the days in which blood samples were drawn without preservatives and urine parameters were measured as described in Chapter 2 of this thesis (*Section* 2.2).

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5.3 Determination of plasma vitamin E

Plasma α-tocopherol levels were determined as described in Chapter 4, Section 4.3 of this thesis.

5.4 Determination of plasma malondialdehyde (MDA)

Commercially available kits BIOXYTECHMDA-586 (*Oxis Research, Portland, USA*) were used to measure plasma MDA (Gérard-Monnier *et al.* 1997). This method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1, NMPI), with MDA at 45 °C. The stoichiometry is such that one molecule of MDA reacts with 2 molecules of NMPI to yield a stable carbocyanine dye.

Reagents

The following reagents were provided with the kit: reagent 1 (R1), which contains N-methyl-2-phenylindole in acetonitrile (3x18ml), reagent 2 (R2), which contains concentrated hydrochloric acid (HCl) (1x16.5ml), MDA standard, which contains 1,1,3,3-

tetramethoxypropane (TOMP) in Tris-HCl (1x1ml), butylated hydroxytoluene (BHT) in acetonitrile (1x2ml), methanol (1x30ml).

Preparation of solutions

The R1 solution was diluted before use, one volume (6ml) of 100% methanol was added to three volumes (18ml) of reagent R1. This solution is stable for 2 days at 4 °C. The MDA standard was provided as tetramethoxypropane (TOMP) because MDA is not stable. The TMOP was hydrolyzed during the acid incubation step at 45 °C, which generated MDA. The TOMP standard was provided as a 10 mM stock solution, which was diluted 1/500 (v/v) in MilliQ water to give a 20 µM stock solution. This standard was diluted as shown in table 5.1.

Mean data obtained from absorbance (586 nm) values of standard solutions (*table 5.1*) were used to prepare a calibration curve as shown in figure 5.1 and are expressed as (*µM*) (Richard *et al.* 1992, Yagi 1998). The calibration curve was then used to calculate the respective plasma free MDA concentrations (*section 5.8*).

Table 5.1 Dilution scheme of TOMP standard for preparations of MDA standards

Standard	Volume of 20 µM standard (µl)	Volume of MilliQ water (µl)	MDA (µM)	Mean Absorbance (586 nm)
0	0	200	0	0
1	25	175	0.5	0.057
2	50	150	1.0	0.148
3	100	100	2.0	0.243
4	150	50	3.0	0.356
5	200	0	4.0	0.461

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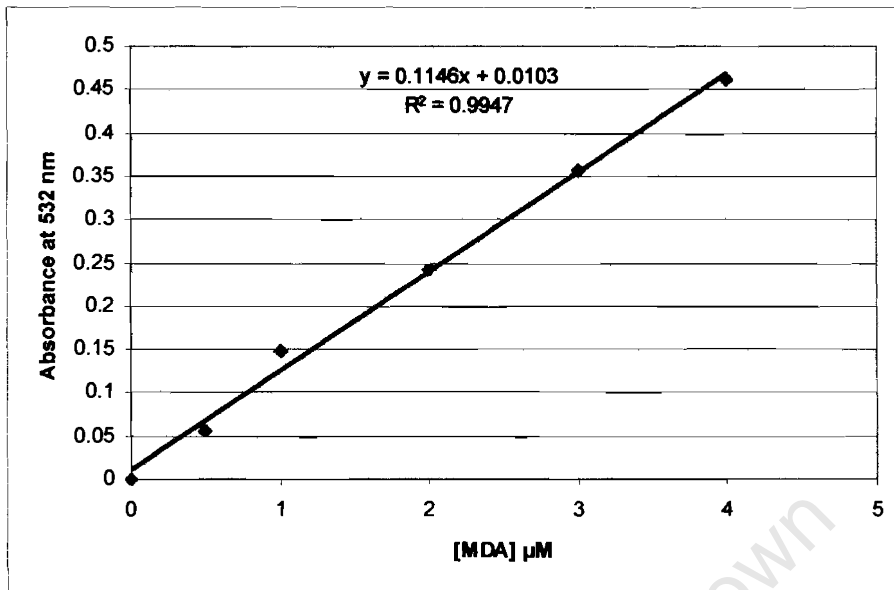

Figure 5.1 Standard curve for MDA determinations

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Assay procedure

10 µl probucol was added to each assay tube and 200 µl sample or standard was added to the respective assay tubes. 640 µl diluted R1 reagent was added to each tube. The assay tubes were vortexed briefly. 150 µl R2 reagent was added to assay tubes, which were stoppered and mixed well by vortex. The tubes were then incubated at 45 °C for 60 min. Turbid samples were centrifuged at 10 000 x *g* for 10 minutes using an eppendorf minispin microcentrifuge (*Lab depot, inc, Georgia, USA*) to obtain a clear supernatant, which was then transferred to a cuvette and the respective absorbances were measured at 586 nm using a spectrophotometer (*Spectronic Unicam Helios, Cambridge, England*).

5.5 Determination of plasma uric acid

Plasma uric acid concentration was measured by a timed-endpoint method using Synchron LX® systems kits (*Beckman Coulter, Inc, Fullerton, CA)* (Fossati *et al.* 1980). In this method, uric acid is oxidized by uricase to produce allantoin and hydrogen peroxide (H_2O_2). The hydrogen peroxide reacts with 4-aminoantipyrine (*4-AAP*) and 3,5-dichloro-2-hydroxybenzene sulfonate (*DCHBS*) in a reaction catalyzed by peroxidase to produce a coloured product (*quinoneimine*).

Reagents

Each kit contains the following items: Two uric reagent cartridges (A and B) (2 x 300 tests), 4-Aminoantipyrine (0.85 mmol/l), 3.5 Dichloro-2-hydroxy-benzene sulfonate (3.4 mmol/l), uricase (240 IU/l) and horseradish peroxidase (961 IU/l).

Assay procedure

No reagent preparation was required. The system automatically proportions the appropriate sample and reagent volumes into a cuvette. The volumes per test are plasma (*12 µl*), cartridge A (*270 µl*) and cartridge B (*30 µl*). The ratio used for uric acid was one part sample to 25 parts for plasma. The system monitors the change in absorbance at 520 nanometers, which is directly proportional to the concentration of analyte in the sample and was used by the Synchron LX® systems to perform all calculations internally to produce the final reported plasma uric acid concentration.

5.6 Determination of plasma ferric reducing antioxidant power (FRAP)

Colorimetric FRAP assay kits (*PolyU Technology and Consultancy Co. Ltd, Hong Kong*) were obtained from collaborators at the Hong Kong Polytechnic University (*Prof Iris F Benzie*). In this assay, the electron-donating antioxidants in the sample reduce a Fe^{3+}/tripyridyltriazine complex, which is present in excess, to the blue coloured product. The increase of absorbance at 593 nm is proportional to the total ferric reducing/antioxidant power (*FRAP value*) (Benzie and Strain 1996, Choi *et al.* 2004, Rabovsky *et al.* 2006).

Preparation of solutions

Four bottles were provided with the kit. FRAP assay reagent A and B, FRAP acid diluent and FRAP buffer. 10ml FRAP assay acid diluent reagent was added to one bottle of FRAP assay reagent A and mixed thoroughly while 10 ml of MilliQ water was obtained from MilliQ water purification system (*Millipore, Berdford, USA*) and added to one bottle of FRAP reagent B. FRAP reagent A, FRAP reagent B and FRAP buffer were then

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mixed in the ratio 1:1:10 to prepare the working FRAP assay reagent immediately before use.

Assay procedure

1ml MilliQ water, was added to clean labeled test tubes (*one for each standard, control and test samples*) and placed in water bath at 37 °C for 5min. 25 µL of standard, control and test samples was added to each tube and mixed. 1ml of the freshly prepared FRAP assay working reagent was pipetted, with mixing, to each test tube at timed intervals (~ *20 seconds*). After 15 minutes' incubation time, the absorbance was then read (*593nm*) against the reagent blank using a spectrophotometer (*Spectronic Unicam Helios, Cambridge, England*). The FRAP value (*µmol/L*) of controls or test samples were then calculated as follows:

$$\textbf{FRAP value} = \frac{\text{absorbance of test}}{\text{absorbance of calibrator}} \times \text{FRAP value of calibrator (1000 µmol/L)}$$

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5.7 Statistical analysis

Data were analysed using repeated measures (ANOVA) Statistica 8.0. Results were considered statistically significant if $p \leq 0.05$.

5.8 Results

5.9 Protocol I: Fish oil and urolithiasis

Dietary analysis

Comparisons of the mean nutrient intakes derived from the 24hr dietary records from black and white subjects for protocol 1 are given in tables 5.2a and 5.2b while the summary of the comparisons is given in table 5.3. The raw data are presented in appendix CD/Chapter 5/Dietary analysis (*protocol 1*).

Table 5.2a Comparisons of mean dietary intakes (SE) of black and white subjects (protocol 1)

Nutrients	**Blacks (B)**			**Whites (W)**			**B vs W, p-values**	
	Day 0	**Day 30**	**p-values**	**Day 0**	**Day 30**	**p-values**	**Day 0**	**Day 30**
BMI (*kg/m²*)	25.51(1.01)	——	——	26.07(1.01)	——	——	0.7045	——
Energy (*kJ*)	8751(1727)	8955(1805)	0.7367	14143(1727)	14204(1805)	0.9206	0.0611**	0.0670**
Moisture(*g/day*)	1828(356)	1798(349)	0.7137	3030(397)	2950(349)	0.3411	0.0419*	0.0490*
Total Protein (*g/day*)	87.99 (13.24)	87.38 (12.76)	0.7653	136.29 (13.23)	134.96 (12.76)	0.5183	0.0300*	0.0319*
Carbohydrate (*g/day*)	332.28 (53.67)	316.57 (59.95)	0.5615	369.37 (53.67)	360.72 (59.95)	0.7476	0.6560	0.5967
Fibre (*g/day*)	27.98(2.43)	28.47(2.33)	0.6518	32.53(2.43)	32.58(2.33)	0.9635	0.2093	0.2535
Total Sugar (*g/day*)	28.31 (6.76)	27.89 (7.76)	0.8679	59.54 (6.76)	58.65 (7.76)	0.7245	0.0152*	0.0163*
Oxalate (*mg/day*)	279.75 (55.80)	261.99 (55.60)	0.2847	128.85 (55.80)	123.54 (55.60)	0.7407	0.0903	0.1155
Calcium (*mg/day*)	729 (62)	710 (78)	0.4522	985 (62)	962 (78)	0.3662	0.0312*	0.0330*
Magnesium (*mg/day*)	327 (30)	317 (25)	0.2530	407 (30)	399 (25)	0.4270	0.0745**	0.0647**
Phosphate (*mg/day*)	1567 (179)	1427 (194)	0.2013	1750 (179)	1763 (194)	0.8977	0.5061	0.2338
Potassium (*mg/day*)	2813 (247)	2833 (240)	0.6818	3749 (247)	3662 (240)	0.1048	0.0257*	0.0421*
Sodium *mg/day*)	2750 (724)	2789 (725)	0.3600	3340 (724)	3274 (725)	0.1353	0.5805	0.6488
Vitamin A (*RE/day*)	1257 (223)	1257 (202)	0.9952	1470 (223)	1452 (202)	0.6245	0.4996	0.5366
Vitamin B6 (*mg/day*)	2.21 (0.34)	2.32 (0.34)	0.5503	3.14 (0.34)	2.96 (0.34)	0.3040	0.0830	0.2101
Vitamin C (*mg/day*)	102 (22)	100 (25)	0.6837	114 (22)	115 (25)	0.8466	0.7329	0.6684
Vitamin D (*µg/day*)	4.73 (0.99)	4.75 (0.97)	0.7904	3.06 (0.99)	3.14 (0.97)	0.4224	0.2636	0.2785
Vitamin E (*mg/day*)	11.83 (1.36)	11.62 (1.04)	0.8267	13.75 (1.36)	12.87 (1.04)	0.3668	0.2869	0.4832

(**Significance at $p \leq 0.05$, **approaching significance*)

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Table 5.2b Comparisons of mean dietary intakes (SE) of fatty acids of black and white subjects (protocol 1)

Nutrients (*g/day*)	Blacks (B)			Whites (W)			B vs W, P-values	
	Day 0	Day 30	p-values	Day 0	Day 30	p-values	Day 0	Day 30
Total fat	96.33 (16.08)	91.96 (15.82)	0.1780	136.21 (16.08)	134.95 (15.82)	0.6812	0.1144	0.0925
Saturated Fat	15.00 (2.92)	15.68 (2.64)	0.3702	15.88 (2.92)	16.37 (2.64)	0.5118	0.8293	0.8654
MUFA	12.65 (3.22)	12.53 (2.52)	0.9104	15.73 (3.22)	14.55 (2.520	0.2945	0.4720	0.6335
PUFA	7.57 (2.55)	7.60 (2.200	0.9693	11.32 (2.55)	10.67 (2.200	0.3862	0.2965	0.3867

Comment

There were no significant changes or differences in mean dietary intakes of fatty acids in both race groups before and following protocol 1(*table 5.2b*).

Table 5.3 Summary of comparisons of mean dietary intakes between the two race groups (protocol 1)

Nutrients	Comparison	
	Day 0	Day 30
Energy**	B < W	B < W
Moisture*	B < W	B < W
Total protein*	B < W	B < W
Total sugar*	B < W	B < W
Calcium*	B < W	B < W
Magnesium**	B < W	B < W
Potassium*	B < W	B < W

** Statistically significant $p \leq 0.05$,*

*** Approaching significance*

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Comment

Differences in the mean dietary intakes between the two groups on both day 0 and day 30 followed similar sequence B<W (*table 5.3*). It is noted that the intake of oxalate in blacks and whites on both day 0 and day 30 was not statistically different.

Urinary analysis (protocol 1)

Comparison of mean 24hr urine data from the two race groups before and following protocol 1 is given in table 5.4 while comparison for mean plasma parameters are given in table 5.5. The raw data from each subject are presented in appendix CD/Chapter 5/urinary analysis/plasma parameters (protocol 1).

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Table 5.4 Comparisons of mean 24hr urine parameters (SE) from urines of black and white subjects (protocol 1)

Urinary parameters	Blacks (B)			Whites (W)			B vs W, p-values	
	Day 0	Day 30	p-values	Day 0	Day 30	p-values	Day 0	Day 30
pH	5.92 (0.16)	6.03 (0.17)	0.4781	6.17 (0.16)	6.18 (0.17)	0.9495	0.3043	0.5403
Volume *(ml/24h)*	1474 (235)	1495 (235)	0.8078	1295 (235)	1446 (235)	0.1082	0.6036	0.8861
Citrate *(mmol/24h)*	1.34 (0.29)	1.77 (0.26)	0.1208	1.54 (0.29)	1.93 (0.26)	0.1559	0.6068	0.6814
Oxalate *(mmol/24h)*	0.21 (0.03)	0.19 (0.02)	0.6664	0.25 (0.03)	0.24 (0.02)	0.7621	0.2686	0.2094
Calcium *(mmol/24h)*	1.36 (0.40)	1.81 (0.22)	0.3984	3.79 (0.40)	2.79 (0.22)	0.0806	<0.0001*	0.0468*
Magnesium *(mmol/24h)*	0.96 (0.27)	1.55 (0.28)	0.2672	3.11 (0.27)	2.94 (0.28)	0.7391	0.0002*	0.0040*
Sodium *(mmol/24h)*	138 (21)	136 (15)	0.9260	152 (21)	184 (15)	0.2912	0.6051	0.0787**
Potassium *(mmol/24h)*	54 (11)	45 (7)	0.5360	69 (11)	66 (7)	0.8022	0.2520	0.1296
Urate *(mmol/24h)*	2.26 (0.39)	3.24 (0.60)	0.1714	3.30 (0.39)	3.44 (0.60)	0.8354	0.1657	0.7834
Creatinine *(mmol/24h)*	13.84 (0.80)	14.34 (0.86)	0.5771	15.00 (0.80)	15.08 (0.86)	0.9282	0.3394	0.5380
Phosphate *(mmol/24h)*	18.08 (3.41)	22.26 (3.28)	0.3290	26.80 (3.41)	30.78 (3.28)	0.3514	0.0857	0.0924
RS CaOx (COM)	7.46 (0.24)	7.13 (0.38)	0.3441	6.96 (0.24)	7.02 (0.38)	0.8641	0.2849	0.8098
RS brushite	1.8E-9 (0.6E-9)	2.6E-9 (1.0E-9)	0.4417	4.3E-9 (0.6E-9)	3.0E-9 (1.0E-9)	0.2279	0.0434*	0.7549
RS uric acid	5.69(0.85)	5.05(1.13)	0.4926	3.75(0.85)	4.10(1.13)	0.7053	0.1965	0.5163
Tiselius Risk Index	141 (35)	133 (21)	0.7236	256 (35)	190 (21)	0.1869	0.0201*	0.1850

**Significance at $p \leq 0.05$, **approaching significance*

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Comment

When making within-group and inter-group comparisons for urine parameters, data showed that there were no statistically significant changes or differences at baseline (day 0) and after ingestion of n-3 fish oil (day 30) within the two race groups. It is noted that urinary calcium and magnesium were significantly different in the two race groups at baseline (day 0), (*p<0.0001 for calcium and p=0.0002 for magnesium*) and at day 30 (*p=0.0468 and p=0.0040, respectively*).

There were no significant changes relative to baseline values in RS CaOx (COM) (*p=0.3441 for blacks and p=0.8641 for whites*), RS brushite (*p=0.4417 for blacks and p=0.2279 for whites*) and RS uric acid (*p=0.4926 for blacks and p=0.7053 for whites*). Although RS brushite was significantly lower in blacks than in whites at baseline (day 0) (*p=0.0434*), there were no significant differences between the two race groups following protocol 1 (*p=0.7549*). With regard to relative Tiselius risk index values, there were no significant differences when making within-group comparisons (*p=0.7236 for blacks and p=0.1869 for whites*). However, inter-group comparisons showed that this parameter was significantly lower in blacks than whites at baseline (*p=0.0201*). Following protocol 1, there were no significant differences between the two race groups for the latter parameter (*p=0.1850*).

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Analysis of plasma biochemistry profiles (protocol 1)

Table 5.5 Comparisons of mean plasma parameters (SE) of black and white subjects

Plasma parameters	Blacks (B)			Whites (W)			B vs W, p-values	
	Day 0	Day 30	p-values	Day 0	Day 30	p-values	Day 0	Day 30
α-tocopherol (*mg/L*)	8.68 (0.72)	8.88 (0.66)	0.6987	10.56 (0.72)	11.22 (0.66)	0.2219	0.0828	0.0372*
FRAP (*μmol/L*)	1359 (101)	1331 (103)	0.2623	1386 (101)	1355 (103)	0.3011	0.8558	0.8692
Uric acid (*mmol/L*)	0.31 (0.03)	0.33 (0.04)	0.4014	0.37 (0.03)	0.37 (0.04)	0.7482	0.2298	0.4199
Free MDA (*μM*)	0.056 (0.01)	0.060 (0.02)	0.7109	0.054 (0.01)	0.058 (0.02)	0.7047	0.9127	0.9162

**Significance at $p \leq 0.05$*

Comment

Experimental data showed that supplementation with n-3 fish oil for 30 days did not significantly change plasma α-tocopherol, FRAP, uric acid and free MDA in both race groups (*$p>0.05$*). When making inter-group comparisons, data showed that plasma α-tocopherol was significantly higher in whites than in blacks at day 30 (*$p=0.0372$*).

5.10 Protocol 2: Fish oil, vitamin E and urolithiasis

Dietary analysis

Comparisons of the mean nutrient intakes derived from the 24hr dietary records from black and white subjects for protocol 2 are given in tables 5.6a and 5.6b while the summary of the comparisons is given in table 5.7. The raw data are presented in appendix CD/Chapter 5/Dietary analysis (*protocol 2*).

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Table 5.6a Comparisons of mean dietary intakes (SE) of black and white subjects (protocol 2)

Nutrients	Blacks (B)			Whites (W)			B vs W, p-values	
	Day 0	Day 30	p-values	Day 0	Day 30	p-values	Day 0	Day 30
BMI (*kg/m²*)	26.31(0.97)	____	____	26.68(0.97)	____	____	0.7922	____
Energy (*kJ*)	10675 (1670)	10780 (1667)	0.9101	15944 (1670)	16750 (1667)	0.3962	0.0517**	0.0316*
Moisture (*g/day*)	1881(222)	1862(186)	0.7366	2781(222)	2681(186)	0.1077	0.0139*	0.0215*
Total Protein (*g/day*)	89.89 (7.76)	95.00 (6.82)	0.2370	116.33 (7.76)	117.51 (6.82)	0.7749	0.0300*	0.0564*
Carbohydrate (*g/day*)	276 (40)	289 (38)	0.1086	315 (40)	304 (38)	0.1753	0.4989	0.7906
Fibre (*g/day*)	29.29 (6.36)	27.85 (4.13)	0.6490	34.29 (6.36)	32.02 (4.13)	0.4788	0.5260	0.5953
Total Sugar (*g/day*)	29.39 (4.92)	28.71 (5.16)	0.7560	78.65 (4.92)	77.45 (5.16)	0.5913	<0.0001*	<0.0001*
Oxalate(*mg/day*)	248(35)	246(35)	0.6081	138(35)	143(35)	0.3476	0.0578**	0.0706**
Calcium (*mg/day*)	745 (67)	728 (63)	0.1281	989 (67)	977 (63)	0.2580	0.0291*	0.0269*
Magnesium (*mg/day*)	302 (22)	300 (20)	0.8841	419 (22)	404 (20)	0.2856	0.0027*	0.0057*
Phosphate (*mg/day*)	1244 (169)	1183 (137)	0.3214	2019 (169)	2050 (137)	0.6125	0.0066*	0.0035*
Potassium (*mg/day*)	1569 (272)	1537 (304)	0.5597	3236 (272)	3216 (304)	0.7178	0.0034*	0.0032*
Sodium (*mg/day*)	2877 (525)	3013 (524)	0.0903	3215 (525)	3127 (524)	0.2499	0.6607	0.8821
Vitamin A (*RE/day*)	1543 (367)	1509 (360)	0.3797	2407 (367)	2423 (360)	0.6629	0.1311	0.1128
Vitamin B6 (*mg/day*)	2.82 (0.48)	2.92 (0.45)	0.4701	3.59 (0.48)	3.43 (0.45)	0.2715	0.2679	0.4627
Vitamin C (*mg/day*)	96(40)	92(41)	0.4266	172(40)	175(41)	0.3894	0.2207	0.1879
Vitamin D (*μg/day*)	4.21 (0.94)	4.16 (0.91)	0.3721	6.31 (0.94)	6.21 (0.91)	0.1060	0.1451	0.1532
Vitamin E (*mg/day*)	11.54 (1.71)	12.13 (1.37)	0.7139	11.37 (1.71)	13.10 (1.37)	0.2969	0.9417	0.6634

(**Significance at p≤0.05, **approaching significance*)

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Table 5.6b Comparisons of mean dietary intakes of fatty acids of black and white subjects (protocol 2)

Nutrients (g/day)	Blacks (B)			Whites (W)			B vs W, P-values	
	Day 0	Day 30	p-values	Day 0	Day 30	p-values	Day 0	Day 30
Total fat	102.84 (19.72)	100.54 (18.60)	0.3197	118.42 (19.72)	117.35 (18.60)	0.6350	0.5813	0.5524
Saturated Fat	16.92 (2.78)	17.06 (2.890	0.8828	17.94 (2.77)	17.21 92.89)	0.4396	0.8064	0.9699
MUFA	14.33 (2.190	13.76 (2.35)	0.3234	16.16 (2.19)	16.28 (2.35)	0.8408	0.5834	0.4549
PUFA	8.57 (1.630	8.82 (1.67)	0.7187	8.29 (1.63)	8.91 (1.67)	0.3780	0.9048	0.9721

Comment

There were no significant changes or differences in mean dietary intakes of fatty acids in both race groups before and following protocol 2 (*table 5.6b*).

Table 5.7 Summary of comparisons of mean dietary intakes of the two race groups (protocol 2)

Nutrients	Comparison	
	Day 0	Day 30
Energy**	B < W	B < W
Moisture*	B < W	B < W
Total protein*	B < W	B < W
Total sugar*	B < W	B < W
Oxalate**	B > W	B > W
Calcium*	B < W	B < W
Magnesium*	B < W	B < W
Phosphate*	B < W	B < W
Potassium*	B < W	B < W

* Significantly different $p \leq 0.05$

** Approaching significance

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Comment

Comparisons of mean dietary intakes between the two groups for day 0 and day 30 followed the sequence B<W (*table 5.7*), the only exception was oxalate which followed the sequence B>W.

Urinary analysis (protocol 2)

Comparisons of mean 24hr urine data from the two race groups before and following protocol 2 are given in table 5.8 while comparisons for mean plasma parameters are given in table 5.9. The raw data for individual subjects are presented in appendix CD/Chapter 5/urinary analysis/plasma parameters (protocol 2). The summary of changes in urinary and plasma parameters for both protocol 1 and 2 is given in table 5.10.

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Table 5.8 Comparisons of mean 24hr urine parameters (SE) from urines of black and white subjects (protocol 2)

Urinary parameters	Blacks (B)			Whites (W)			B vs W, p-values	
	Day 0	Day 30	p-values	Day 0	Day 30	p-values	Day 0	Day 30
pH	6.25 (0.16)	6.25 (0.27)	0.9775	6.11 (0.16)	6.24 (0.27)	0.3511	0.6462	0.9801
Volume *(ml/24h)*	1416 (144)	1521 (184)	0.3685	1398 (144)	1603 (184)	0.0998	0.9402	0.7334
Citrate *(mmol/24h)*	2.20 (0.44)	2.41 (0.51)	0.6762	2.50 (0.44)	2.52 (0.51)	0.9649	0.6661	0.8730
Oxalate *(mmol/24h)*	0.24 (0.03)	0.20 (0.02)	0.0998	0.29 (0.03)	0.28 (0.02)	0.5919	0.2451	0.0738**
Calcium *(mmol/24h)*	2.01 (0.24)	1.61 (0.36)	0.2357	3.89 (0.24)	3.52 (0.36)	0.2668	0.0009*	0.0008*
Magnesium *(mmol/24h)*	1.34 (0.22)	1.45 (0.31)	0.7793	3.25 (0.22)	3.51 (0.31)	0.4877	0.0002*	<0.0001*
Sodium *(mmol/24h)*	158 (13)	132 (11)	0.1545	166 (13)	156 (11)	0.5581	0.6342	0.1647
Potassium *(mmol/24h)*	53 (6)	38 (5)	0.1284	77 (6)	64(5)	0.1793	0.0095*	0.0057*
Urate *(mmol/24h)*	2.70 (0.51)	2.22 (0.52)	0.1104	3.34 (0.51)	3.58 (0.52)	0.3956	0.4011	0.0935
Creatinine *(mmol/24h)*	14.04 (1.60)	13.44 (1.55)	0.4379	17.50 (1.60)	17.62 (1.55)	0.8744	0.1557	0.0941
Phosphate *(mmol/24h)*	18.48 (3.19)	16.72 (5.70)	0.7224	32.52 (3.19)	36.28 (5.70)	0.4544	0.0510*	0.0102*
RS CaOx (COM)	6.72 (0.35)	6.35 (0.70)	0.3671	7.20 (0.35)	6.90 (0.70)	0.4658	0.5557	0.4972
RS brushite	3.72E-9 (9.1E-10)	4.38E-9 (1.6E-9)	0.4346	2.49E-9 (9.1E-10)	3.31E-9 (1.6E-9)	0.3411	0.5160	0.5689
RS uric acid	3.66 (0.97)	3.78 (1.20)	0.8115	4.67 (0.97)	3.61 (1.20)	0.1282	0.5236	0.9172
Tiselius Risk Index	190 (13)	140 (11)	0.0163*	228 (13)	198 (11)	0.1002	0.0429*	0.0047*

**Significance at $p \leq 0.05$, **approaching significance*

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Comment

Comparisons of mean urine parameters between the two race groups showed that urinary calcium, magnesium, potassium and phosphate were significantly higher in whites than in blacks (*$p<0.05$*) at baseline (day 0) and after ingestion of n-3 fish oil and vitamin E (day 30). It is interesting to note that urinary oxalate tended to be lower in blacks at day 30 while no such trend occurred at baseline.

There were no significant changes or differences in RS CaOx (COM), RS brushite and RS uric acid in the two race groups following protocol 2. However, Tiselius risk index values were significantly reduced in blacks following protocol 2 (*$p=0.0163$*) while there were no significant changes for this parameter in white subjects (*$p=0.1002$*). When making inter-group comparisons, data showed that the latter parameter was significantly lower in blacks than in whites at baseline (*$p=0.0429$*) as well as following protocol 2 (*$p=0.0047$*).

Analysis of plasma biochemistry profiles (protocol 2)

Table 5.9 Comparisons of mean plasma parameters (SE) of black and white subjects (protocol 2)

Plasma parameters	Blacks (B)			Whites (W)			B vs W, p-values	
	Day 0	Day 30	p-values	Day 0	Day 30	p-values	Day 0	Day 30
α-tocopherol (*mg/L*)	8.66 (0.76)	12.30 (0.76)	0.0196*	14.70 (1.83)	18.76 (1.83)	0.0144*	0.0855	0.0577*
FRAP (*μmol/L*)	1371 (105)	1409 (97)	0.2509	1392 (105)	1429 (97)	0.2607	0.8905	0.8951
Uric acid (*mmol/L*)	0.30 (0.02)	0.31 (0.02)	0.7679	0.33 (0.02)	0.34 (0.02)	0.4390	0.3848	0.2248
Free MDA (*μM*)	0.062 (0.01)	0.052 (0.01)	0.0905	0.054 (0.01)	0.046 (0.01)	0.1622	0.5357	0.6407

**Significance at $p\leq 0.05$*

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Comment

Supplementation with n-3 fish oil in tandem with vitamin E for 30 days significantly increased plasma α-tocopherol in both race groups (*p=0.0196 for blacks and p=0.0144 for whites*). Whites also had a significantly higher plasma α-tocopherol than blacks on day 30 (*p=0.0577*). No other significant changes were observed.

Summary of results (protocols I and 2)

Table 5.10 Summary of changes in parameters following protocols 1 and 2 (*30 days*)

Parameter	Fish oil (Protocol 1)		Fish oil + vitamin E (Protocol 2)	
	Blacks	Whites	Blacks	Whites
Plasma				
α-tocopherol	—	—	↑ *	↑ *
FRAP	—	—	—	—
Uric acid	—	—	—	—
Free MDA	—	—	—	—
Urine				
Calcium	—	—	—	—
Oxalate	—	—	—	—
RS CaOx (COM)	—	—	—	—
RS brushite	—	—	—	—
RS uric acid	—	—	—	—
Tiselius risk index	—	—	↓ *	↓ NS

**Significance at $p \leq 0.05$, NS not statistically significant*

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5.11 Discussion

A substantial body of literature suggests that n-3 fish oil ingestion regulates lipid metabolism as they are incorporated into cell membranes where they can influence membrane functions and enhance susceptibility to peroxidation due to a high degree of insaturation (Mills *et al.* 1995, Calder 2003, Accinni *et al.* 2006). Following fish oil ingestion, lipid peroxidation is thought to proceed by radical mediated abstraction of a hydrogen atom from a methylene carbon on a PUFA (*EPA and DHA*) or PUFA side chain (Yagi 1987, Dreher and Junod 1996, McCall and Frei 1999, Saygili *et al.* 2003). The resulting carbon-centred radical may then undergo molecular rearrangement followed by interaction with molecular oxygen to form a peroxyl radical, which then culminates in oxidative injury (Chow 1991, Mazor *et al.* 1997, Wander and Du 2000). The latter action is a pathogenic factor in a variety of diseases including stone formation (Halliwell 1987, Thamilselvam *et al.* 2003). According to Ricciarelli and co-workers (2000), this drawback can be theoretically minimized by antioxidants such as vitamin E. In fact, as mentioned in the previous chapter of this thesis, the best known function of vitamin E relates to its capacity to scavenge reactive oxygenated species, thus acting as a chain-breaking antioxidant inhibiting lipid peroxidation, a predisposing factor for renal stone formation (Sundl *et al.* 2007).

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In the present chapter, antioxidant status, lipid peroxidation as well as urinary risk factors for stone formation were evaluated in South African black and white subjects before and following ingestion of fish oil alone (protocol 1) as well as fish oil in tandem with vitamin E (protocol 2).

Data showed that plasma α-tocopherol levels were not significantly affected by n-3 fish oil ingestion for 30 days (protocol 1) in both race groups. This supports the findings by Luostarinen and co-workers (1995), who observed no change in this parameter following protocol 1 (*8.6g n-3 FA, 30 ml/day, 4 weeks*) in mildly hypertriglyceridemic males. In contrast, in a study by Shapiro and co-workers (1991) an increase in plasma α-tocopherol was observed following protocol 1 (*MaxEPA, 18g/day, 6 weeks*) in normolipidemic males while Nair and co-workers (1993), reported a decrease in this parameter following protocol 1 (*15 g/day fish oil, 10 weeks*) in healthy males.

On the other hand, in the present study, plasma *α-tocopherol* levels were favourably and significantly elevated in both race groups (*p=0.0196 for blacks and p=0.0144 for whites*) when fish oil was ingested in tandem with vitamin E. Although this is in agreement with a study by Nair and co-workers (1993), in which protocol 2 (*15g/day FO in tandem with 200 mg all-rac-α-tocopherol, 8 weeks*) increased plasma α-tocopherol in healthy men, it contradicts findings in a double-blinded, parallel design study by Turley and co-workers (1998), in which plasma α-tocopherol concentrations were raised compared with baseline values after protocol 1 (*2.4 g/day, providing 0.72g of EPA and DHA*) or protocol 2 (*2.4 g/day, providing 0.72g of EPA and DHA in tandem with 3 IU vitamin E/g oil*) for 28 days in healthy women.

As mentioned in the previous Chapter, studies usually associate decreased plasma α-tocopherol with increased susceptibility to lipid peroxidation and increased plasma α-tocopherol levels with prevention of membrane injury and consequently, the risk of stone formation (Belcher *et al.* 1993, Srinivisan *et al.* 2004). Thus, a favourable and significant increase in plasma α-tocopherol for both race groups is apparent in both studies following vitamin E ingestion protocols.

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It has been postulated that the overall antioxidant status of the biological system may be more important than the levels of any single antioxidant (Wayner *et al.* 1987, Selvam 2002, Panza *et al.* 2008). Some studies have shown that the total '*antioxidant power*' of a variety of biological fluids and tissues can be evaluated using the ferric reducing antioxidant power (FRAP) assay (Benzie and Strain 1996, Choi *et al.* 2004, Rabovsky *et al.* 2006). In the present study, plasma FRAP was investigated in the two race groups before and following ingestion of n-3 fish oil alone and in tandem with vitamin E. Data showed that this parameter was not significantly affected by either protocol 1 or 2 in both race groups. There is in general, scant data in the literature, of studies that have investigated plasma FRAP as a measure of total antioxidant power. In particular, the present author could not find human studies that have measured this parameter following protocols 1 and 2. Thus, no comparisons of the present result with those of others could be made in this regard.

The FRAP values obtained in this study are comparable to those obtained in a study by Benzie and Strain (1996) who measured this parameter in healthy Chinese subjects.

The baseline range of fresh plasma FRAP values in the latter study was 612-1634 µmol/L in healthy Chinese subjects while in the present study the baselines ranges of fresh plasma FRAP values were 992-1662 µmol/L for blacks and 1207-1692 µmol/L for whites (protocol 1). In protocol 2, the respective ranges were 1042-1768 µmol/L for blacks and 1181-1481 µmol/L for whites.

In addition, although not directly applicable to the present study, supplementation with vitamin E (400IU) on its own in healthy Caucasian and Chinese males did not significantly change plasma FRAP values 90 and 180 min post supplementation (Choi *et al.* 2004). In a study by Rabovsky and co-workers (2006), on healthy male and female subjects, it was also observed that supplementation with different antioxidants including vitamin E (*1000 IU, 4hrs*) on its own did not significantly change plasma FRAP values. The reasons for the lack of effects of antioxidants on plasma FRAP values is not clear in literature (Choi *et al.* 2004, Rabovsky *et al.* 2006). It was noted in the latter study that measures of antioxidant capacity in plasma have not been standardized or validated and therefore results are also inconsistent from lab to lab.

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Plasma uric acid, a purine degradation product and a major water soluble antioxidant present in blood has also been previously investigated (Maxwell *et al.* 2006, Feig *et al.* 2008). It has been reported that uric acid contributes up to 60% of the total plasma FRAP and that its increased levels might also interfere with calcium oxalate solubility (Serafini *et al.* 1998, Dotan *et al.* 2004, Panza *et al.* 2008). It has also been suggested that uric acid must be evaluated when measuring plasma FRAP in order to accurately predict antioxidant capacity. Thus, once it is removed, the antioxidant protection of other antioxidants present in the blood such as vitamins, phytochemicals, carotenoids and others can then be measured (Rabovsky *et al.* 2006).

In the latter study, in order to measure the effect of antioxidant supplementation on plasma FRAP, uric acid was removed by the addition of uricase so that the effects of only supplemented antioxidants were measured and not the activity due to uric acid. In the present study, plasma uric acid did not undergo any statistically significant change in either race group in both protocols 1 and 2. Thus, the contribution of plasma uric acid was deemed to be similar in both groups. Hence comparisons of non-urate FRAP in the two race groups were not evaluated in this study.

Owing to the limitations of the TBARS (*MDA equivalents*) assay mentioned in Chapter 4, section 4.8, it was deemed more appropriate and prudent to investigate lipid peroxidation using the more specific MDA-586 assay in the present study. It is recognized that even with this more specific method, there is evidence that peroxidates and aldehydes in food can be absorbed through the gut to a limited extent and can also potentially confound measures of MDA and perhaps of other plasma peroxides as well (Frietsche and Johnston 1988, Gonzalez *et al.* 1992, Allard *et al.* 1997). Nonetheless, of particular importance, in the MDA-586 method, is that the assay conditions serve to minimize interference from other lipid peroxidation products, such as 4-hydroxyalkenals (Gérard-Monnier *et al.* 1997).

In the present study, data showed that n-3 fish oil ingestion (protocol 1) did not significantly change plasma free MDA in either race group (*p=0.7109 for blacks and p=0.7047 for whites*). This finding suggests that supplementation with n-3 fish oil for 30 days did not induce lipid peroxidation in the studied groups of subjects. This is in contrast to a study by Nair and co-workers (1993) in which protocol 1 (*15 g/day* FO, *10 weeks*) raised plasma MDA in healthy males inducing lipid peroxidation. On the other hand, the present result supports findings of a study by Turley and co-workers (1998) that have reported that protocol 1 (*18% EPA and 12% DHA, 28 days*) does not significantly change this parameter in healthy women as well as those of Hansen and co-workers (1998) in which protocol 1 (*providing 4g EPA and DHA)* was administered in healthy males with no significant change in lipid peroxidation.

A lack of consistency in results involving protocol 2 are also apparent. In the present study, protocol 2 (*FO plus 400IU dl--α-tocopheryl acetate, 30 days*) did not significantly change plasma free MDA levels in either race group (*p=0.0905 for blacks and p=0.1622 for whites*). Similarly, protocol 2 (*FO plus 3IU vitamin E /g oil*) did not significantly change plasma MDA levels in the study by Turley and co-workers (1998) while in contrast, in the study by Nair and co-workers (1993), protocol 2 (*FO* plus *200mg all-rac-α-tocopherol, 8 weeks*) counter-acted the rise in plasma MDA observed in protocol 1. According to Wander and Du (2000), many studies investigating increased consumption of n-3 fatty acids report no changes in lipid peroxides.

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It has been reported that long-chain n-3 PUFA dose, duration of exposure, differences between subjects and differences in experimental design might contribute to different findings (Lapointe *et al.* 2006, Yusof *et al.* 2008). For example, in a study by Higdon and co-workers (2001), it was shown that fish oil (*providing EPA and DHA*) does not increase overall oxidation as compared to sunflower oil (*providing oleate*) and safflower oil (*providing linoleate*). It is worth mentioning that although the study by Turley and co-workers (1998) involved healthy women, they administered fish oil (*providing EPA and DHA*) as in the present study as opposed to the study by Nair and co-workers (1993), in which a 50% concentrate of refined anchovy oil (ROPUFA 50%) was administered (*contents were not identified*).

The duration of the study by Turley and co-workers (1998) was also comparable to the present study (*28 days in their study vs 30 days in the present study*) while the study by Nair and co-workers (1993) was for a longer period (*10 weeks*). It is also noted that different dosages of both fish oil and vitamin E supplements were administered in these three studies as indicated above. Moreover, while plasma free MDA was measured in the present study, the other studies did not indicate whether they measured plasma free MDA, protein-bound MDA or total MDA. Thus, it is perfectly reasonable to conclude that the differences in design, dosage and duration may explain the different observations in these studies.

There are reports that suggest that vitamin E may also act as a prooxidant molecule when it reacts with lipid radicals *in vivo* and form the α-tocopheroxyl radical, which might promote lipid peroxidation (Kontush *et al.* 1996, Brigelius-Flohé and Traber 1999, Carr *et al.* 2000, Lapointe *et al.* 2006, Thabet and Chan 2006). However, there is no evidence in the present study that lipid peroxidation occurred as demonstrated by plasma free MDA levels that were not altered significantly in the two protocols for both race groups.

Data in the present study also showed that urinary parameters (*notably urinary calcium and oxalate*) were not significantly altered by either protocol 1 or 2 in both race groups. This is in contrast with studies that have reported reductions of these parameters in stone patients (Claassen *et al.* 1995, Kruger *et al.* 1995, Kruger *et al.* 1998, Schlemmer *et al.* 1999, Kelly *et al.* 2003, Kruger and Schollumb 2005), although it is in agreement with studies that have reported unchanged levels of these parameters following protocol

1 in stone patients and healthy subjects (Rothwell *et al.* 1993, Yasui *et al.* 2001). In the study by Rothwell (1993) on 18 hypercalciuric recurrent stone patients, it was reported that urinary calcium excretion was reduced but urinary oxalate levels were unchanged, while in a study by Yasui and co-workers (2001), a significant reduction in urinary calcium was only observed in the hypercalciuric group but not in the normocalciuric group after administration of a highly purified preparation of EPA (*1800 mg/day*) for 3 months (*short term*) and 18 months (*long term*). The reasons for the lack of significant effects of protocol 1 and 2 on urinary parameters such as calcium and oxalate in the present study as well as in other studies (Rothwell *et al.* 1993, Yasui *et al.* 2001) are not clear.

It has been reported that it is possible that lipid peroxidation can occur in the capsules during storage (Sanders and Hinds 1992). However, this seems unlikely in the present study as the capsules were provided in dark sealed opaque containers and subjects were instructed to keep them refrigerated at 4 °C (Wander *et al.* 1996).

Although urinary parameters were not significantly altered in both protocol 1 and 2 in the two race groups in this Chapter, it was deemed prudent to investigate whether these protocols had any effects on the relative supersaturation values of stone forming salts and on the Tiselius risk index values because subtle changes in individual urinary parameters may have significant effects on the more complex indices. Data showed that there were no significant changes in RS CaOx (COM) (*$p=0.3441$ for blacks and $p=0.8641$ for whites*), RS brushite (*$p=0.4417$ for blacks and $p=0.2279$ for whites*) and RS uric acid (*$p=0.4926$ for blacks and $p=0.7053$ for whites*) following protocol 1. Although RS brushite was significantly lower in blacks than in whites at baseline (day 0) (*$p=0.0434$*), there were no significant differences between the two race groups following protocol 1 (*$p=0.7549$*). The significantly lower value of RS brushite in blacks at baseline is in conformity with the low stone incidence in this group as opposed to in the white subjects. However, the lack of significant differences in the two race groups following protocol 1, is noteworthy as it points towards a more therapeutic effect of this protocol on this parameter for white subjects. It is noted that there were no significant changes or differences in the RS CaOx (COM), RS brushite and RS uric acid in the two race groups following protocol 2.

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Tiselius risk index values were not significantly altered in both race groups following protocol 1. However, this parameter was significantly lower in blacks than in whites at baseline (*p=0.0201*), which is also in conformity with the low incidence of stone formation in the black group. Following protocol 1, there were no significant differences between the two race groups for this parameter (*p=0.1850*). This could be attributable to the more profound effects of protocol 1 in whites in reducing the Tiselius risk index values (*from 256±35 to 190±21*) as opposed to the low reduction of this parameter in blacks for protocol 1 (*from 141±35 to 133±21*). However, it is noted that these reductions of Tiselius risk index values following protocol 1 were not statistically significant in both race groups. Following protocol 2, Tiselius risk index values were significantly reduced in blacks (*p=0.0163*) while there were no significant changes for this parameter in whites (*p=0.1002*). This suggests a more profound effect of protocol 2 for this parameter in the black group. Although this parameter was also significantly lower in blacks following protocol 2 (*p=0.0047*), this could not be attributable to the effects of protocol 2 as this parameter was also significantly lower in blacks than in whites at baseline (day 0) (*p=0.0429*).

In the present study, comparisons of mean dietary intakes within the two race groups revealed that subjects maintained their habitual intake throughout the respective study periods as demonstrated by the absence of significant differences when making comparisons at baseline and postsupplementation in both protocols 1 and 2 and for both race groups. The mean intakes of other nutrients followed similar sequences as described in Chapters 2, 3 and 4 of this thesis, with blacks having significantly higher intakes of oxalate while the other nutrients namely energy, moisture, total protein, total sugar, calcium magnesium and potassium were significantly lower in blacks than in whites at baseline and postsupplementation. The only exceptions were the mean dietary intakes of oxalate and phosphate which were similar between the two race groups in protocol 1 of this Chapter. It is possible that this anomaly might be due to the small number of subjects (*n=5*) who participated in this protocol. The implication of the differences in mean dietary intakes of the aforementioned nutrients in the incidences of stone formation between the two race groups has been fully described in Chapter 2 of this thesis (*section 2.4*).

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There were no statistically significant differences with regard to mean dietary intakes of vitamin E between the two race groups in protocols 1 and 2 described in this Chapter, compared to those reported in the previous Chapter of this thesis. In addition, since both protocols 1 and 2 of the present study involved ingestion of essential fatty acids (*n-3 fish oil*), it was deemed appropriate and prudent to also investigate regular mean dietary intakes of essential fatty acids in the two race groups. Data showed that there were no significant differences in the mean dietary intakes of total fat, saturated fat, monounsaturated fat and polyunsaturated fat in both race groups for both protocols 1 and 2 of this Chapter. Therefore, the results obtained in the present Chapter could be attributed to the effects of protocols 1 and 2 with some degree of confidence. It is noted that the absence of significant differences in these parameters (*vitamin E, total fat, saturated fat, monounsaturated fat, polyunsaturated fat*) between healthy black and white subjects is in agreement with observations of a previous study by Allen and co-workers (2003) in the two race groups.

It is widely accepted that the metabolic pathway of n-3 fatty acids occurs as shown in figure 5.2 and that n-3 PUFAs inhibit the conversion of linoleic acid into arachidonic acid and directly influence the conversion from arachidonic acid into a series of prostaglandins (Calder 2003, Accinni *et al.* 2006). These analytes were not measured in the present study as they were deemed to be beyond its scope. Future studies in this area should address these pathways.

Despite this limitation, the results of the present study have shown that protocol 1 was neither beneficial (*e.g. no favourable significant changes in plasma α-tocopherol, FRAP, urinary calcium*) nor harmful (*e.g. no adverse or unfavourable significant changes in plasma uric acid, plasma free MDA, urinary oxalate*) in the two race groups. Protocol 2 favourably and significantly raised plasma α-tocopherol levels in both groups but did not have any effect on the other parameters. Although this supports findings in other studies (Turley *et al.* 1998, Higdon *et al.* 2000, Wander and Du 2000, Higdon *et al.* 2001, Rabovsky *et al.* 2006), it is apparent that same limitations as observed in Chapter 4 apply due to the small number of subjects and lack of dietary control. Large cohort studies with dietary control are needed before firm conclusions can be drawn.

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It is noted that although the overall responses to the two protocols appear to be in concert in the two race groups, the findings of a significantly higher plasma α-tocopherol post-supplementation (*day 30*) in whites than in blacks in protocol 1 (*p=0.0372*) and protocol 2 (*p=0.0577*) is counter-intuitive considering the lower incidence of stone formation in the black group. Nonetheless, it is worthy of attention as it tentatively points towards therapeutic properties of this nutrient in this small groups of white subjects. Further research is undoubtedly warranted in this area.

According to Choi and co-workers (2004), although oxidative stress is implicated in the aetiology of many diseases, most supplementation trials with antioxidant micronutrients have also not shown expected beneficial effects. In fact, results of large-scale, long-term intervention studies have also cast some doubts on the oxidative modification hypothesis (Stocker and Keaney 2004, Niki *et al.* 2005).

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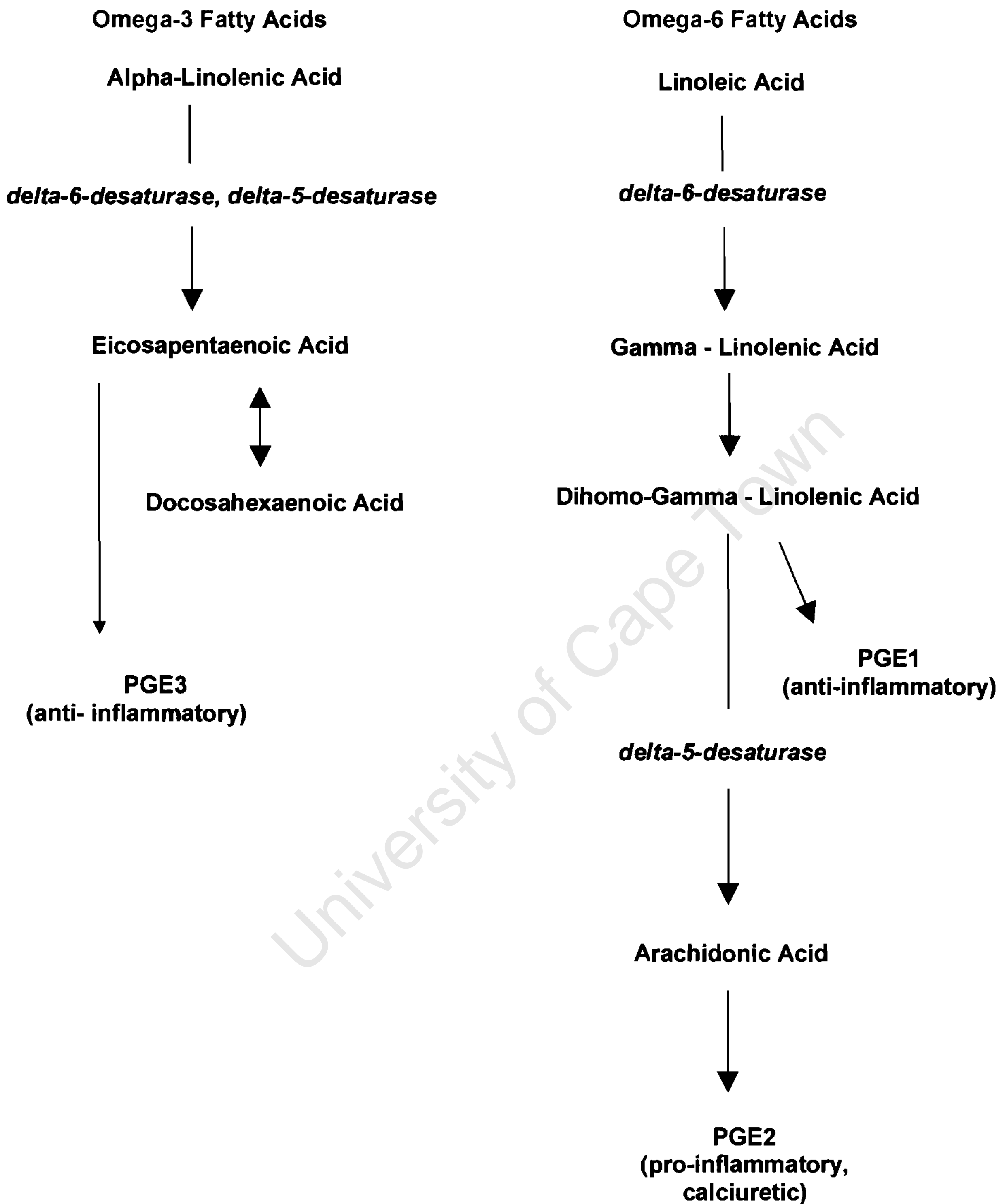

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Figure 5.2 Essential fatty acid pathway (*Rodgers et al. 2009*)

5.12 References

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Chapter Six

General Discussion / Concluding remarks

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6.1 General discussion

6.2 Dietary analysis

In all of the studies described in this thesis, dietary intakes were assessed by means of 24hr dietary recalls and/or food frequency questionnaires. Although it has been shown that this method seems to be precise (Fellström *et al.* 1989), it has also been suggested that the 24hr dietary recall tends to underestimate and the food frequency questionnaires tends to overestimate nutrients intakes (Kennedy *et al.* 2004).

Comparisons of mean dietary intakes from the various Chapters of this thesis (*table 6.1*) shows that energy, moisture, total protein, total sugar, calcium, magnesium, phosphate, potassium were significantly lower in blacks than in whites while there were no significant differences in the mean dietary intakes of total fat, carbohydrate, fibre, sodium, vitamins A, B6, C, D and E. In fact, the intakes of saturated fat, monounsaturated fat and polyunsaturated fat in protocols 1 and 2 of Chapter 5 were also not significantly different between the two race groups. The intakes of these parameters (*saturated fat, monounsaturated fat and polyunsaturated fat*) were only investigated in protocols 1 and 2 of Chapter 5 of this thesis because these were the only protocols in which the effects of ingestion of essential fatty acid (*omega-3 fish oil*) in the two race groups were investigated.

The only mean nutrient intake that was significantly higher in blacks than in whites in all studies described in this thesis was dietary intake of oxalate albeit its intake was similar for black and white subjects who participated in protocol 1 of Chapter 5 of this thesis.

The clinical significance of the aforementioned observed differences in mean dietary intakes between the two race groups with regard to kidney stone disease in general and more specifically with the low incidence of stone formation in the black population as compared to in the white population were fully described in Chapter 2 of this thesis (*section 2.4*). Moreover, the aforementioned observations of different mean dietary nutrient intakes between the two race groups are in conformity with findings in other studies (Lewandowski *et al.* 2001, Rodgers and Lewandowski 2002, Allie and Rodgers 2003, Lewandowski and Rodgers 2004).

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It is noteworthy that slight differences were observed in the mean dietary intakes of black and white subjects who participated in the various studies of this thesis. For example, although the mean dietary intakes of energy was consistently significantly lower in blacks than in whites in Chapters 2, 3, 4 and in protocol 2 of Chapter 5 of this thesis, this difference was only approaching significance in protocol 1 of the latter Chapter. Furthermore, the mean dietary intakes of phosphate were significantly lower in blacks than in whites in most studies of this thesis. The exceptions were in Chapter 4 and in protocol 1 of Chapter 5, where although the mean dietary intakes of this parameter were lower in blacks than in whites, this difference was not statistically significant. Similarly, the mean dietary intakes of potassium were significantly lower in blacks than in whites in most studies of this thesis albeit this was not statistically different in Chapter 4 of this thesis. The reasons for the aforementioned minor discrepancies are not clear. It is probable that it could be attributable to the small and varying number of subjects who participated in the various studies/Chapters of this thesis.

Table 6.1 Comparisons of mean dietary intakes (SE) of black and white subjects in the various studies/Chapters of this thesis

Nutrients	**Chapter 2**	**Chapter 3**	**Chapter 4**		**Chapter 5**			
					FO protocol		**FOE protocol**	
	Day 0	**Day 0**	**Day 0**	**Day 60**	**Day 0**	**Day 30**	**Day 0**	**Day 30**
Energy	SB<W*	SB<W*	SB<W*	SB<W*	SB<W**	SB<W**	SB<W*	SB<W*
Moisture	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*
Total Protein	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*
Fat	SB<W*	SB<W**	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}
Carbohydrate	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}
Fibre	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}
Total sugars	SB<W*	SB<W*	SB<W**	SB<W**	SB<W*	SB<W*	SB<W*	SB<W*
Oxalate	SB>W*	SB>W*	SB>W*	SB>W**	SB>W^{NS}	SB>W^{NS}	SB>W*	SB>W**
Calcium	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*	SB<W*
Magnesium	SB<W*	SB<W*	SB<W*	SB<W*	SB<W**	SB<W**	SB<W*	SB<W*
Phosphate	SB<W*	SB<W*	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W*	SB<W*
Potassium	SB<W*	SB<W*	SB<W^{NS}	SB<W^{NS}	SB<W**	SB<W**	SB<W*	SB<W*
Sodium	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}
Vitamin A	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}
Vitamin B6	SB<W^{NS}	SB<W**	SB=W^{NS}	SB=W^{NS}	SB<W^{NS}	SB=W^{NS}	SB<W^{NS}	SB<W^{NS}
Vitamin C	SB<W^{NS}	SB>W^{NS}	SB>W^{NS}	SB>W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}	SB<W^{NS}
Vitamin D	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB>W^{NS}	SB>W^{NS}	SB<W^{NS}	SB<W^{NS}
Vitamin E	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}	SB=W^{NS}

*(*Significance at $p \leq 0.05$, ** approaching significance, ^{NS}no significant differences)*

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6.3 Urinary analysis

It is uncertain whether the trends with respect to urine compositions for the various studies described in this thesis can be reliably compared due to the relatively small sample sizes. Moreover, it has been suggested that irrespective of differences or similarities in mean dietary intakes, differences in urinary outputs might occur due to variations in gastrointestinal uptake of nutrients between subjects irrespective of race group (Fellström *et al.* 1989).

Analysis of spot urine samples

In the present thesis, data of mean urine parameters obtained from spot morning urine samples in Chapter 2 could not be reliably compared with the data obtained from 24hr urine samples in Chapters 3 to 5. Instead, it was compared with those of a study by Ogawa and co-workers (2003) which have also reported measurements of urine parameters in spot morning urine samples in healthy subjects.

It is noted that more parameters (*pH, volume, sodium, potassium, urate, phosphate*) were measured in the present study as compared to the latter study. Therefore, only the parameters (*calcium, oxalate, citrate and magnesium*) that were measured in the latter study were compared between the two studies. These parameters were corrected for creatinine excretions and were expressed as mg parameter/mg creatinine in both studies.

As shown in table 6.2, data for healthy black subjects obtained in the present study are similar to that of the study by Ogawa and co-workers (2003) albeit urinary excretion of magnesium appears to be higher in the latter study. In addition, the values for urinary excretions of citrate of these two groups were relatively higher than those obtained for healthy white subjects in the present study. The observation of a relatively higher mean urinary citrate excretion in black subjects than in whites has been previously reported (Lewandowski *et al.* 2001) although studies have not been consistent in this regard (Modlin 1967, Whalley *et al.* 1998). Given that citrate is one of the most widely recognized natural inhibitors of calcium oxalate stone formation (Fleisch 1978, Pak 1994, Ryall 1997, Chow *et al.* 2004, Rodgers *et al.* 2005). The observation of higher values of

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this parameter in blacks although not statistically significant in the present study (*p=0.1588*), is consistent with their lower stone incidence. However, as has been previously pointed in this thesis, values lie within the normal range. As such higher urinary citrate in blacks could account for a relatively lower stone incidence in this group, but cannot adequately account for absolute rarity of this disease in this group. It is extremely interesting that although the race of participants was not identified in the study by Ogawa and co-workers (2003), their study was conducted in Okinawa, Japan, where the inhabitants also have a low incidence of stone formation (Yoshida and Okada 1990).

Table 6.2 Comparisons of mean spot urine parameters from urines of black and white subjects in Chapter 2 of this thesis (SE) with that from a previous study (SD)

Urine parameters *(mg parameter /mg creatinine)*	**Present study** *(Chapter 2)*		**Previous study***
	Blacks (SB)	Whites (W)	Healthy Subjects
Calcium	0.0650(0.02)	0.0412(0.008)	0.116(0.078)
Oxalate	0.0105(0.002)	0.0116(0.003)	0.020(0.015)
Citrate	0.3198(012)	0.1634(0.06)	0.480(0.324)
Magnesium	0.0116(0.003)	0.0141(0.005)	0.074(0.043)

* Ogawa *et al.* 2003

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Analysis of 24hr urine samples

Data comparisons of mean urine parameters from 24hr urine samples of black and white subjects in Chapters 3 to 5 of the present thesis (*table 6.3*) show that the parameters pH, volume, oxalate, creatinine and uric acid were not significantly different between the two race groups while urinary excretions of calcium and magnesium were significantly lower in blacks than in whites in studies of Chapters 3, 4 and 5 of this thesis.

There were variable differences in the mean urinary excretions of 24hr urine parameters in the various studies of this thesis. For example, although urinary potassium and phosphate excretions were lower in blacks than in whites, these differences only reached statistical significance in protocol 2 of Chapter 5. In addition, there were no significant differences in the urinary excretions of citrate between blacks and whites in Chapters 4 and 5 of this thesis, although this parameter was significantly lower in blacks than in whites in the latter Chapter.

The mean urinary excretions of sodium of black and white subjects who participated in the various studies of this thesis were inconsistent. It was significantly lower in blacks than in whites in Chapter 3 and in protocol 1 of Chapter 5, while it was significantly higher in blacks than in whites in Chapter 4 and there were no significant difference for this parameter in protocol 2 of Chapter 5. These aforementioned observations of differences in the urinary excretions of these risk factors of stone formation in black and white subjects are in conformity with values reported by other workers who have investigated 24hr urine parameters in the two race groups (Modlin 1967, Whalley *et al.* 1998, Lewandowski *et al.* 2001, Rodgers and Lewandowski 2002, Lewandowski and Rodgers 2004).

The inconsistencies in the findings of urinary excretions of sodium and citrate in the two race groups in the present study as well as in the studies by other workers are noteworthy. Some authors have observed a significantly higher urinary excretion of sodium in blacks than in whites (Modlin 1967, Whalley *et al.* 1998, Lewandowski and Rodgers 2004) while in a study by Rodgers and Lewandowski (2002), it was reported that blacks had significantly lower mean urinary sodium excretions than whites. It is noted that the urinary excretions of this parameter were not significantly different in the latter two studies. Equally inconsistent is the findings of urinary excretions of citrate in the two race groups. Studies have observed significantly lower (Modlin 1967, Whalley *et al.* 1998), no significant differences (Rodgers and Lewandowski 2002) or significantly higher (Lewandowski and Rodgers 2004) excretions of this parameter in blacks than in whites. The reasons for these discrepancies are not clear in literature. Further research is warranted in this area.

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Table 6.3 Comparisons of mean 24hr urine parameters from urines of black and white subjects obtained at baseline in Chapters 3 to 5 of this thesis

Urine parameters	**Chapter 3** SB vs W	**Chapter 4** SB vs W	**Chapter 5**	
			Protocol 1 SB vs W	**Protocol 2** SB vs W
pH	$SB = W^{NS}$	$SB = W^{NS}$	$SB = W^{NS}$	$SB = W^{NS}$
Volume	$SB < W^{NS}$	$SB = W^{NS}$	$SB < W^{NS}$	$SB = W^{NS}$
Citrate	$SB < W^{*}$	$SB = W^{NS}$	$SB = W^{NS}$	$SB = W^{NS}$
Oxalate	$SB = W^{NS}$	$SB = W^{NS}$	$SB = W^{NS}$	$SB = W^{NS}$
Calcium	$SB < W^{*}$	$SB < W^{*}$	$SB < W^{*}$	$SB < W^{*}$
Magnesium	$SB < W^{*}$	$SB < W^{*}$	$SB < W^{*}$	$SB < W^{*}$
Sodium	$SB < W^{*}$	$SB > W^{*}$	$SB < W^{**}$	$SB = W^{NS}$
Potassium	$SB < W^{NS}$	$SB < W^{NS}$	$SB < W^{NS}$	$SB < W^{*}$
Urate	$SB < W^{NS}$	$SB < W^{NS}$	$SB < W^{NS}$	$SB < W^{NS}$
Creatinine	$SB < W^{NS}$	$SB = W^{NS}$	$SB = W^{NS}$	$SB < W^{NS}$
Phosphate	$SB < W^{NS}$	$SB < W^{NS}$	$SB < W^{NS}$	$SB < W^{**}$

*(*Significance at p≤0.05, **approaching significance, NS no significant differences)*

6.4 Concluding remarks

The present thesis was focused at investigating the genetic, gastrointestinal, renal and dietary factors in white and black South African subjects as a possible key to understanding the relative absence of calcium oxalate kidney stone disease in the black population. It is deemed appropriate to appraise the degree to which the main objectives of the various studies of this thesis were accomplished.

The absence of any genetic studies attempting to explain this anomaly prompted the present author to investigate whether the frequency of the alanine:glyoxylate aminotransferase Proline11Leucine polymorphism (*a genetic aspect*) might be a contributory factor towards explaining the differences in stone incidences between the two race groups. The most important finding of Chapter 2 of this thesis was that the frequency of this polymorphism is similar in the two race groups and therefore does not appear to explain the variation in stone occurrence between subjects from the two race groups who participated in this study. This observation is being reported for the first time in the present thesis. Thus, researchers of stone risk factors in the two race groups can in future focus their attention on other genetic and non-genetic factors in attempts to

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elucidate the reasons for the differences in stone incidences between the two race groups with some degree of confidence in the knowledge that this polymorphism does not seem to play a key role in this anomaly.

Previous studies that have investigated urinary oxalate excretion (*the most important risk factor for calcium oxalate stone formation*) in the two race groups have yielded surprising results in light of the low incidence of stone formation in the black group. In most cases, the results have been contrary to that which might have been reasonably expected when comparing relatively stone-free and stone-prone population groups. For example, it has been shown that despite the hyperoxalurogenic eating habits of the black population, urinary oxalate excretion is within the normal range in this group.

In Chapter 3 of this thesis, the role of gut permeability as a possible key in identifying possible differences in the handling mechanisms of oxalate in the two race groups following various dietary challenges was investigated. The most important finding of this study was that the gut permeability index was slightly higher in black subjects than in white subjects when on a high oxalate standardized diet. This is counter-intuitive considering the low incidence of stone formation in the black group. Equally surprising was the finding that blacks handled oxalate more transiently than their white compatriots. The factors that might contribute to this anomaly were fully described in Chapter 3 of the present thesis (*section 3.5*). Further research is warranted in this area as it is the present author's contention that elucidation of factors contributing to the different handling mechanisms of oxalate in the two race groups might shed more light in the low incidence of stone formation in the black group.

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The important finding in Chapter 4 of the present thesis was that administration of vitamin E in the two race groups provoked a favourable and significant increase in the mean urinary excretions of citrate coupled with a favourable and significant increase of plasma α-tocopherol levels in both groups. This supports the protective role of vitamin E ingestion in calcium oxalate urolithiasis albeit it does not explain the differences in stone incidences in the two South African race groups. Nonetheless, clinicians can be assured that this intervention can be offered as another mode of therapy in the quest for treatment modalities in urolithiasis. However, caution should be exercised, as

undesirable side effects have been attributed to prolonged administration of vitamin E in a daily dose greater than 300 mg (Miller III *et al.* 2005).

In consideration of the fact that there is a theoretical concern, that overwhelming the human system with one constituent could damage it as a whole in the long term (Herbert 1998, Emmert and Kirchner 1999) and the potential for increased oxidative stress following fish oil ingestion (Yamamoto *et al.* 1985, Bartoli *et al.* 1988, Meydani *et al.* 1993, Wander *et al.* 1996, Wood *et al.* 2003) as well as the present author's interest in vitamin E therapy, the effects of n-3 fish oil ingestion alone and in tandem with vitamin E ingestion was investigated in Chapter 5 of this thesis. The important finding in this Chapter was that following administration of fish oil alone and in tandem with vitamin E, there were significantly higher plasma α-tocopherol levels in whites than in blacks in both protocols. Although this is counter-intuitive considering the lower incidence of stone formation in the black group, it is worthy of attention as it tentatively points towards therapeutic properties of this nutrient in this small group of white subjects.

The aforementioned synopsis depicts findings of various investigations in the two race groups that are being reported for the very first time in this thesis. Thus, many other researchers in the urolithiasis field might benefit a lot from the knowledge generated in these studies. In retrospect, the present thesis has made significant contributions to the body of scientific knowledge.

With regard to the overall objective of this thesis, that is gaining more insights into the complex nature of the physicochemical, biochemical and physiological mechanisms in black South Africans which provide this group with a natural protection against urolithiasis in contrast to their white compatriots, it is noted that profound revelations did not emerge. However, the type of studies described in this thesis are "core" for gaining a fundamental, basic understanding of this anomaly and have the potential to provide the "key" mentioned in the title of this thesis. The philosophy and the approach remain sound. Clearly, many more subjects are needed for firm conclusions to be drawn.

The results of this thesis have demonstrated that explanation of the phenomenon of stone rarity in blacks is extremely complex – no simple answers exist. Synergies of multiple physicochemical, physiological, gastrointestinal, renal and other mechanisms

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are likely to be involved. Perhaps, an aspect that could be responsible for the observations from studies in this thesis is the origin of selected individuals of the black group. It is possible that changes in the ancient habits of the black population to the habits of modern industrial civilization had also implied an increase of the risk of stone formation capacity in blacks, similar to that of white population. The results of this thesis serve to motivate further studies to elucidate this intriguing phenomenon.

One such study might be to investigate whether the difference in stone incidence between blacks and whites reflects reduced Randall's plaques formation in blacks or whether they have similar amounts of plaques to whites but fail to grow calcium oxalate upon it. It might also be interesting to evaluate the morphoanatomic features of the kidneys of black population. The lack of differences between common urinary biochemical parameters of the two populations seems to point out that the black population has a specially protected kidney against stone development. This could involve, for example, the presence of a slight deep calyx and with few void spaces in the interior of their kidneys. Another aspect that should not be forgotten, is the Modlin hypothesis about the role that phytate, as a powerful crystallization inhibitor, could play as an important protective function in the black population, due to their dietetic habits. Further studies are warranted in these areas.

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6.5 References

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