Dietary and Microbiological Investigation in South African Black and White Subjects as a Key to Understanding the Difference in the Incidence of Kidney Stone Disease in the Two Race Groups

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Thesis presented for the degree of
Doctor of Philosophy
in the Department of Chemistry
University of Cape Town

September 2003

Acknowledgements

I would like to extend my sincere gratitude to the following:

Professor Allen Rodgers for all his guidance, mentoring and expert advice.

Dr Val Abratt and Dr Sharon Reid for all their enthusiasm and boundless patience.

Diane Pinnock, Gretchen Baretta, Jeanette Durbach for all their assistance in the Lab, caring support and friendship.

All my colleagues and friends in the Kidney Stone Lab and the Anaerobe unit for their assistance, friendship and encouragement.

Special thanks to my mom Elisabeth Burda for her endless love, trust and financial support.

Publications

Parts of the thesis published in refereed journals

- Lewandowski S, Rodgers AL, Schloss I. The influence of a high-oxalate diet on calcium oxalate renal stone risk factors in non-stone-forming black and white South African subjects. Br J Urol 2001, 87: 1-5.

- Rodgers AL, Lewandowski S. Effects of five different diets on urinary risk factors for calcium oxalate kidney stone formation: Evidence of different renal handling mechanisms in different race groups. J Urol 2002, 168: 931-936.

- Lewandowski S, Rodgers AL. Evidence of renal resistance to dietary challenges in a stone-free population. J Urol (submitted to Kidney International).

Invited review article

- Lewandowski S, Rodgers AL. Idiopathic Calcium Oxalate Urolithiasis: Risk Factors And Conservative Treatment. Clin Chim Acta 2003 (in press).

Parts of the thesis presented at international conferences (poster)

- Lewandowski S, Rodgers AL, Schloss I. The influence of a high oxalate diet on the risk of calcium oxalate renal stone formation in healthy black and white South Africans. In: Rodgers AL, Hibbert B, Hess B, Khan S, Preminger GM, editors, Urolithiasis 2000: Proceedings of the 9th International Symposium on Urolithiasis. Volume 1. Cape Town, South Africa, 2000: 300.

- Lewandowski Sonja, Rodgers AL: Investigation of the effect of two separate lithogenic diets on calcium oxalate urinary risk factors in healthy black and white South African subjects. In: Kok DJ, Romijn HC, Verhagen PCMS, Verkoelen CF, editors, Eurolithiasis: Proceedings of the 9th European Symposium on Urolithiasis. Rotterdam, Netherlands, 2001: 90.

- Lewandowski Sonja, Rodgers AL. The effect of different dietary supplements on the urine chemistry of black and white South African subjects. Urol Res 2003; 31(2): 117 (A154).
or
- Lewandowski Sonja, Rodgers AL: The effect of different dietary supplements on the urine chemistry of black and white South African subjects. In: Sarica K, Yağci F, Erbağci A, İnal Y, editors, Urolithiasis: Proceedings of the 10th European Symposium on Urolithiasis. Istanbul, Turkey, 2003: 242.

Parts of the thesis presented at international conferences (oral)

- Lewandowski S, Rodgers AL. Calcium Oxalate Kidney Stones: Dietary Advice as First-line Treatment. SAJCN 2002; 15: S20.

- Lewandowski S, Rodgers AL. The Effect of two Lithogenic Diets on Calcium Oxalate Kidney Stone Risk Factors in Healthy Black and White South African Subjects. SAJCN 2002; 15: S20.

Summary

Kidney stones occur in the South African white population to the same extent as in other western countries. However, the incidence of this disease in the South African black population is extremely rare. In this thesis, investigations were undertaken to establish whether different renal handling mechanisms occur in the two race groups in response to different dietary challenges and whether there might be qualitative and quantitative differences in the colonization and utilization of oxalate-degrading bacteria in the two race groups which might account for this phenomenon.

With respect to the dietary effects, lithogenic and antilithogenic dietary and supplemental protocols were administered to healthy volunteer subjects from both race groups. The different challenges which were investigated are low calcium/high oxalate (in combination), low calcium, high calcium, high oxalate, vegetarian, vitamin C-rich, high salt, supplemental calcium, supplemental vitamin B_6, supplemental L-cysteine and supplemental L-glutamine. These protocols were administered under controlled conditions. Urine samples (24h) were collected at baseline and at various times during each protocol. These were rigorously analyzed for biochemical risk factors and were also subjected to crystallization studies in which calcium oxalate metastable limits and crystal volume-size distributions were determined. Physicochemical risk factors such as the relative supersaturation and Tiselius Index were computed from the urinary parameters.

The results of the different challenges were the following: for the low calcium/high oxalate (in combination) the only urinary parameter which changed significantly was urinary oxalate which increased by 57% ($p=0.022$) in whites; the low calcium diet caused statistically significant changes only in blacks: urinary oxalate increased ($p=0.01$), the relative supersaturation of calcium oxalate decreased ($p=0.03$) and the relative supersaturation of brushite increased ($p=0.03$). The high oxalate dietary challenge caused statistically significant changes in both race groups. However, these changes were different in the two groups. In whites, urinary pH increased ($p=0.01$), potassium excretion increased ($p=0.01$) and relative supersaturation of brushite increased ($p=0.05$). In blacks, urinary citrate was raised ($p=0.01$). None of the high dietary calcium, calcium supplement, vitamin B_6 supplement, L-glutamine supplement and L-cysteine supplement protocols altered the urine biochemistry in black subjects. However In whites, the high Ca 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 ($p=0.014$); the vitamin B_6

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); the L-cystine supplement significantly decreased urinary calcium (p=0.031) and the Tiselius Risk Index (p=0.013).

With respect to microbiological aspects, urine and faecal samples were obtained from healthy volunteer subjects of both race groups as well as from white calcium oxalate kidney stone formers. The participating subjects were on their free diets. Urines were analyzed as previously described. Faecal samples were analyzed for oxalate degrading bacteria by using a selective oxalate medium and characterized by gram staining. Bacterial growth was determined spectrophotometrically while oxalate utilization was determined using a calcium chloride precipitation assay. Black subjects were found to have significantly higher counts of oxalate-degrading bacteria (ODB) as well as *Lactobacillus* bacteria relative to whites (p=0.014, p=0.05 respectively) and to stone formers (p=0.014, p=0.048 respectively), while stone formers had significantly less ODB (p=0.001) than healthy whites. The oxalate-degrading bacteria isolated from blacks utilized significantly more oxalate relative to healthy whites (p=0.001) and stone formers (p=0.048).

In each of the above investigations, dietary histories were recorded by means of different food frequency and recall questionnaires. Data collected in this way allowed for the analysis of food and nutrient intakes in the two race groups. The dietary intakes of blacks and whites differed significantly in the amount of total protein, animal protein, calcium, magnesium, total sugar, vitamin B_6 and vitamin C they consumed. Consumption in all cases was higher in the whites. This signifies that dietary factors which are important for assessing urinary risk factor for stone formation are different in the two groups, and it is likely that other gastrointestinal or renal oxalate handling mechanism exist that provide blacks with stone immunity despite their lithogenic eating habits.

Results for the dietary and supplemental challenges provided compelling evidence for the hypothesis that different renal handling mechanisms operate in the two race groups thereby enabling South African blacks to achieve a homeostatic balance of urinary solutes that does not occur in white subjects. The microbiological results showed that black subjects have significantly more oxalate degrading bacteria as well as other lactic acid bacteria relative to healthy and stone-forming white subjects and that the oxalate-degrading capability of the bacteria isolated from black subjects is significantly greater than that of the other two groups.

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Abbreviations

abs	absorbance
BHI	Brain heart infusion
bp	base pairs
CaOx	calcium oxalate
CF	cystic fibrosis
cfe	cell free extract
cfu	colony forming units
dH_2O	distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	gram(s)
GI	gastro-intestinal
hrs	hours
ht	height
l	litre(s)
m	metre(s)
min	minute(s)
MRS	De Man Rogosa Sharpe
msl	metastable limit
nm	nanometres
OD	optical density
OX	oxalate medium
p	p-value
PCR	polymerase chain reaction
RCA-17	Reinforced clostridium agar
RE	retinol equivalents
RS brushite	relative supersaturation of brushite
RS CaOx	relative supersaturation of calcium oxalate
RS uric acid	relative supersaturation of uric acid
SE	standard error
SF	stone formers
t	time
THM	Tamm-Horsfall mucoprotein
UPTF1	urinary prothrombin fragment 1
VIS	visible light
w/v	weight per volume
wt	weight
µ	micro

ChapterOne

General Introduction

1.1 Introduction

There are several types of urinary stones based on chemical composition. Pure calcium oxalate stones or those which contain calcium phosphate are by far the most common, occurring in 70 to 80 percent of stone sufferers. The majority is idiopathic, indicating the absence of any known clinical cause of the disease.

Idiopathic calcium oxalate urolithiasis is not only common, painful and costly but also multifactorial in its pathogenesis. In this general introduction four main aspects of this disease will be discussed. Firstly, epidemiological factors such as climate, occupation and its variable occurrence in different countries and races will be presented. Secondly important physiochemical factors that influence the formation of kidney stones, including pH and urinary concentrations of oxalate, calcium, uric acid and citrate will be discussed. Thirdly, dietary *patterns and dietary nutrients which have been directly or indirectly implicated with* lithogenicity and conservative treatment will be discussed. Finally the role of oxalate-degrading bacteria, specifically *Oxalobacter formigenes* will be presented.

1.2 Epidemiology

Prevalence, Recurrence Rate and Economics

The incidence of renal calculus in economically developed western countries has increased *considerably, far greater than can be explained merely by the improved methods of diagnosis* during the last 90 years. It has been postulated that the steady increase of calcium oxalate urolithiasis in the last century correlates with the rising economic development in some countries [Rao *et al.* 1982b] and with the net income of population groups [Robertson *et al. 1981*].

In wealthy countries like Saudi Arabia the prevalence of kidney stone formation is greater than 20% [Robertson *et al.* 1994]. In other developed countries the risk of developing stones is reported to be 13% in North America, 12% in Canada and in 5-9% in Europe [Ramello *et al.*

2000]. While epidemiological studies have shown that the average prevalence of renal stones in males is between 7 and 15%, it is only between 3 and 6% in females [Scott *et al.* 1977, Ljunghall 1977, Robertson *et al.* 1983a]. It is not clear why women have a lower incidence of renal stones. Women could be protected from stone disease by very high citrates, which act as urinary inhibitor [Parks and Coe 1986]. Others, however, have not always corroborated this finding [Conte *et al.* 1989]. Furthermore, the occurrence of elevated uric acid excretion rates in a greater proportion of male (34%) than female (14%) stone-forming patients and in more control males (12%) than females (0%) could be a possible explanation [Coe 1978]. There is new evidence that oestrogen protects women against kidney stone formation by lowering the urinary saturation of stone-forming salts [Heller *et al.* 2002]. Conversely androgens appear to increase urinary oxalate and urinary calcium-oxalate saturation [Fan *et al.* 1999].

The recurrence rate of calcium oxalate renal stones is reported to be common, ranging from 40% within 3 years, 74% within 10 years to 98% within 25 years [Robertson 1999a, Sutherland *et al.* 1985, Ljunghall and Danielson 1984]. This poses a major strain on the budget of health care and research institutions. The estimated cost for treating urolithiasis varies among countries, choice of treatment and stone-frequency. The estimated annual cost for treating stone disease ranges from $1.83 billion in the United States [Chandohke 2002], £111.333million in the United Kingdom [Robertson 1999a] to DM103.32 million (54.38 million euros) in Germany [Strohmaier and Hörmann 2000]. With the introduction of new medical techniques like extracorporeal shock-wave lithotripsy, the cost-effectiveness of conservative and/or medical treatment has become debateable. Taking into consideration the high frequency of stone recurrence, recent studies suggest that medical prophylaxis can save $2.158 per patient per year in the United States [Parks and Coe 1996], £64.624 in the United Kingdom [Robertson 2000], and DM 333.1 (175.31 euros) in Germany [Strohmaier and Hörmann 2000].

Climate

Although renal stones are common among the western economically developed races, the incidence is far from uniform. There are areas in Europe, United States and Africa in which the incidence is considerably higher than the average. One of these so-called "stone belts" stretches through the south-eastern United States including Florida, Georgia, South Carolina and North Carolina. It is postulated that the hot climate in such geographical areas contributes to dehydration and hence decreased urinary volume, increased urine osmolality, increased concentration of calcium and oxalate, and increased urine pH. These are all urinary variables known to increase the risk of stone formation [Schwille and Herrmann 1992]. Another theory is that prolonged exposure of the skin to the UV portion of sunlight activates the conversion of 7-dehydrocholesterol to vitamin D_3 (also called cholecalciferol). Vitamin D_3 and its hydroxylated derivative 25-hydroxyvitamin D_3, act on intestine and bone to increase plasma Ca^{2+} and PO_4^{3-} levels resulting in increased intestinal calcium absorption, calciuria and stone frequency [Broadus *et al.* 1984]. However, there are countries like South Africa in which there is a hot climate and yet the stone incidence is low [Modlin 1967, Wise and Kark 1961]. In addition, the 25-hydroxyvitamin D_3 is generally normal in the Saudi population where the climate is extremely hot and stone prevalence is the highest [Sedrani *et al.* 1991]. Thus, the hypothesis that climate has an affect on the risk of calcium oxalate stone formation must be investigated further.

Occupation

There is evidence suggesting that people in certain occupations experience higher risk of calcium oxalate urolithiasis [Borghi *et al.* 1993, Ferrie and Scott 1984]. It seems that certain nephrotoxic effects of hot-metal fumes increase the incidence of kidney stones in some occupations [Ferrie and Scott 1984]. In addition, sedentary occupations like aviation pilots or truck drivers have been noted as having a higher incidence of stone formation [Borghi *et al.* 1993, Zheng *et al.* 2002]. A possible explanation for the latter could be that infrequent voiding

increases the urinary concentration of stone forming salts. However, further studies are required to understand the occupational aspects of stone formation.

Race

The incidence of renal stones varies remarkably between countries as well as within different races in the same country. For example, in South Africa 15% of the white population are at risk of developing CaOx kidney stone disease in their lifetime. In the same country less than 1% of the black population form CaOx stones [Modlin 1967, Wise and Kark 1961, Meyers 1994]. South African blacks appear to have a natural immunity to this disease [Wise and Kark 1961, Muskat 1951]. This phenomenon will be discussed more fully in chapter 1.6. Although other race groups like Eskimos, Aborigines and American blacks have also been associated with lower prevalence for stones, comprehensive research data is lacking [Bateson 1977, Rodgers 1991, Widdowson and McCance 1970]. Currently, only South African blacks are being investigated (see chapter 1.6).

1.3 Urinary Physiochemical Risk Factors And Inhibitors

Urinary Calcium

Until recently, raised urinary calcium was considered the most important urinary risk factor for calcium oxalate urolithiasis [Rao *et al.* 1982b, Marshall *et al.* 1972, Vahlensieck 1986, Bleich *et al.* 1979, Rose 1987, Goldfarb 1994]. Researchers reached this conclusion after observing that the calcium ion is common both in calcium oxalate and calcium phosphate stones and that there is a significantly higher incidence of idiopathic hypercalciuria in stone formers (approx 50%) compared to their normal controls [Pak 1998, Heilberg 2000]. A factor that had been overlooked for a long time is that urinary excretion of oxalate is fifteen times more potent in terms of urinary supersaturation than urinary calcium [Robertson and Peacock 1980]. A simple chemical experiment demonstrated that only a small quantity of sodium

oxalate was required to initiate spontaneous crystallization, whereas it was almost impossible to initiate crystallization by adding calcium chloride [Robertson 1999b].

In the 1970's and 1980's various studies supported the view that patients with idiopathic hypercalciuria presented with either absorptive or renal hypercalciuria [Pak *et al.* 1975, Coe and Favus 1986]. Patients with hyperabsorption of calcium absorbed more calcium from food than healthy subjects, thus raising blood levels to such an extent that increased renal calcium excretion followed. On the other hand renal hypercalciuria presented with increased PTH and calcitriol levels. Since then, Coe *et al.* (1982, 1997) demonstrated that absorptive and renal hypercalciuria are inherently the same condition resulting from an abnormal hydroxylation of 1,25 vitamin D_3 [Coe *et al.* 1982, Coe *et al.* 1997].

Urinary Oxalate

Oxalate is a dicarboxylate anion and it forms an insoluble salt with calcium in urine. At neutral pH, only 0.67mg of calcium oxalate salt will dissolve per 100ml of water. Owing to the extreme insolubility of the latter, urine is often supersaturated with this salt and stone formation occurs. It is thus not surprising that 65%-80% of all urinary stones contain calcium oxalate.

Many 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]. In a comprehensive review, Robertson (1999) reports a linear relationship between stone disease and urinary oxalate, as well as between stone prevalence in various countries and the urinary oxalate excretion in the population. He concludes that urinary oxalate is the most critical determinant of stone prevalence by far, compared to other risk factors like hypercalciuria, hyperuricuria, and hypocitraturia.

Four mechanisms have been identified that account for increased urinary oxalate excretion. Firstly increased dietary intake of oxalate allows for more oxalate to reach the colon, increasing the availability of more oxalate for absorption. Secondly intestinal hyperabsorption

of oxalate is an important mechanism. Factors promoting oxalate absorption in the gut include bile acid malabsorption (which increases the permeability of the colon to oxalate), malabsorption of fatty acids (which may decrease the amount of intraluminal calcium available for binding with oxalate as for example in patients with ileal resection [Earnest *et al.* 1974]) and certain fatty acids that increase colon permeability to oxalate absorption. Thirdly, increased endogenous production of oxalate is yet another mechanism. However, data suggest that this is unlikely to be the cause of raised urinary oxalate in idiopathic calcium oxalate stone formers [Robertson 1999b]. Finally, a deficiency of oxalate-degrading bacteria (in particular *Oxalobacter formigenes*) has been suggested as causing an increase in the risk of hyperoxaluria and subsequently calcium oxalate urolithiasis [Allison *et al.* 1986]. By utilizing oxalate in the digestive tract, oxalate-degrading bacteria regulate oxalate homeostasis and prevent excessive enteric absorption of oxalate. This aspect of stone disease is discussed more fully in section 1.5 (p 22)

Urinary Uric Acid

Increased urinary uric acid is another risk factor for the formation, growth and aggregation of calcium oxalate crystals [Robertson *et al.* 1978, Griffith *et al.* 1986]. Approximately one third of patients with calcium oxalate stones have raised urinary uric acid excretion [Coe 1978]. Coe (1978) have suggested two possible mechanisms for the latter: excessive intake of dietary protein (70% of cases) and endogenous overproduction of uric acid (30% of cases). The most plausible hypothesis explaining the link between uric acid and calcium oxalate is the "salting-out" effect described by Ryall *et al.* (1986) in which uric acid increases the tendency for calcium oxalate crystals to aggregate into large masses.

There is evidence that calcium oxalate kidney stone patients with hyperuricosuria respond to Allopurinol treatment, decreasing urinary uric acid by 40 percent [Pak *et al.* 1978, Ettinger 1991]. This drug has a direct effect on uric acid by blocking its production [Ettinger 1991] and although it has no effect on urinary calcium or oxalate saturation [Tiselius *et al.* 1986, Baggio

et al. 1983] evidence shows it decrease CaOx stone recurrence rate in patients with hyperuricosuria [Ettinger 1991, Pak 1982].

Urinary Citrate

Citrate is recognized to inhibit nucleation [Hallson *et al.* 1983, Nicar *et al.* 1987, Schwille *et al.* 1999], growth [Ryall *et al.* 1981, Bek-Jensen *et al.* 1996] and aggregation [Ryall *et al.* 1981, Kok *et al.* 1987, Tiselius *et al.* 1993] of calcium oxalate and calcium phosphate crystallization. By complexing calcium, citrate reduces the ionic calcium concentration in urine. In addition it inhibits spontaneous precipitation of calcium oxalate, retards calcium oxalate crystal agglomeration, and inhibits crystal growth of calcium phosphate [Nicar *et al.* 1987, Kok *et al.* 1987, Meyer and Smith 1975]. Furthermore several studies have demonstrated hypocitraturia in stone formers (19 to 63%), thus demonstrating the importance of citrate as an inhibitor of urolithiasis [Rudman *et al.* 1982, Nicar *et al.* 1983, Pak *et al.* 1985, Laminski *et al.* 1990, Cupisti *et al.* 1992]. Hypocitraturia is also associated with acidosis (as for example in cases of chronic diarrhoea and renal tubular acidosis, strenuous exercise and high animal protein diets), hypercalciuria and hyperuricosuria [Nicar *et al.* 1983].

In view of the above-mentioned factors, hypocitraturia is considered an important risk factor for renal stone formation. The general consensus among researchers is that calcium oxalate stone-formers with lower urinary citrate excretion compared to controls should undergo alkali therapy to correct their citrate status [Parks and Coe 1986, Conte *et al.* 1989].

Urinary pH

An alkaline urinary pH seems to reduce the risk of calcium oxalate crystallization [Kohri *et al.* 1991, Pak 1994]. This is to a great extent because phosphate and citrate ions become dissociated at higher pH increasing the complexation of calcium, which lowers the urinary saturation of calcium oxalate [Pak 1994]. This increased complex formation between calcium and phosphate that occurs with increasing alkalinity of the urine may increases the risk of calcium phosphate (brushite) crystallization especially if hypercalciuria is present [Tiselius *et*

al. 1978]. Since the majority of urinary stones are composed of CaOx and CaP in mixture the risk of CaP crystallization in patients undergoing therapeutic alkalinization in order to reduce CaOx formation should be monitored [Tiselius 1984]. On the other hand a pH less than 5.5 may be associated with uric acid stone formation because of the predominance of undissociated uric acid [Finlayson and Smith 1974]. Thus it would be desirable to keep the urinary pH between 6-7 because firstly, in this range, the concentration of undissociated uric acid is low and secondly, there is an enhanced inhibitory activity of citrate and pyrophosphate because more of these species would be in active ionic form [Pak 1994, Messa et al. 1997].

1.4 Diet, Drinking Water and Supplements

As stated earlier, urolithiasis is a complex, multifaceted disease in which diet is one of the most important determinants of urine chemistry. Many excellent research studies have analyzed the relationship between dietary intake of various nutrients and the excretion of lithogenic and inhibitory substances in the urine [Curhan 1993, Hesse *et al.* 1993, Goldfarb 1994, Massey *et al.* 1993, Trinchieri *et al.* 1991]. Body weight is another factor to be considered [Curhan et al. 1998].

Dietary Oxalate

Urinary oxalate is derived from exogenous and endogenous sources. The exogenous amount of oxalate absorbed from the gastrointestinal tract is relatively small, ranging in values from 2.3%-12% [Earnest *et al.* 1974]. There is evidence that dietary oxalate is directly related to urinary oxalate excretion [Finch *et al.* 1981, Brinkley *et al.* 1990]. The average contribution of dietary oxalate to urinary oxalate excretion differs among studies, ranging from 10-20% (earlier studies) [Finch *et al.* 1981] to 40% (more recent studies) [Holmes *et al.* 1999]. Holmes *et al.* (1999) demonstrated that increasing the dietary oxalate intake from 50mg to 250mg increased its urinary excretion by 24%. This provides evidence that urinary oxalate excretion can be modified by dietary intake.

Endogenous oxalate is produced from 2 major sources: approximately 40% is derived from the end product of ascorbate metabolism and approximately 40%-50% is derived from the

metabolic reaction involving glyoxalic acid [Massey *et al.* 1993, Robertson 1999b]. Approximately half of ingested oxalate is used as a substrate by enteric bacteria, and about 25% is excreted unchanged in the faeces [Robertson 1999b]. Oxalate absorption can occur anywhere along the gastrointestinal tract, but is found largely in the colon [Dobbins and Binder 1977].

Several studies have emphasized the influence that dietary calcium has on urinary oxalate. Data suggest that as dietary oxalate increases, urinary excretion thereof increases only when calcium is restricted [Marshall *et al.* 1972, Hess 1996, Breslau *et al.* 1988]. For example Hess *et al.* (1998) has demonstrated that a 20-fold increase of dietary oxalate increased urinary oxalate when it was administered with a normal calcium intake, but that a normal urinary oxalate excretion occurred when the oxalate load was given with a 3-fold increase of dietary calcium. This demonstrates the important influence dietary calcium has on oxalate at all levels - intestinal, renal and urinary. Similarly Marshall *et al.* (1972) studied the restriction of calcium, oxalate and calcium/oxalate in stone formers versus controls. They found that urinary oxalate significantly decreased only after dietary oxalate and calcium were restricted together [Marshall *et al.* 1972]. A more recent study did not find any such decrease in urinary oxalate after the combined restriction of calcium and oxalate in hypercalciuric patients [Bataille *et al* 1983]. Thus, Heilberg (2000) urges further investigation of the relationship between calcium intake and its colonic oxalate binding in hypercalciuric versus normocaliuric patients.

New insights into the gut colonization of the oxalate-degrading bacterium *Oxalobacter formigenes* calls for the re-examination of dietary oxalate restriction in CaOx stone formers. Besides being a fastidious anaerobic bacterium, *O. formigenes* is very selective, depending on a constant supply of oxalate as its carbon source. The question arises if the absence of O. *formigenes* which was shown in stone formers [Sidhu *et al.* 1999a] is related to the restriction of dietary oxalate. Furthermore the successful recolonisation of this bacterium as a preventative probiotic [Sidhu *et al.* 1999a] depends on dietary oxalate. As far as it is known,

there has not been a study investigating the relationship between dietary oxalate intake and optimal *O. formigenes* oxalate-utilization. Further studies are needed to clarify the dietary oxalate and/or calcium interaction and their influence on gastrointestinal and renal oxalate and/or calcium.

Researchers are generally in agreement that oxalate in the diet influences the level of urinary oxalate [Hesse *et al.* 1993, Kasidas and Rose 1980, Holmes and Kennedy 2000] and therefore it is important to advise calcium oxalate stone patients to reduce their intake of this nutrient. Foods which contain high levels of oxalate and which cause a significant increase in urinary oxalate excretion are spinach, rhubarb, beetroot, peanuts, chocolates, parsley, strawberries, wheat bran and tea [Massey *et al.* 1993, Hesse *et al.* 1993, Finch *et al.* 1981, Brinkley *et al.* 1990, Holmes and Kennedy 2000]. Spinach and rhubarb in particular lead to increases of urinary oxalate excretion of 300%-400% in the circadian excretion curve [Hesse *et al.* 1993]. Considerable variations in the oxalate content in plants can occur depending on the season, the species, variety, age, maturity and the part of the plant [Myers 1947, Kohman 1939], soil conditions during growth [Oke 1969], and also on measures taken to reduce its levels [Hodgkinson 1978]. The amount of oxalate in tea varies with the duration of the infusion time [Zarembski and Hodgkinson 1969]. Reliable methods to measure the oxalate content of foods are lacking and need to be addressed urgently [Massey *et al.* 1993].

Dietary Calcium

In the same way that reduced dietary oxalate minimizes urinary oxalate concentration and thus decreases calcium oxalate crystal formation, it would seem obvious that dietary calcium should be similarly decreased. Although this advice has been the mainstay for the prevention of recurrent kidney stone formation [Bleich *et al.* 1979, Robertson *et al.* 1987, Galosy *et al.* 1980] recent studies have demonstrated that this was inappropriate and even potentially dangerous [Messa *et al.* 1997, Curhan 1993, Bataille *et al.* 1983]. The most convincing study in this regard is that conducted by Curhan *et al.* (1993), in which the relationship between dietary calcium intake and the risk of kidney stones was investigated in a cohort study of

45,619 healthy men. After a period of 4 years, during which 8.7 percent of subjects developed stones, an inverse relationship between calcium intake and idiopathic calcium oxalate urolithiasis was demonstrated. Similarly, studies have found that dietary calcium restriction increases the relative supersaturation of calcium oxalate [Messa *et al.* 1997, Marshall *et al.* 1972]. Restricting dietary calcium reduces the binding (by calcium) of oxalate in the gut rendering more oxalate to be absorbed and excreted in the urine. Since urinary oxalate is the most important determinant for urinary calcium oxalate supersaturation, dietary calcium restriction will increase the risk of stone formation by increasing the supersaturation of this salt. Dietary calcium is not only important for minimizing urinary oxalate but also for keeping a positive calcium balance to prevent bone resorption observed in hypercalciuric patients [Heilberg *et al.* 1994, Zanchetta *et al.* 1996, Bataille *et al.* 1983, Jaeger *et al.* 1994]. A calcium intake of less than 400mg per day can increase in the risk of developing osteoporosis [Coe *et al.* 1982, Fuss *et al.* 1990]. Dietary advice should be focused on maintaining a calcium intake between 800mg and 1200mg of dietary calcium per day rather than restricting or exceeding its input [Wahl and Hess 2000].

Dietary Animal Protein

Other dietary regimes extensively studied are those which are high or low in animal protein. A high animal protein diet has been associated with the increased incidence of urolithiasis in the western world [Robertson *et al.* 1981]. Robertson *et al.* (1979) showed that an increase of 34g/day of animal protein in the diet significantly increased urinary calcium by 23%. In the same study it was reported that dietary animal protein increases urinary oxalate. More recent studies support the finding that purine-rich animal protein diets increase urinary calcium and urinary uric acid and decrease urinary citrate [Curhan 1993, Trinchieri *et al.* 1991, Brockis *et al.* 1982, Fellström *et al.* 1984, Nguyen *et al.* 2001].

Brockis and colleagues (1982) compared the dietary habits and urinary excretion patterns of 30 meat-eating subjects with those of vegetarian subjects. It was demonstrated that urinary calcium excretion increases as animal protein intake increases. The proposed mechanism is

that a dietary protein load increases the endogenous acid production with consequent metabolic acidosis resulting in increased calcium reabsorbtion of bone and increased urinary calcium excretion. In the same study animal protein consumption did not affect oxalate excretion. However, Nguyen *et al.* (2001) postulated that in approximately one third of idiopathic calcium oxalate stone formers urinary oxalate increase after an animal protein load.

Breslau *et al.* (1988) examined whether different types of dietary protein might have different effects on urinary calcium excretion, and therefore an effect on renal stone formation. The 15 subjects in the study received food containing vegetable protein, vegetable protein and egg protein, or animal protein. Each protocol was administered for 12 days. The diets were constant with respect to Na, K, Ca, P, Mg, and quantity of protein, but they had progressively higher sulphur contents. As the sulphur content increased from the vegetable protein diet to the animal protein diet, the urinary calcium excretion increased from 103 mg/day to 150 mg/day. It was hypothesized that sulphur-containing amino acids, derived mainly from animal protein, prevent calcium reabsorption by renal tubules by forming a complex, thereby increasing its urinary excretion [Schuette *et al.* 1980].

Further lithogenic effects of animal protein intake include an increased urinary excretion of uric acid [Breslau *et al.* 1988, Siener and Hesse 2002]. Conversely, vegetarian diets were associated with the reduction in urinary uric acid [Breslau *et al.* 1988, Robertson *et al.* 1979, Giannini *et al.* 1999]. In addition it is interesting that transient acidosis following a dietary protein load increases the tubular reabsorption of citrate [Fellström *et al.* 1984, Siener and Hesse 2002]. The resultant decrease in urinary citrate increases the risk of calcium oxalate urolithiasis. Siener and Hesse. (2002) reported the same result in a study where a vegetarian diet significantly increased the urinary citrate, pH and potassium, thereby decreasing the risk of urolithiasis. Another study observed no benefit in consuming a lacto-ovo-vegetarian diet [Massey and Kynast-Gales 2001].

Considering the importance of bone metabolism in CaOx urolithiasis the potential consequence of protein-induced bone resorption of calcium is of concern. Recent studies

questioned the hypothesis that the additional urinary calcium following a high protein diet stems from bone [Kerstetter *et al.* 2003, Dawson-Hughes 2003]. Kerstetter *et al.* (2003) administered low, medium and high protein diets to healthy subjects and found that the high protein diet (>75g/day) resulted in hypercaliuria without change in the serum parathyroid hormone and the low protein diet (0-43g/day) caused secondary hyperparathyroidism which resulted from a reduced intestinal calcium absorption. These findings suggest that dietary protein influences GIT calcium absorption and that very low protein diets are potentially harmful.

Ingesting more than 1.2 g of meat protein per kg body weight per day increases urinary excretion of uric acid and calcium and decreases urinary excretion of citrate [Breslau *et al.* 1988, Siener and Hesse. 2002]. In addition, recent data demonstrated that low protein diets might be potentially harmful to skeletal health [Kerstetter *et al.* 2003, Dawson-Hughes 2003]. Advice given to kidney stone formers is therefore to consume between 0.8 and 1.2 g of meat protein per kg body weight per day [Kerstetter *et al.* 2003, Wahl and Hess 2000].

Conversely, the intake of fruit and vegetables (except those rich in oxalate) is encouraged to provide many essential minerals/nutrients like magnesium, potassium, citrate and phytate that have been shown to positively affect urinary risk factors [Wahl and Hess 2000]. It is recommended to have at least 3 portions of fruit and vegetables per day.

Dietary Sodium Chloride

Sodium Chloride has received considerable attention due to its calciuric action. Researchers are in agreement that for each increase in 100mmol (2300mg) dietary NaCl, there is an approximate increase of 1.0mmol (40mg) in urinary calcium excretion [Blackwood *et al.* 1999, Lemann *et al.* 1995]. Studies by Kok *et al.* (1990) and Sakhaee *et al.* (1993) found that increasing dietary sodium chloride (from 140 to 310mmol/d and 50 to 250mmol/day respectively) increased urinary calcium by 34% and 31% respectively and decreased urinary citrate by 10% and 20% respectively. Thus, calciuria and hyopcitraturia are important

lithogenic consequences of excessive intake of dietary salt, thereby increasing the risk of calcium oxalate stone formation. In addition hypercaliuric stone patients seem to be twice as sensitive to salt loading as normocalciuric stone formers [Brutis *et al.* 1994].

Salt intake in westernized diets typically ranges between 150mmol/day (3.45grams) and 300mmol/day (6.9grams) largely due to the consumption of processed foods [Massey and Whiting 1996]. The average consumption of salt in stone formers is reported to be 200mmol/day [Trinchieri *et al.* 1998, Fuss *et al.* 1990]. It is important to note that the dietary assessment of salt is very inaccurate; therefore Massey *et al.* (1996) suggest that the urinary excretion of sodium is a more reliable indictor of salt intake. Also, Brutis *et al.* (1994) reported that, dietary salt is a much stronger predictor of urinary calcium than dietary calcium and protein, therefore stressing its importance.

Reducing dietary salt in kidney stone formers has a positive effect not only on urinary risk factors but also on bone turnover. The importance of bone metabolism in CaOx urolithiasis was highlighted when hypercalciuric stone formers on restricted calcium diets presented with negative calcium balance [Fuss *et al.* 1990]. Melton *et al.* (1998) found that calcium oxalate stone formers had 3.9 times the risk of bone fractures than control subjects. In addition when studying post-menopausal women, Devine *et al.* (1995) found that dietary salt intake was directly related to urinary calcium and bone mineral loss; similarity Matkovic *et al.* (1995) correlated peak bone mass to high salt intake in adolescent women.

Idiopathic calcium oxalate stone formers are advised to decrease their table salt intake to less than 100 mmol/day i.e. less than 4 grams per day. Calcium oxalate stone formers with hypercalciuria seem to benefit the most from NaCl restriction [Brutis *et al.* 1994].

Given the important effect that dietary salt and urinary sodium have on urinary risk factors for CaOx urolithiasis, more diet modification studies are called for. It might be warranted to include urinary sodium in computed relative risk indices (for example Tiselius risk index and relative supersaturation programs).

Dietary Carbohydrate / Refined Sugar

Some researchers have claimed that the higher incidence of stones in wealthier countries is due to an increased refined sugar intake [Robertson *et al.* 1978, Thom *et al.* 1981, Robertson 1987]. Leman *et al.* (1969) observed that glucose increases the rate of urinary calcium excretion both in normal and calcium oxalate stone forming subjects, although this increase was more prominent in the latter group. Similarly, other studies have revealed that glucose increases intestinal calcium absorption in a dose-dependent manner [Wood *et al.* 1987, Knowles *et al.* 1988].

Thom *et al.* (1981) observed very rapid urinary calcium excretion following ingestion of sucrose solutions and suggested that this was a hormonal phenomenon rather than a result of increased calcium absorption in the gut, which would have had a less immediate effect. Rao *et al.* (1982a) found a higher resting insulin level and a higher, more prolonged insulin peak in response to a carbohydrate-rich test meal in renal stone formers. Furthermore, in healthy subjects postprandial oxaluria in response to a refined-carbohydrate-rich test meal as well in response to glucose was demonstrated [Nguyen *et al.* 1986] but not in response to fructose [Nguyen *et al.* 1989]. An explanation could be that refined carbohydrates stimulate endogenous synthesis of oxalate, which is then eliminated via the urine. The nature of hyperinsulinemia is still unknown and to date there is no evidence that calcium oxalate renal stone formers have an increased intake of refined sugar.

On the basis of present knowledge, it can be summarized that because carbohydrates induce calciuria and oxaluria, their ingestion could increase the risk of stone formation. However, this warrants further investigation. Refined sugars (except fructose) may pose more of a risk than complex carbohydrates.

Dietary Fat

At cellular level, fats are thought to play an important secondary role to urolithiasis. Researchers suggest an abnormal renal phospholipid composition in calcium oxalate stone

formers that results in hyperoxaluria and hypercalciuria [Baggio *et al.* 1998, Kurien and Selvam 1989]. A disturbance in the cell membranes composition has a direct effect on ion channels, membrane bound receptors and membrane protein functions. Stone formers have been found with increased arachidonic acid in the membrane phospholibids which facilitates intestinal calcium absorption, hypercalciuria and an alteration in urinary excretion of prostaglandin 2 (PGE_2; phospholipid metabolite) [Kurien and Selvam 1989, Henriquez-La Roche *et al.* 1992]. Furthermore, essential fatty acid supplementation has been shown to reduce calcium, oxalate and PGE_2 in urine of calcium oxalate stone formers [Henriquez-La Roche *et al.* 1992, Hirayama *et al.* 1988]. For example Buck *et al.* (1991) administered dietary fish oil to hypercalciuric calcium oxalate stone patients for 8 weeks that resulted in a significant reduction in urinary oxalate and calcium. Essential fatty acids are probably beneficial because by increasing the eicosapentaenoic and docosahexaenoic polyunsaturated fatty acid proportion, they decrease the arachidonic acid and PGE_2 proportion of cell membranes. This may contribute to the cell membranes fluidity, flexibility and permeability, to the regulation of ion transport ions such as calcium, sodium and potassium and to the transmembrane exchange of substances like oxalate [Baggio *et al.* 1998].

Thus, to optimize the integrity of the cell wall at the level of the kidney, intestine and bone tissue, idiopathic calcium oxalate stone formers are sometimes advised to consume a source of omega-3 essential fats daily [Henriquez-La Roche *et al.* 1992, Hirayama *et al.* 1988]. This essential fat is found in fatty fish, fish oil, flax seed oil, walnut oil and as supplemental form. There are no published data on the recommended dosage of omega-3 for kidney stone sufferers but 1-2 oily fish portions per week or 1-1.5g/day as cold-pressed oil or supplement (omega6:omega3 should be 4:1) is suggested.

Dietary Fibre / Phytic acid

Several studies have shown that stone formation decreases with increased consumption of bran [Jarrar *et al.* 1984, Shar *et al.* 1980]. A study by Griffith (1986) assessing dietary intakes

of stone formers compared to age and sex matched controls, found an association between a low dietary fibre intake and a high stone incidence. This view was supported by an earlier study [Griffith *et al.* 1981]. However, other studies found no significant difference in dietary fibre intakes between renal stone cases and age and sex matched controls [Rao *et al.* 1982b, Jahnen *et al.* 1992]. Interestingly, the South African black population which is virtually stone-free, ingest a diet low in fibre compared to the white stone-prone population [Walker *et al.* 1994].

Studies that administered bran gave conflicting results: the consumption of 15mg to 30mg bran decreased the risk of calcium oxalate stone formation in some intervention studies [Messa *et al.* 1997, Gleeson *et al.* 1990] and had no effect in others [Jahnen *et al.* 1992, Hiatt *et al.* 1996, Rotily *et al.* 2000]. Therefore there is no convincing evidence to proof the efficacy of bran or dietary fibre in reducing the risk of renal stone formation.

Modlin (1980) found an association between a high stone incidence and low phytic acid intake. He postulated that a reduction in dietary phytic acid decreases the urinary phosphorylated inositols which are inhibitors of stone formation. More recent studies confirm that phytate is one of the most important inhibitors of calcium oxalate crystallization [Grases *et al.* 1996, Grases *et al.* 1998]. In support of this, Grases *et al.* (2000) found significantly lower urinary phytate levels in calcium oxalate stone formers compared to healthy people.

Phytic acid may also reduce the risk of developing kidney stones by forming calcium phytate complexes in the intestine, thereby reducing the absorption of calcium [Messa *et al.* 1997, Shar *et al.* 1980]. Similarly, Hesse *et al.* (1993) found that different kinds of bran, depending on their phytic acid content, bind calcium, leading to an increased urinary excretion of oxalic acid. This could be due to the oxalate content of the bran or due to the intestinal reduction of calcium oxalate complexes [Parivar *et al.* 1996]. However, the result of the increased urinary oxalate does not seem in this study to increase calcium oxalate crystallization.

Drinking Water

It is common practice to advise all stone forming patients, regardless of the stone type, to consume between 2.5 and 3 litres of fluid per day to maintain a urine output of 2 litres per day [Goldfarb 1990, Pak *et al.* 1984]. A urine volume of less than 1 litre per day is the only unique factor known to increase the risk of calcium oxalate stone formation [Goldfarb 1990]. Any condition that brings on frequent chronic dehydration episodes like in the case of marathon runners, increases the risk of developing renal stones [Milvy *et al.* 1981]. Chronic dehydration raises saturation of stone forming salts and also decreases urinary pH, all of which are risk factors for crystal formation. Rigorous hydration will keep the urine diluted, thus delaying the onset of supersaturation and consequently the crystallisation of stone forming minerals [Goldfarb 1990, Goldfarb 1988]. Borghi et al demonstrated that by increasing fluid intake to achieve a urinary volume of 2 litres, significantly decreased calcium and oxalate concentrations in the urine and as a consequence reduced the stone recurrence rate from 27 percent to 12 percent [Borghi *et al.* 1996]. Similarly a large cohort retrospective study by Curhan (1993) involving more than 45000 subjects, found an inverse relationship between fluid intake and calcium oxalate urolithiasis. In another example, Goldfarb (1988) advises regular fluid intake of 250ml every 4 hours. Adequate hydration can decrease approximately 60 percent of new stone cases [Pak *et al.* 1984] but does not guarantee the cessation of stone formation [Goldfarb 1990, Goldfarb 1988].

Although the term 'fluid intake' most often implies water intake, other fluids like lemon juice are also recommended [Seltzer *et al.* 1996, Curhan *et al.* 1996]. Other beverages shown to be beneficial include cranberry juice [McHarg and Rodgers 2003], apple juice [Vahlensieck 1986] and herbal teas [Hesse *et al.* 1993]. Curhan *et al.* (1996) conducted a cohort study of 45,289 men investigating 21 different beverages and the risk of urolithiasis and concluded that the beverage type may determine the risk of stone formation. In the same study Curhan *et al.* (1996) found apple juice (35%) and grapefruit juice (37%) to increase the risk of kidney stone formation.

The effects of solutes in mineral water like calcium or calcium in combination with magnesium may have a beneficial effect on preventing kidney stone formation. [Bellizzi *et al.* 1999, Rodgers 1998, Rodgers 1997]. Rodgers (1998) compared urinary risk factors after the ingestion of mineral water and tap water. In male stone formers, nine urinary risk factors changed favourably after the ingestion of mineral water containing magnesium and calcium making this a possible prophylactic agent.

Calcium Supplement

Studies investigating calcium supplementation and its risk for CaOx urolithiasis can be divided into two groups: population studies [Curhan *et al.* 1997, Curhan 1993] and dietary intervention studies [Sakhaee *et al.* 1994, Levine *et al.* 1994, Williams *et al.* 2001, Domrongkitchaipom *et al.* 2002]. Results are conflicting; Curhan *et al.* (1993,1997) compared *dietary calcium and supplemental calcium in two large cohort studies* with female and male subjects respectively and showed that supplemental calcium increased the risk of stone formation in women (increased by 20%) [Curhan *et al.* 1997] but not in men [Curhan 1993]. The author explains the latter result by suggesting that supplemental calcium, when taken between meals rather than with them, increases GIT calcium absorption, thereby increasing urinary calcium [Curhan *et al.* 1997, Harvey *et al.* 1985]. On the other hand, calcium supplement intervention studies resulted in no long-term significant increase in risk of CaOx stone formation [Sakhaee *et al.* 1994, Levine *et al.* 1994, Williams *et al.* 2001, Domrongkitchaipom *et al.* 2002]. Sakhaee *et al.* (1994) and Levine *et al.* (1994) administered calcium citrate to stone-forming women and healthy women respectively. No significant increase in calcium oxalate saturation was found after 3 and 6 month respectively. Similarly, a study that administered calcium carbonate to postmenopausal women with osteoporosis found no significant increase of calcium oxalate stone formation after 3 month of treatment [Domrongkitchaipom *et al.* 2002]. Therefore, the available data on the effect of calcium supplement on the risk of CaOx urolithiasis is inconclusive and calls for more research. Future dietary intervention studies should take the following into consideration: the dietary

protocol should be taken on a constant metabolic diet, the type and timing of the supplement must be strictly defined and the duration of the study is important to account for intestinal adaptation.

Citrate Supplement

A variety of citrate-containing preparations have been investigated for the management of CaOx urolithiasis. Among these are potassium citrate [Pak *et al.* 1985, Pak and Fuller 1986, Abdulhadi *et al.* 1993], sodium potassium citrate [Schwille *et al.* 1987, Schwille *et al.* 1987, Rumenapf and Schwille 1987, Berg *et al.* 1990, Hofbauer *et al.* 1994, Ogawa 1994], calcium citrate [Sakhaee *et al.* 1994, Levine *et al.* 1994], calcium sodium citrate [Schwille *et al.* 1997] and potassium-magnesium citrate [Pak *et al.* 1992, Sakhaee *et al.* 1983]. Of these, the most extensively studied preparations are potassium citrate and sodium potassium citrate. They have been shown to consistently increase urinary citrate and pH and have been effective in reducing stone formation [Pak and Fuller 1986, Abdulhadi *et al.* 1993, Schwille *et al.* 1985, Schwille *et al.* 1987, Rumenapf and Schwille 1987, Berg *et al.* 1990, Hofbauer *et al.* 1994, Ogawa 1994].

Magnesium Supplement

Magnesium has been shown to inhibit nucleation, growth and aggregation of calcium phosphate and calcium oxalate crystals [Li *et al.* 1985, Achilles and Ulshöfer 1985, Siener and Hesse 1995]. Moreover, dietary magnesium is suggested as being beneficial by binding intestinal oxalate, rendering less to be excreted in the urine [Barilla *et al.* 1978, Berg *et al.* 1986]. Some studies have shown a lower urinary magnesium excretion in stone formers compared to stone free controls [Tiselius *et al.* 1978, Yendt 1970]. Although the oral administration of magnesium successfully increased urinary magnesium in some studies [Fetner *et al.* 1978, Johnason *et al.* 1980], it brought little success in others because the benefit was counteracted by the associated increase in urinary calcium excretion [Fetner *et al.* 1978, Ettinger *et al.* 1984]. A more recent study by Lieberman *et al.* (2000) compared the

effects of magnesium oxide and calcium carbonate on gastrointestinal oxalate absorption in healthy subjects and found the efficiency of oxalate absorption to be similar for the two supplements (7.6% vs 5.1% respectively). In addition, the study demonstrated a decrease in the magnesium-calcium ratio after MgO supplementation. The magnesium-calcium ratio has been negatively correlated with urolithiasis [Kohri *et al.* 1993]. Supporting this finding, Rodgers (1997) found that the Mg/Ca ratio of mineral water was more beneficial than that of tap water (0.178 and 0.098 respectively). Allie *et al.* (2003) proposed a positive synergistic effect of magnesium supplement combined with citrate on lowering the relative supersaturation of brushite. No significant changes in urinary calcium were observed.

Vitamin C (ascorbic acid) and Vitamin B_6 (pyridoxine) Supplement

Both vitamin C and vitamin B6 are involved in the metabolic pathway of oxalate. While 40% of dietary vitamin C undergoes a non-enzymatic conversion to oxalate, vitamin B_6 metabolizes oxalate [Menon and Mahle 1982]. Since vitamin C has been shown to increase urinary oxalate researchers concluded that it might play a detrimental role in increasing the risk of kidney stone formation [Griffith *et al.* 1986, Power *et al.* 1984]. This finding was challenged by several studies carried out in humans and in experimental animals [Auer *et al.* 1998, Curhan *et al.* 1999, Johnston 1999]. Indeed, the study by Auer *et al.* (1998) showed that failure to preserve urine specimens in investigations by other workers had resulted in erroneously high oxalate determinations.

Several studies have shown that prolonged vitamin B_6 deficiency can induce urinary oxalate excretion leading to a higher incidence of calcium oxalate stones [Faber *et al.* 1963, Tommaso *et al.* 2002]. Treating calcium oxalate stone patients with a pyridoxine supplement has given contradictory results with urinary oxalate being reported as decreased [Nguyen *et al.* 2001, Gill and Rose 1986] and unchanged [Tolomelli *et al.* 1991].

A recent cohort study by Curhan *et al.* (1999) surveyed 85.557 women, evaluating the relationship between vitamin B_6 and C intake and the risk of calcium oxalate kidney stone

formation. The results showed high vitamin B_6 intake inversely associated with the risk of stone formation and vitamin C showed no association with stone risk.

1.5 Oxalate-Degrading Bacteria

Dawson *et al.* (1980a) was the first to isolate anaerobic ruminal oxalate-degrading bacteria. These bacteria were described as gram-negative rods and the production of CO_2 and formate from oxalate is its *only* energy source [Dawson *et al.* 1980a, Anantharam *et al.* 1989]. The same anaerobic bacterium was later isolated by Allison *et al.* (1985) and described as *Oxalobacter formigenes.* It is believed that this bacterium colonizes the human intestine anything from complete absence to up to 10^8 cfu/gm. wet weight of faeces. *O. formigenes* regulates intestinal oxalate homeostasis by utilizing oxalate and by creating a transepithelial gradient, facilitating its secretion in the faeces [Sidhu *et al.* 1997]. Intestinal utilization of oxalate prevents the accumulation of excess oxalate that would otherwise be harmful and thus oxalate-degrading bacteria are important for the prevention of oxalate related diseases [Allison *et al.* 1977]. Thus, it is not surprising that the lack of the intestinal bacterium *Oxalobacter formigenes* has been directly associated with hyperoxaluria and consequently the risk of calcium oxalate urolithiasis [Sidhu *et al.* 1998, Kumar *et al.* 2002]. In addition, the number of O. *formigenes* per gram of faeces was directly correlated with the frequency of urolithiasis episodes [Kleinschmidt *et al.* 1993, Sidhu *et al.* 1999]. In the case of cystic fibrosis patients (CF), the absence of *O. formigenes* appears to be directly linked to absorptive hyperoxaluria and the risk of CaOx urolithiasis [Sidhu *et al.* 1998]. Sidhu *et al.* (1998) investigated 43 CF patients and 21 healthy subjects for the presence of *O. formigenes* using a quantitative competitive-template PCR method. Results showed that 71% of healthy subjects and only 16% of CF patients tested positive for *O. formigenes* [Sidhu *et al.* 1998]. In addition, urinary oxalate excretion was significantly different in CF patients colonized and CF patients not colonized with the latter bacterium. In another study Sidhu *et al.* (1999) that enteric hyperoxaluria can be reversed by the administration of O. *formigenes* or a preparation of oxalate-degrading enzymes (cloned from *O. formigenes*) in rats. *O. formigenes* absent rats

were put on diets that differed in the amount of oxalate and then inoculated with high doses of the latter bacterium. Subsequent faecal analysis showed that *O. formigenes* was successfully colonized in rats on the high oxalate diet (1.5% ammonium oxalate) followed after 4 days by rats on the low oxalate (0.5% ammonium oxalate) diet. Furthermore in more recent study Sidhu *et al.* confirmed the results of the former study demonstrating that the probiotic recolonization of *O. formigenes* may reduce hyperoxaluria in rats [Sidhu *et al.* 2001].

Considering the vast, unknown numbers of gastrointestinal bacteria and the relatively new discovering of anaerobic oxalate-degrading bacteria, it seems probable that similar oxalate-degrading bacteria to *O. formigenes* are abundant in the gastrointestinal tract of humans and animals. Besides *O. formigenes,* oxalate-degraders isolated are *Eubacterium lentum* [Ito *et al.* 1994] and *Enterococcus faecalis* [Hokama *et al.* 2000]. In addition, Campieri *et al.* (2001) postulated that lactic acid bacteria (*Bifodobacterium* and *Lactobacillus*) behave as "generalist" in terms of oxalate degradation.

1.6 Urolithiasis in South Africa

The prevalence of CaOx urolithiasis in South African whites is similar to that in other westernized countries ranging between 12 and 15 percent [Meyers *et al.* 1994]. Among blacks with a partially westernized life style, the disease remains uncommon with a frequency of less than 1 percent [Meyers *et al.* 1994, Whalley *et al.* 1998]. Past research that has investigated urinary variables and diet in the two race groups has been inconclusive.

The low incidence of renal stones in the black population has been associated to their lower calcium [Muskat *et al.* 1951, Modlin 1967, Whalley *et al.* 1998] and protein consumption [Muskat *et al.* 1951]. The dietary anomalies that were found in black subjects were significantly increased intake of sodium [Modlin 1967, Whalley *et al.* 1998] and a greater oxalate intake (significantly different only in black women) [Whalley *et al.* 1998]. Furthermore, the significantly higher sodium intake corresponded to a significantly higher urinary sodium excretion but had no effect on the urinary calcium excretion [Modlin 1967, Whalley *et al.*

1998] and the significantly lower calcium intake together with the greater oxalate intake had no effect on the urinary oxalate excretion [Whalley *et al.* 1998].

Studies of the urine composition of the two ethnic groups reveal intriguing differences. Two independent studies found that blacks have lower urinary citrate compared to whites [Modlin 1967, Whalley *et al.* 1998]. Since urinary citrate acts as a urinary inhibitor this result is surprising. The reason for the lower level of citrate is unknown but it indicates that the protective mechanism of citrate in whites is not a factor in blacks. Of some interest however, is the observation of lower urinary calcium in blacks which was the only other urinary variable that was reported in the above-mentioned studies. Although blacks have significantly lower urinary calcium compared to whites, their values nevertheless lie within the normal range [Modlin 1967, Whalley *et al.* 1998]. Therefore it is unlikely that urinary calcium is the crucial factor protecting blacks against calcium oxalate kidney stones. It therefore seems that the important urinary factors for assessing stone forming risk are different in the two race groups. In addition previous studies have suggested that the absence of stones in blacks could be related to their relatively high urinary sodium-to-calcium ratio, their decreased urinary cystine [Whalley *et al.* 1998] or to different interactions between sodium and the calcium-to-cystine ratio [Whalley *et al.* 1998].

Recently, the kidney stone research group at the University of Cape Town (of which the present investigator is a member) adopted a new approach in which the inhibitory role of urinary proteins in the two race groups are being investigated. Urinary prothrombin fragment 1 (UPTF1) and Tamm-Horsfall mucoprotein (THM) are two such proteins [Webber *et al.* 2002, Craig *et al.* 1999]. The results of both studies suggest that the composition of urine in black subjects enhances the inhibitory activity of the respective urinary protein. Thus, identification of the optimum urinary factors and the mechanism by which they might be manipulated and controlled (diet, probiotic or other) would be of considerable interest in the management of calcium oxalate stone disease.

1.7 Objectives

Based on the risk factors identified in the General Introduction, several objectives were identified for this thesis.

Overall Objectives

In designing this project, the overall objective was to investigate dietary and microbiological factors in South African black and white subjects with a view to gaining insights into why the incidence of calcium oxalate kidney stone disease is extremely rare in the former race group.

Specific Objectives

In attempting to achieve the overall goal, specific objectives were identified:

to assess, evaluate and compare the diet of groups of black and white South African subjects with special reference to lithogenic and anti-lithogenic components

to investigate and compare the effect of a low calcium/high oxalate dietary protocol on urine biochemistry and renal handling in both race groups

to investigate and compare the effect of several other potentially lithogenic and anti - lithogenic diets on urine biochemistry and renal handling in both race groups

to investigate and compare the effect of several potentially lithogenic and anti lithogenic dietary supplements on urine biochemistry and renal handling in both race groups.

to isolate, identify and quantify oxalate degrading bacteria and to quantify their relative oxalate utilization in healthy subjects from both race groups as well as in white stone formers.

The studies in which these objectives were pursued are described in the chapters which follow (chapters 2 – 6 inclusive). Each study is treated independently and is presented with its own unique introduction, methods, results and conclusions to achieve focus. The final chapter (no 7) attempts to consolidate all the results and to provide a global overview.

1.8 References

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ChapterTwo

Analysis of dietary intake in black and white South African subjects

1.1 Introduction

As has been stated in the General Introduction, kidney stone disease is recognized as a disease of lifestyle, its prevalence steadily increasing in industrialized communities the last 90 years [Rao *et al.* 1982, Robertson *et al.* 1981]. The current prevalence of CaOx urolithiasis in South African males is 15% in whites and less than 1% in blacks [Wise and Kark 1961, Meyers 1994]. It has been suggested that the low incidence of CaOx urolithiasis in blacks is reflected in their different dietary habits [Wise and Kark 1961, Muskat 1951]. The traditional diet of this race group is high in complex carbohydrates, fibre and vegetables and low in fat and animal protein [Viljoen and Gericke 2001], all of which contribute to lower risk of kidney stone formation [Trinchieri *et al.* (1991)]. Besides urolithiasis, other diseases of lifestyle that occur rarely in blacks are gallstones, coronary heart disease, Crohn's disease, diverticulitis and intestinal adenomas [Segal 1998, Oettle and Segal 1985, Walker and Segal 1997].

Urbanisation and socio-economic changes have led to a more Western lifestyle in the black population. The term 'nutrition in transition' has been used in South Africa to describe ethnic groups that change from traditional to westernised eating habits [Viljoen and Gericke 2001, Bourne 2002]. With changing eating habits it is fascinating to observe the changes in disease patterns. The identification and characterization of these dietary changes will have significant clinical implications to clinical conditions like kidney stone disease.

1.2 Material and Methods

The dietary habits of healthy black and white men were assessed as part of two comprehensive studies (chapter 3 and chapter 6). The studies differed with regard to dietary assessment methods and black population groups.

The food intake of both groups was assessed using a semi-quantitative food frequency questionnaire combined with a 24-hour dietary recall questionnaire (examples of these

questionnaires are given in appendix CD: chapter 2/diet quest). The principal application of a single 24-hour recall is to record all the food and beverages consumed within the 24-hour period prior to completing the questionnaire itself. To ensure validity, a well-trained interviewer (dietician) should ask probing questions to identify food additions such as butter, snacks and beverage breaks [Campbell and Dodds 1967]. The first advantage of a 24-hour recall is that literacy of the respondent is not required. (In the present study, the black subjects spoke mainly Xhosa). The second advantage is that respondents are generally able to recall most of their dietary intake because of the immediacy of the recall period [Thompson and Byers 1994]. The disadvantages of a 24- hour dietary recall are that most individual's diets vary greatly from day to day and therefore it is not appropriate to use data from a single 24-hour period to characterise an individual's usual diet [Thompson and Byers 1994].

A food frequency questionnaire consists of a list of food items in which respondents are required to indicate their frequency of consumption. To estimate relative or absolute nutrient intake, many food frequency questionnaires also require respondents to indicate portion sizes. This allows semi-quantitative data to be accrued [Zulkifli and Yu 1992]. The underlying principle of the food frequency approach is that an average dietary intake over weeks, months or years is more accurate than an intake which has been recorded on a few specific days. Food frequency methods are used most commonly to rank a study group in order to assess the dietary intake and compare it with disease risk [Thompson and Byers 1994]. The advantage of a semi-quantitative food frequency questionnaire is that the researcher is able to categorise individuals according to their usual consumption of foods or groups of foods and nutrient intake [Thompson and Byers 1994]. This kind of questionnaire can be designed to be self-administered and quick and easy to complete. Also, the data collection and processing is easy and not as time consuming as other dietary assessment methods e.g. dietary records. Furthermore, it is flexible, allowing for different groups with different dietary habits to use the same questionnaire. The disadvantage of a semi-quantitative food

frequency questionnaire is that the details of dietary intake are difficult to measure; hence full quantification of intake is not as accurate as with dietary records or dietary recalls [Thompson and Byers 1994]. Possible errors with this questionnaire include incomplete listing of all foods, error in frequency estimation, and incorrect estimation of serving sizes. Krebs-Smith *et al.* (1994) found that longer food frequency lists overestimated intake, and shorter lists underestimated intake. The food list cannot possibly include all the variables in an individual's diet such as brands, preparation practices, and the many different foods themselves.

Dietary Assessment in Ethnic Populations

The content of the dietary assessment questionnaire must be specific to the cultural background of a population [Hankin *et al.* 1994]. The language and slang used for certain food items must be known. For example South African's black population refer to sunflower oil as "fish oil". It is also important to identify the nutrient compositions of ethnic-specific foods. For example the staple food for the black population group is maize meal porridge. Thus, the nutrient composition database should include all ethnic foods of the population group under investigation.

Dietary Survey # 1

Two experimental groups, consisting of 11 black males and 11 white males were age matched (ages ranging form 16 - 61). The group of black subjects was drawn from a peri-urban population group attending an orthopaedic ward of a state hospital. Recruited patients had only minor bone injuries that did not require strong pain medication. The group of white subjects was drawn from an urban population group resident in the Cape Town area. None of the subjects in either group had a metabolic illness or any clinical problem.

As stated earlier, the aim of the present study was to obtain information on nutritional factors which might play a role in the low incidence of urolithiasis in blacks and might thus be

important in the aetiology and treatment of renal stone disease. Nutrients which have been identified as playing a role in the pathogenisis of CaOx calculi (discussed in the General Introduction) and which were of particular interest were oxalate [Holmes and Assimos 1999, Finch *et al.* 1981, Hesse *et al.* 1993, Holmes and Kennedy 2000], calcium [Jaeger *et al.* 1994, Messa *et al.* 1997, Curhan 1993, Bataille *et al.* 1983], total protein [Robertson *et al.* 1979, Fellström *et al.* 1984] and animal protein [Nguyen *et al.* 2001, Brockis *et al* 1982, Massey and Kynast-Gales 2001, Breslau *et al.* 1988]; carbohydrate (in particular refined carbohydrate) [Leman *et al.* 1969, Wood *et al.* 1987, Knowles *et al.* 1988, Blacklock 1987] and fibre [Griffith *et al* 1986, Gleeson *et al.* 1990, Jahnen *et al* 1992]; vitamin C [Johnston 1999, Auer *et al* 1998, Curhan *et al.* 1999], magnesium [Berg *et al.* 1986, Zimmermann *et al* 2003] and vitamin B_6 [Curhan *et al.* 1999, Tommaso *et al.* 2002]. The dietary intake of salt is an important risk factor for CaOx urolithiasis but its dietary assessment is very inaccurate; therefore Massey et al suggest that the urinary excretion of sodium is a more reliable indictor of salt intake [Massey and Whiting 1996].

All the data captured from the dietary questionnaires were combined and analysed for macro- and micronutrients using the computer programme "Foodfinder" version 5 that uses Food Composition Tables [Langenhoven MJ *et al.* 1991a]. The oxalate content of foods was determined using the Food Finder Tables [Kruger *et al.* 1998].

Dietary Survey # 2

The protocol was similar to that described for the first dietary survey. Briefly, 10 black and 10 white South African males between the ages 18-28, with no metabolic disorder or history of kidney stones, participated in the study. All of the subjects were students at the University of Cape Town. The black population group differed to that described in Dietary Survey # 1 as it comprised urbanized students rather than peri-urban hospital patients.

The food intake of black and white subjects was assessed using a semi-quantitative food frequency questionnaire combined with a 2-day dietary record. All the data captured from the dietary questionnaires were analysed for macro- and micronutrients using the computer programme "Foodfinder" version 6 [Langenhoven ML *et al* 1991b]. Statistical analysis was performed by analysis of variance at statistical significance of $p \leq 0.05$.

2.3 Results

Dietary Survey # 1

The two experimental groups, consisting of 11 black males and 11 white males had matched ages [31.36 vs 29.54, $p>0.100$] ranging between 16 and 61 years. The mean baseline intake of nutrients for the two race groups was determined from the completed dietary questionnaires. Values are given in table 2-1. It is noted that total protein [86.93 vs 109.82, $p=0.046$), animal protein (45.06 vs 64.92, $p=0.037$), vitamin C (122.54 vs 247.0, $p=0.033$) and vitamin B_6 (2.13 vs 3.28, $p=0.043$) were the only nutrients which were significantly different between the groups; consumption in all cases was higher in white subjects. The means with standard errors of the nutrients that show significance are illustrated graphically (Figure 2-1, 2-2, 2-3, 2-4)

Dietary Survey # 2

Dietary intake data computed from 2-day food dairies and food-frequency questionnaires are tabulated in table 2-2. In the context of CaOx kidney stone risk factors, attention is drawn to the significantly higher intake of oxalate in black subjects [297.89 vs 128.30, $p=0.025$] and their significantly lower intakes of calcium [663.11 vs 1080.10 mg, $p=0.011$], magnesium [325.56 vs 468.00 mg, $p=0.013$] and total sugar [29.14 vs 63.49 gm, $p=0.011$] relative to healthy white subjects (table 2-2). Vitamin B6 intake was lower in blacks than whites but not significantly [2.06 vs 3.06 mg, $p=0.063$] (table 2-2, figure 2-9). Attention is drawn to dietary sodium intake which was the same in both groups [2736 mg vs 3973 mg, $p=0.072$]. Other nutrient intakes which were significantly lower in blacks were phosphate [1252 vs 1997 mg,

p=0.002] and potassium [2613 vs 3922 mg, p=0.022] (table 2-2). The means (with standard errors) of the nutrients that show significant differences in the groups are illustrated graphically (figure 2.5, 2-6, 2-7, 2-8).

Table 2-1: Dietary survey # 1: Mean and Median daily intake of nutrients for black and white subjects

	Blacks	Whites	p-value
Moisture (%)	80.17 (1.04) 79.3 [77.3; 82.2]	80.67 (1.25) 81.0 [77.9; 83.2]	p = 0.763 p = 0.818
Protein (g/day)	86.93 (6.48) 86.6 [77.1; 98.6]	109.82 (7.35) 108.5 [92.2; 132.8]	p = 0.046* p = 0.066
Animal (g/day) Protein	45.06 (5.58) 43.1 [34.1; 59.7]	64.92 (6.84) 67.7 [62.1; 80.6]	p = 0.037* p = 0.048*
Fat (g/day)	115.97 (12.09) 110.2 [84.3; 160.5]	120.35 (11.31) 106.8 [94.5; 157.9]	p = 0.794 p = 0.922
Carbohydrate (g/day)	375.26 (31.98) 369.6 [308.1; 444.8]	360.47 (36.74) 357.9 [283.0; 381.7]	p = 0.764 p = 0.599
Fibre (g/day)	34.93 (4.49) 30.8 [23.8; 47.2]	37.32 (4.88) 35.4 [25.5; 41.2]	p = 0.721 p = 0.693
Added sugar (g/day)	97.53 (17.00) 77.8 [59.8; 123.1]	80.53 (8.52) 67.5 [56.1; 111.8]	p = 0.386 p = 0.554
Oxalate (mg/day)	322.0 (36.73) 320.1 [203.8; 392.5]	329.38 (74.14) 277.1 [115.0; 442.2]	p = 0.930 p = 0.645
Ca /mg (mg/day)	1071.81 (141.41) 898.0 [730.0; 1452.0]	1162.82 (131.61) 1002.0 [814.0; 1471.0]	p = 0.643 p = 0.511
Mg (mg/day)	469.0 (35.26) 445.0 [402.0; 580.0]	546.54 (59.58) 489.0 [386.0; 643.0]	p = 0.276 p = 0.450
PO_4 (mg/day)	1599.09 (130.92) 1532.0 [1337.0; 1793.0]	1957.0 (190.32) 1735.0 [1546.0; 2600.0]	p = 0.137 p = 0.178
K (mg/day)	4070.18 (417.70) 3470.0 [3281.0; 4954.0]	4636.73 (411.26) 4402.0 [3515.1; 5806.0]	p = 0.345 p = 0.264
Na (mg/day)	3900.82 (307.72) 4080.0 [3338.0; 4639.0]	3775.18 (355.81) 3529.0 [2924.0; 4824.0]	p = 0.792 p = 0.646
Vitamin A (RE/day)	2799.91 (583.46) 2399.0 [246.0; 5159.0]	806.82 (197.77) 2200.0 [1233.0; 2273.0]	p = 0.132 p = 0.293
VitaminB_6 (mg/day)	2.13 (0.34) 2.05 [1.4; 2.9]	3.28 (0.41) 3.0 [2.29; 3.9]	p = 0.043* p = 0.049*

Vitamin C (mg/day)	122.54 (23.23) 101.0 [77.0; 123.0]	247.0 (47.25) 183.0 [147.0; 385.0]	p = 0.033* p = 0.026*
Vitamin D (ug/day)	6.34 (1.58) 4.65 [2.9; 11.6]	4.12 (0.69) 3.59 [2.23; 5.88]	p = 0.220 p = 0.470

*Significance at p<0.05; ( ): standard error; [ ]: lower quartile, upper quartile

Figure 2-1: Comparison of the means of total protein intake (Dietary Survey # 1)

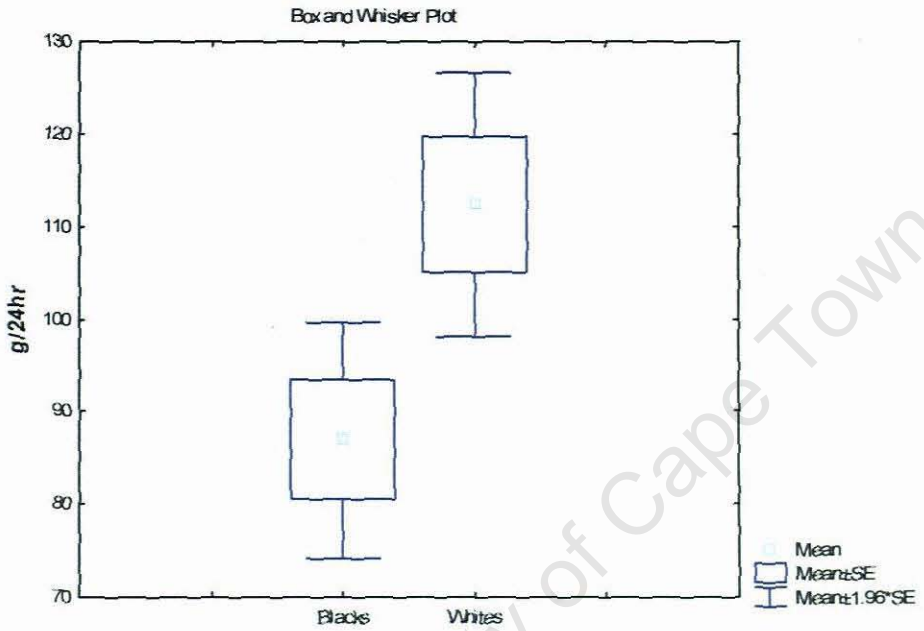


Figure 2-2: Comparison of the means of animal protein intake (Dietary Survey # 1)

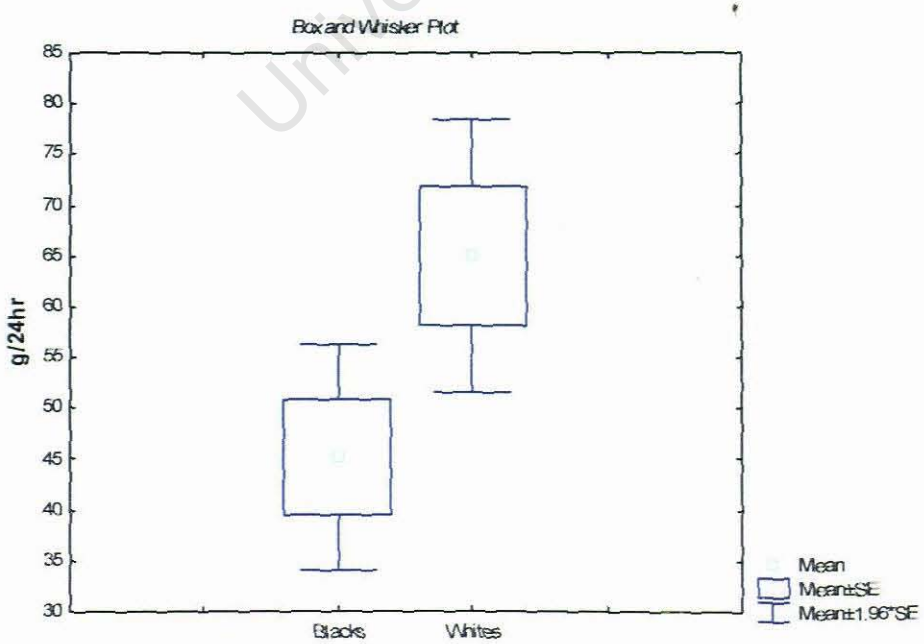


Figure 2-3: Comparison of the means of vitamin C intake (Dietary Survey # 1)

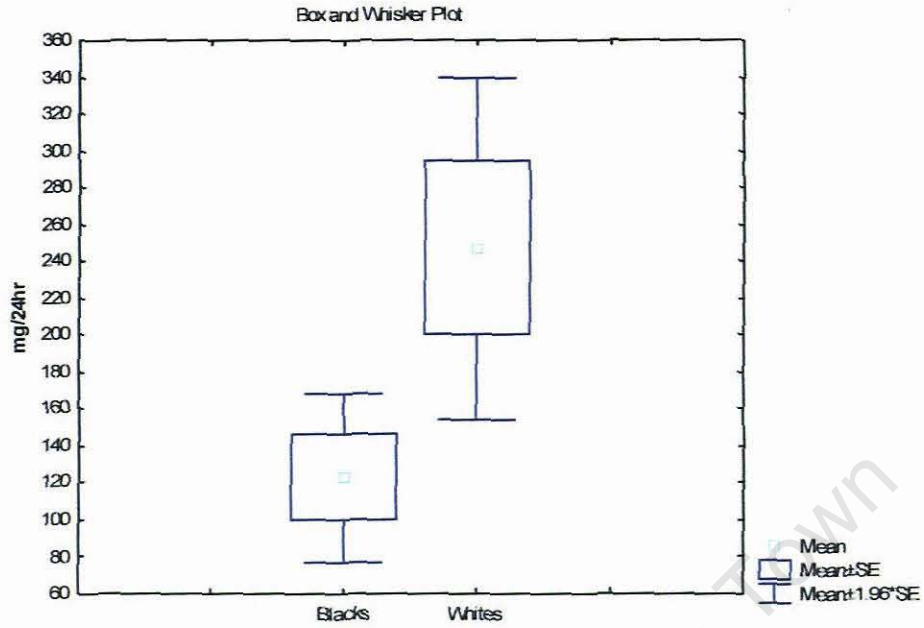


Figure 2-4: Comparison of the medians of vitamin B_6 intake (Dietary Survey # 1)

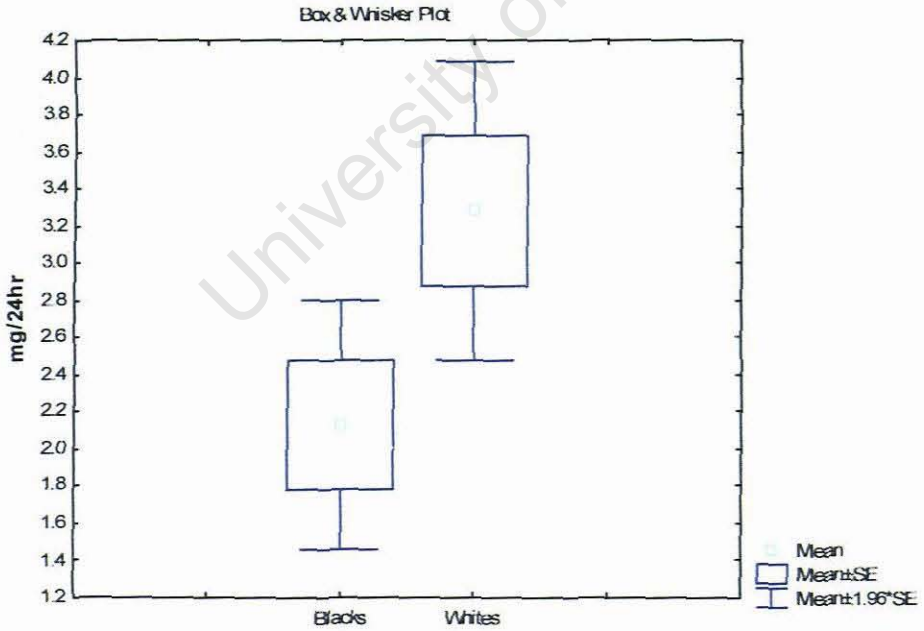


Table 2-2: Dietary survey # 2: Mean and Median Daily Intake of Nutrients for Black and White Subjects

	Blacks	Whites	p-value
Moisture (g/day)	1897 (224.31) 1951 [1819, 2251]	2950 (361.68) 2930 [1873, 4193]	0.027* 0.043*
Protein (g/day)	79.56 (9.41) 75.2 [65.8, 99.2]	120.15 (12.71) 106.5 [97.3, 140.3]	0.022* 0.049*
Animal (g/day) Protein	42.70 (9.41) 40.1 [23.6, 51.9]	71.78 (11.79) 66.4 [48.5, 75.7]	0.075 0.041*
Fat (g/day)	81.94 (12.56) 75.2 [65.8, 113.6]	126.45 (20.60) 91.6 [80.0, 189.5]	0.091 0.058
Carbohydrate (g/day)	285.70 (40.71) 273 [219.8, 380.2]	362.95 (42.91) 306 [276, 393]	0.211 0.201
Fibre (g/day)	24.50 (3.07) 23.0 [16.5, 60.6]	29.62 (4.60) 28.5 [19.4, 33.9]	0.378 0.779
Total Sugar (g/day)	29.14 (5.84) 23.9 [17.6, 34.9]	63.49 (10.19) 62.6 [32.2, 93.2]	0.011* 0.010*
Added sugar (g/day)	25.39 (6.27) 23.2 [17.8, 24.8]	57.41 (13.60) 50.1 [25.0, 98.6]	0.055 0.072
Oxalate (mg/day)	297.89 (68.94) 234.29 [137.5, 413.9]	128.3 (21.44) 109.24 [78.3, 212.25]	0.025* 0.052
Ca /mg (mg/day)	663.11 (99.78) 666.0 [415, 817]	1080.10 (105.55) 1022.0 [908, 1194]	0.011* 0.015*
Mg (mg/day)	325.56 (31.18) 305.0 [264, 388]	468.00 (39.67) 420.0 [352, 539]	0.013* 0.023*
$P0_4$ (mg/day)	1252.30 (138.08) 1275.0 [930, 1680]	1996.70 (154.01) 1857.5 [1593, 2426]	0.002* 0.007*
K (mg/day)	2613 (309.28) 2771.0 [1871, 3008]	3921.80 (404.46) 3740.5 [2998, 4678]	0.022* 0.022*
Na (mg/day)	2736 (420.46) 3049 [1727, 3605]	3973.1 (480.03) 3691.5 [2607, 5755]	0.072 0.070*
Vitamin A (RE/day)	967 (527.85) 531 [345, 578]	2028.8 (815.67) 1088 [590, 2188]	0.301 0.359
Vitamin B_6 (mg/day)	2.06 (0.41) 1.85 [1.2, 2.4]	3.06 (0.31) 3.06 [2.43, 3.31]	0.063 0.020*
Vitamin C (mg/day)	132.44 (85.63) 33.0 [14.0, 96.0]	101.30 (20.13) 89.5 [54.0, 144.0]	0.714 0.727
Vitamin D (ug/day)	5.69 (1.47) 4.18 [3.2, 9.8]	5.26 (0.99) 5.04 [2.99, 7.2]	0.808 0.930

*significance at p<0.05; ( ): std error; [ ]: lower quartile, upper quartile

Figure 2-5: Comparison of the Means of Oxalate Intake (Dietary Survey # 2)

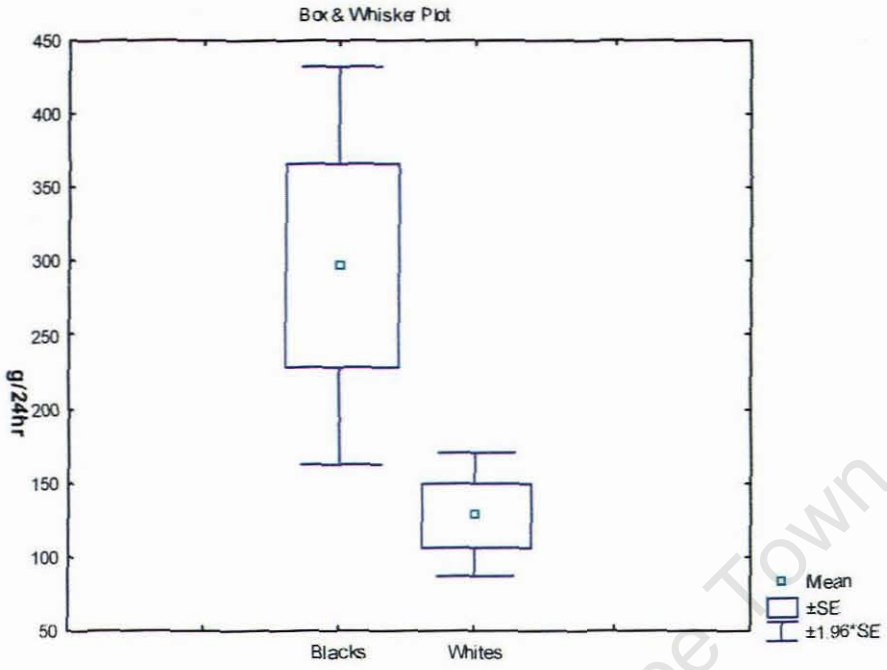


Figure 2-6: Comparison of the Means of Protein Intake (Dietary Survey # 2)

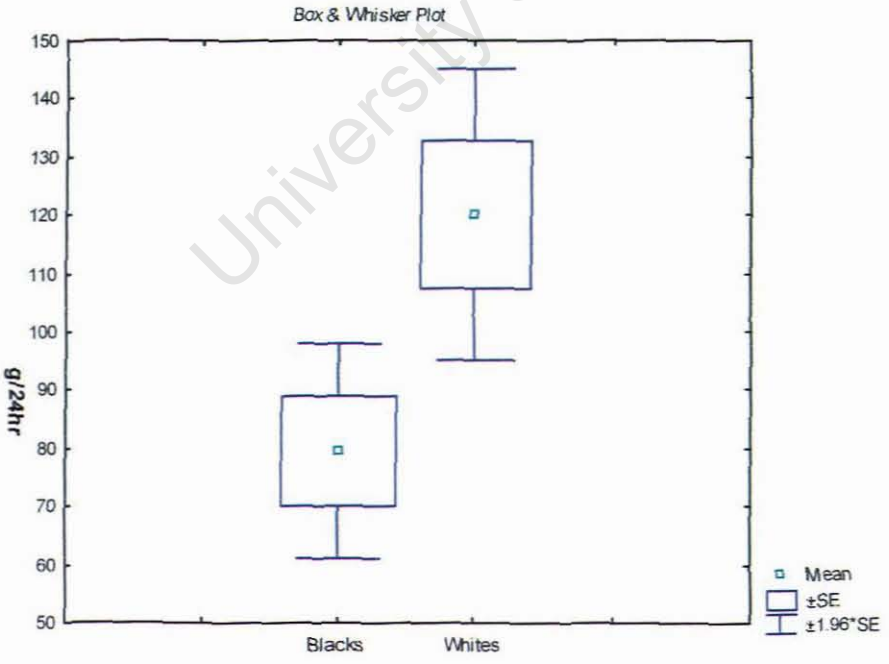


Figure 2-7: Comparison of the Means of Calcium Intake (Dietary Survey # 2)

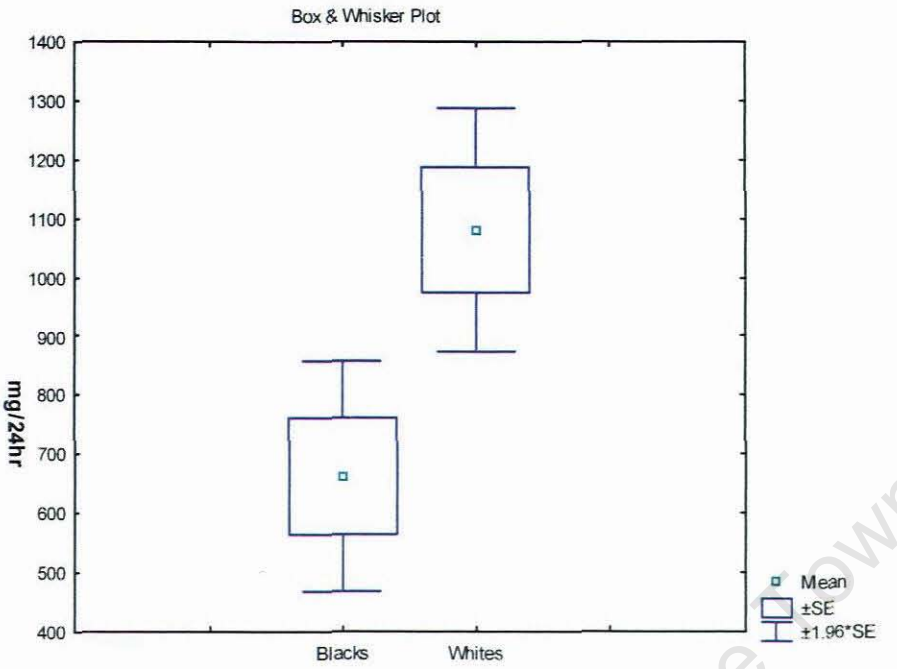


Figure 2-8: Comparison of the Means of Magnesium Intake (Dietary Survey # 2)

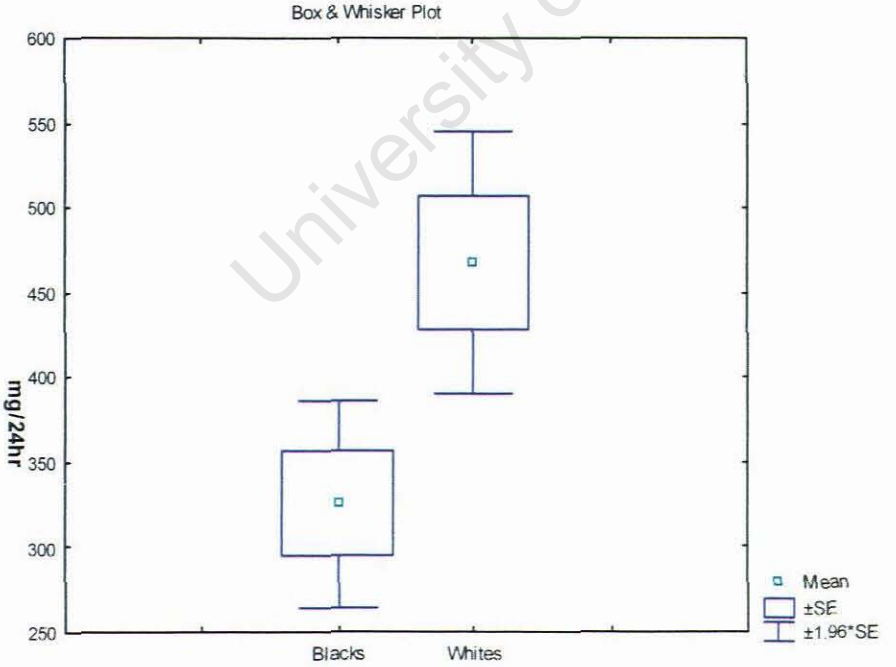
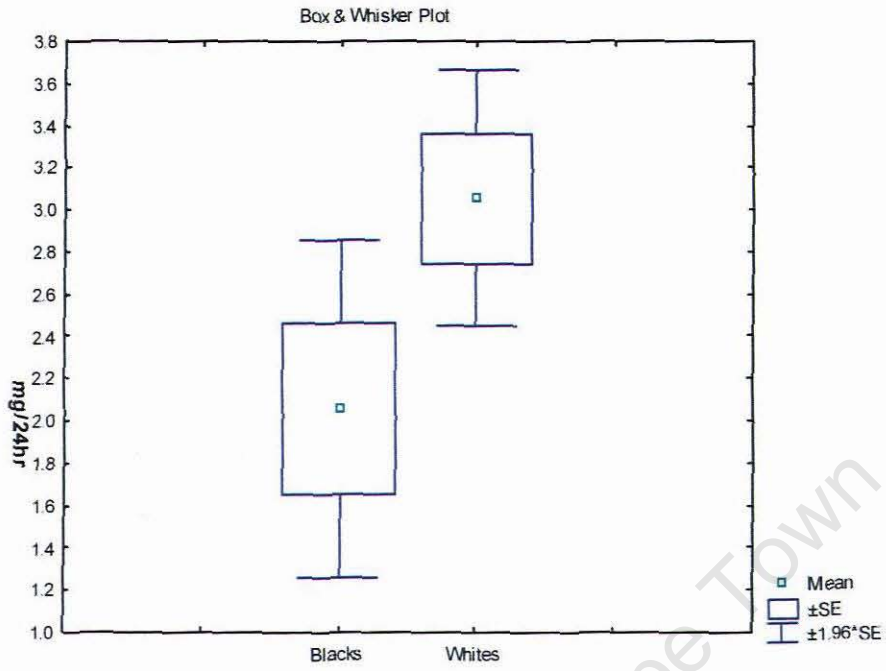


Figure 2-9: Comparison of the Means of Vitamin B_6 Intake (Dietary Survey # 2)



1.4 Discussion

In South Africa there occurs the unique existence of stone-free and stone-prone populations. Despite this near-ideal research situation, only a few attempts have been made to identify dietary factors that contribute to the apparent stone immunity in the black population group [Wise and Kark 1961, Muskat 1951, Whalley *et al.* 1998, Modlin 1967]. The objective of the dietary surveys was to evaluate the diet of black and white subjects by means of dietary assessment methods. Dietary survey # 2 was conducted differently to dietary survey # 1 because the latter survey had the following limitations: Firstly, the two subject groups were not homogenous - the black subjects were hospital patients with a peri-urban background while the white subjects were free-living with an urban background. Secondly, the black subjects in dietary survey # 1 were mostly Xhosa speaking (with no English literacy) - thus the use of an interpreter might have been a confounding factor. On the other hand, for dietary survey # 2, subjects of both race groups were urbanized, free-living students with good English literacy. Since urolithiasis has been directly correlated with westernisation [Robertson *et al.* 1978, Robertson 1987], the different biographical data in the two surveys of the present study, made possible the comparison between black peri-urban and urban population groups.

As early as 1951 Muskat *et al.* (1951) associated the low incidence of renal stones in this group to their calcium and protein consumption. More recent studies confirmed the observation that blacks have lower calcium intake relative to whites [Whalley *et al.* 1998, Modlin 1967]. Furthermore, Modlin (1967) reported that blacks have a significantly higher sodium intake which corresponds to significantly higher urinary sodium excretion. A direct correlation between urinary sodium and calcium has been reported [Sakhaee *et al* 1993] as a risk factor for CaOx urolithiasis. It is therefore of interest that a direct correlation between urinary sodium and urinary calcium has been found in whites but not in blacks [Whalley *et al.* 1998]. This was attributed to the significantly lower intake of calcium in blacks [Whalley *et al.*

1998]. The present study is in agreement with previous studies [Muskat 1951, Whalley *et al.* 1998, Modlin 1967], showing a lower intake of calcium in blacks (table 2-2, figure 2-7). Unlike previous studies [Whalley *et al.* 1998, Modlin 1967] the present study did not find any difference in sodium intake between the two groups (table 2-1 and table 2-2). As mentioned before dietary sodium intake cannot be accurately measured by dietary intake questionnaires because of the inaccurate estimation of added dietary salt [Massey and Whiting 1996].

The only other dietary intake in the present study that confirms the results of previous studies was that of a significantly lower intake of total protein in blacks (table 2-1, table 2-2) [Wise and Kark 1961, Muskat 1951, Whalley *et al.* 1998]. Furthermore, dietary survey # 1 showed blacks to have significantly lower intake of animal protein as well as total protein. Epidemiological studies have correlated a high protein diet and an increase in the risk of stone formation [Curhan 1993, Robertson *et al.* 1979, Fellström *et al.* 1984]. The mechanism by which dietary protein increases the risk of stone formation is complex and includes an increase in urinary risk factors (calcium [Brockis *et al.* 1982], oxalate [Robertson *et al* 1979, Brockis *et al.* 1982], uric acid [Brockis *et al.* 1982, Breslau *et al* 1988, Siener and Hesse 2002]), facilitation of an optimal environment for renal stone growth (low pH [Breslau *et al* 1988, Siener and Hesse 2002] and decreased urinary citrate excretion [Nguyen *et al.* 2001]). On the other hand, studies have associated vegetarian diets with a lower risk of stone formation [Siener and Hesse 2002, Brockis *et al.* 1982]. Thus, the significantly lower incidence of renal stones in blacks could be related to their lower protein intake.

The effect of dietary calcium and oxalate on the urinary risk factors for CaOx urolithiasis has received much attention. As discussed in the General Introduction, studies have demonstrated that calcium restriction increases the risk of kidney stone formation by increasing oxalate absorption and excretion [Jaeger *et al.* 1994, Messa *et al.* 1997, Curhan 1993, Bataille *et al.* 1983]. Thus, bearing in mind blacks' imperviousness to stone formation, it is surprising that blacks have lower calcium intake than whites. In addition, dietary survey #

2 showed that urbanized blacks have a significantly higher intake of oxalate (table 2-2), which would increase their risk of developing calcium oxalate kidney stones even further. Since dietary oxalate intake has not been reported previously, validation of the present result is not possible. However, spinach (a high oxalate food) is a traditional part of the meal pattern of South African blacks as reported in a nutrition survey conducted in 1994 [Viljoen and Gericke 2001], thereby lending a degree of confidence to the present result. Despite a lower calcium and higher oxalate intake in blacks relative to whites, the former group has lower urinary calcium and normal urinary oxalate values [Whalley *et al.* 1998, Modlin 1967], suggesting that different renal handling mechanisms may occur in the two race groups.

Other nutrients involved with oxalate handling are magnesium [Berg *et al.* 1986, Zimmermann *et al.* 2003], vitamin B_6 [Menon and Mahle 1982] and vitamin C [Menon and Mahle 1982]. Magnesium has the same ability as calcium to bind oxalate in the intestine forming complexes that prevent excessive urinary oxalate excretion [Johnston 1999, Auer *et al.* 1998, Sakhaee *et al.* 1993]. The results of dietary survey # 2 demonstrated that blacks have significantly lower intake of magnesium relative to whites. Although further research is needed to confirm this data it appears that intestinal oxalate handling in blacks does not depend on magnesium (or calcium, as suggested in the previous paragraph).

The dietary intake analysed from survey # 1 showed a significantly lower amount of vitamin C and vitamin B_6 intake in the black group. Data from survey # 2 confirmed the significantly lower intake of vitamin B_6 in black subjects ($p=0.02$) (figure 2-9) but the results for vitamin C could not be reproduced in survey # 2. Both vitamin C and vitamin B_6 are involved in the metabolic pathway of oxalate. While 40% of dietary vitamin C undergoes a non-enzymatic conversion to oxalate, vitamin B_6 metabolizes oxalate [Menon and Mahle 1982]. Since vitamin C has been shown to increase urinary oxalate, researchers concluded that it might play a detrimental role in increasing the risk of kidney stone formation [Griffith *et al.* 1986, Power *et al.* 1984]. This finding has been challenged in several studies [Johnston 1999, Auer

et al. 1998, Curhan *et al.* 1999]. A recent cohort study by Curhan *et al.* (1999) in which 85.557 women were surveyed, evaluated the relationship between vitamin B_6 and C intake and the risk of calcium oxalate kidney stone formation. The results showed that high vitamin B_6 intake is inversely associated with the risk of stone formation while vitamin C showed no such association [Curhan *et al.* 1999]. The fact that both vitamin C and vitamin B_6 are involved in the oxalate metabolic pathway again draws attention to a different oxalate handling in blacks.

It seems that despite consuming a diet which could be considered as lithogenic, (high oxalate/ low calcium/low magnesium/low vitamin B_6), it appears that blacks are able to keep urinary oxalate at normal levels by unidentified protective mechanisms and to avert urolithiasis.

In the context of calcium oxalate urolithiasis it is of interest that according to dietary survey # 2, urbanized blacks seem to consume significantly less total sugar ($p=0.011$) and less added sugar ($p=0.055$). Similarly to dietary salt, it is difficult to ascertain dietary intake of sugar because of its accompaniment with meals. Therefore special precautions were made to include an additional table to the food-frequency questionnaire assessing sugar added to food/drinks as for example coffee, cereal etc. [appendix CD: chapter 2/diet quest].

Some researchers have claimed that the higher incidence of stones in wealthier countries is due to an increased refined sugar intake [Blacklock 1987, Robertson *et al.* 1978, Robertson 1987]. Leman *et al.* (1969) observed that glucose increases the rate of urinary calcium excretion both in normal and calcium oxalate stone forming subjects, although this increase was more prominent in the latter group. Similarly, other studies have revealed that glucose increases intestinal calcium absorption in a dose-dependent manner [Wood *et al.* 1987, Knowles *et al.* 1988]. Furthermore, Blacklock (1987) suggested that in over 70 per cent of idiopathic stone formers, the excretion of urinary risk factors increases when sucrose is consumed. Also, there is evidence that refined carbohydrates stimulate endogenous

synthesis of oxalate, which is then eliminated via the urine [Nguyen *et al.* 1986]. The nature of the mechanism is still unknown and to date there is no evidence that calcium oxalate renal stone formers have an increased intake of refined sugar. Nevertheless, on the basis of present knowledge, it could be postulated that because refined carbohydrates induce calciuria and oxaluria, their decreased ingestion in blacks could decrease the risk of stone formation in this race group.

In view of the results described in this chapter, it can be said that the apparent immunity to calcium oxalate urolithiasis demonstrated by South African blacks cannot be attributed to a simple dietary relationship. The lower intakes of calcium and magnesium in this population group suggest a different intestinal handling of oxalate to that reported in Caucasians. Furthermore, the lower intake of protein and refined carbohydrates in this group could be an additional contributing factor to their lower risk of CaOx urolithiasis.

It is recognized that the present study had limitations. Firstly, both surveys involved a relatively small number of subjects, all of whom were males. Dietary recall questionnaire are considered accurate only with a sample number larger than 50. Secondly the two dietary surveys differed in design. However, despite these shortcomings, the results obtained are of significant interest to warrant in-depth investigation of hypotheses which have been promulgated in this chapter. Such investigations are discussed in chapters 3 to 6 of this thesis.

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ChapterThree

Effects of a high oxalate/low calcium diet on calcium oxalate renal stone risk factors in black and white South African subjects

3.1 Introduction

It is well established that diet plays a key role in determining urine chemistry and, as a consequence, can influence the risk of stone formation. Several different lithogenic and prophylactic diets have been investigated in previous studies. In the context of calcium oxalate urolithiasis, diets that have received the most attention are those that have been high and low in Ca [Messa *et al.* 1997, Hess 1996, Jaeger *et al.* 1994], high and low in oxalate [Hess *et al.* 1998, Finch *et al.* 1981] and combinations thereof [Hess *et al.* 1998]. Indeed, the effect of dietary calcium and oxalate on the urinary risk factors associated with calcium oxalate stone formation has been the subject of several excellent reviews [Hess 1996, Massey *et al.* 1993, Heller 1999, Curhan *et al.* 1993, Curhan *et al.* 1997]. These studies have shown that a restriction in calcium enhances oxalate absorption and excretion, whereas an increase in calcium intake will reduce urinary oxalate by binding more oxalate in the gut. Evidence in support of such a mechanism is provided by two large prospective studies which showed a decrease in the risk of stone formation with increasing intake of dietary calcium [Curhan *et al.* 1993, Curhan *et al.* 1997]. On the other hand, other studies have shown that there is a direct correlation between dietary oxalate and oxalate excretion [Massey *et al.* 1993].

Since previous studies involving low calcium / high oxalate protocols have proven to increase stone risk, the response of stone free blacks to such protocols would be of considerable interest. Furthermore, the possibility that the two groups handle renal mechanisms differently is an additional factor to be considered. The present study was undertaken to address this issue.

3.2 Materials and Methods

Study Design

The present study had a cross-sectional analytical design.

Study Population

Two experimental groups, consisting of 11 black males and 11 white males were age matched. The black group was recruited from a peri-urban population attending the orthopaedic ward in the Groote Schuur Hospital. All had only orthopaedic-related minor injuries, which did not require any medication. Subjects in the white group were resident in the Cape Town area. None of the subjects in either group had a metabolic illness or any clinical problem. The protocol was approved by the Ethical and Research council of the University of Cape Town.

Biographical Data

For each subject, brief information about their social and medical history was collected. The purpose and procedure of the study was explained to each subject. Subjects who agreed to participate in the study signed a letter of consent (see appendix CD: chapter3/diet quest).

Dietary Data

The subjects' normal home-diet food intake was assessed using a semi-quantitative food frequency questionnaire. To determine nutrient content, all the data captured from the dietary questionnaire were analyzed using Food Composition Tables [Langenhoven *et al.* 1991, Kruger *et al.* 1998].

Experimental Procedure

Subjects in hospital consumed a full ward diet prescribed by the hospital kitchen. This diet, which was varied over a 14-day cycle, was comprehensively analyzed using Food

Composition Tables. An equivalent diet was designed for the non-hospitalized group. Dietary intake was controlled by instructing subjects to follow a dietary eating plan that clearly defined food groups. Additional guidelines were given on food choices and portion sizes (appendix CD: chapter 3/diet quest). Each subject in both groups was given an oxalate-rich snack on each of 3 successive days – day 1: 2 X 180g Greek spinach pie; day 2: 100g beetroot salad; day 3: 110g rhubarb apple pie. The corresponding total daily intake during the three experimental days was 318-334 mg calcium, 510 mg oxalate, 21-31 g animal protein and 28-48 mg ascorbic acid (table 3-1) (for recipes refer to appendix CD: chapter3/diet recipes).

Table 3-1: Nutrient content of the Full Ward Diet (FWD), with and without snack, during the experimental days.

	Day 1		Day2		Day3	
	FWD	FWD + snack	FWD	FWD + snack	FWD	FWD + snack
Calcium	278mg	323mg	329mg	334mg	290mg	318mg
Oxalate	30mg	510mg	15mg	510mg	15mg	510mg
Animal Protein	30g	31g	28g	28g	21g	21g
Vitamin C	44mg	48mg	25mg	28mg	29mg	31mg

Urine Analysis

Urine Collection and Preparation

Twenty-four hour urine samples were collected for each subject at baseline and during the last day of the supplemental period. Urines were tested for the presence of blood and infection (Combur 10 test strips, Boehringer Mannheim, Mannheim, Germany). All urine samples that tested positive for haematuria or the presence of nitrite were discarded. Urine pH and volume were routinely measured. Aliquots were filtered through a 0.74µm filter to remove cellular debris and proteinaceous material. Urinary sodium, potassium, calcium and magnesium were then determined by atomic absorption spectroscopy while oxalate, citrate, phosphate, urate, chloride and creatinine were determined using commercially available assay kits (Boehringer Mannheim).

Calcium Oxalate Metastable Limit

The metastable limit (MSL) of each urine specimen was determined according to the method described by Ryall et al [Ryall *et al*. 1985].

To induce spontaneous crystallisation, the filtered urine samples were treated with sodium oxalate of increasing concentration (0.0 - 2 mmol/dm^3) every 5 minutes. After 30 minutes incubation at 37°C, particle number was measured using a Coulter Counter Multisizer (Beckman Coulter, Johannesburg, South Africa) (140 µm orifice, 2.8-90.0 µm particle size range, ± 0.5% instrument error). A graph of particle number versus sodium oxalate concentration was plotted and the metastable limit determined by interpolation of the line to the abscissa (figure 3-1):

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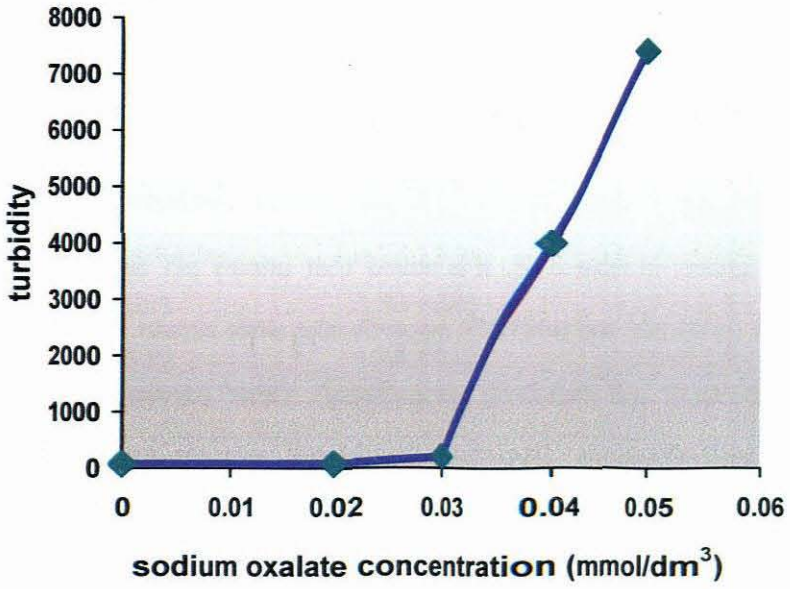
Figure 3-1: Example of Metastable Limit Graph



Risk Index and Relative Supersaturation

Urinalysis data were used to calculate the Tiseluis risk index $\{(Ca/Cr)^{0.71} \times (Ox/Cr) / (Mg/Cr)^{0.14} \times (Cit/Cr)^{0.1}\}$ [Tiselius 1982], while relative urinary supersaturations of calcium oxalate (RS CaOx), uric acid (RS uric acid) and calcium phosphate (RS brushite) were obtained using the computer program EQUIL [Werness *et al.* 1985].

Statistical analysis

All data were statistically analysed by the method of analysis of variance using the SAS statistical package. Results were considered statistically significant if $p \leq 0.05$.

3.3 Results

The mean baseline intake of nutrients for the two race groups was determined from the completed dietary questionnaires and was presented in the previous chapter.

Urinary parameters and computed risk indices for both race groups prior to implementation of the dietary protocol are given in table 3-2. It is noted that urinary pH and oxalate are significantly higher in blacks (p=0.008 and p=0.009 respectively) while citrate and phosphate are significantly lower (p=0.0003 and p=0.010 respectively). Other parameters (volume, calcium, magnesium, sodium, potassium, uric acid, creatinine and chloride) showed no significant differences at baseline. Relative supersaturation values (which are computed from all the urinary parameters shown in table 3-2) for calcium oxalate and brushite were significantly higher in blacks (p=0.0009 and p=0.018 respectively) while the value for uric acid was significantly lower (p=0.027). The Tiselius risk index (computed from urinary calcium, magnesium, citrate, oxalate and creatinine only) was significantly higher in blacks (p=0.023). Metastable limits were not different at baseline.

Comparison of the pre- and post-dietary protocol parameters within each group (table 3-3) shows that the only urinary variables which changed significantly were oxalate excretion which increased in whites and the Tiselius risk index which increased in blacks after ingestion of the special diet (p=0.022 and p=0.019 respectively).

Table 3-2: Comparison of mean urinary parameters (+/- SE) between black and white subjects prior to the dietary protocol

Variable	Black	White	p-value
pH	6.50 ±0.07	6.21 ± 0.07	0.008*
Volume (ml/24hr)	1554 ± 201	1726 ± 201	0.547
Citrate (mmol/24hr)	1.47 ± 0.40	3.69 ± 0.40	<0.001*
Oxalate (mmol/24hr)	0.23 ± 0.02	0.14 ± 0.02	0.008*
Ca (mmol/24hr)	3.83 ± 0.80	3.05 ± 0.80	0.492
Mg (mmol/24hr)	4.38 ± 0.52	4.35 ± 0.52	0.968
Na^+ (mmol/24hr)	83.96 ± 19.19	102.50 ± 19.19	0.498
K^+ (mmol/24hr)	67.42 ± 7.33	60.04 ± 7.33	0.481
Uric acid (mmol/24hr)	3.02 ± 0.37	3.69 ± 0.37	0.209
Creatinine (mmol/24hr)	12.80 ± 0.87	14.63 ± 0.87	0.153
$P0_4$ (mmol/24hr)	19.93 ± 3.03	31.45 ± 3.03	0.010*
Cl^- (mmol/24hr)	101.90 ± 17.5	145.30 ± 17.5	0.095
MSL	0.42 ± 0.10	0.66 ± 0.10	0.100
Tiselius risk index	281 ± 44	134 ± 44	0.023*
RS CaOx	5.79 ± 0.80	1.71 ± 0.80	0.001*
RS brushite	2.04 ± 0.34	0.87 ± 0.34	0.018*
RS uric acid	0.61 ± 0.26	1.45 ± 0.26	0.027*

*significance at p≤0.05; ± std error

Table 3-3: Comparison of pre-protocol and post-protocol urinary parameters (± SE) in both race groups

	Blacks			Whites		
Variables	Baseline	high oxalate /low calcium	p	Baseline	high oxalate /low calcium	p
pH	6.50 ± 0.07	6.58 ± 0.07	0.458	6.21 ± 0.07	6.17 ± 0.07	0.662
Volume (ml/24h)	1554 ± 201	1873 ± 201	0.269	1726 ± 201	1783 ± 201	0.547
Citrate (mmol/24h)	1.47 ± 0.40	2.08 ± 0.40	0.289	3.69 ± 0.40	2.76 ± 0.40	0.111
Oxalate (mmol/24h)	0.23 ± 0.02	0.28 ± 0.02	0.114	0.14 ± 0.02	0.22± 0.02	0.022*
Ca (mmol/24h)	3.83 ± 0.80	4.97 ± 0.80	0.322	3.05 ± 0.80	3.34 ± 0.80	0.795
Mg (mmol/24h)	4.38 ± 0.52	4.25 ± 0.52	0.862	4.35 ± 0.52	4.37 ± 0.52	0.974
Na+ (mmol/24h)	83.96± 19.19	110.05± 19.19	0.342	102.50 ± 19.17	131.85 ± 19.17	0.286
K+ (mmol/24h)	67.42 ± 7.33	51.85 ± 7.33	0.141	60.04 ± 7.33	50.37 ± 7.33	0.357
Urate (mmol/24h)	3.02 ± 0.37	3.03 ± 0.37	0.986	3.69 ± 0.37	3.73 ± 0.37	0.945
Creatinine (mmol/24h)	12.80 ± 0.87	10.96 ± 0.87	0.151	14.63 ± 0.87	14.03 ± 0.87	0.635
PO4 (mmol/24h)	19.93 ± 3.03	17.01 ± 3.03	0.499	31.45 ± 3.03	30.45 ± 3.03	0.816
Cl- (mmol/24h)	101.90± 18.37	129.40± 17.51	0.286	145.30 ± 17.51	149.30 ± 17.51	0.875
msl	0.42 ± 0.10	0.409 ± 0.10	0.963	0.66 ± 0.10	0.46 ± 0.10	0.183
Tiselius risk index	281 ± 44	434 ± 44	0.017*	134 ± 44	191 ± 44	0.363
RS CaOx	5.79 ± 0.80	4.93 ± 0.80	0.453	1.71 ± 0.80	3.02 ± 0.80	0.252
RS brushite	2.04 ± 0.34	1.44 ± 0.34	0.232	0.87 ± 0.34	1.05 ± 0.34	0.709
RS uric acid	0.61 ± 0.26	0.41 ± 0.26	0.586	1.45 ± 0.26	1.46 ± 0.26	0.976

*significance at p≤0.05; ± std error

3.4 Discussion

The baseline urinalysis data for the two race groups in the present study reveal an intriguing anomaly: South African Blacks seldom form stones, yet the urinary pH and oxalate values in this group of peri-urban blacks are higher than in whites and their urinary citrate is lower! Since CaOx solubility decreases with increasing pH [Tiselius 1981] and high oxalate is widely regarded as a powerful predisposing factor in CaOx stone formation [Borsati 1991], these risk factors would have been expected to show precisely the reverse trends to those observed. Similarly, citrate is well known as an inhibitor by virtue of its ability to chelate calcium [Fleisch 1990] and to moderate crystallization [Tiselius 1993], and would therefore have been expected to be higher in blacks.

During the past 40 years, only two comprehensive studies of urine composition in South African blacks and whites have been conducted [Whalley *et al.* 1998, Modlin 1967]. The present result for pH differs to that reported by Modlin who found this parameter to be lower in blacks; pH values were not reported by Whalley *et al.* (1998). Although CaOx solubility decreases with increasing pH [Werness *et al.* 1985], a contrary, pH-dependent effect has also been reported in which the inhibitory action of citrate and pyrophosphate increases as the pH rises [Hesse *et al.* 1997]. However, this is unlikely to be contributory to the black populations' stone immunity, especially since urinary citrate was found to be relatively low in this race group. Nevertheless, citrate excretion increased in blacks after the dietary oxalate load (albeit insignificantly), suggesting that there may be some synergism here.

The finding of relatively lower urinary citrate in blacks agrees with the results of both previous studies [Whalley *et al.* 1998, Modlin 1967]. The reason for the lower citrate is unknown. Whalley and co-workers provide compelling arguments in eliminating mechanisms such as high protein diet, decreased urinary magnesium and potassium depletion, any one of which might have explained the relative hypocitraturia in blacks [Whalley *et al.* 1998]. However,

irrespective of the mechanism by which this occurs, citraturia in blacks does not appear to be a key factor in stone prevention or pathogenesis as it is in whites.

The relatively higher oxalate in this group of blacks is equally intriguing. This also is difficult to explain. 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]. However, although other studies have shown that blacks ingest less calcium [Whalley *et al.* 1998, Wise and Kark 1961], dietary survey # 1 discussed in chapter 2 did not demonstrate a significant difference between the two races. Thus, the higher urinary oxalate in this group of blacks cannot be explained in this way. A possible reason for the higher urinary oxalate could be due to the different population groups of blacks used in the present study; as discussed in chapter 2 the black subject group in the present study was peri-urban compared to the urbanized black population groups used in other studies. Another possible explanation of the higher urinary oxalate in this group of blacks might be related to the respective vitamin B_6 intake in the two groups. Dietary patterns analyzed in the present study revealed a significantly lower B_6 intake in blacks than in whites. This vitamin is a cofactor in the oxalate metabolic pathway. Studies in humans [Gershoff *et al.* 1959] and animals [Faber *et al.* 1963] refer to a B_6 deficiency leading to increased oxalate production and urinary excretion. Although no such deficiency was demonstrated in blacks, the *relatively* lower B_6 intake might be the cause of the relatively higher oxalate excretion in blacks. However, irrespective of this argument, it appears that oxaluria in blacks does not provide any obvious clues which might explain this race group's stone immunity.

Although not significantly different at baseline, it is noted that the metastable limit decreased (57%) following the dietary protocol only in whites. This indicates that the low calcium / high oxalate protocol had more of an effect on the white subject group than it had on the black subject group, once again supporting the view that the renal handling of a dietary oxalate load is different in the two groups.

The empirically computed Risk Index [Tiselius 1982] and relative supersaturation ratio [Werness *et al.* 1985] are widely used as predictors of crystallization probability. Here too, the results are contrary to those expected: values for the risk index, relative supersaturation of calcium oxalate and relative supersaturation of brushite are higher in blacks, supposedly indicating a greater probability of stone formation than in whites. A possible explanation for this anomaly is that both of these mathematical predictors have limitations as neither incorporates the concentration of urinary proteins and other macromolecules in their computations nor do these risk indices take into account physiological mechanisms such as oxalate absorption. Therefore, it might be postulated that the black population's immunity towards stones may be linked to such factors. The higher relative supersaturation of uric acid in whites is not surprising in the light of this group's higher dietary intake of animal protein. This parameter is not regarded as being critically important in assessing stone immunity in blacks.

Thus, the results of this study in which a potently lithogenic dietary protocol was administered, indicate that the conventional urine parameters and empirical indices which have been used widely and routinely to assess stone formation have not provided clues to explain the immunity towards this disease in blacks and are of little value in this regard. Indeed, as discussed earlier, some results have been totally contrary to those which might have been reasonably expected. One is therefore forced to look elsewhere for an explanation. A possible insight is afforded by the present results. It is noted that following the high oxalate – low calcium dietary load, oxalate excretion in whites increased significantly (ie by 57%), while no significant change occurred in this parameter in blacks. Oxalate-rich foods such as those used in this study have been reported as causing a significant increase (>100%) in urinary oxalate excretion [Massey and Roman-Smith 1993] while dietary calcium restriction has had the same effect [Massey and Roman-Smith 1993]. It is therefore of some considerable interest that no such effect was observed in the black subjects suggesting that

different renal handling mechanisms may occur in the two race groups in response to lithogenic and anti-lithogenic dietary challenges. This aspect is explored in chapters 4 and 5 which follow.

3.5 References

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ChapterFour

Effects of different, potentially lithogenic and antilithogenic dietary protocols, on urinary risk factors for calcium oxalate kidney stone formation in black and white South African subjects:
Low calcium
High oxalate
Vegetarian
Vitamin C – rich
High salt

Evidence of different renal handling mechanisms in different race groups

4.1 Introduction

In the previous chapter, a high oxalate/low calcium protocol induced different urinary effects in the two race groups, prompting the suggestion that different renal handling mechanisms might be occurring. Clearly, investigation of the *individual* effects of high oxalate and low calcium diets in the two race groups is warranted. In addition, other lithogenic diets discussed in the General Introduction (vitamin C – rich and high salt) also warrant investigation in the South African context. Furthermore, the effects of a vegetarian diet (also discussed in the General Introduction) promise to be of some interest.

4.2 Material and Methods

The study comprised 10 black and 10 white South African males between the ages 18-28. All the subjects were healthy without any history of kidney stones or any metabolic disorder.

Experimental Procedure

The five protocols (low calcium, high oxalate, vegetarian, vitamin C – rich and high salt) were administered using a complete Latin Square design. Each subject followed each protocol for 4 days. A washout period of 7 days was observed before each protocol. Protocols were based on a standardized diet with prescribed and clearly defined daily portions of all the major food groups (appendix CD:chapter4/diet sheets). All of the subjects received detailed dietary instructions in this regard. Nutrients were added or excluded for the respective protocols. For the "low calcium diet", all dairy products were excluded; for the "high oxalate diet", 230g beetroot per day was included; for the "vitamin C diet", 1000mg ascorbic acid per day was added; for the lacto-vegetarian diet", meat, chicken, fish and eggs were excluded; for the "high salt diet", 15g sodium chloride per day was added. The overall nutrient content per day of each protocol was 400 mg calcium ("low calcium"); 510mg oxalate ("high oxalate"); 1000mg ascorbic acid supplement ("vitamin C"); 15 to 18g sodium chloride ("high

salt") taken in three equal doses at breakfast, lunch and dinner. Only the aforementioned nutrients were controlled. However, subjects were instructed to follow a dietary eating plan which standardized their nutrient intake. To allow for metabolic adaptation the subjects started following the standardized diet 7 days prior each protocol. The oxalate content of foods was determined using the Food Finder Tables [Kruger *et al.* 1998]. A 24-hour dietary recall questionnaire was recorded on the day of the baseline urine collection and analyzed for macro- and micronutrients using the computer programme "Foodfinder" version 5.

Urine Analysis

24 hr urine samples were collected from each subject at baseline and after 4 days on the prescribed diet. The choice of a four day period of consumption was regarded as being long enough to achieve metabolic stabilization and was based on that used in other studies. For example, in their study of the effects of three different diets on urinary excretion of calcium and oxalate, Juuti and Alhava administered the protocol for at least three days [Juuti and Alhava 1980]. Urinary sodium, potassium, calcium and magnesium were determined by atomic absorption spectroscopy while oxalate, citrate, phosphate, urate, chloride and creatinine were determined using commercially available assay kits. Urine pH and volume were measured routinely. The urinary excretion values were used to determine the Tiselius risk index [Tiselius 1982] and the relative urinary supersaturations of calcium oxalate, uric acid and calcium phosphate (brushite) [Werness *et al.* 1985].

The CaOx metastable limit of each urine sample was determined using a Coulter Multisizer (Beckman Coulter, Johannesburg, South Africa) [Ryall *et al.* 1985]. An oxalate load of 30mmol/l in excess of the metastable limit was then added to each filtered urine sample and incubated at 37°C for 90 min. The particle volume - size distribution was measured at 90 min using a Coulter Multisizer to assess the effect of the protocols on each urine's potential to support crystallization of calcium oxalate.

Statistical Analysis

Statistical analysis was performed by analysis of variance using the SAS statistical package. Results were considered statistically significant if $p \leq 0.05$.

4.3 Results

Dietary Analysis

The mean baseline intake of nutrients for the two groups was determined from the completed dietary questionnaires. Values are given in table 4-1. There were no significant differences between any of the nutrients except for vitamin C which was lower in blacks (105.3 ± 20.9 mg/day vs 207.1 ± 19.6 mg/day, p=0.002) (table 4-1).

Table 4-1: Mean and Median daily intake of nutrients for black and white subjects on 'controlled diet'

	Blacks	Whites	p-value
Age	23.5 ± 2.72	22.87 ± 0.62	0.072
BMI	22.64 ± 0.31	22.87 ± 0.62	0.745
Energy (kJ)	10260.9 ± 1391.81	12742.3 ± 816.51	0.141
Total Protein (g/day)	86.84 ± 9.51	108.34 ± 8.98	0.118
Animal Protein (g/day)	57.14 ± 7.71	59.55 ± 6.06	0.809
Total Fat (g/day)	93.63 ± 18.49	100.06 ± 7.84	0.752
Added sugar (g/day)	76.39 ± 16.33	74.03 ± 9.77	0.900
Oxalic acid (mg/day)	208.34 ± 50.16	124.18 ± 25.60	0.158
Fibre (g/day)	19.02 ± 4.02	33.71 ± 9.13	0.158
Ca (mg/day)	786.20 ± 170.79	1026.10 ± 148.25	0.303
Mg (mg/day)	301.80 ± 37.08	448.10 ± 89.61	0.149
P04 (mg/day)	1337.80 ± 199.86	1801.10 ± 257.86	0.174
K (mg/day)	3071.10 ± 489.66	3878.30 ± 437.91	0.235
Na (mg/day)	4237.00 ± 537.39	3748.2 ± 618.4	0.559
Zn (mg/day)	12.55 ± 1.63	13.84 ± 1.92	0.615
Vitamin A (RE/day)	1181.20 ± 394.64	1008.60 ± 174.30	0.694
Vitamin B6 (mg/day)	2.18 ± 0.35	3.03 ± 0.86	0.379
Vitamin C (mg/day)	105.30 ± 20.93	207.10 ± 19.57	0.002*
Vitamin D (µg/day)	6.46 ± 1.62	5.80 ± 1.73	0.782

*significance at $p \leq 0.05$; ± std error

Urine Analysis

At baseline (table 4-2) blacks had significantly lower urinary calcium, urate and phosphate (p=0.03, p=0.04, and p=0.01 respectively), while their calcium oxalate metastable limit was significantly higher (p=0.01). Other urinary variables were not significantly different at baseline, although the lower relative supersaturation of CaOx in blacks approached significance.

The low calcium protocol (table 4-3) caused statistically significant changes only in blacks where it increased the urinary oxalate (p=0.01), decreased the relative supersaturation of CaOx (p=0.03) and raised the relative supersaturation of brushite (p=0.03).

The high oxalate protocol (table 4-4) significantly raised the urinary pH and potassium excretion (p=0.01 and p=0.01 respectively), and significantly increased the supersaturation of brushite (p=0.05) in whites. In blacks urinary citrate and volume were significantly raised by this protocol (p=0.01 and p=0.04 respectively).

The vegetarian protocol (table 4-5) caused a significant increase in the pH of blacks (p=0.01) while the vitamin C protocol (table 4-6) raised the risk index in blacks (p=0.02). As expected the NaCl protocol (table 4-7) increased the urinary sodium and chloride in blacks (p=0.03 and p=0.02) and the urinary chloride in whites (p<0.01).

Table 4-2: Urine variables in black and white subjects at baseline

Variables	Blacks		Whites		p
pH	6.22	± 0.09	6.24	± 0.10	0.902
Volume (ml/24h)	1178	± 117.88	1092	± 123.63	0.615
Citrate (mmol/24h)	1.94	± 0.28	2.01	± 0.29	0.858
Oxalate (mmol/24h)	0.17	± 0.02	0.17	± 0.02	0.961
Ca (mmol/24h)	2.00	± 0.34	3.11	± 0.36	0.028*
Mg (mmol/24h)	3.05	± 0.59	4.37	± 0.62	0.128
Na^+ (mmol/24h)	165.30	± 31.67	201.62	± 33.23	0.431
K^+ (mmol/24h)	35.20	± 8.39	40.01	± 8.81	0.693
Urate (mmol/24h)	2.57	± 0.26	3.37	± 0.27	0.039*
Creatinine (mmol/24h)	11.91	± 0.001	13.71	± 0.001	0.305
PO_4 (mmol/24h)	19.69	± 2.54	28.89	± 2.66	0.014*
Cl^- (mmol/24h)	129.18	± 14.61	144.00	± 15.33	0.486
Tiselius risk index	173	± 64.51	190	± 67.66	0.858
MSL	0.11	± 0.01	0.06	± 0.01	0.014*
RS CaOx	1.88	± 0.30	2.75	± 0.32	0.050*
RS brushite	0.85	± 0.26	1.34	± 0.28	0.207
RS uric acid	1.17	± 0.27	1.43	± 0.28	0.500
Ca/Na ratio	0.012	± 0.002	0.017	± 0.003	0.160

*significance at $p \leq 0.05$; ± std error

Table 4-3: Urine variables in black and white subjects following the "low calcium" protocol

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	Low Calcium Diet	p	Baseline	Low Calcium Diet	p	Low Calcium Diet p
pH	6.22	6.25	0.811	6.24	6.35	0.411	0.465
Volume (ml/24h)	1178	1170	0.961	1092	1018	0.675	0.377
Citrate (mmol/24h)	1.94	1.89	0.902	2.01	1.84	0.672	0.861
Oxalate (mmol/24h)	0.17	0.23	0.008*	0.17	0.16	0.811	<0.01*
Ca (mmol/24h)	2.00	1.14	0.079	3.11	2.31	0.118	0.020*
Mg (mmol/24h)	3.05	2.75	0.724	4.37	4.43	0.942	0.050*
Na^+ (mmol/24h)	165.30	181.23	0.723	201.62	168.16	0.478	0.777
K^+ (mmol/24h)	35.20	36.55	0.910	40.01	38.83	0.925	0.851
Urate (mmol/24h)	2.57	2.54	0.922	3.37	3.16	0.590	0.104
Creatinine (mmol/24h)	11.91	14.44	0.150	13.71	13.14	0.750	0.478
PO_4 (mmol/24h)	19.69	21.13	0.690	28.89	25.36	0.351	0.253
Cl^- (mmol/24h)	129.18	118.27	0.600	144.00	131.30	0.559	0.540
Tiselius risk index	173.00	231.00	0.523	190.00	140.00	0.605	0.330
MSL	0.11	0.11	0.991	0.06	0.08	0.297	0.154
RS CaOx	1.88	0.97	0.034*	2.75	3.01	0.767	<0.01*
RS brushite	0.85	1.69	0.027*	1.34	1.33	0.987	0.352
RS uric acid	1.17	1.32	0.685	1.43	1.01	0.300	0.432
Ca/Na ratio	0.012	0.008	0.061	0.017	0.018	0.742	0.920

*significance at $p \leq 0.05$

Table 4-4: Urine variables in black and white subjects following the "high oxalate" protocol

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	High Oxalate Diet	p	Baseline	High Oxalate Diet	p	High Oxalate Diet p
pH	6.22	6.42	0.136	6.24	6.62	0.007*	0.142
Volume (ml/24h)	1178	1520	0.043*	1092	1206	0.514	0.069
Citrate (mmol/24h)	1.94	2.99	0.008*	2.01	2.17	0.690	0.043*
Oxalate (mmol/24h)	0.17	0.15	0.344	0.17	0.14	0.234	0.731
Ca (mmol/24h)	2.00	1.89	0.816	3.11	2.66	0.378	0.123
Mg (mmol/24h)	3.05	3.51	0.588	4.37	5.90	0.085	0.006*
Na^+ (mmol/24h)	165.30	248.00	0.068	201.62	264.45	0.184	0.721
K^+ (mmol/24h)	35.20	40.10	0.681	40.01	73.49	0.008*	0.007*
Urate (mmol/24h)	2.57	2.75	0.642	3.37	3.34	0.940	0.121
Creatinine (mmol/24h)	11.91	10.80	0.516	13.71	14.88	0.514	0.021*
PO_4 (mmol/24h)	19.69	20.35	0.856	28.89	27.90	0.793	0.043*
Cl^- (mmol/24h)	129.18	162.36	0.112	144.00	149.20	0.811	0.536
Tiselius risk index	173.00	318.00	0.626	190.00	150.00	0.677	0.469
MSL	0.11	0.11	0.855	0.06	0.06	0.943	0.011*
RS CaOx	1.88	1.19	0.110	2.75	2.07	0.133	0.047*
RS brushite	0.85	0.60	0.497	1.34	2.12	0.050*	<0.01*
RS uric acid	1.17	0.66	0.190	1.43	0.76	0.095	0.802
Ca/Na ratio	0.012	0.012	0.791	0.017	0.013	0.431	0.224

*significance at $p \leq 0.05$

Table 4-5: Urine variables in black and white subjects following the "vegetarian" protocol

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	Lacto-Vegetarian Diet	p	Baseline	Lacto-Vegetarian Diet	p	Lacto-Vegetarian Diet p
pH	6.22	6.57	0.009*	6.24	6.43	0.155	0.315
Volume (ml/24h)	1178	1301	0.462	1092	1316	0.203	0.932
Citrate (mmol/24h)	1.94	2.19	0.528	2.01	2.12	0.790	0.868
Oxalate (mmol/24h)	0.17	0.16	0.850	0.17	0.19	0.404	0.322
Ca (mmol/24h)	2.00	1.66	0.475	3.11	3.02	0.868	0.007*
Mg (mmol/24h)	3.05	3.19	0.873	4.37	5.05	0.439	0.032*
Na^+ (mmol/24h)	165.30	217.32	0.248	201.62	248.96	0.316	0.493
K^+ (mmol/24h)	35.20	38.75	0.765	40.01	60.47	0.104	0.077
Urate (mmol/24h)	2.57	2.05	0.158	3.37	2.69	0.083	0.093
Creatinine (mmol/24h)	11.91	11.73	0.915	13.71	10.43	0.070	0.459
PO_4 (mmol/24h)	19.69	19.63	0.986	28.89	24.26	0.222	0.211
Cl^- (mmol/24h)	129.18	138.27	0.661	144.00	146.6	0.905	0.695
Tiselius risk index	173.00	183.00	0.915	190.00	357.00	0.083	0.065
MSL	0.11	0.11	0.839	0.06	0.06	0.829	0.015*
RS CaOx	1.88	1.61	0.519	2.75	3.01	0.578	0.002*
RS brushite	0.85	0.72	0.712	1.34	1.40	0.864	0.074
RS uric acid	1.17	0.52	0.095	1.43	0.72	0.081	0.607
Ca/Na ratio	0.012	0.008	0.043*	0.017	0.015	0.740	0.043*

*significance at $p \leq 0.05$

Table 4-6: Urine variables in black and white subjects following the "vitamin C" protocol

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	Vitamin C Diet	p	Baseline	Vitamin C Diet	p	Vitamin C Diet p
pH	6.22	6.34	0.358	6.24	6.31	0.592	0.821
Volume (ml/24h)	1178	1407	0.173	1092	1185	0.596	0.196
Citrate (mmol/24h)	1.94	1.91	0.935	2.01	1.96	0.911	0.886
Oxalate (mmol/24h)	0.17	0.18	0.622	0.17	0.16	0.662	0.330
Ca (mmol/24h)	2.00	1.87	0.787	3.11	2.62	0.340	0.132
Mg (mmol/24h)	3.05	3.31	0.763	4.37	4.93	0.524	0.061
Na^+ (mmol/24h)	165.30	198.31	0.463	201.62	188.49	0.781	0.831
K^+ (mmol/24h)	35.20	44.25	0.450	40.01	55.12	0.228	0.374
Urate (mmol/24h)	2.57	2.11	0.214	3.37	3.07	0.442	0.013*
Creatinine (mmol/24h)	11.91	11.38	0.758	13.71	11.44	0.207	0.973
PO_4 (mmol/24h)	19.69	17.15	0.480	28.89	27.28	0.670	0.007*
Cl^- (mmol/24h)	129.18	150.90	0.310	144.00	134.30	0.655	0.448
Tiselius risk index	173.00	393.00	0.018*	190.00	192.00	0.978	0.035*
MSL	0.11	0.10	0.881	0.06	0.06	0.906	0.015*
RS CaOx	1.88	1.78	0.800	2.75	1.99	0.090	0.634
RS brushite	0.85	0.56	0.431	1.34	1.11	0.570	0.148
RS uric acid	1.17	0.94	0.563	1.43	1.51	0.850	0.154
Ca/Na ratio	0.012	0.011	0.070	0.017	0.019	0.661	0.160

*significance at $p \leq 0.05$

Table 4-7: Urine variables in black and white subjects following the "high salt" protocol

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	NaCl Diet	p	Baseline	NaCl Diet	p	NaCl Diet p
pH	6.22	6.25	0.785	6.24	6.48	0.090	0.109
Volume (ml/24h)	1178	1413	0.162	1092	1308	0.219	0.541
Citrate (mmol/24h)	1.94	2.64	0.075	2.01	2.33	0.442	0.434
Oxalate (mmol/24h)	0.17	0.14	0.174	0.17	0.15	0.578	0.476
Ca (mmol/24h)	2.00	2.06	0.913	3.11	3.20	0.851	0.022*
Mg (mmol/24h)	3.05	3.29	0.782	4.37	3.83	0.539	0.529
Na^+ (mmol/24h)	165.30	263.21	0.031*	201.62	273.55	0.129	0.822
K^+ (mmol/24h)	35.20	51.06	0.185	40.01	53.89	0.278	0.817
Urate (mmol/24h)	2.57	2.69	0.751	3.37	3.75	0.331	0.006*
Creatinine (mmol/24h)	11.91	11.99	0.962	13.71	12.43	0.476	0.802
PO_4 (mmol/24h)	19.69	19.58	0.976	28.89	31.12	0.555	0.002*
Cl^- (mmol/24h)	129.18	177.91	0.021*	144.00	208.20	0.004*	0.156
Tiselius risk index	173.00	139.00	0.707	190.00	201.00	0.902	0.503
msl	0.11	0.12	0.491	0.06	0.04	0.441	<0.001*
RS CaOx	1.88	1.29	0.168	2.75	1.98	0.087	0.120
RS brushite	0.85	0.48	0.317	1.34	1.82	0.217	<0.001*
RS uric acid	1.17	1.05	0.772	1.43	1.49	0.883	0.268
Ca/Na ratio	0.012	0.009	0.211	0.017	0.013	0.243	0.190

*significance at $p \leq 0.05$

Particle Volume - Size Distribution

The mean particle volume - size distribution of black subjects (measured at 90 min after the addition of sodium oxalate) is shown in figure 4-1. All of the diets induced a decrease in mean particle size (diameter) relative to the baseline control (table 4-8). The low calcium and high oxalate protocol increased the mean particle volume (23.5% and 27.8% respectively) while the vegetarian, vitamin C and high salt protocol decreased the mean particle volume (47.9%, 11.4% and 24.7% respectively) (table 4-9). Furthermore, the total particle volume (depicted by the total area under the curve) increased for the high oxalate protocol (5.9%) and decreased for all other protocols, the greatest change resulting from the low calcium protocol (60.7% decrease) followed by the vegetarian protocol (52.3% decrease). The differences were not significant due to the large variation in particle volume between each urine sample (figure 4-2).

Figure 4-1: Particle Volume - Size Distribution in the Urine of Black Subjects

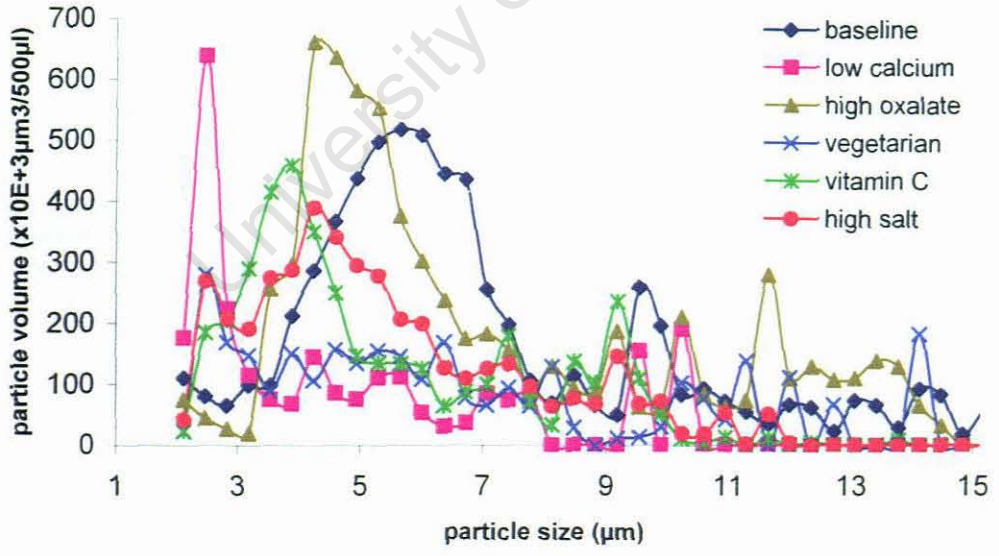


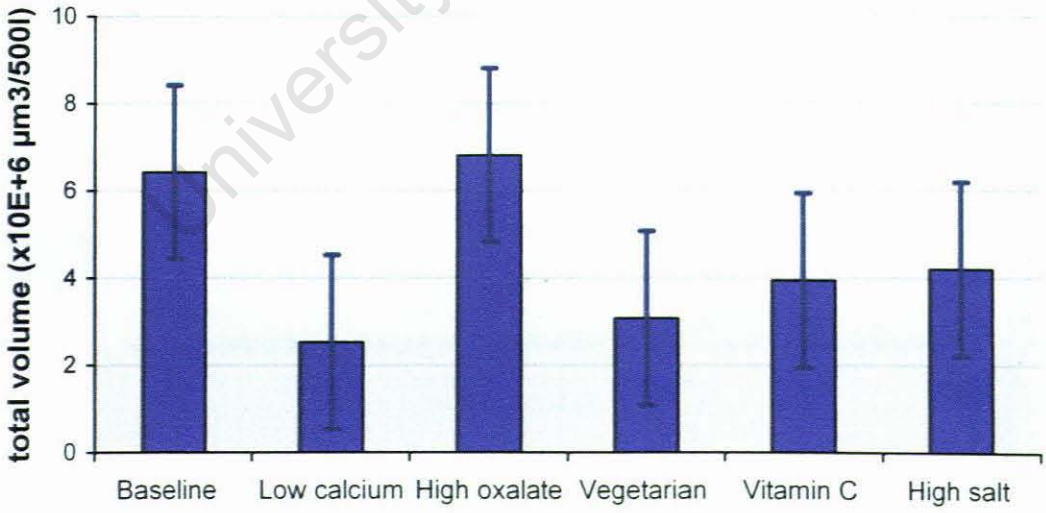
Table 4-8: Particle Volume - Size Distribution in Black Subjects

	Mean Particle Size (µm)	Mean Particle Volume ($x10^3$ μm^3/500 µl)	Total Volume ($x10^6$ μm^3/500 µl)
Baseline	5.65	516.87	6.43
Low calcium protocol	2.47	638.17	2.53
High oxalate protocol	4.24	660.66	6.81
Vegetarian protocol	2.47	268.99	3.07
Vitamin C protocol	3.89	457.87	3.94
High salt protocol	4.24	388.94	4.20

Table 4-9: Percentage Change (Relative to Baseline) in Mean Particle Parameters – Black Subjects

Variables	% decrease Mean Particle Size	% decrease Mean Particle Volume	% decrease Total Volume
Low calcium protocol	56.3	-23.5	60.7
High oxalate protocol	25.0	-27.8	-5.9
Vegetarian protocol	56.3	47.9	52.3
Vitamin C protocol	31.2	11.4	38.7
High salt protocol	25.0	24.7	34.7

Figure 4-2: Total Volume ($X10^3$ μM^3/500 µL)– Black Subjects



The mean particle volume – size distribution in the urine of white subjects (measured at 90 min after the addition of sodium oxalate) is shown in figure 4-3. As was observed in blacks, all of the diets induced a decrease in mean particle size (table 4-10). Mean particle volume relative to the baseline urine increased for the low calcium, high oxalate and high salt protocol (154%, 68.7% and 73.7% increase respectively) and decreased for the vegetarian and vitamin C protocol (69.3% and 75.4% decrease respectively) (table 4-11). In the same group the total volume followed the same trend: it increased for the low calcium, high oxalate and high salt protocols (11.8%, 218.7% and 184.9% increase respectively) and decreased for the vegetarian and vitamin C protocol (76.0% and 82.9% decrease respectively) (table 4-11). Differences for the respective increases for the low calcium, high oxalate and high salt protocol were significant (figure 4-4).

Figure 4-3: Particle Volume - Size Distribution in the urine of white subjects

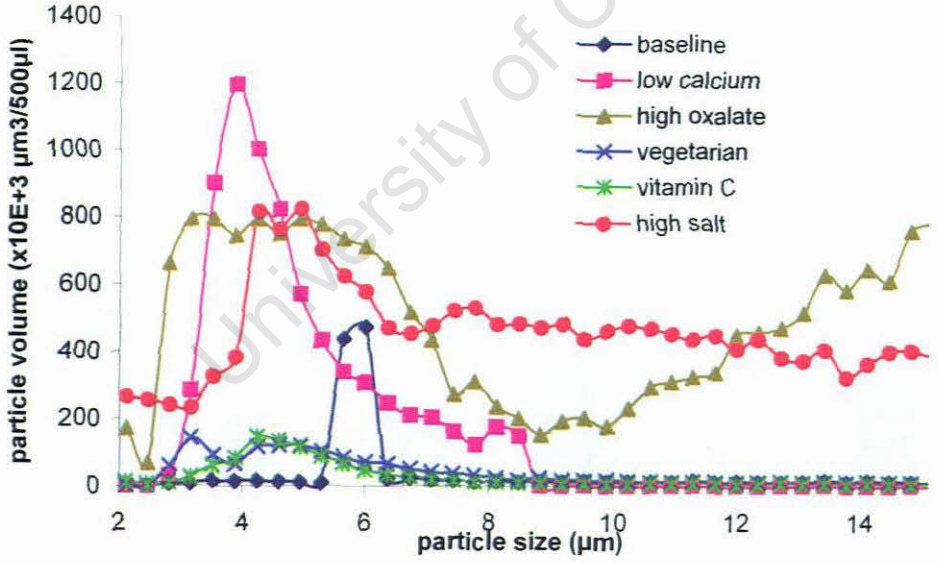


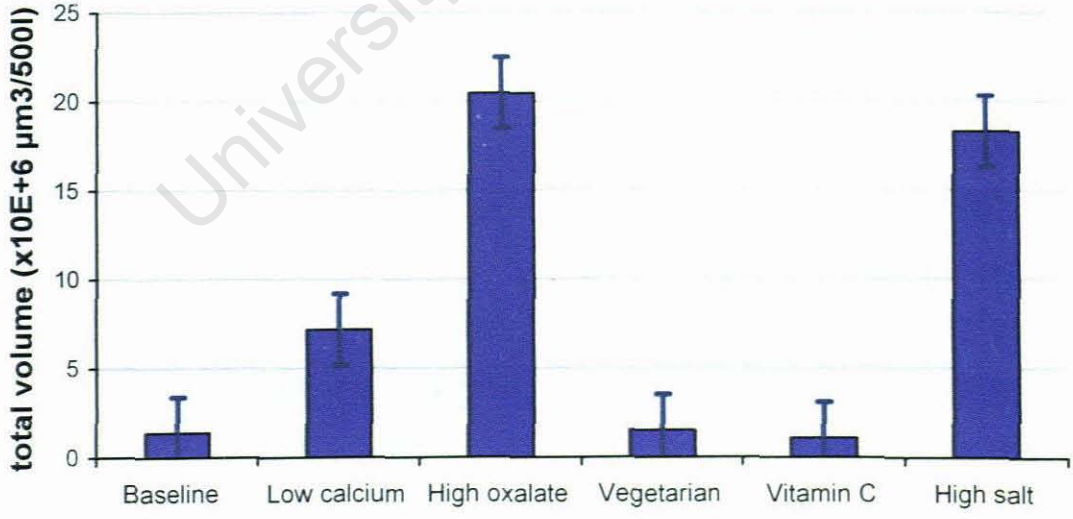
Table 4-10: Particle Volume - Size Distribution In White Subjects

	Mean Particle Size (µm)	Mean Particle Volume ($x10^3$ μm^3/500 µl)	Total Volume ($x10^6$ μm^3/500 µl)
Baseline	6.01	471.48	1.39
Low calcium protocol	3.89	1197.61	7.19
High oxalate protocol	4.24	795.51	20.49
Vegetarian protocol	3.18	144.70	1.54
Vitamin C protocol	4.24	116.08	1.10
High salt protocol	4.59	818.98	18.32

Table 4-11: Percentage Change (Compared to Baseline) in Mean Particle Parameters– Whites

Variables	% decrease Mean Particle Size	% decrease Mean Particle Volume	% decrease Total Volume
Low calcium protocol	35.3	-154.0	-11.8
High oxalate protocol	29.5	-68.7	-218.7
Vegetarian protocol	47.1	69.3	76.0
Vitamin C protocol	29.5	75.4	82.9
High salt protocol	23.6	-73.7	-184.9

Figure 4-4: Total Volume ($X10^3$ μM^3/500 µL)– White Subjects



4.4 Discussion

The observation of significantly *lower* baseline values for urinary calcium, urate and phosphate in black subjects is of interest as *high* levels of these components are widely regarded as important risk factors for CaOx stone formation [Robertson *et al.* 1978, Öhman *et al.* 1992]. Thus, these results, together with the significantly higher MSL observed at baseline in blacks and the apparently lower RS of CaOx are in accordance with the reported lower incidence of stone formation in blacks than in whites. None of these results (with the exception of that for phosphate) were observed in chapter 3. Indeed, other results – significantly higher urinary pH, significantly higher oxalate excretion and significantly lower citrate excretion in blacks – were reported [chapter 3]. There are two possible explanations for this apparent discrepancy. Firstly, baseline urine values in the previous study were obtained from subjects who were on a low calorie hospital diet with a low fruit and vegetable consumption while in the present study the baseline urines were collected on subjects' self selected diets. Thus the different diets in the two studies might have manifested themselves as different urine parameters. Secondly, subjects in the previous study were of rural origin while in the present study subjects were drawn from the student population of the University of Cape Town. Again, different lifestyles and diet could have caused the discrepancy between the two studies. Another example of the possible influence of lifestyle is the significantly lower urinary phosphate in blacks. Since dietary fructose has recently been shown to increase urinary loss of phosphate [Milne and Nielsen 2000], the difference in the race groups' excretion of this component may reflect different consumption levels of fruit which in turn may be an economy-related lifestyle factor. Indeed, the lower vitamin C intake by blacks at baseline supports the lower fruit consumption hypothesis. Thus the different urine chemistries obtained in these studies may highlight the important role played by short-term metabolic challenges in determining urine composition.

The supposed role of dietary Ca restriction in increasing or decreasing the risk of stone formation has been recently debated at length [Curhan *et al.* 1993, Curhan *et al*, 1997]. In the present study, oxalate excretion in blacks increased significantly after the low Ca diet (0.17 to 0.23, p= 0.01, Table 2). No such increase was observed in whites. Although baseline oxalate excretion did not differ between the groups, values after the diet were significantly higher in blacks than in whites (0.23 vs 0.16, p= 0.01). Thus, since restriction of dietary Ca has been reported to lead to a greater absorption of oxalate [Jaeger *et al.* 1994], it appears that blacks are more sensitive to this mechanism than are whites. This is intriguing, since when low calcium was given *together with* high oxalate in the previous study (chapter 3), oxalate excretion increased significantly in whites but not in blacks. The results of these studies thus support the view that different renal handling of calcium and oxalate occurs in the two race groups and that these mechanisms are dependent on the relative magnitudes of the respective dietary challenges.

It is of some considerable interest that the low Ca diet caused a significant decrease in RS CaOx in blacks, despite the concomitant increase in oxalate excretion. Therefore, it seems that urinary oxalate is not as critical in blacks as it is in whites in determining this important physicochemical risk factor. Although synergistic effects with other urinary components may play a role, a reasonable conclusion is that urinary calcium may be the key factor. This is supported by our baseline data which showed that blacks have significantly lower calcium excretion than whites (but no difference in oxalate excretion) and that their baseline RS CaOx values tend towards being significantly lower.

Equally surprising is the significant increase in the supersaturation of brushite observed after the low calcium protocol in blacks. It is difficult to suggest a mechanism by which this occurred, but it can be speculated that synergism of multiple urinary factors may be involved.

Although the low calcium diet significantly affected three urinary risk factors in blacks as discussed above, no significant changes following this diet occurred in whites. This further supports the proposal that dietary Ca might be handled differently in the two race groups and might be indirectly involved in some, as yet unidentified, protective mechanism in blacks.

The high oxalate diet is also of interest because it elicited different responses in the two race groups. Surprisingly, urinary oxalate was not affected in either group. However in blacks, citrate excretion was significantly raised while in whites pH increased significantly. Both of these changes are synonymous with inhibitory responses in the respective groups: citrate is a well established inhibitor of CaOx crystallization [Pak 1994, Purich *et al.* 1992, Kok *et al.* 1986] both as a chelator and as a modifier [Fleisch 1990], while the raised pH is favourable since the activity of inhibitors has been reported as being enhanced at higher pH values [Tiselius 1981]. Furthermore, since higher urinary potassium has been associated with decreased stone growth, it could be suggested that the raised potassium excretion in whites is another protective mechanism in response to the oxalate challenge [Pierratos *et al.* 2000]. However, since blacks are relatively immune to stones it could be postulated that the citrate response in this race group to the high oxalate protocol is a more effective protective mechanism than the raised urinary pH and potassium in whites. Notwithstanding this conclusion, different renal handling of a dietary challenge (in this case high oxalate) is again apparent.

Although there is no obvious explanation for the increase in the relative supersaturation of brushite in whites following the high oxalate diet it is suggested that it may be related to the increase in pH [Dodds 2003].

The increase in pH following the vegetarian protocol in blacks is considered as a favorable effect with respect to calcium oxalate crystallization because more phosphate and citrate ions become dissociated at higher pH, increasing their inhibitory activity and promoting

calcium complexation [Tiselius 1981, Pak 1994]. Since black South Africans traditionally eat a diet consisting of significantly less animal protein than white South Africans anyway [chapter 2, p50, Modlin 1967, Whalley *et al.* 1998], the observation of this unique alteration in pH in blacks demonstrates a greater renal sensitivity to the absence of animal protein in the diet of this race group.

The only other statistically significant change caused by any of the diets was a raised risk index following the Vitamin C diet in blacks. Vitamin C was the only dietary variable that was indicative of non-compliance with respect to the dietary instructions for fruit intake– blacks consuming significantly less at baseline. This was not regarded as a confounding factor as studies have shown that vitamin C intake does not affect urinary oxalate [Auer *et al.* 1998], Nevertheless, it appears that the Vitamin C protocol in blacks had some effect on the synergism of the factors in the Tiselius risk index which did not occur in the white subjects. A different renal handling mechanism in the two race groups is again suggested.

The high salt diet raised sodium in blacks only and chloride in both groups but had no effect on urinary calcium. This latter observation is surprising in the light of previous studies which have associated raised calcium excretion with salt intake [Sakhaee *et al.* 1993]. A possible explanation could be that the increased urinary calcium is only apparent with a long-term (>7days) dietary salt challenge. Notwithstanding, as observed by Dodds (2003), the ratio of urinary calcium to sodium is higher in whites than in blacks in all dietary groups (table 4-2 – 4-7). Although the difference was not significant it was reported [Dodds 2003] that the higher ratio of urinary calcium to urinary sodium in whites relative to blacks is a common occurrence [Modlin 1967, Whalley *et al.* 1998, Bell *et al* 1985, Dibba *et al.* 1999, O'Brien *et al.* 1996, Pratt *et al.* 1996, Widdowson and McCance 1970]. As a possible explanation Dodds (2003) proposes that a different competitive reabsorption of calcium and sodium in the renal tubules occurs between the two races.

4.5 References:

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ChapterFive

Effects of different, potentially lithogenic
and anti lithogenic dietary supplements,
on urinary risk factors for
calcium oxalate kidney stone formation
in black and white South African subjects:
High Ca diet
Ca supplement
Vitamin B6 supplement
L-glutamine
L-cysteine

Evidence of renal resistance to dietary challenges
in a stone-free population

5.1 Introduction

In chapters 3 and 4 of this thesis, studies on the effects of different lithogenic dietary protocols provided evidence of potentially different renal handling mechanisms in black and white subjects. Since dietary supplements have also been reported as playing a role in urolithiasis, an investigation of their effects in the two race groups was regarded as being worthwhile and meaningful.

As stated in the General Introduction, Curhan *et al.* (1993) reported an increased risk associated with ingestion of supplemental calcium. Interestingly, an inverse relationship between risk and high *dietary* calcium was also reported. Thus in undertaking a study of the effects of supplemental calcium in the two race groups, it seemed prudent to conduct a parallel investigation involving a high calcium dietary protocol.

Also discussed in the General Introduction is the relationship between vitamin B_6 and CaOx urolithiasis. Curhan *et al.* (1999) reported that vitamin B_6 intake related inversely to the risk of calcium oxalate kidney stone formation in women. Vitamin B_6 is a cofactor in the oxalate metabolic pathway. Deficiency of this vitamin has been shown to increase oxalate production and contribute to calcium oxalate urolithiasis [Williams and Smith 1968, Di Tommaso *et al.* 2002]. Conversely, supplementation reduced the recurrence of calcium oxalate kidney stone formation in hyperoxaluric stone formers [Rattan *et al.* 1994, Balcke *et al.* 1983]. Thus, it is of some considerable interest that in chapter 2 of this thesis, a significantly lower dietary B_6 (pyridoxine) intake and a concomitantly higher urinary oxalate were reported in a small cohort of black subjects compared to whites. Hence, the present study also undertook to investigate the effect of vitamin B_6 supplementation on the urinary biochemical risk factors in the two race groups.

Urinary cystine is also of interest in the South African context. Whalley *et al.* found that South African blacks have significantly lower urinary levels than whites [Whalley *et al.* 1998]. Furthermore, an in vitro study by Martins et al demonstrated that cystine increased growth

and aggregation of calcium oxalate crystals in urine [Martins *et al.* 2002]. This confirms the observation of other studies which have associated high urinary cystine with increased risk of calcium oxalate calculi [Resnick *et al.* 1979, Carpentier *et al.* 1983]. In addition, some studies have described an anticystinuric effect of oral supplementation of the amino acid L-glutamine [Jaeger *et al.* 1986, Miyaji *et al.* 1979]. Thus, investigation of the effects in both race groups of ingestion of the amino acid supplement L-cysteine (which is metabolically converted to cystine and is eliminated in the urine [Kachmar and Grant 1970] and L-glutamine was also deemed as being of potential interest and value.

5.2 Material and Methods

The protocol is similar to that which was described in the previous chapter. Briefly, 10 black and 10 white South African males between the ages 18-28, with no metabolic disorder or history of kidney stones, participated in the study. All of the subjects are students at the University of Cape Town. Four different supplemental challenges as well as a high dietary calcium challenge were administered using a complete Latin Square design. Each subject ingested each challenge for 5 days in conjunction with a standardized diet with prescribed and clearly defined daily portions of all the major food groups. A washout period of 7 days was observed between the protocols. For the "high dietary calcium" challenge, 500mg of plain low fat yoghurt (915mg calcium per day) was added to the standardized diet; taken in three equal doses at breakfast, lunch and dinner; for the "calcium supplement" challenge, 925 mg per day calcium was administered in the form of calcium carbonate (SOLGAR, U.S.A), taken in three equal doses at breakfast, lunch and dinner; for the "vitamin B_6 supplement" challenge, 50mg pyridoxine (SOLGAR, U.S.A) twice daily between meals was added; for the "L-glutamine supplement" challenge, 1000mg L-glutamine (SOLGAR, U.S.A) twice daily between meals was added; for the "L-cysteine supplement" challenge, 500mg L-cysteine (SOLGAR, U.S.A) per day between meals was added. The dosage and time of intake of the challenges was strictly controlled. The amount of yoghurt to be ingested was determined to match the calcium content of the supplement as closely as possible using the

'Food Quantities Manual' of the South African Medical Research Council [Langenhoven *et al.* 1991]. A 24-hour dietary recall questionnaire was recorded on the day of each urine collection to monitor compliance with the dietary protocols.

24 hr urines were collected from each subject at baseline and after 5 days on the prescribed dietary supplement. Urinary sodium, potassium, calcium and magnesium were determined by atomic absorption spectroscopy while urinary oxalate, citrate, phosphate, urate, chloride and creatinine were determined using commercially available assay kits. Urinary sulphate was analysed using a turbidimetric method [Berglund and Sörbo 1960]. Urinary cystine and cysteine were determined using a spectrophotometic assay [Chrastil 1990] (described in more detail below) but were measured only in the baseline urines and in those collected after ingestion of the L-glutamine and the L-cysteine supplements. Urine pH and volume were measured routinely. The urinary excretion values were used to determine the Tiselius risk index [Tiselius 1982] and the relative urinary supersaturations of calcium oxalate, uric acid and calcium phosphate (brushite) [Werness *et al.* 1985]. As a further measure of the effects of each supplemental / dietary challenge on the potential of urine to support crystallization, CaOx metastable limits and particle volume – size distributions (90 minutes after administration of an aqueous sodium oxalate load) were determined in each urine using a Coulter Multisizer [Ryall *et al.* 1985]. The 24-dietary recall questionnaires were analysed for macro- and micronutrients using the computer programme "Foodfinder" version 6 [Wolmarans *et al.* 2001]. Statistical analysis was performed by analysis of variance at statistical significance of $p \leq 0.05$ using the SAS statistical package.

Urinary cystine and cysteine determination

Urinary cystine and cysteine were determined according to the spectrophotometric method of Chrastil (1990). Experiments were carried out as described in the latter paper, except the concentration of cystine and cysteine were determined using a standard curve instead of the reported formulae (figure 5-1 and figure 5-2). The absorbance was read at optical density (OD) 335nm.

Figure 5-1: Standard Curve of urinary cystine determination

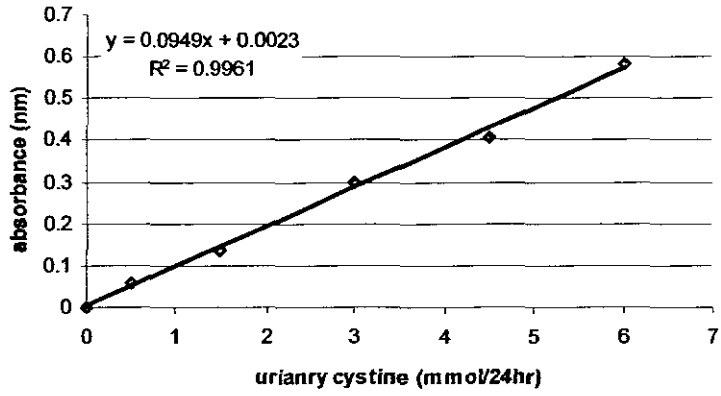
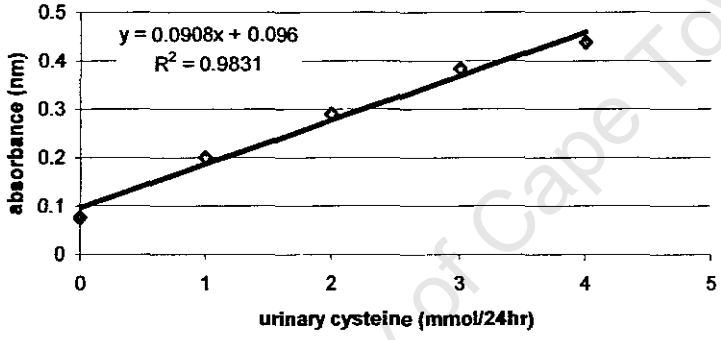


Figure 5-2: Standard Curve of urinary cysteine determination



5.3 Results

Dietary Analysis

There were no significant differences between the nutrient intakes at baseline and after the 5 days ingestion period proving compliance to dietary instructions (table 5-1).

Table 5-1: Mean dietary intake of blacks and white subjects on 'standardized diet'

	Blacks		Whites		p-value
Age	23.50	± 2.72	21.60	± 1.578	0.072
BMI	22.70	± 1.71	22.87	± 1.19	0.799
Moisture (g)	2588.35	± 523.12	2151.47	± 444.21	0.059
Energy (kJ)	13805.80	± 3250.11	12576.50	± 1747.88	0.306
Total Protein (g/day)	126.56	± 31.64	121.14	± 18.24	0.644
Animal Protein (g/day)	86.45	± 28.11	68.88	± 23.97	0.150
Total Fat (g/day)	118.09	± 33.85	112.40	± 41.95	0.742
Carbohydrates (g/day)	405.02	± 89.30	347.12	± 41.25	0.079
Total sugar (g/day)	81.66	± 17.43	68.06	± 23.19	0.155
Added sugar (g/day)	108.07	± 52.97	77.62	± 21.48	0.109
Fibre (g/day)	23.37	± 8.10	26.78	± 10.36	0.423
Phytate (mg/day)	331.00	± 146.36	325.70	± 65.16	0.918
Ca (mg/day)	1387.40	± 324.69	1236.30	± 306.92	0.299
Mg (mg/day)	403.80	± 102.68	409.30	± 161.12	0.928
PO_4 (mg/day)	1993.60	± 395.35	1815.20	± 328.35	0.287
K (mg/day)	3850.50	± 1275.73	3201.60	± 551.49	0.157
Na (mg/day)	3021.10	± 1012.43	3329.60	± 1639.15	0.619
Cl (mg/day)	3411.70	± 1872.24	3169.10	± 901.44	0.716
Zn (mg/day)	16.68	± 5.13	14.93	± 3.17	0.372
Vitamin A (RE/day)	1240.90	± 617.69	814.80	± 298.73	0.065
Vitamin B_6 (mg/day)	2.82	± 1.44	2.19	± 0.56	0.210
Vitamin C (mg/day)	72.30	± 46.15	95.80	± 121.42	0.574
Vitamin D (µg/day)	7.41	± 3.50	6.51	± 3.69	0.582
Citric acid (mg/day)	2154.90	± 754.77	1645.70	± 568.54	0.106
Oxalic acid (mg/day)	38.60	± 24.29	40.00	± 26.52	0.903

Significance at $p \leq 0.05$; ± std error

Figure 5-1: Standard Curve of urinary cystine determination

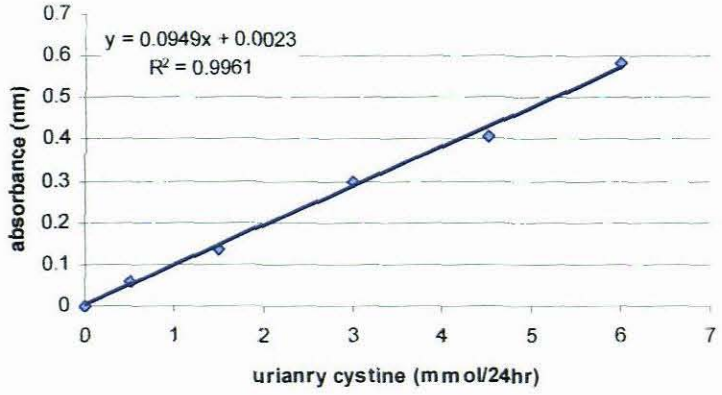
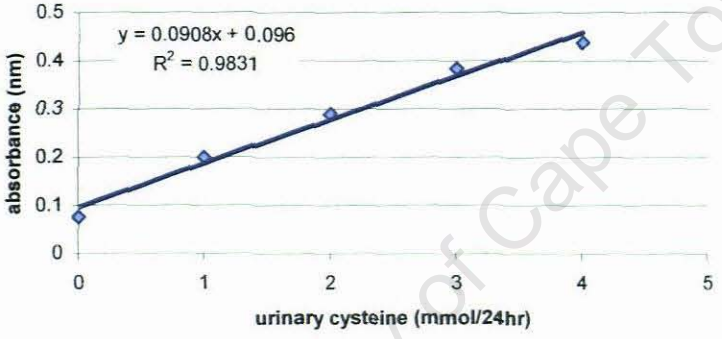


Figure 5-2: Standard Curve of urinary cysteine determination



5.3 Results

Dietary Analysis

There were no significant differences between the nutrient intakes at baseline and after the 5 days ingestion period proving compliance to dietary instructions (table 5-1).

Table 5-1: Mean dietary intake of blacks and white subjects on 'standardized diet'

	Blacks		Whites		p-value
Age	23.50	± 2.72	21.60	± 1.578	0.072
BMI	22.70	± 1.71	22.87	± 1.19	0.799
Moisture (g)	2588.35	± 523.12	2151.47	± 444.21	0.059
Energy (kJ)	13805.80	± 3250.11	12576.50	± 1747.88	0.306
Total Protein (g/day)	126.56	± 31.64	121.14	± 18.24	0.644
Animal Protein (g/day)	86.45	± 28.11	68.88	± 23.97	0.150
Total Fat (g/day)	118.09	± 33.85	112.40	± 41.95	0.742
Carbohydrates (g/day)	405.02	± 89.30	347.12	± 41.25	0.079
Total sugar (g/day)	81.66	± 17.43	68.06	± 23.19	0.155
Added sugar (g/day)	108.07	± 52.97	77.62	± 21.48	0.109
Fibre (g/day)	23.37	± 8.10	26.78	± 10.36	0.423
Phytate (mg/day)	331.00	± 146.36	325.70	± 65.16	0.918
Ca (mg/day)	1387.40	± 324.69	1236.30	± 306.92	0.299
Mg (mg/day)	403.80	± 102.68	409.30	± 161.12	0.928
PO_4 (mg/day)	1993.60	± 395.35	1815.20	± 328.35	0.287
K (mg/day)	3850.50	± 1275.73	3201.60	± 551.49	0.157
Na (mg/day)	3021.10	± 1012.43	3329.60	± 1639.15	0.619
Cl (mg/day)	3411.70	± 1872.24	3169.10	± 901.44	0.716
Zn (mg/day)	16.68	± 5.13	14.93	± 3.17	0.372
Vitamin A (RE/day)	1240.90	± 617.69	814.80	± 298.73	0.065
Vitamin B_6 (mg/day)	2.82	± 1.44	2.19	± 0.56	0.210
Vitamin C (mg/day)	72.30	± 46.15	95.80	± 121.42	0.574
Vitamin D (µg/day)	7.41	± 3.50	6.51	± 3.69	0.582
Citric acid (mg/day)	2154.90	± 754.77	1645.70	± 568.54	0.106
Oxalic acid (mg/day)	38.60	± 24.29	40.00	± 26.52	0.903

Significance at $p<0.05$; ± std error

Urine Analysis

At baseline (table 5-2), black subjects on the standardized diet had significantly lower urinary calcium, urate, phosphate and cystine excretions as well as significantly lower relative supersaturations of CaOx and brushite. Urinary excretion of citrate in blacks and their calcium oxalate metastable limits were significantly higher than in whites. Other urinary variables were not significantly different at baseline.

Urine variables after each of the ingested challenges are given in table 5-3 – 5-7. None of the protocols significantly altered any of the urinary parameters in South African black subjects, except creatinine which increased after the L-glutamine supplement. However, urinary parameters in whites changed significantly after each of the protocols. In this race group, the dietary calcium challenge increased urinary potassium and decreased RS brushite (table 5-3); the calcium supplement significantly decreased the Tiselius risk index (table 5-4); the vitamin B_6 supplement significantly decreased urinary calcium, urinary phosphate and RS brushite (table 5-5); the L-glutamine supplement significantly decreased RS of CaOx (table 5-6); finally, the L-cysteine supplement significantly decreased urinary calcium and the Tiselius risk index (table 5-7).

Table 5-2: Urine variables in black and white subjects at baseline

Variables	Blacks		Whites		p
pH	6.46	± 0.10	6.39	± 0.10	0 .608
Volume (ml/24h)	1688	± 110.76	1538	± 110.76	0.341
Citrate (mmol/24h)	2.8	± 0.33	1.78	± 0.33	0.031*
Oxalate (mmol/24h)	0.13	± 0.01	0.15	± 0.01	0.307
Ca (mmol/24h)	2.41	± 0.40	3.89	± 0.40	0.011*
Mg (mmol/24h)	3.324	± 0.37	4.12	± 0.37	0.128
Na^+ (mmol/24h)	135.52	± 27.36	112.58	± 27.36	0.555
K^+ (mmol/24h)	47.01	± 9.25	47.15	± 9.25	0.991
Urate (mmol/24h)	2.4	± 0.24	3.43	± 0.24	0.003*
Creatinine (mmol/24h)	12.28	± 0.83	14.54	± 0.83	0.059
PO_4 (mmol/24h)	18.59	± 2.29	28.89	± 2.29	0.002*
Cl^- (mmol/24h)	133.64	± 17.63	138.2	± 17.63	0.855
SO_4 (mmol/24h)	16.643	± 1.74	20.76	± 1.74	0.097
Cystine (mmol/24h)	1.495	± 0.08	2.217	± 0.24	0.012*
Cysteine (mmol/24h)	0.606	± 0.15	1.237	± 0.25	0.053
Ca/Na ratio	0.028	± 0.01	0.086	± 0.05	0.228
Tiselius risk index	267.47	± 40.84	315.37	± 40.84	0.409
MSL	0.094	± 0.01	0.053	± 0.01	0.004*
RS CaOx	1 .45	± 0.33	2.63	± 0.33	0.013*
RS brushite	0.75	± 0.23	1.81	± 0.23	0.002*
RS uric acid	0.55	± 0.23	0.84	± 0.23	0.362

*significance at $p \leq 0.05$; ± std error

Table 5-3: Urine variables in black and white subjects following the high calcium diet challenge

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	Calcium Diet	p	Baseline	Calcium Diet	p	Calcium Diet p
pH	6.46	6.58	0.402	6.39	6.49	0.477	0.522
Volume (ml/24h)	1688	1723	0.834	1538	1695	0.319	0.859
Citrate (mmol/24h)	2.8	2.56	0.601	1.78	2.27	0.296	0.541
Oxalate (mmol/24h)	0.13	0.14	0.733	0.15	0.14	0.776	0.691
Ca (mmol/24h)	2.41	3.02	0.28	3.89	3.41	0.401	0.500
Mg (mmol/24h)	3.32	3.14	0.728	4.12	3.37	0.151	0.665
Na^+ (mmol/24h)	135.52	121.31	0.714	112.58	174.18	0.115	0.175
K^+ (mmol/24h)	47.01	40.83	0.638	47.15	104.35	<0.001*	<0.001*
Urate (mmol/24h)	2.4	2.73	0.330	3.43	3.82	0.25	0.002*
Creatinine (mmol/24h)	12.28	12.87	0.619	14.54	15.11	0.631	0.061
PO_4 (mmol/24h)	18.59	21.32	0.401	28.89	31.85	0.363	0.002*
Cl^- (mmol/24h)	133.64	146.6	0.616	138.2	175.46	0.139	0.266
SO_4 (mmol/24h)	16.643	19.04	0.332	20.76	24.48	0.134	0.030*
Ca/Na ratio	0.028	0.030	0.826	0.086	0.026	0.209	0.615
Tiselius risk index	267.47	345.99	0.178	315.37	215.66	0.088	0.027*
MSL	0.094	0.093	0.971	0.053	0.078	0.075	0.283
RS CaOx	1.45	1.35	0.848	2.63	1.77	0.071	0.374
RS brushite	0.75	0.87	0.715	1.81	1.09	0.035*	0.497
RS uric acid	0.55	0.44	0.740	0.84	0.77	0.839	0.299

*significance at $p \leq 0.05$

Table 5-4: Urine variables in black and white subjects following the calcium supplement challenge

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	Calcium Supp	p	Baseline	Calcium Supp	p	Calcium Supp p
pH	6.46	6.57	0.434	6.39	6.46	0.618	0.426
Volume (ml/24h)	1688	1671	0.914	1538	1535	0.987	0.389
Citrate (mmol/24h)	2.8	3.01	0.659	1.78	2.48	0.136	0.262
Oxalate (mmol/24h)	0.13	0.16	0.055	0.15	0.12	0.114	0.014
Ca (mmol/24h)	2.41	3.42	0.076	3.89	4.08	0.737	0.253
Mg (mmol/24h)	3.32	3.55	0.665	4.12	4.24	0.816	0.185
Na^+ (mmol/24h)	135.52	118.41	0.659	112.58	114.75	0.955	0.925
K^+ (mmol/24h)	47.01	37.16	0.454	47.15	64.29	0.194	0.041*
Urate (mmol/24h)	2.4	2.65	0.460	3.43	3.83	0.238	0.001*
Creatinine (mmol/24h)	12.28	13.6	0.267	14.54	16.03	0.210	0.049*
PO_4 (mmol/24h)	18.59	17.23	0.675	28.89	25.68	0.324	0.324
Cl^- (mmol/24h)	133.64	132.5	0.964	138.2	158.9	0.409	0.293
SO_4 (mmol/24h)	16.643	18.59	0.431	20.76	19.27	0.545	0.783
Ca/Na ratio	0.028	0.032	0.648	0.086	0.054	0.536	0.295
Tiselius risk index	267.47	345.74	0.179	315.37	169.77	0.014*	0.003*
MSL	0.094	0.077	0.224	0.053	0.044	0.495	0.020*
RS CaOx	1.45	2.34	0.057	2.63	1.79	0.078	0.242
RS brushite	0.75	0.87	0.731	1.81	1.43	0.254	0.094
RS uric acid	0.55	0.42	0.708	0.84	1.26	0.201	0.012*

*significance at $p \leq 0.05$

Table 5-5: *Urine variables in black and white subjects following the vitamin B_6 supplement challenge*

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	Vitamin B_6 supp	p	Baseline	Vitamin B_6 supp	p	Vitamin B_6 supp p
pH	6.46	6.41	0.722	6.39	6.43	0.803	0.926
Volume (ml/24h)	1688	1596	0.558	1538	1287	0.113	0.052
Citrate (mmol/24h)	2.8	2.58	0.639	1.78	2.26	0.305	0.493
Oxalate (mmol/24h)	0.13	0.14	0.427	0.15	0.13	0.330	0.460
Ca (mmol/24h)	2.41	2.69	0.619	3.89	2.50	0.016*	0.734
Mg (mmol/24h)	3.324	3.11	0.685	4.12	3.17	0.070	0.914
Na^+ (mmol/24h)	135.52	97.23	0.325	112.58	120.6	0.836	0.547
K^+ (mmol/24h)	47.01	39.48	0.566	47.15	47.45	0.982	0.544
Urate (mmol/24h)	2.4	2.87	0.167	3.43	3.26	0.615	0.250
Creatinine (mmol/24h)	12.28	13.53	0.293	14.54	13.18	0.252	0.768
PO_4 (mmol/24h)	18.59	20.19	0.622	28.89	21.62	0.027*	0.659
Cl^- (mmol/24h)	133.64	116.3	0.616	138.2	135.7	0.920	0.439
SO_4 (mmol/24h)	16.643	16.05	0.809	20.76	17.25	0.157	0.625
Ca/Na ratio	0.028	0.033	0.674	0.086	0.027	0.230	0.610
Tiselius risk index	267.47	300.86	0.565	315.37	213.84	0.082	0.135
MSL	0.094	0.096	0.857	0.053	0.08	0.059	0.238
RS CaOx	1.45	1.47	0.953	2.63	1.94	0.145	0.317
RS brushite	0.75	0.91	0.638	1.81	0.83	0.004*	0.825
RS uric acid	0.55	0.65	0.741	0.84	1.24	0.221	0.073

*significance at $p \leq 0.05$

Table 5-6: Urine variables in black and white subjects following the L-glutamine supplement challenge

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	L-glutamine supp	p	Baseline	L-glutamine supp	p	L-glutamine supp p
pH	6.46	6.47	0.983	6.39	6.59	0.167	0.394
Volume (ml/24h)	1688	1558	0.409	1538	1390	0.347	0.286
Citrate (mmol/24h)	2.8	2.42	0.418	1.78	1.68	0.820	0.112
Oxalate (mmol/24h)	0.13	0.13	0.864	0.15	0.13	0.281	0.820
Ca (mmol/24h)	2.41	3.03	0.274	3.89	3.04	0.139	0.987
Mg (mmol/24h)	3.324	3.71	0.455	4.12	4.29	0.739	0.266
Na^+ (mmol/24h)	135.52	122.52	0.738	112.58	126.27	0.724	0.923
K^+ (mmol/24h)	47.01	41.87	0.695	47.15	72.92	0.052	0.020*
Urate (mmol/24h)	2.4	3.02	0.069	3.43	4.09	0.053	0.002*
Creatinine (mmol/24h)	12.28	14.65	0.048*	14.54	15.96	0.232	0.270
PO_4 (mmol/24h)	18.59	22.36	0.247	28.89	28.89	1.000	0.046*
Cl^- (mmol/24h)	133.64	136.6	0.906	138.2	163.1	0.321	0.291
SO_4 (mmol/24h)	16.64	18.38	0.481	20.76	18.41	0.341	0.991
Cystine (mmol/24h)	1.41	1.25	0.289	2.07	2.21	0.596	0.002*
Cysteine (mmol/24h)	0.61	0.61	1.000	1.24	1.09	0.654	0.013*
Ca/Na ratio	0.028	0.051	0.148	0.086	0.034	0.290	0.360
Tiselius risk index	267.47	276.05	0.882	315.37	210.28	0.072	0.258
MSL	0.094	0.095	0.943	0.053	0.068	0.296	0.056
RS CaOx	1.45	1.78	0.481	2.63	1.41	0.010*	0.429
RS brushite	0.75	1.06	0.351	1.81	1.45	0.280	0.249
RS uric acid	0.55	0.68	0.672	0.84	0.67	0.608	0.981

*significance at $p \leq 0.05$

Table 5-7: Urine variables in black and white subjects following the L-cysteine supplement challenge

	Blacks			Whites			Blacks vs Whites
Variables	Baseline	L-cysteine supp	p	Baseline	L-cysteine supp	p	L-cysteine supp p
pH	6.46	6.3	0.236	6.39	6.32	0.609	0.870
Volume (ml/24h)	1688	1717	0.853	1538	1242	0.062	0.003*
Citrate (mmol/24h)	2.8	2.9	0.839	1.78	1.73	0.913	0.014*
Oxalate (mmol/24h)	0.13	0.15	0.234	0.15	0.15	0.909	0.955
Ca (mmol/24h)	2.41	2.75	0.550	3.89	2.64	0.031*	0.855
Mg (mmol/24h)	3.324	3.39	0.902	4.12	3.72	0.450	0.519
Na+ (mmol/24h)	135.52	148.42	0.740	112.58	143.8	0.422	0.905
K+ (mmol/24h)	47.01	45.66	0.918	47.15	70.2	0.082	0.064
Urate (mmol/24h)	2.4	2.84	0.195	3.43	3.3	0.701	0.176
Creatinine (mmol/24h)	12.28	13.31	0.385	14.54	14.51	0.970	0.312
PO4 (mmol/24h)	18.59	21.21	0.420	28.89	27.43	0.653	0.058
Cl- (mmol/24h)	133.64	156.9	0.353	138.2	142.9	0.851	0.576
SO4 (mmol/24h)	16.643	19.51	0.247	20.76	18.54	0.368	0.693
Cystine (mmol/24h)	1.41	1.53	0.361	2.07	2.24	0.628	0.076
Cysteine (mmol/24h)	0.606	0.433	0.372	1.237	1.303	0.888	0.050*
Ca/Na ratio	0.028	0.041	0.368	0.086	0.026	0.214	0.317
Tiselius risk index	267.47	303.81	0.531	315.37	168.18	0.013*	0.021*
MSL	0.094	0.081	0.390	0.053	0.065	0.410	0.224
RS CaOx	1.45	1.92	0.310	2.63	2.09	0.254	0.716
RS brushite	0.75	0.62	0.690	1.81	1.32	0.147	0.037*
RS uric acid	0.55	0.82	0.400	0.84	0.95	0.728	0.677

*significance at $p \leq 0.05$

Particle Volume – Size Distribution

The particle volume – size distribution in urines from black subjects after administration of a sodium oxalate load, is shown in figure 5-3 while numerical data and analysis thereof are given in tables 5-8 and 5-9 respectively. In this group, the mean particle size relative to the baseline urine showed an increasing trend (not statistically significant) for the high calcium diet, the L-glutamine supplement and the L-cysteine supplement (11% increase in all cases) (table 5-9). In the same group the mean particle volume showed an increasing trend relative to baseline, for the high Ca diet challenge, and for all supplemental challenges except for the L-cysteine supplemental challenge for which the mean particle volume did not change. Of these, the only statistically significant increase was for the L-glutamine supplement challenge (58.8% increase) (table 5-9). The total particle volume showed an increasing trend (not statistically significant) relative to baseline for all challenges except for the L-cysteine supplement for which the total volume decreased (table 5-9).

Figure 5-3: Particle Volume - Size Distribution in the Urine of Black Subjects

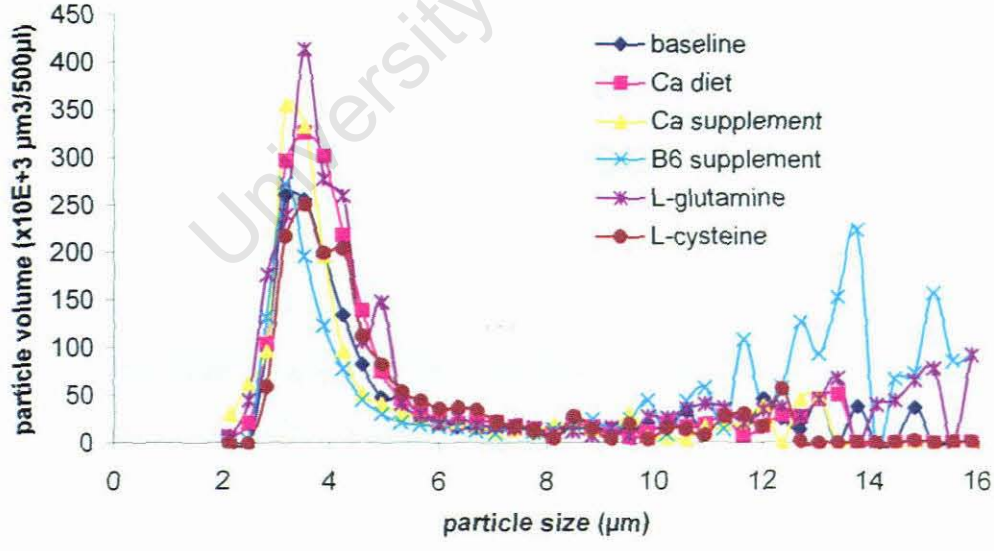


Table 5-8: Particle Volume - Size Distribution in the Urine of Black Subjects

Variables	Mean Particle Size (µm)	Mean Particle Volume ($x10^3$ μm^3/500 µl)	Total Volume ($x10^6$ μm^3/500 µl)
Baseline	3.18	260.94	1.63
High Ca diet	3.53	327.15	2.04
Ca supplement	3.18	356.42	1.74
Vitamin B_6 supplement	3.18	272.28	2.51
L-glutamine supplement	3.53	414.40	2.63
L-cysteine supplement	3.53	251.20	1.34

Table 5-9: Percentage Change (Compared to Baseline) in Mean Particle Parameters– Black Subjects

Variables	% increase Mean Particle Size	% increase Mean Particle Volume	% increase Total Volume
High Ca diet	11.1	25.4	25.2
Ca supplement	0	36.6	6.7
Vitamin B_6 supplement	0	4.3	54.0
L-glutamine supplement	11.1	58.8	61.3
L-cysteine supplement	11.1	-3.7	-17.8

The particle volume – size distribution in urines from white subjects after administration of a sodium oxalate load, is shown in figure 5-4 while numerical data and analysis thereof are given in tables 5-10 and 5-11 respectively. In this group, the only change (not statistically significant) in the mean particle size was observed for the high Ca diet challenge (25.8% increase) (table 5-11). The mean particle volume relative to baseline showed an increasing trend for all supplement challenges (table 5-11); these increases were statistically significant for the Ca supplement, vitamin B_6 supplement and the L-cysteine supplement (38%, 44.1% and 48.8% respectively). In addition, all dietary / supplemental challenges showed an increasing trend relative to baseline in the total volume; however, only the difference for the Ca supplement challenge was statistically significant (85.6% increase).

Figure 5-4: Particle Volume - Size Distribution in the Urine of White Subjects

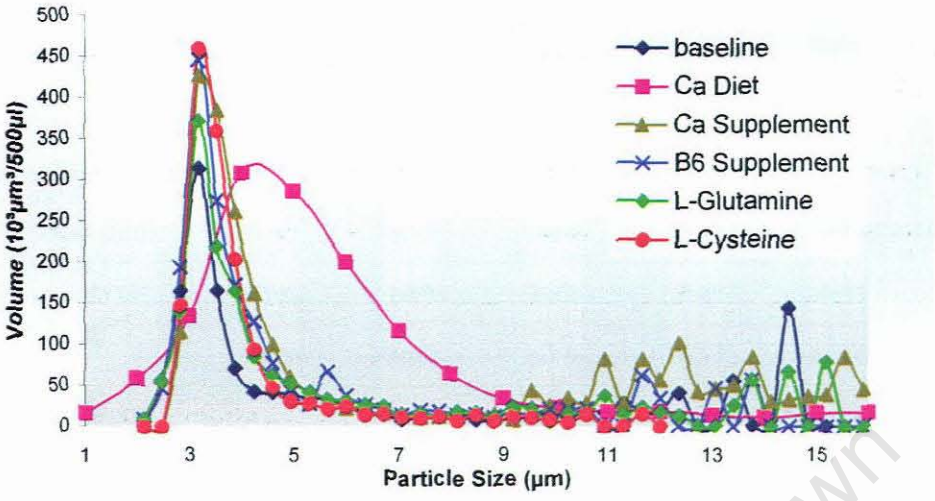


Table 5-10: Particle Volume - Size Distribution in the Urine of White Subjects

Variables	Mean Particle Size (µm)	Mean Particle Volume ($x10^3$ µm³/500 µl)	Total Volume ($x10^6$ µm³/500 µl)
Baseline	3.18	308.68	1.46
High Ca diet	4.00	308.68	1.86
Ca supplement	3.18	426.03	2.71
Vitamin B_6 supplement	3.18	444.89	1.94
L-glutamine supplement	3.18	370.24	1.81
L-cysteine supplement	3.18	459.20	1.56

Table 5-11: Percentage Change (Compared to Baseline) in Mean Particle Parameters– White Subjects

Variables	% increase Mean Particle Size	% increase Mean Particle Volume	% increase Total Volume
High Ca diet	25.8	0.0	27.4
Ca supplement	0	38.0	85.6
Vitamin B_6 supplement	0	44.1	32.9
L-glutamine supplement	0	19.9	24.0
L-cysteine supplement	0	48.8	6.8

Figure 5-4: Particle Volume - Size Distribution in the Urine of White Subjects

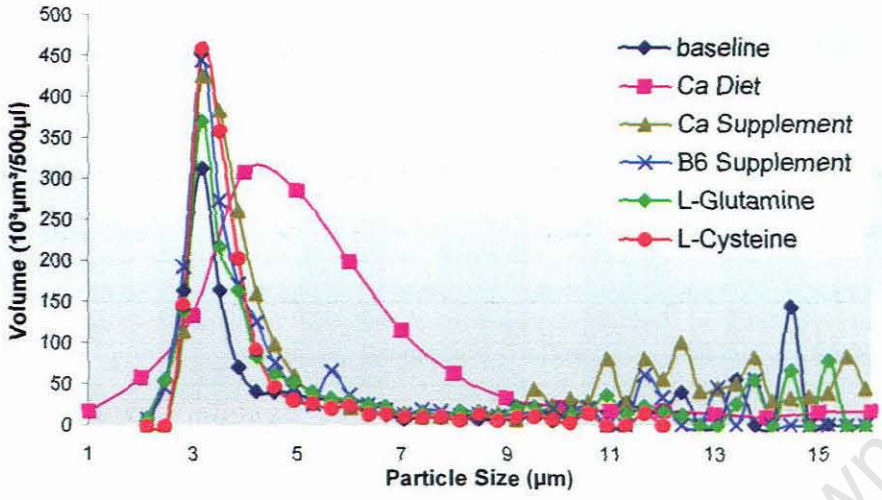


Table 5-10: Particle Volume - Size Distribution in the Urine of White Subjects

Variables	Mean Particle Size (µm)	Mean Particle Volume ($x10^3$ µm^3/500 µl)	Total Volume ($x10^6$ µm^3/500 µl)
Baseline	3.18	308.68	1.46
High Ca diet	4.00	308.68	1.86
Ca supplement	3.18	426.03	2.71
Vitamin B_6 supplement	3.18	444.89	1.94
L-glutamine supplement	3.18	370.24	1.81
L-cysteine supplement	3.18	459.20	1.56

Table 5-11: Percentage Change (Compared to Baseline) in Mean Particle Parameters– White Subjects

Variables	% increase Mean Particle Size	% increase Mean Particle Volume	% increase Total Volume
High Ca diet	25.8	0.0	27.4
Ca supplement	0	38.0	85.6
Vitamin B_6 supplement	0	44.1	32.9
L-glutamine supplement	0	19.9	24.0
L-cysteine supplement	0	48.8	6.8

Figures 5-5, 5-6 and 5-7 allow inter-group comparisons of the effects of each challenge on particle volume – size parameters. Intra-group comparisons were presented in tables 5-8 – 5-11. Figure 5-5 shows that there were no statistically significant differences in the mean particle diameter between the groups at baseline or after any of the challenges. However, figure 5-6 shows two significant effects. Firstly, it is apparent that the mean particle volumes after the vitamin B_6 and L-cysteine supplemental challenges are greater in whites. Attention is drawn to the fact that these differences arise because the mean particle volume in white subjects increased following these challenges while in blacks there was no change. Figure 5-7 shows a similar effect following the Ca supplemental challenge.

Figure 5-5: Mean Particle Size ($X10^3$ μM^3/500 µL) in The Urine of Black and White Subjects

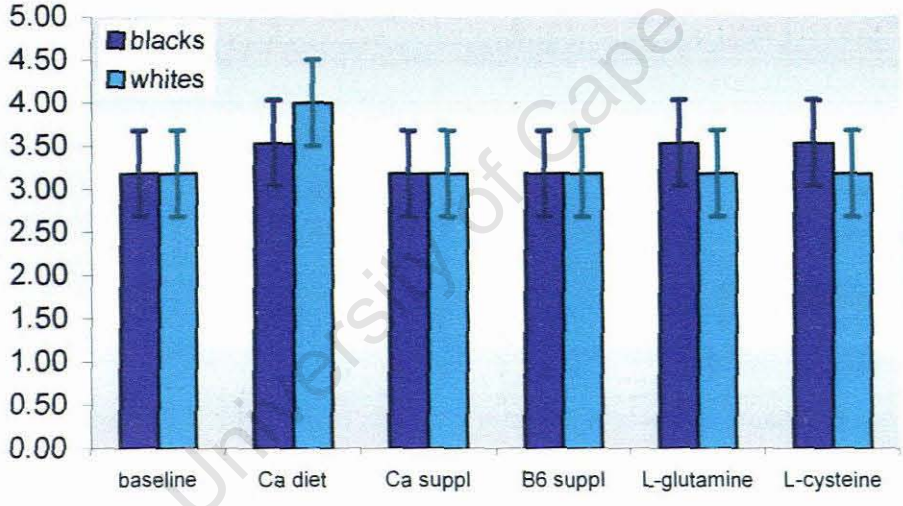


Figure 5-6: Mean Particle Volume (x10^3 µm^3/500 µl) in the Urine of Black and White Subjects

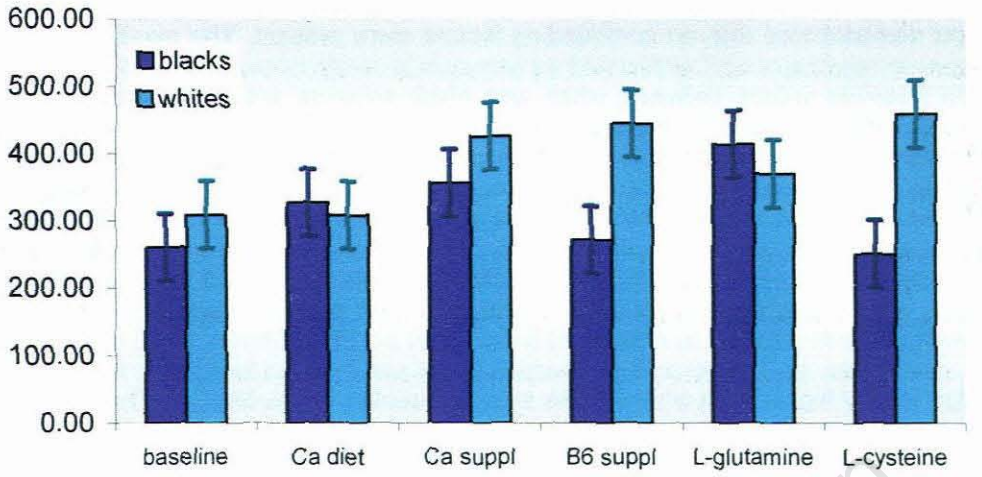
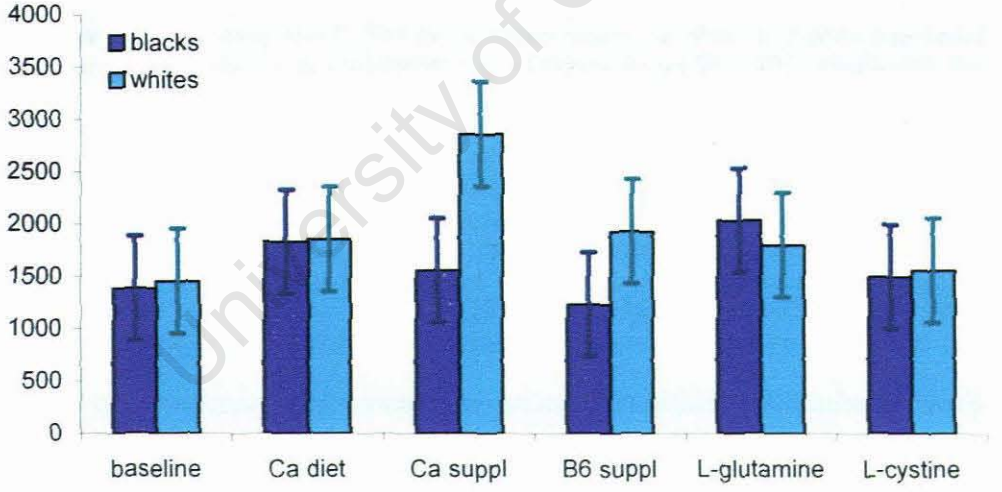


Figure 5-7: Total Particle Volume (x10^3 µm^3/500 µl) in the Urine of Black and White Subjects



5.4 Discussion

The analysis of nutrient intake confirms compliance by the subjects to the dietary regimens and demonstrates that no confounding factors were present. The significant differences in the baseline values between white and black subjects are in accordance with the low incidence of kidney stones in this race group since all of these parameters are inversely related to the risk of CaOx urolithiasis. All of these differences have been previously reported (chapters 3 and 4), except for that of citrate which has been found to be consistently lower in black subjects [chapter 3, chapter 4] [Whalley *et al.* 1998, Modlin 1967] but was found to be significantly higher than whites in the study presented in this chapter. The latter discrepancy might be due to the fruit and vegetable intake having been standardized in the present study whereas in chapter 4, dietary questionnaires revealed non-compliance with fruit ingestion, reflected in the low dietary vitamin C intake of the black subjects. Thus, urinary citrate in black subjects might be very sensitive to dietary intake whereas urinary calcium might depend on more complex renal or gastrointestinal mechanisms.

Urinary values obtained from the cystine and cysteine determinations were in the range 1.1 - 3.6 mmol/24hr (260 – 870mg/l) and 0.1 – 2.3 mmol/24hr (8 – 300mg/l) respectively which are in accordance with previously reported values [Chrastil 1990]. Also, the lower urinary cystine in blacks relative to whites agrees with previously reported values [Whalley *et al.* 1998] and is noteworthy, as its possible correlation with low stone incidence in South African blacks has been suggested before [Martins *et al.* 2002].

Recent studies have provided compelling evidence of dietary calcium being inversely related to CaOx stone formation [Curhan *et al.* 1993, Messa *et al.* 1997, Williams *et al.* 2001]. In the present study the dietary calcium challenge did not induce any statistically significant changes in the urinary parameters of black subjects. However in whites, urinary potassium increased significantly and the relative supersaturation of brushite decreased significantly (table 5-2). The increase in potassium is accounted for by the high potassium content of the yoghurt (1700 mg potassium per day). Interestingly, the same response was not observed in

blacks, despite the fact that they consumed the identical amount of yoghurt. Since urinary potassium has been inversely linked to calcium oxalate urolithiasis (because it supposedly alkalanizes the urine making it less favourable for crystallization [Pierratos *et al.* 2000, Martini *et al.* 1998]) the response in whites is regarded as favourable. The significant decrease in the relative supersaturation of brushite is also favourable and could be due to a host of synergistic factors including the raised urinary volume and lower urinary calcium, albeit that these changes were not statistically significant. Other changes which tended towards significance are the decrease in the relative supersaturation of CaOx, the decrease in Tiselius Risk Index and the increase in the metastable limit (table 5-2). All of these changes are favourable and are in agreement with recent research which suggest that a high calcium diet decreases the risk of stone formation [Curhan *et al.* 1993, Messa *et al.* 1997]. Paradoxically, none of these changes are indicated in black subjects who are inherently at low risk anyway.

Curhan has demonstrated that supplemental calcium increases the risk of calcium oxalate stone formation when taken between meals but that it does not pose any threat when taken with meals [Curhan *et al* 1997]. The explanation which he offers is that in the former case, more unbound calcium (and hence, oxalate) is available for absorption while in the latter case, the converse is true. The results of the present study show that the risk of stone formation (Tiselius risk index) in whites actually decreases when the calcium supplement is taken with meals, despite there not being any concomitant decrease in urinary calcium or oxalate. This suggests that a more complex mechanism needs to be invoked to explain the effects of calcium supplementation than has been previously mooted. Moreover, the absence of a similar response in the black subjects suggests different renal handling of calcium supplements in the two race groups.

As stated earlier, prolonged vitamin B_6 deficiency can induce hyperoxaluria [Di Tommaso *et al.* 2002], while intake of this vitamin appears to be inversely related to CaOx stone formation [Curhan *et al.* 1997]. It is interesting that blacks have a diet which is traditionally high in

oxalate [Viljoen and Gericke 2001], low in calcium [Whalley *et al.* 1998] and low in vitamin B_6 [chapter 2] relative to whites. It is therefore surprising that blacks do not form CaOx kidney stones and that, in the present study, black subjects did not experience any significant changes in their urine biochemistry after vitamin B_6 supplementation. There are two possible explanations for the latter observation. Firstly, blacks may have more calcium available to bind oxalate in the gut, forming complexes that are excreted in the faeces before being absorbed. However, this is unlikely since calcium intake was controlled in the present study; as such, it was approximately equal in the two groups. Interestingly, when a low calcium/high oxalate diet was administered to both race groups, urinary oxalate increased in whites but not in blacks [chapter 3], again highlighting this puzzling difference between the groups. Secondly, blacks may have more oxalate degrading bacteria (*Oxalobacter formigenes*) thereby resulting in less oxalate being absorbed through the gut. Such a mechanism would also explain their normal baseline urinary oxalate despite their traditional hyperoxalurogenic diet. This hypothesis will be explored and discussed in chapter 6 of this thesis.

Intriguingly, the vitamin B_6 supplement had three significant effects on urinary risk factors in whites, all of which were beneficial: it decreased urinary calcium, phosphate and the relative supersaturation of brushite. Since vitamin B_6 is involved with the oxalate metabolic pathway, one would have expected urinary oxalate to decrease after its supplementation. Indeed, some studies have demonstrated this effect [Balcke *et al.* 1983, Gill and Rose 1986] while in another, no effect was observed [Tolomelli *et al.* 1991]. It is difficult to account for the decrease in urinary calcium. However, with respect to the decrease in urinary phosphate, attention is drawn to a study in which vitamin B_6 deficiency was induced in adult rats resulting in the deposition of phosphate and oxalate crystals in the parenchymal connective tissue of the kidney [Di Tommaso *et al.* 2002]. Thus, it is postulated that pyridoxine supplementation in the present study might have induced a related but reverse effect, i.e. a decrease in urinary phosphate. This effect, together with the decreased excretion of calcium accounts for the decrease in RS brushite. Pyridoxine has also been found to exert an overall positive effect on urinary risk factors [Curhan *et al* 1999, Tolomelli *et al.* 1991]. In this context it is worthwhile to

note that in the present study, the metastable limit increased by 34% and the Tiselius risk index decreased by 32% in whites, albeit that these changes were not statistically significant.

Previous studies have shown that the administration of glutamine with sodium has an anti-cystinuric effect [Jaeger *et al.* 1986, Aunsholt and Ahlbom 1990]. However, other studies have not been able to reproduce this effect [Van der Berg *et al.* 1980, Joost and Jarosch 1981]. The absence of an anticystinuric effect in the present study might be due to the absence of sodium in the glutamine protocol. Surprisingly, this protocol induced a reduction in RS CaOx. This has not been previously reported. It is difficult to offer an explanation for this effect but it is speculated that it may be due to several subtle (but not statistically insignificant) changes which act in concert. The decrease in calcium excretion is an example of such a change. However, of interest in the present study is that the reduction in RS CaOx occurred in the white race group but not in the black group.

Although there are no previous reports on the effects of L-cysteine supplementation on urinary risk factors, a direct correlation between sulphur amino acids and urinary calcium has been recorded [Tschope 1985]. In the present study the observation of a decrease in calcium excretion (with a concomitant decrease in the Tiselius risk index) following ingestion of this supplement is therefore surprising and unexpected and is probably due to an unknown hepatic or renal mechanism. Notwithstanding this interesting effect, attention is again drawn to its absence in the black subjects.

It is of some interest to consider inter group differences in the urine parameters after each challenge and to compare them to those differences which were originally identified in the baseline values. It is noted that some differences which occurred in the baseline values of the two groups were abolished after various diets, while others were retained. The difference in urinary calcium and the concomitant difference in RS CaOx observed at baseline did not manifest itself after any of the challenges. In most other cases, differences in urinary parameters which occurred originally in baseline samples also tended to disappear after most of the dietary challenges, although the difference in urinary urate was maintained after

three such challenges. None of the original differences were reversed. However, new differences were identified after some of the challenges. Noticeable among these were urinary potassium which was significantly lower in blacks after the high dietary calcium, supplemental calcium and glutamine challenges, urinary cysteine which was significantly lower in blacks after the L-cysteine supplement challenge and the Tiselius risk index which was significantly higher in blacks after the high dietary calcium, supplemental calcium and L-cysteine challenges. While it is recognised that complex mechanisms might be involved in the genesis of these differences, the mere existence of the latter are indicative of different renal handling mechanisms in the two groups. More specifically, since calcium ingestion (either via diet or via supplement) appears to be a common factor, perhaps it is in the handling of this nutrient where the main difference may lie. Furthermore, since none of the challenges significantly altered any of the urinary parameters in black subjects, it is reasonable to speculate that the inter group differences which occurred after ingestion of these challenges are due to urinary changes in the white group. This, in turn, suggests that the black group was able to invoke a renal mechanism of resistance to the challenges which the white subjects were unable to do.

Finally, the volume – size distribution curves showed no significant changes in the mean particle size and only a few changes in the mean particle volume and total volume in the two subject groups. In blacks, the only statistically significant change was in the mean particle volume which increased following the L-glutamine protocol (figure 5-6). (Similarly, L-glutamine was the only protocol that affected urinary factors in black subjects by significantly increasing urinary creatinine (table 5-6)). Although the exact mechanism relating to how these changes occurred is not known, attention is drawn to the absence of similar changes in white subjects. On the other hand statistically significant increases in the mean particle volume occurred in the urine of white subjects but not black subjects, after administration of the calcium, vitamin B_6 and L-cysteine supplements (figure 5-6). In addition the total particle volume increased significantly in whites following the Ca supplemental challenge, but not in blacks (figure 5-7). These data (i.e. increases in mean and total particle volume) suggest

increased nucleation and/or growth of urinary crystals, but are inconsistent with results obtained for urine parameters in the text. This apparent discrepancy might have arisen because volume measurements using the Coulter Counter can be misinterpreted as increased deposition of calcium oxalate crystals when they are in fact due to intra-crystalline incorporation of urinary proteins [Ryall *et al.* 2001]. As discussed in the previous chapter, the mechanisms by which these changes actually occurred are not necessarily important in the context of this particular study. Of greater importance is that the solitary change in the urine of black subjects (increase in particle volume after L-glutamine protocol) *did not occur in whites*, while the particle volume changes observed in the urine of white subjects after various supplemental protocols did not occur in blacks. This provides further evidence in support of the contention that different renal handling mechanisms occur in the two race groups.

In conclusion, the present study has provided convincing evidence of different renal or gastrointestinal handling mechanisms in response to a high dietary calcium and four different supplemental challenges in black and white South African subjects. It would seem that the former group (by virtue of its apparent immunity to urolithiasis) is able to maintain a homeostatic balance of urinary solutes and is able to offer renal resistance to challenges of this nature. Further investigation of a host of factors such as gastrointestinal transport and absorption, the role of oxalate degrading bacteria and the renal interactions of calcium, sodium and cystine in the two race groups is clearly warranted. One of these factors – the role of oxalate degrading bacteria – is described in the next chapter.

5.5 References

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ChapterSix

Gut colonisation of Oxalobacter formigenes and Lactic acid bacteria in South African blacks, whites and calcium oxalate renal stone formers as a key to understanding the differences in the incidence of calcium oxalate urolithiasis in the three groups

6.1 Introduction

Kidney stone research in South Africa has focused on the 'stone-free' black population and the factors which might provide protection against the formation of renal calculi. Previous studies described in earlier chapters in this thesis have investigated the effect of various dietary challenges and supplements on the urinary biochemistry of blacks and whites and have provided evidence of different renal handling mechanisms between the two races [chapters 3,4 and 5]. However, some results have been surprising and others inconclusive, suggesting that the low incidence of urolithiasis in blacks is more complex than a simple dietary or renal relationship.

One such unexplained anomaly in blacks is their oxalate handling mechanism: The dietary intake of blacks is traditionally high in oxalate [Viljoen and Gericke 2001] because of regular intake of spinach, low in calcium because of wide spread lactose intolerance [Viljoen and Gericke 2001] and low in vitamin B_6 [chapter 2, p50 & 53]. Despite these hyperoxalurogenic eating habits, urinary oxalate is within the normal range [chapter 4, p86; chapter 5, p111]. Furthermore, when a high oxalate/low calcium diet was administered to both race groups, the urinary oxalate increased in whites but not in blacks [chapter 3, p74]. Thus it is postulated that oxalate-degrading bacteria might be more prevalent in blacks relative to whites resulting in less oxalate being absorbed despite the lithogenic diet.

The lack of the intestinal bacterium *Oxalobacter formigenes* has been directly associated with hyperoxaluria and consequently the risk of calcium oxalate urolithiasis [Sidhu *et al.* 1998, Kumar *et al.* 2002]. Oxalate-degrading bacteria were first isolated by Dawson *et al* (1980a) and later *O. formigenes* was described by Allison *et al* (1985). *O. formigenes* regulates intestinal oxalate homeostasis by utilizing oxalate, but is unique since this process is its *only* energy source [Dawson *et al.* 1980a, Anantharam *et al.* 1989]. Utilization of oxalate creates a transepithelial gradient, facilitating its excretion in the faeces [Sidhu *et al.* 1997]. This total dependence on oxalate degradation as a metabolic energy source emphasizes the need of

this bacterium for a constant supply of dietary oxalate in order to survive [Dawson *et al.* 1980a, Allison *et al.* 1985].

Certain diseases and drugs have been shown to increase the risk of hyperoxaluria and of subsequent calcium oxalate urolithiasis. Examples of such diseases are cystic fibrosis (CF) [Sidhu *et al.* 1998, Hoppe *et al.* 1998], intestinal disorders like Crohn's disease [Goldkin *et al.* 1986], steatorrhea [Hylander *et al.* 1979], short-bowel syndrome [Worcester *et al.* 2002] and ulcerative colitis [Trinchieri *et al.* 2002], while examples of drugs include antibiotics [Argenzio *et al.* 1988, Sidhu *et al.* 1999b, Duncan *et al.* 2002]. It has been suggested that disturbance of the intestinal bacterial milieu, including *O. formigenes*, disrupts the epithelial integrity and thus increases oxalate absorption by the body [Sidhu *et al.* 1998a, Hylander *et al.* 1979, Allison *et al.* 1986]. In the case of cystic fibrosis patients, the absence of *O. formigenes* appears to be directly linked to absorptive hyperoxaluria and the risk of CaOx urolithiasis [Sidhu *et al.* 1998]. Campieri *et al.* (2001), commenting on the former study, noted that not all CF patients who tested negative for *O. formigenes* were hyperoxaluric and thus postulated that the intestinal handling of oxalate is influenced not only by the presence of *O. formigenes* but also by the integrity of the entire gut microflora, specifically anaerobic bacteria. These workers found a significantly decreased urinary excretion of oxalate (30 mg/day) after the administration of a mixture of lactic acid bacteria including *Lactobacillus* and *Bifidobacterium* species to kidney stone formers with mild hyperoxaluria [Campieri *et al.* 2001]. In the latter study none of the lactic acid bacteria tested positive for the genes responsible for oxalate degradation (oxalate:formate antiporter (Ox1T), *frc* and *oxc*) usually used to identify the ability of a bacterium to utilize oxalate [Lung *et al.* 1994]. However, Campieri *et al.* (2001) used *O. formigenes* DNA specific primers and so there is still the possibility of these genes being present in lactic acid bacteria, which have a higher G + C DNA ratio. *Lactobacillus* and *Bifidobacterium* were described as "generalists", in terms of oxalate utilization meaning that as compared to *O. formigenes,* a "specialist", they are able to utilize carbon sources in addition to oxalate [Rasic 1983]. These beneficial anaerobic bacteria favourably re-colonize

and maintain the gastrointestinal microflora [Rasic 1983] and thereby may provide an optimal environment for the colonization of *O. formigenes*. The later explanation highlights the observation that maximal intestinal oxalate utilization depends on the integrity and health of the gastrointestinal tract.

Anaerobic lactic acid bacteria play an important part in maintaining intestinal health. As end products of their carbohydrate metabolism, they produce organic compounds like lactic acid, hydrogen peroxide, and acetic acid which increase the intestinal pH which then inhibits the reproduction of harmful microorganisms [Rasic 1983]. These kinds of anaerobic organisms have complex nutritional requirements for amino acids and vitamins and therefore, their abundance depends on lifestyle factors such as dietary intake, drugs and intestinal health [Arunachalam *et al.* 2000, Perdigon *et al.* 1995].

As in the case of nephrolithiasis, the prevalence of bowel diseases such as ulcerative colitis and Crohn's disease are very uncommon in black South Africans [Walker and Segal 1979, Oettle and Segal 1985, Walker and Segal 1997]. In addition, favourable differences in faecal pH and intestinal microflora were reported in a study investigating the low incidence of colorectal adenomas in South African blacks relative to whites [Segal 1998].

The intestinal colonization of oxalate-degrading bacteria, specifically *O. formigenes* has not been previously investigated in South Africans. It is hypothesized in the present investigation that the very low stone incidence in blacks lies within the ability of blacks to degrade oxalate in the gut. It is further hypothesized that the colonization of oxalate-degrading bacteria, *Lactobacillus* and *Bifidobacterium* in South African blacks may be greater than that in whites, that these bacteria may have greater oxalate-degrading capabilities and/or that other strains of oxalate–degrading bacteria may occur in the former but not in the latter. This chapter describes an investigation of these hypotheses.

6.2 Materials and Methods

Study Population

Ten South African healthy black males, ten healthy white males and ten calcium oxalate stone forming patients (white males) participated in this study. The inclusion criteria for the healthy black and white subjects were the absence of any history of kidney stones, gastrointestinal or kidney disease. Stone forming patients had to have had at least one calcium oxalate kidney stone episode within the last 2 years and no other metabolic disease. All subjects completed an informed consent form and a medical history questionnaire [appendix CD:chapter6/questionnaire]. In addition, subjects received sterile containers and an instruction sheet on how to collect urine and stool samples [appendix CD:chapter6/instructions].

Dietary Analysis

A 2-day food diary and semi-quantitative food-frequency questionnaire [appendix CD:chapter2/survey#2diet], designed by the present investigator, had to be completed and were analysed for macro- and micronutrients using the computer programme "Foodfinder" version 6 [Wolmarans *et al.* 2001]. This data (dietary survey # 2) was presented in chapter 2 of this thesis.

Urine Analysis

On day 1, a 24-hour urine sample was collected from each subject. Urinary sodium, potassium, calcium and magnesium were determined by atomic absorption spectroscopy (Varian Techtron AA5) while oxalate, citrate, phosphate, urate, chloride and creatinine were determined using commercially available assay kits (Sigma) and citrate using the commercially available assay kit (Boehringer Mannheim). Urine pH and volume were measured routinely. The urinary excretion values were used to determine the Tiselius risk index [Tiselius 1982] and the relative urinary supersaturations of calcium oxalate, uric acid

and calcium phosphate (brushite) [Werness *et al.* 1985]. The CaOx metastable limit of each urine sample was determined using a Coulter Counter Multisizer (Beckman Coulter, Johannesburg, South Africa) (140 µm orifice, 2.8-90.0 µm particle size range, ± 0.5% instrument error) [Ryall *et al.* 1985]. Statistical analysis was performed by analysis of variance at statistical significance of $p \leq 0.05$ (Statistica version 6).

Stool Analysis

On the morning of day 2 (coinciding with the final voiding of the 24hr urine sample collection), faecal samples were obtained from each subject. They were processed within 1 hour, under strict anaerobic conditions inside an anaerobic cabinet (Forma Scientific, gas composition (5% H_2, 15% CO_2, 80% N_2). One gram of the faecal sample was suspended in 10ml anaerobic sterilized distilled water. After vigorous shaking the solid matter was allowed to settle. The supernatant was diluted (anaerobic dH_20) and plated out on the following bacterial culture media: Brain Heart Infusion (BHI) (DIFCO) [Ithoh K and Mitsuokat 1985] agar medium to obtain the total number of culturable bacteria per gram of wet weight of faeces, Mann-Rogosa Sharpe (MRS) (BIOLAB) [Dave and Shah 1996] agar medium to select for *Lactobacillus* species, Reinforced Clostridium Agar (RCA-17) (DIFCO) [Roy 2001] medium to select for *Bifidobacterium* species, and on minimal oxalate agar medium (OX) [Allison *et al.* 1985] to detect oxalate utilizers. The latter was modified as follows (grams per litre): KH_2PO_4: 0.25; K_2HPO_4: 0.25; Na acetate: 0.082; yeast extract (DIFCO): 0.5; ammonium oxalate: 5.68; Na_2CO_3: 4.0; cysteine·HCL·H_2O: 0.5; agar: 15; resazurin: 0.001; and trace element solution SL-10 (http://www.dsmz.de/media/med320.htm): 1.0. All ingredients except the trace elements were mixed and pH adjusted to 6.8. The mixture was sterilised by autoclaving and immediately transferred to the anaerobic cabinet. After the media had cooled to approximately 50°C, the trace element solution (sterile) was added. Stringent anaerobic conditions were ensured by pouring and incubating OX medium plates inside the anaerobic cabinet. The presence of oxygen, as indicated by the slight pink colour of resazurin in the plates, disappeared completely within 2 hours, thereby confirming anaerobic conditions. All

plates were incubated at 37°C. On day 6, the number of colonies on each media plate were counted and recorded as the number of colony forming units per 1 gram of (wet weight) faeces. Mean values and standard errors were calculated for all subjects within subject groups. Student t-tests were calculated for statistical difference using the computer package Statistica version 6.

Bacterial Identification (Oxalate-Degrading Bacteria)

Oxalate-degrading bacteria were characterised by gram staining performed according to standard procedures [Gillies and Dodds 1984]. Growth conditions were analysed as follows: bacteria were grown in an anaerobic chamber (Forma Scientific containing an atmosphere of 5% H_2, 10% CO_2, and 85% N_2) at 37°C, or incubated aerobically at 37°C; testing for oxalate dependent bacterial growth was compared using complete medium (BHI) and minimal medium which provided oxalate as only energy source (OX).

PCR and Sequence Analysis

A single colony of an oxalate-degrading bacterium was grown in BHI broth for 18 hours and genomic DNA was prepared according to the method of Wehnert *et al.* (1992). The universal 16S rRNA gene primers for PCR were: Forward 5'- GCC AGC AGC CGC GGT AAT AC-3' and Reverse 5'- CAC GAG CTG ACG ACA TCC ATG C -3'. PCR was performed (50 µl) with 0.3µ Taq I polymerase (Supertherm); 1µl chromosomal DNA; 2.5 µl of each primer; 2 µl of $MgCl_2$; 5 µl buffer and 0.3 µl of each dNTP. The PCR was carried out in a GeneAmp 9700 machine (Applied Biosystems). The amplification program consisted of one cycle of 96°C for 2 min; 96°C for 45 sec; 25 cycles of 55°C for 30 sec, 72°C for 90 sec, 51°C for 30 sec and finally one cycle of 72°C for 3 min. The reaction was cooled down to 4°C and the PCR product was purified using the High Pure Purification Kit (Roche Diagnostics). Nucleotide sequences were determined directly from plasmids by the fluresence dideoxy chain termination method, using Thermo-Sequencing kit (USB) and 5'-flurorescently labelled universal forward and reverse primers. The reaction products were analysed on the

MegaBACE 500 Sequencing System (Amersham Biosciences). The nucleotide sequences were determined using the MegaBACE 500 Sequence Analyser v2.4 software.

Oxalate Utilization Assay

To quantitate bacterial growth and oxalate utilization in oxalate broth, plates of each medium from the stool analysis with approximately 100 colonies were selected and colonies scraped off with 1 ml oxalate broth medium [Campieri *et al.* 2001] which had been prepared as follows: proteose peptone no 3 (DIFCO): 20g; yeast extract (DIFCO): 10g; Tween '80: 2ml; KH_2PO_4,: 4g; Na acetate: 10g; di-Ammoniumhydrogen-citrate: 4g; $MgSO_4$.7H2O: 0.1g; $MnSO_4$,: 0.1g; water made up to 1000mL and sterilised at 121°C for 15 min). Of this basal broth medium 5ml was mixed with 2.5ml 4% glucose and 2.5ml 80mM Ammonium Oxalate (filter sterilized). The broth culture had a final concentration of 20mM oxalate. Fresh oxalate broth medium (10 ml) was inoculated with 5µl of the bacterial suspension prepared as described above. Oxalate broths were incubated anaerobically at 37°C for 5 days. Bacterial growth was determined by comparing spectrophotometer readings (VIS_{600}) at time the time of inoculation (t=0) and on day 5 (120 hrs). Results were presented as OD_{600} on day 5 minus OD_{600} on day 0 (t=0) (for individual results refer to the appendix CD:chapter6/ox utilization/sheet1).

At time t=0 and on day 5, oxalate utilization was determined using a $CaCl_2$ precipitation assay described by Dawson *et al* (1980b) [Dawson *et al.* 1980b]. Culture broth (1ml) was centrifuged at 5000 rpm for 5 minutes. $CaCl_2$ {stock 1% (w/v)}, 200µl was then added to 100µl culture supernatant followed by 0.9 ml distilled water. For the blank, $CaCl_2$ was omitted and 1.1ml distilled water and 100µl culture supernatant was used. The absorbance was taken (*Beckman DU® Series 64 Spectrophotometer, Memory Pac™ Module*) at VIS 600nm and the presence of oxalate was indicated by the development of a milky precipitate within 5 minutes. Mean values, standard errors of the mean and statistical significance at $p<0.05$ was calculated using the computer package Statistica version 6.

6.3 Results

Medical History and Analysis of Dietary Questionnaires

The stone frequency of the calcium oxalate stone formers was recorded and found to be 3.3 stone episodes within 3 years (table 6-1).

Table 6-1: Medical History of Calcium Oxalate Stone Formers (SF)

Patient	Year of last episode	No of episodes	Other complications
SF1	2002	3	
SF2	2003	7	Athletes foot, hemorrhoids
SF3	2003	3	
SF4	2001	1	
SF5	2003	3	
SF6	2002	3	
SF7	2003	6	
SF8	2003	2	Diverticulitis, Heart Attack
SF9	2000	1	
SF10	2000	4	Dermatitis

Dietary intake data computed from 2-day food dairies and food-frequency questionnaires of the black and white subjects was discussed in chapter 2. In the context of the present chapter only relevant nutrients are presented of black and white subjects in addition to stone forming patients. Attention is drawn to the significantly higher intake of oxalate [317.82 vs 123.99, p=0.010] and significantly lower intake of calcium [663.11 vs 991.09 mg, p=0.011] and magnesium [352.56 vs 434.64 mg, p=0.013] in black relative to healthy white subjects (table 6-2). Vitamin B_6 intake was lower in blacks than whites but the difference was not significant [2.06 vs 11.96 mg, (p=0.063)] (table 6-2). Stone formers were found to have significantly lower intake of carbohydrates (p=0.005), fibre (p=0.029), calcium (p=0.015), magnesium (p=0.003) and vitamin B_6 (p=0.001) compared to healthy white subjects (table 6-3). Nutrient intakes are given in tables 6-2, 6-3 and 6-4.

Table 6-2: Nutrient intake of black and white subjects from dietary questionnaires on normal diet

	Blacks		Whites		p-value
Total Protein (g/day)	79.56	± 9.41	120.15	± 12.71	0.022*
Total Fat (g/day)	81.94	± 12.56	126.45	± 20.60	0.091*
Carbohydrates (g/day)	285.70	± 40.71	362.95	± 42.91	0.211
Added sugar (g/day)	29.14	± 5.84	63.49	± 10.19	0.011*
Oxalate (mg/day)	297.89	± 68.94	128.30	± 21.44	0.025*
Fibre (g/day)	24.50	± 3.07	29.62	± 4.60	0.378
Ca (mg/day)	663.11	± 99.78	1080.10	± 105.55	0.011*
Mg (mg/day)	325.56	± 31.18	468.00	± 39.68	0.013*
Vitamin B_6 (mg/day)	2.06	± 0.41	3.06	± 0.31	0.063
Vitamin C (mg/day)	132.44	± 85.63	101.30	± 20.13	0.714

*significance at $p<0.05$; ± std error

Table 6-3: Nutrient intake of white subjects and stone formers from dietary questionnaires on normal diet

	Whites		Stone Formers		p-value
Total Protein (g/day)	120.15	± 12.71	95.17	± 9.77	0.155
Total Fat (g/day)	126.45	± 20.60	112.35	± 13.24	0.595
Carbohydrates (g/day)	362.95	± 42.91	194.26	± 22.64	0.005*
Added sugar (g/day)	63.49	± 10.19	31.23	± 4.85	0.018*
Oxalate (mg/day)	128.30	± 21.44	127.60	± 34.72	0.986
Fibre (g/day)	29.62	± 4.60	15.85	± 2.77	0.029*
Ca (mg/day)	1080.10	± 105.55	703.63	± 80.92	0.015*
Mg (mg/day)	468.00	± 39.68	292.13	± 27.42	0.003*
Vitamin B_6 (mg/day)	3.06	± 0.31	1.48	± 0.21	<0.01*
Vitamin C (mg/day)	101.30	± 20.13	51.75	± 14.20	0.001*

*significance at $p<0.05$; ± std error

Table 6-4: Nutrient intake of black subjects and stone formers from dietary questionnaires on normal diet

	Blacks		Stone Formers		p-value
Total Protein (g/day)	79.56	± 9.41	95.17	± 9.77	0.268
Total Fat (g/day)	81.94	± 12.56	112.35	± 13.24	0.117
Carbohydrates (g/day)	285.70	± 40.71	194.26	± 22.64	0.078
Added sugar (g/day)	29.14	± 5.84	31.23	± 4.85	0.791
Oxalate (mg/day)	297.89	± 68.94	127.60	± 34.72	0.042*
Fibre (g/day)	24.50	± 3.07	15.85	± 2.77	0.561
Ca (mg/day)	663.11	± 99.78	703.63	± 80.92	0.761
Mg (mg/day)	325.56	± 31.18	292.13	± 27.42	0.439
Vitamin B_6 (mg/day)	2.06	± 0.41	1.48	± 0.21	0.246
Vitamin C (mg/day)	132.44	± 85.63	51.75	± 14.20	0.395

*significance at $p<0.05$; ± std error

Baseline Urine Results

Urinary baseline results are given in table 6-5. It is noted that calcium (p=0.002), urate (p=0.016), phosphate (p=0.01), sulfate (p=0.008) and magnesium (p=0.003) were significantly lower in blacks than in whites. Urinary oxalate was not significantly different between any of the groups. In addition, blacks had a significantly higher metastable limit (p=0.005) (table 6-5). No difference between urinary variables could be found in healthy whites and stone forming whites.

Table 6-5: Urinary Baseline Results

Variables	Blacks	Whites	Stone Formers	B vs W p-value	B vs S p-value	W vs S p-value
pH	5.95	5.91	5.83	0.750	0.479	0.638
Volume (ml/24h)	1137.78	1599.09	1614.00	0.105	0.070	0.953
Citrate (mmol/24h)	2.03	2.16	2.30	0.766	0.618	0.747
Oxalate (mmol/24h)	0.16	0.15	0.17	0.952	0.763	0.648
Ca (mmol/24h)	2.25	5.01	5.27	0.002*	0.006*	0.798
Mg (mmol/24h)	2.37	4.00	3.10	0.003*	0.224	0.150
Na^+ (mmol/24h)	124.66	111.94	153.40	0.674	0.370	0.163
K^+ (mmol/24h)	56.74	52.53	78.51	0.812	0.278	0.104
Urate (mmol/24h)	2.76	4.19	4.19	0.016*	0.013*	0.999
Creatinine (mmol/24h)	15.16	17.12	16.41	0.396	0.461	0.704
PO_4 (mmol/24h)	20.19	31.98	35.10	0.010*	0.008*	0.579
Cl^- (mmol/24h)	131.89	144.64	155.00	0.682	0.445	0.701
SO_4 (mmol/24h)	12.76	25.03	29.76	0.008*	<0.001*	0.300
Tiselius risk index	142.83	171.99	197.27	0.661	0.375	0.705
RS CaOx	3.61	3.61	3.89	0.865	0.790	0.759
RS brushite	0.95	1.32	3.52	0.475	0.062	0.246
RS uric acid	2.16	2.62	1.05	0.444	0.824	0.543
msl	0.11	0.04	0.05	0.005*	0.026*	0.609

*Significance at p≤0.05, (B): Blacks, (W): Whites, (S): Stone formers

Bacterial Isolation and Identification

Faecal samples were diluted and plated on various enrichment media. Although the media are designed to be selective for a specific genus, a number of other strains can grow on these. The MRS and RCA-17 pool therefore represents a mixture of bacterial strains, although they will be predominantly *Lactobacillus* bacteria and *Bifidobacteria* respectively.

Bacterial counts from the 3 groups of subjects are given in table 6-6 and figures 6-1 and 6-2. The results show that blacks have significantly more bacterial numbers per mg wet weight faeces as seen on the media plate selecting for *Lactobacillus* bacteria (5.84×10^9 vs 1.16×10^9, p=0.05) and for the media plate selecting for oxalate-dependent bacteria (including *O. formigenes*) (7.24×10^9 vs 1.40×10^9, p=0.014) (table 6-6). Although the number of *Bifidobacteria* isolated from the RCA-17 agar plates was not significantly different between blacks and whites (7.62×10^9 vs 2.77×10^9, p=0.128), figure 6-1 demonstrates the tendency for blacks to have more of this bacterium. Stone formers had significantly less oxalate-degrading bacteria isolated from OX media plates relative to healthy white subjects (2.52×10^8 vs 1.40×10^9 respectively, p=0.001) (table 6-6 and figure 6-1).

Allison *et al* (1985) described the detection of oxalate-degrading bacteria by the formation of clear zones around colonies. This phenomenon was not observed in the present study. Instead, three bacteria strains with different colony morphologies were seen growing on the oxalate medium (OX). The colonies that were believed to be *O. formigenes* (microscopically equal to a positive control donated by Sidhu and colleagues) were approximately 0.5 µm in size and the cells were characterised as gram-negative rods [Gillies and Dodds 1984], strictly anaerobic and oxalate dependent. Besides the putative O. *formigenes*, two other colony types were detected. Both colonies were approximately 2 mm in size and both consisted of gram-negative rods. In addition, the bacteria which were isolated from these colonies were not able to grow aerobically and did not depend on oxalate as the sole carbon substrate since they grew well on BHI medium with no added oxalate. DNA was prepared from two bacterial strains and used in PCR with universal 16S rRNA primers to isolate the 16S rRNA genes.

DNA sequence of the PCR product was compared to rRNA databases to obtain an identification of the bacteria.

Table 6-6: Mean of total count of bacteria per 1 gram (wet weight) of faeces for each culture medium

Bacteria Isolated on:	Blacks	Whites	Stone Formers	p B vs W	p B vs SF	p W vs SF
BHI	$8.97x10^9$	$1.57x10^{10}$	$4.76x10^9$	0.523	0.321	0.300
MRS	$5.84x10^9$	$1.16x10^9$	$1.07x10^9$	0.050*	0.048*	0.859
RCA-17	$7.62x10^9$	$2.77x10^9$	$1.43x10^9$	0.128	0.055	0.257
OX	$7.24x10^9$	$1.40x10^9$	$2.52x10^8$	0.014*	0.014*	0.001*

Significance at $p<0.05$

Figure 6-1: Mean of total count of bacteria isolated per 1 gram (wet weight) of faeces plotted for each culture medium

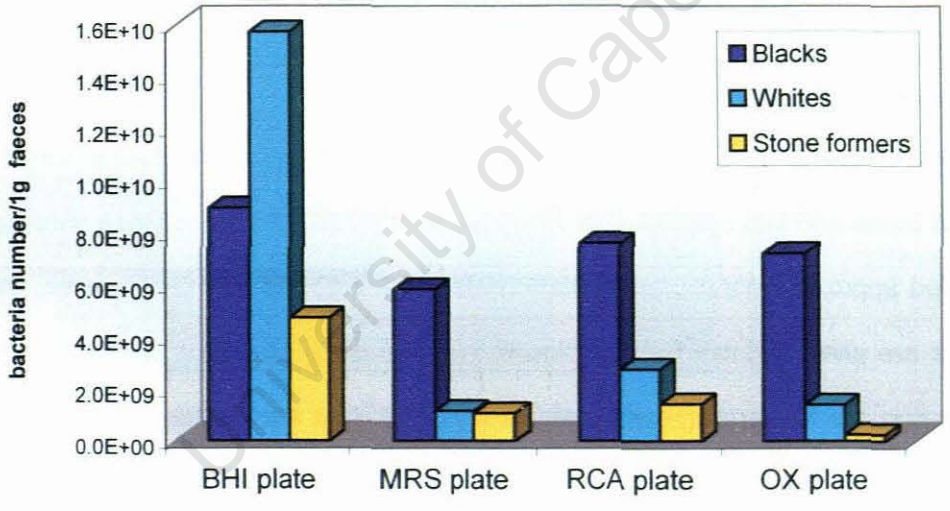
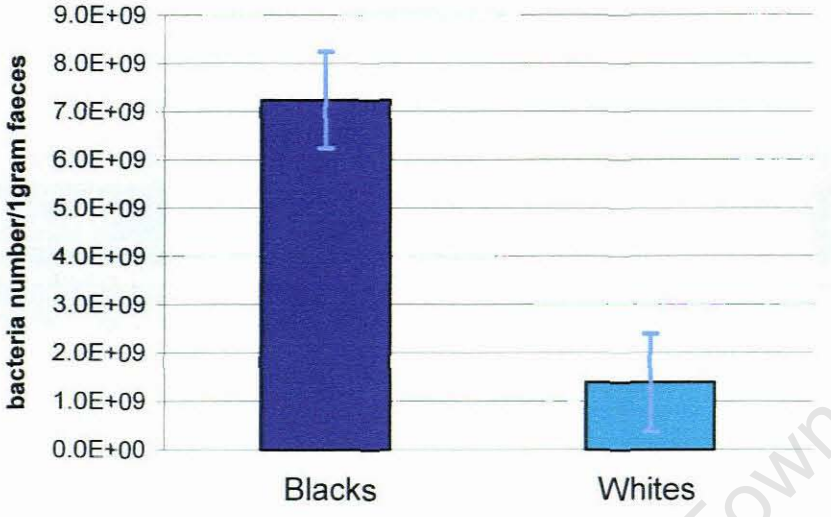


Figure 6-2: Mean total count and deviation from mean across group of oxalate degrading bacteria isolated per gram wet weight of faeces in black and white subjects



Bacterial Growth in Oxalate Medium

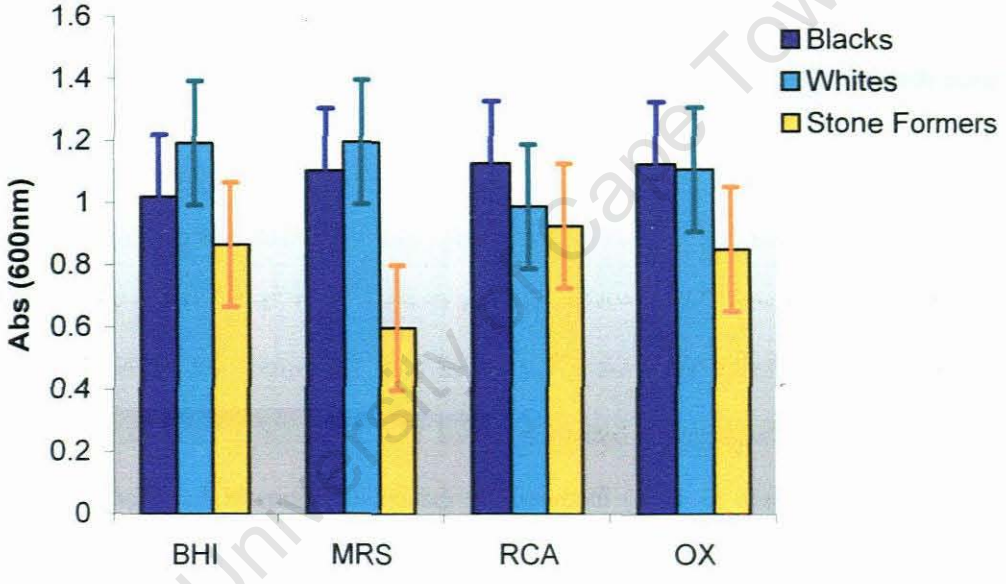
After five days of growth in 20mM oxalate broth, the absorbance (VIS 600nm) of the bacterial species was measured and compared to the initial absorbance. An absorbance reading of 1.0 corresponded approximately to a microbial content of 2×10^{12} cfu/ml broth media. Bacterial growth data are given in table 6-7 and figure 6-3. The only significant difference observed when analyzing the bacterial growth over 5 days was that bacteria isolated on the MRS medium from stone formers grew to a significantly less extent relative to both blacks and whites ($p=0.018$ and $p=0.004$ respectively) (table 6-7, figure 6-3).

Table 6-7: Mean total bacterial growth (A_{600}) in oxalate broth for each group of isolates (difference between day 0 and day 5)

Bacteria Isolated on:	Blacks /Abs	Whites /Abs	Stone Formers /Abs	p B vs W	p B vs S	p W vs S
BHI	1.021	1.194	0.866	0.271	0.389	0.087
MRS	1.104	1.196	0.599	0.541	0.018*	0.004*
RCA-17	1.127	0.987	0.924	0.538	0.308	0.801
OX	1.125	1.108	0.851	0.920	0.191	0.258

*Significance at $p<0.05$

Figure 6-3: Mean total and deviation from mean of bacterial growth (A_{600}) in oxalate broth for each group of isolates (difference between day 0 and day 5)



Oxalate Utilization

To test for oxalate utilization, bacterial species isolated from different media plates were grown in oxalate culture broth [Campieri *et al.* 2001]. An oxalate concentration of 20mM corresponded to an absorbance of approximately 0.700 on the standard curve (appendix CD:chapter6/stand curve, figure A6-1 and figure A6-2). The linear portion of the standard curve was between 10 and 20mM. Different media broth concentrations of oxalate for bacterial degradation varies between 10mM and 20mM for lactic acid bacteria [Campieri *et al.* 2001] and 20mM [Kevin Millsap, personal communication], 30mM [Sidhu *et al.* 1999b], 40mM [Allison *et al.* 1985], and 75mM [Allison *et al.* 1985] for *O. formigenes*. In the present experiment a concentration of 20mM ammonium oxalate was chosen because this concentration is within the range previously investigated for both lactic acid bacteria and *O. formigenes* and because the linear portion in the standard curve ranged from 10 to 20mM.

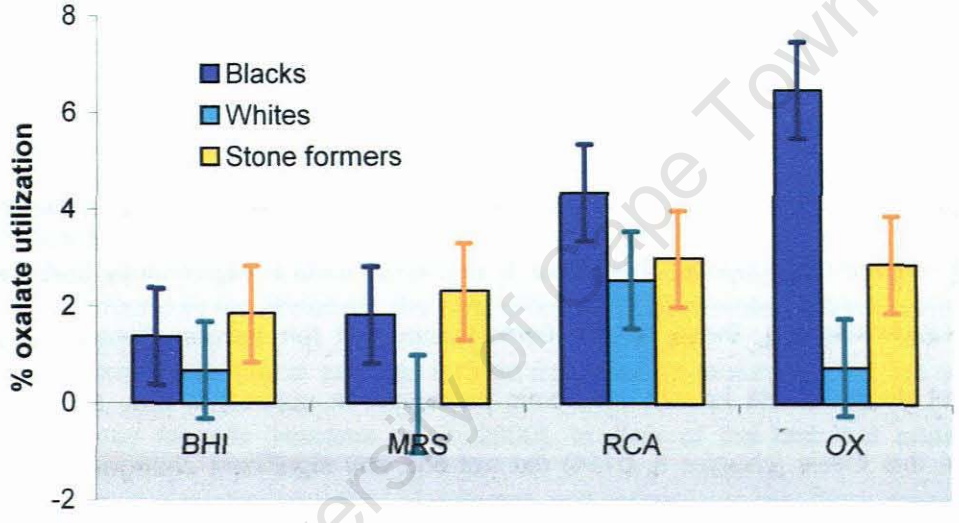
Oxalate utilization data are given in table 6-8 and figure 6-4. The experiments testing for percentage oxalate utilization showed significantly more oxalate being utilized by oxalate-degrading bacteria in blacks compared to whites (6.48% vs 0.75 % respectively, p=0.0009) and in blacks compared to stone formers (6.48% vs 2.88 % respectively) (table 6-8 and figure 6-4). Within-group comparisons in blacks showed that bacteria isolated from medium OX degraded the most oxalate (6.48%) followed by *Bifidobacterium* (4.35%) and *Lactobacillus* bacteria (1.82%) (table 6-8 and figure 6-4). In whites, *Bifidobacterium* isolated from RCA-17 media showed the highest utilisation (2.54%) followed by oxalate-dependent bacteria (0.75%). *Lactobacillus* bacteria isolated from MRS media tested negative for oxalate utilization in this group (table 6-5, figure 6-3). In stone forming subjects, *Bifidobacterium* isolated from the RCA-17 media plate had the highest oxalate utilization (3.01%) followed by bacteria isolated from OX medium (2.88%) and *Lactobacillus* bacteria isolated from MRS media (2.33%) (table 6-8 and figure 6-4).

Table 6-8: Percentage Oxalate Utilization of 20mM oxalate culture broth over 5 days

Original media	Blacks %	Whites %	Stone Formers %	p B vs W	p B vs SF	p W vs SF
BHI	1.38	0.68	1.86	0.480	0.720	0.377
MRS	1.82	0	2.33	0.071	0.750	0.083
RCA-17	4.35	2.54	3.01	0.452	0.641	0.875
OX	6.48	0.75	2.88	<0.001*	0.048*	0.133

*Significance at p≤0.05, B: black subjects, W: white subjects, SF: stone patients

Figure 6-4: Percentage (and deviation of the mean percentage) of Oxalate Utilization of 20mM oxalate culture broth over 5 days



6.4 Discussion

Urinary oxalate is the most important determinant and predictor of crystallization and extensive research has therefore tried to identify factors that reduce urinary oxalate. Calcium and magnesium have been shown to bind oxalate in the intestine forming complexes that prevent excessive urinary oxalate excretion [Curhan *et al.* 1993, Messa *et al.* 1997]. Since the relatively stone-free black population has a significantly lower dietary intake of both calcium and magnesium as well as a significantly higher dietary intake of oxalate relative to stone-prone whites, it is surprising that this population group has a urinary oxalate excretion within the normal range. This anomaly is compounded even further by comparison of the intake of vitamin B_6 in both race groups. Vitamin B_6 is a cofactor in the oxalate pathway helping oxalate hepatic degradation and thus decreases urinary excretion of oxalate [Gill and Rose 1986, Tolomelli *et al.* 1991]. Thus it is surprising that blacks have been shown in chapter two (chapter 2, p50 & 53) to ingest considerably less vitamin B_6 relative to whites. In the present cohort of 10 stone-free subjects, there was a tendency towards significantly lower values ($p=0.06$). Notwithstanding, these results demonstrate that the excess dietary oxalate of blacks is not degraded via hepatic metabolic pathways. In support of this, another study described in this thesis (chapter 5, p114) did not find any significant changes in the urine biochemistry of black subjects after vitamin B_6 supplementation. In addition, in yet another study in this thesis (chapter 3, p74), a low calcium/high oxalate diet was administered to both race groups caused an increase in urinary oxalate in whites but not in blacks, again highlighting that a diet which elevates oxaluria in whites does not achieve the same result in blacks. These observations allow one to postulate that the puzzling difference in the oxalate handling between the two race groups could be attributed to the ability of blacks to degrade oxalate more effectively in the gut. Indeed the present results lend strong support to this hypothesis, having demonstrated that blacks have significantly more oxalate-degrading bacteria (ODB) including *O. formigenes*, greater numbers of lactic acid bacteria and greater oxalate-degrading capability.

In the present study, oxalate-dependent bacteria (isolated using OX media plates) were found to be present in the faeces of healthy white and black subjects ranging from 1.40 x 10^9 to 7.24 x 10^9 cfu/gm wet weight of faeces respectively. Stone formers on the other hand, had a significance of 10 fold less oxalate degrading bacteria (2.52 x 10^8 cfu/gm wet weight of faeces) than black subjects. Medium OX is extremely stringent, with oxalate being the only carbon source besides a minimal amount of yeast (0.05%). Therefore, all bacterial species isolated on this medium use oxalate as a substrate. This represents *all* oxalate-degrading bacteria, and possibly includes *O. formigenes*. Three morphologically different bacteria were recognized and although preliminary identifications have been made, full characterization of these bacteria requires further study. Since these organisms currently form part of a patent application, their identity cannot be divulged here. In contrast to *O. formigenes,* but in common to other oxalate degraders [Ito *et al.* 1996, Hokama *et al.* 2000], the newly isolated oxalate-degrading bacteria are able to use substrates other than oxalate for growth. Besides *O. formigenes,* which is the most extensively researched anaerobic oxalate-degrading bacterium found in the literature, the only other well-documented oxalate-degrading bacteria isolated from human faecal samples include *Eubacterium lentum WYH-1* [Ito *et al.* 1996] and *Enterococcus faecalis* [Hokama *et al.* 2000]. In view of the fact that anaerobic oxalate-degradation is a relative new field of research and recognizing that there are vast numbers of intestinal bacteria which are yet to be isolated [Wilson 1996], relatively few species which utilize intestinal oxalate as a substrate have been identified.

The methods used for isolating oxalate-degrading bacteria have been time-consuming (enrichment procedures [Dawson *et al.* 1980a]) and complicated (anaerobic roll-tube method [Allison *et al.* 1985, Dawson *et al.* 1980a, Dawson *et al.* 1980b, Duncan *et al.* 2002) and it has been previously proposed that the development of improved enrichment and isolation methods may facilitate the identification of other oxalate-degrading bacteria [Dawson *et al.* 1980a, Hokama *et al.* 2000]. Thus, in the present study, it appears that changes to the medium and method of isolation made possible the detection of other oxalate-degraders. The

method described in the present study differed to previous reports as follows: viable counts were taken using medium OX agar plates and an anaerobic cabinet instead of using the roll-tube method described previously [Allison *et al.* 1985, Dawson *et al.* 1980b]. The roll-tube method is an outdated technique but is still used when strict anaerobic conditions are required [Eller *et al.* 1971]. Instead, for the present study an anaerobic cabinet was used which was totally oxygen free. Furthermore the culture medium (OX) used for the present investigation is a modification of medium A described by Allison *et al.* (1985) and differed mainly by the use of different trace elements [appendix CD:chapter6/media].

The occurrence of other bacterial species with oxalate-degrading capability could explain that the total numbers of ODB isolated in the present study (1.40×10^9 to 7.24×10^9 cfu/gm wet weight of faeces) is higher than the reported number of *O. formigenes* colonizing the intestine (0 to 10^8 cfu/gm. wet weight of faeces) [Campieri *et al.* 2001]. *O. formigenes* had been reported as being absent in cystic fibrosis patients with hyperoxaluria [Sidhu *et al.* 1998], while in patients with calcium oxalate urolithiasis, numbers have been reported as ranging between 0 and 10^3 cfu/g wet weight of faeces [Han *et al.* 1995]. Therefore, it is surprising that all of the calcium oxalate stone forming patients used in the present study had large numbers of oxalate-degrading bacteria averaging 1.8×10^8 per gram of faeces. The same explanation as given above could explain this discrepancy; that is in the present study the total number of oxalate-degrading bacteria were investigated. Furthermore, an apparent link exists between the quantity of O. *formigenes* and kidney stone recurrence rate [Han *et al.* 1995, Kleinschmidt *et al.* 1993, Kumar *et al.* 2002]. In previous studies, patients who were tested negative for *O. formigenes* had at least five stone recurrences [Kleinschmidt *et al.* 1993] whereas in the present study only 2 out of 10 patients had more than 5 recurrent episodes (table 6-1). Notwithstanding, it is of significance that the total number of oxalate-degrading bacteria per gram wet weight of faeces was found to be significantly lower in CaOx stone formers compared to both healthy black and white subjects (figure 6-1).

A further objective of the present study was to isolate and quantify *Bifidobacterium* and *Lactobacillus* bacteria in all three subject groups. The enrichment media of choice were MRS for the isolation of *Lactobacillus* bacteria [Dave and Shah 1996] and RCA-17 for the isolation of *Bifidobacterium* [Roy 2001]. However, although these media enrich specifically for *Bifidobacterium* and *Lactobacillus* bacteria respectively, other bacteria can grow on these media to a small extent. Therefore, a more rigorous analysis of the composition of each pool of isolates is required before definite conclusions can be made.

The present study demonstrated that black subjects have significantly more *Lactobacillus* bacteria relative to both healthy whites and stone formers. It has to be recognized that this study involved only 10 subjects of each group and therefore the trend towards significance observed for *Bifidobacterium* cannot be ignored (figure 6-1). In addition, black subjects have significantly more oxalate-degrading bacteria than the other groups (5-fold increase). Furthermore, in the present study calcium oxalate stone formers were found to have significantly less oxalate degrading bacteria relative to non-stone-forming whites (table 6-6). This agrees with previous reports [Han *et al.* 1995, Kleinschmidt *et al.* 1993, Troxel *et al.* 2003], however, there was no significant difference (in the present study) in number for both *Lactobacillus* and *Bifidobacterium* isolated between the two groups (table 6-6). In the study by Campieri *et al* (2001), oral lactic acid bacteria were administered to calcium oxalate kidney stone formers with mild hyperoxaluria and subsequently a decrease in urinary oxalate was observed. Although further studies are required to analyse the composition of the *Bifidobacteria* and *Lactobacillus* bacteria pools before definite conclusions can be drawn, possible explanations for the apparently equal levels of *Lactobacillus* and *Bifodobacterium* could be that the patients participating in the present study were not screened for hyperoxaluria nor do their baseline urinary results depict elevated urinary oxalate. In addition, the bacteria used to test for oxalate utilization in the present study were isolated from human subjects whereas Campieri *et al.* (2001) used commercially available freeze-dried bacteria.

The difference in the numbers of beneficial bacteria (including oxalate-degraders) colonizing the intestines between the two race groups is of great importance because South African blacks are known to have low incidence of those diseases related to intestinal health such as inflammatory bowel disease (Crohn's disease), colorectal adenomas [Segal 1998], and kidney stone disease [Modlin 1967, Whalley *et al.* 1998]. It is especially interesting that both Crohn's disease [Goldkin *et al.* 1986] and kidney stone disease are related to hyperoxaluria and the absence of *O. formigenes* [Han *et al.* 1995, Kleinschmidt *et al.* 1993]. Moreover, it has been reported that South African blacks have a mean faecal pH significantly lower than whites (6.12 vs 6.29, $p<0.01$) [Walker and Segal 1979, Walker and Segal 1997, Segal 1998] indicating the presence of more lactic acid bacteria [Segal 1998] and a more optimal environment for the colonization of beneficial bacteria.

To test for oxalate utilization, bacterial species isolated from different media plates were grown in oxalate broth adapted from Campieri *et al* (2001). Comparisons across all groups investigated showed that bacteria isolated from medium OX in blacks, utilized the most oxalate (6.48% or 0.18µmol/min/ml). Previous research data report that *O. formigenes* isolated from humans, degrades up to 0.56µmol/min/ml [Allison *et al.* 1985]. The difference in oxalate-utilisation between previous accounts and the present data could be due to the fact that a mixed culture was being assayed, or that different culture media and a different oxalate degradation assay were used. The former study used oxalate broth specific for *O. formigenes* and oxalate utilization was estimated using [^{14}C] oxalate degradation rates. In the present study 'Campieri' broth culture was used which is a complete medium (oxalate containing yeast extract and glucose as well as oxalate) and oxalate utilization was estimated using a $CaCl_2$ precipitation assay.

The average oxalate utilisation percentage of *Lactobacillus* and *Bifidobacterium* in the present study agreed with that of Campieri *et al.* (2001) who observed a degradation between 0 and 11.79 percent. But unlike the latter study, the results of the current study demonstrated a relationship between bacterial growth and oxalate utilization in blacks and in calcium oxalate

stone formers. It is surprising that in whites, bacteria isolated from media plates selecting for *Bifidobacterium* had the highest growth (A_{600} 1.196) but no oxalate degradation. In contrast, Campieri *et al* (2001) demonstrated 2.18% oxalate utilization for *Bifidobacterium infantis*. Thus, the ability to use oxalate might be species specific. With limited data available on the oxalate-degrading capability of lactic acid bacteria, it is difficult to draw firm conclusions but this does not minimize the value of the interesting results which have been reported here.

A significant finding of the present study was that the oxalate-degrading bacteria (OX medium) isolated from blacks utilized significantly more oxalate than that of white normal subjects and stone forming patients. One can therefore speculate that blacks have oxalate-degrading bacteria that might be qualitatively superior in utilizing oxalate. Another explanation could be that the integrity of the gastrointestinal tract, which as discussed earlier in the text, is healthier in blacks relative to the whites, optimises the microbiological flora and oxalate-degradation.

In conclusion, the present study successfully isolated oxalate-degrading bacteria, and *Lactobacillus* and *Bifidobacteria* in healthy black, white and calcium oxalate stone forming South Africans and demonstrated that blacks have significantly more oxalate-degrading bacteria and other lactic acid bacteria relative to healthy and stone-forming whites. Furthermore, the oxalate-degrading capability of the bacteria isolated from black subjects was found to be significantly greater when compared to the other groups. This suggests that the gastrointestinal handling of oxalate in blacks is kept in homeostatic balance by the adequate colonisation of beneficial intestinal bacteria, thereby minimizing urinary oxalate exertion. This could be a contributing factor to the extreme rarity of calcium oxalate urolithiasis in black South Africans.

It seems reasonable to speculate whether *O. formigenes* is the most significant intestinal bacterium regulating oxalate homeostasis, however, at present it is the only bacterium which is safe to administer orally as a probiotic [Sidhu *et al.* 2001, Sidhu *et al.* 1999]. Oxalate

degrading bacteria such as *Eubacterium lentum WYH-1* [Ito *et al.* 1996] and *Enterococcus faecalis* [Hokama *et al.* 2000] are potential pathogens. A new isolate may be more suitable for safe oral administration. In addition, a bacterium which is a "generalist" would have the benefit of not being solely dependent on oxalate as a substrate; therefore a continuous oxalate supply does not have to be considered (as in the case of *O. formigenes*).

Future work should focus on the identification and quantification of these different oxalate-degrading bacteria, in order to ascertain whether there are other bacteria which can utilize oxalate as efficiently as *O. formigenes* with possible probiotic potential.

6.5 References

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ChapterSeven

Conclusion

This thesis has addressed the phenomenon of the extremely low incidence of urolithiasis in South Africa's black population relative to the white population by focussing on dietary, urinary and gastrointestinal risk factors in the two race groups. Interesting and meaningful results have been obtained.

As part of two larger studies discussed in chapters 3 and 6 of this thesis, the dietary intakes of urban and peri-urban black subjects and urban white subjects have been analyzed using dietary questionnaires. Decreased intake of protein and refined carbohydrates in blacks are the only two factors that could be correlated with their decreased risk of stone formation. All of the other results were surprising as they would usually indicate increased risk of CaOx urolithiasis. This demonstrates that dietary factors which are important for assessing urinary risk factor for stone formation are different in the two groups, and that it is likely that other gastrointestinal or renal oxalate handling mechanisms may exist which provide blacks with stone immunity despite their lithogenic eating habits.

While some urinary risk factors (chapters 3 and 4) in black subjects were found to be totally contrary to those which might have explained low stone incidence (urinary citrate, pH and oxalate), others support the apparent immunity in this race group (urinary calcium, uric acid and cystine). However the latter are unlikely to be the key factors to explain this phenomenon. Thus it was concluded that conventional urine parameters which have been routinely used to assess stone formation in western population groups have little value in explaining the rarity of this disease in South African blacks. As such it was argued that explanations would have to be sought elsewhere.

In this context, the possibility that different renal handling mechanisms might be occurring in the two race groups was investigated (chapters 3, 4 and 5). The results provided convincing evidence in support of this hypothesis. These results prompted the further hypothesis that blacks are able to maintain a homeostatic balance of urinary solutes and are able to offer

renal resistance to dietary and supplemental challenges. The apparently different renal handling of such challenges has not been previously reported and is of considerable significance. Future studies will need to investigate and characterize the renal interactions of calcium, sodium and cystine (and their ratios) as well as the gastrointestinal transport and absorption of oxalate.

As has been stated throughout this thesis, urinary and dietary oxalate are key determinants of calcium oxalate renal stones. Of these, urinary oxalate has emerged as the most important determinant and predictor of crystallization and therefore extensive research has tried to identify intestinal, hepatic and renal- oxalate handling mechanism. In this context, calcium and magnesium have been shown to bind oxalate in the intestine forming complexes that prevent excessive urinary oxalate excretion. Since the relatively stone-free black population has a significantly lower dietary intake of calcium, magnesium and vitamin B6 as well as a significantly higher dietary intake of oxalate relative to stone-prone whites [chapter 2], it is surprising that this population group has a urinary oxalate excretion within the normal range [chapter 4,5 and 6]. Furthermore, results from chapters 3, 4, and 5 highlight the different intestinal handling of oxalate in the two race groups. Thus it was postulated that oxalate-degrading bacteria might be more prevalent in blacks relative to whites resulting in less oxalate being absorbed despite the gastrointestinal excess of oxalate. Indeed results from chapter 6 lend strong support to this hypothesis, having demonstrated that blacks have significantly more oxalate-degrading bacteria, greater numbers of lactic acid bacteria and greater oxalate-utilizing capability. As stated in chapter 6, the lack of the intestinal bacterium *Oxalobacter formigenes* has been directly associated with hyperoxaluria and consequently the risk of calcium oxalate urolithiasis. It has been suggested that disturbance of the intestinal bacterial milieu that includes O. *formigenes*, disrupts the epithelial integrity and thus increases oxalate absorption. In addition, favourable differences in faecal pH and intestinal microflora have been reported in a study investigating the low incidence of

colorectal adenomas in South African blacks relative to whites. Furthermore, as in the case of nephrolithiasis, absorptive hyperoxaluria is risk factor of bowel diseases such as ulcerative colitis and Crohn's disease. Therefore it is fascinating that the prevalence of all these diseases is very uncommon in black South Africans. Thus, it could be postulated that those factors which provide blacks with a healthy, well-colonized gastrointestinal tract protect this race group against urolithiasis. More specifically it could be hypothesized that while dietary intake could not provide definite clues to explain the different renal handling mechanism shown to exist in black subjects, the influence of dietary intake on gastrointestinal health might provide clues to their apparent immunity to CaOx urolithiasis.

In summary, the results of the studies described in this thesis have provided compelling and significant evidence in support of two principal hypotheses to explain the very low incidence of CaOx kidney stones in South African blacks relative to whites. Firstly, the thesis has shown that South African blacks respond differently to whites with respect to their urinary biochemical parameters, when challenged which different lithogenic and antilithogenic dietary and supplemental protocols. Furthermore this thesis has shown that the response of the former group to such challenges is more beneficial in some cases and less harmful in others when compared to parallel responses in South African whites.

Secondly, this thesis has shown that South African blacks have a greater level of gut colonization of oxalate degrading bacteria than whites and that oxalate utilization by these bacteria is greater in the former group.

These results, hitherto unreported in previous studies, are likely to contribute significantly to a better understanding of the disparity in the incidence of CaOx kidney stone disease in South African's two main race groups and indicate a potentially promising prognosis for the ultimate management of this disease in general.