Investigation of the potential beneficial effects of supplemental polyunsaturated fatty acids and glycosaminoglycans on the risk factors for calcium oxalate kidney stone formation using theoretical, experimental and human models

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Ephesians 3: 20 - 21

,,,Now to Him who is able to do immeasurably more than all we ask or imagine, according to His power that is at work within us, to Him be glory in the church and in Christ Jesus throughout all generations, for ever and ever! Amen,,,

Abstract

Introduction: Two hypotheses with regard to calcium oxalate (CaOx) renal stone formation were tested in this thesis.

The first hypothesis is that fatty acid (FA) supplementation (n-6 and n-3) and chondroitin sulphate (CS) supplementation may reduce the plasma (FA) and urinary (FA and CS) risk factors for CaOx renal stone formation, and ultimately serve as therapeutic agents in the management of this disease. The notion that FAs may reduce plasma risk factors is based on previous studies which have shown that n-6 and n-3 FA supplementation reduces the concentrations of arachidonic acid, while the notion of an effect on urinary risk factors is based on reports of these supplements decreasing urinary calcium and/or oxalate excretion in animal and human studies. The notion of CS playing a role in reducing CaOx stone risk is based on its chemical structure which presents potential binding sites for calcium and magnesium.

The second hypothesis is that black and white healthy South African subjects may respond differently to these dietary supplements and that these differences may provide insights which could account for the lower stone incidence in the former group compared to the latter. This hypothesis is based on the observation in many previous studies of different renal responses in the two race groups, to different dietary and supplemental challenges. FA's and CS have not been previously investigated in this regard.

Methods: These hypotheses were tested simultaneously by administering n-6 and n-3 FA supplements individually and in combination, and supplemental CS, to different groups of black and white healthy male subjects. For the FA studies, blood samples were analyzed for serum biomarkers (25-hydroxyvitamin D3 and triglycerides) for CaOx stone formation and FA profiles in plasma total phospholipids since arachidonic acid regulates calcium excretion. Urine samples were analyzed for individual CaOx stone risk factors, risk indices (Tiselius risk index and supersaturation (SS) of calcium oxalate, brushite and uric acid); crystallization experiments (metastable limit and crystal growth kinetics) were also conducted.

For the CS studies, thermodynamic binding constants for calcium-CS and magnesium-CS complexes were determined by isothermal titration calorimetry. These constants were then used to model speciation in different urines using the computer program *Joint Expert Speciation System* (JESS), and to calculate supersaturation values under different urinary conditions. This was followed by *in vitro* crystallization experiments in which the effects of exogeneous CS on the CaOx metastable limit and CaOx crystallization kinetics were investigated in artificial and real urine samples. Finally, human studies were performed in which CS supplements were administered to subjects to test their efficacy on reducing the urinary risk factors for CaOx stone formation. Urines were analyzed and crystallization experiments were performed as described for the FA supplementation studies.

Results: In the FA studies, favourable changes in the plasma CaOx stone risk factors were achieved by the supplementation of n-3 FA alone. Post-supplementation, the concentration of arachidonic acid in plasma total phospholipids was significantly reduced in both groups, thereby implying a reduction in urinary calcium excretion. However, FA supplementation had no positive effect on the urinary risk factors or on CaOx metastable limits and CaOx crystallization kinetics.

Abstract

In the CS studies, theoretical modelling showed that the reduction of ionized calcium concentrations can only be attainable at 100 times physiological concentrations of urinary CS and that the formation of the calcium-CS complex does not influence the urinary supersaturation of CaOx. The formation of the magnesium-CS complexes was unfavourable because it resulted in an increase in the concentrations of ionized oxalate, a risk factor for CaOx stone formation.

The *in vitro* crystallization experiments showed that exogeneous CS at physiological and above physiological concentrations had no favourable effect on the metastable limit and crystal growth kinetics in all the tested urine samples. Finally, the human study showed that CS supplementation had no effect on urine chemistry and crystallization kinetics. Speciation calculations also showed that the SS values of CaOx and the concentrations of ionized calcium were not significantly changed by supplementation.

Within groups, the effects of FA supplementation on the urinary risk factors were different. n-6 FA supplementation significantly increased magnesium and significantly decreased urate in the black group whereas in whites citrate, oxalate and potassium were significantly increased while ionized calcium was significantly reduced. In the n-3 FA study, magnesium was significantly increased and SS value of brushite was significantly decreased in whites. In the black group, there was no significant difference in the urinary risk factors after supplementation compared to baseline values. With regards to n-6 & n-3 FA supplementation, citrate was significantly increased while oxalate and SS CaOx were significantly decreased in the black group whereas in the white group, magnesium was significant increased.

With regards to the CS study, SS values for brushite, tribasic calcium phosphate, hydroxylapatite and octacalcium phosphate decreased significantly in black subjects after CS supplementation, whereas the SS value for hydroxylapatite increased significantly in the white group. These effects could not be attributed to complexation of CS with calcium or magnesium. However, they are noteworthy because they are different in the two groups.

Discussion: The results of these studies do not support the hypothesis that supplemental fatty acids or chondroitin sulfate have significant beneficial effects for reducing blood and urinary risk factors for calcium oxalate stone formation. Although the response to FA and CS supplementation was different between the two race groups, these findings did not provide new information to explain the difference in the incidence of calcium oxalate stone disease in the two population groups.

The work described in this thesis provides a foundation for future studies in which CaOx stone patients, rather than healthy individuals are investigated.

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Plasma total phospholipids, serum 25-hydroxyvitamin D3 and urinary risk factors in black and white South African subjects: effect of n-3 fatty acid supplementation

P. Gogwana, A. Rodgers, S. Allie-Hamdulay, D. Webber and P. Van Jaarsveld

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Effect of the ingestion of polyunsaturated fatty acids (PUFA) on urinary prostaglandin E2 (PGE2) and plasma phospholipids in two ethnic groups

P. Gogwana, A. Rodgers, S. Allie-Hamdulay, D. Webber and P. Van Jaarsveld

Experts in Stone Disease 1st Conference, Dubai December 2012

24 hr urinary Ca and Ox in stone-free and stone-prone population groups following fatty acids ingestion

T. Theka, ***P. Gogwana***, A. Rodgers, D. Webber and S. Allie-Hamdulay

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Inhibitory effects of calcium binding to glycosaminoglycans: An isothermal titration calorimetry and chemical speciation study

P. Gogwana, A. Rodgers, S. Allie-Hamdulay and G. Jackson

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Abbreviations

25(OH)D	–	25-hydroxyvitamin D3
AA	–	arachidonic acid
DGLA	–	dihomo-ɣ-linolenic acid
DHA	–	docosahexaenoic acid
EPA	–	eicosapentaenoic acid
FA	–	fatty acid
GLA	–	γ-linolenic acid
HYP	–	hydroxyproline
MUFA	–	monosaturated fatty acid
PGE2	–	prostaglandin E2
PUFA	–	polyunsaturated fatty acid
RBC	–	red blood cell
SFA	–	saturated fatty acid
TAG	–	triglyceride
TRI	–	Tiselius risk index
CaOx	–	calcium oxalate
COM	–	calcium oxalate monohydrate
CaP	–	calcium phosphate
UA	–	uric acid
1,25(OH)2D	–	1,25-dihydroxyvitamin D3
PTH	–	parathyroid hormone
NaCl	–	sodium chloride
LA	–	linoleic acid
ALA	–	α-linoleic acid
CSA	–	chondroitin-4-sulfate
CSC	–	chondroitin-6-sulfate
MSL	–	metastable limit
Na_2Ox	–	sodium oxalate
CS	–	chondroitin sulfate

Units

%	–	percent
μg	–	micrograms
μL	–	microliter
μm	–	micrometer
cm	–	centimeter
g	–	grams
h	–	hour
L	–	liter
m	–	meter
mg	–	milligram
min	–	minutes
mL	–	milliliter
mM	–	millimolar
mm	–	millimeter
mmol	–	millimoles
ng	–	nanograms
nm	–	nanometer
nmol	–	nanomoles
°C	–	degrees Celsius
pg	–	picograms
rpm	–	revolutions per minute
s	–	seconds
k	–	kilo
Da	–	Dalton
K	–	equilibrium binding constant
M	–	moles per liter

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Chapter 1

Introduction

1.1 COMPOSITION OF KIDNEY STONES

Kidney stones are described as crystals that have gathered together to form a hard solid within the kidney. These stones come in different types and are distinguished by the type of chemical components that they contain. Nearly 80 % of all stones are made up of calcium oxalate (CaOx) and calcium phosphate (CaP) [1 - 6]. CaOx is the most encountered stone and can occur either as the monohydrate [$CaOx \cdot H_2O$, whewellite] or dihydrate [$CaOx \cdot 2H_2O$, weddellite) [7 - 10]. Whewellite (COM) stones are the most thermodynamically stable CaOx stones and are more prevalent than weddellite (COD) [11 - 13]. Apatite [$Ca_5(PO_4)_3OH$, basic calcium hydrogen phosphate] and brushite [$CaHPO_4 \cdot 2H_2O$, calcium hydrogen phosphate] are types of calcium phosphate (CaP) stones and are formed to a lesser extent than CaOx [14 - 16]. Uric acid (UA) stones are responsible for about 10 % of all stones [13, 17 - 19]. Both calcium phosphate and uric acid crystals are often found in trace amounts as mixtures with CaOx as the main component [20 - 24]. Struvite and cysteine are the least common types of kidney stones [25 - 28].

Because there is no known cause for idiopathic CaOx stone formation, they are the most frequently studied types of kidney stones, thus the following discussion is focused.

1.2 EPIDEMIOLOGY OF STONE FORMATION

As far back as the first century, people have been developing stones. Although bladder stones were the most common stones found during those periods, nowadays the occurrence of stones formed in the kidney are the most documented [29 – 31]. The risk of developing kidney stones appears to be more frequent in males than in females. In adults, the stone risk ratio has been reported to be about 3.1 times higher in males compared to females [32 – 35]. Recently, an increasing trend in stone incidence among women has been observed, and the ratio has been lowered to 1.3:1, male to female [36, 37].

Stone incidence begins to rise after the age of 20 in both adult men and women, and reaches its peak during middle age and after that declines. Children have been reported to develop kidney stones at any age, from premature to newborn babies and teenagers [38 - 40]. Once an individual forms a stone, he/she is likely to develop another one. It has been estimated that about 10 to 20 % of untreated individuals will form another stone within 1 to 2 years, whereas the recurrence rate increases from 35 % to 60 % within 5 to 10 years, respectively [41 - 43].

Changes in environmental climate have been shown to affect the rate of stone formation in various populations. For example, the combination of warm temperatures and inadequate fluid intakes is associated with increased incidence of stone formation [44 - 48].

The tendency of developing stones varies across the world. The highest prevalence, 20 %, of stone formation has been found in men living in the United Arab Emirates and Saudi Arabia. In Europe, stone formation accounts for about 2 to 8 % of the population whereas in Greece it is estimated to be around 15 %. Approximately 12 % of individuals in the United State are predicted to form stone at least once in their lifetime. Meanwhile, Asian males have been found to have the lowest risk at 5 % [49 - 53].

The prevailing trends in kidney stone disease are highly dependent on the socioeconomic conditions of the communities. For example, changes in social and financial status over time, and the subsequent changes in dietary habits, have affected the incidence of stone formation. Dietary intake influences urinary composition, and can therefore modify the urine components associated with stone formation. The most common nutrients implicated in stone formation include calcium, animal protein, oxalate, sodium, carbohydrates, magnesium, phosphorus, vitamins B, C, D and E, etc [54 - 61].

Regardless of the location, it has been found that stone occurrence differs among race groups. In the USA, the black population has significantly less risk of developing stones compared to the white population [62 - 65]. Similarly, in South Africa the risk of stone formation in the black population is considerably less (<1 %) compared to the white race group (15 %) [66, 67]. Other groups that are known to have less occurrence of stone formation include aboriginal tribes of Australia and the Inuit of Greenland [68, 69].

1.3 URINARY BIOCHEMICAL RISK FACTORS

Kidney stones are created as a consequence of changes in the parameters that are present in urine which regulate the activity of ions that participate in CaOx salt precipitation. Below is the description of the most important urinary determinants that contribute to the process of CaOx stone formation.

Urine volume

Low urinary volume leads to greater saturation of stone-forming salts and is therefore a risk factor for all types of kidney stones. In situations where water is lost through other means than via the kidney, the amount of water that is retained by the kidney increases while the amount that is excreted becomes low. For instance, frequent perspiration due to increased physical activities such as working outdoors and playing sports in high temperatures leads to decreased urinary volume [70 - 74]. Individuals who suffer from long periods of diarrhoea without replacing the amounts of water fluids lost are also likely to have low urinary volumes [75, 76].

When the total urine volume is low, the stone-forming salts become more concentrated and their solubility decreases, which may lead to their being precipitated out of the urine [77 - 80]. In contrast, a high urine output (≥ 2L per day) has been suggested to have the opposite effect where the urinary components become diluted, with subsequent reduction in the saturation of salts that form stones [81 - 83].

Urinary oxalate

In urine, oxalate combines with calcium to form CaOx. Precipitation of this salt under favorable conditions will result in CaOx stone formation [84 - 87]. Urinary oxalate is obtained from absorption of dietary foods that contain oxalate and as an end-product of endogenous metabolism [88 - 90]. Increased absorption of foods that have a high content of oxalate has been shown to result in hyperoxaluria [91 - 96]. Hyperoxaluria is defined as urinary oxalate concentrations greater than 0.45 mol/24h [97]. In addition, certain foods that are precursors to oxalate also contribute to urinary oxalate excretion [98 - 103].

The other source of hyperoxaluria has been suggested to be due to errors in glycolate metabolism. The metabolism of glycolate by the enzymes alanine glyoxylate aminotransferase (AGT) and D-glycerate dehydrogenase leads to the formation of glyoxylate, which is the immediate precursor of oxalate. Under normal circumstances, glyoxylate is catabolized by peroxysomal enzymes into glycine, which is further metabolized to serine. Deficiencies in AGT and the D-glycerate dehydrogenase enzymes have been shown to result in increased oxidation of glyoxylate to oxalate, which ultimately leads to increased urinary oxalate excretion [104 - 108]. In more than 35 % of stone patients, the occurrence of hyperoxaluria has been shown to be due to disorders of glycolate metabolism [109 - 114].

Some studies have demonstrated that the absorption of oxalate in the gut is regulated by oxalate-degrading bacteria. *Oxalobacter formigenes* breaks down oxalate to form carbon dioxide and formate [115 – 120]. Hyperoxaluric stone patients have been shown to have total absence or decreased activity of the *Oxalobacter formigenes* [121 - 125]. The concentration of oxalate in urine is considerably lower than the concentration of calcium. As a result, slight changes in urinary oxalate have the ability to influence the saturation of CaOx salt [126 - 129].

Excess supersaturation of urine with CaOx can be avoided by advising the stone patients to reduce the amounts of oxalate-rich foods that they normally eat [130, 131]. It has been observed that urinary oxalate excretions fall from high levels to low concentrations in rats [132] and stone patients after reduced oxalate diet [133, 134]. Increasing dietary intake of calcium is another way that is used to lower urinary oxalate concentrations. In the gut, oxalate binds to calcium to form a salt that is not easily absorbed in the intestines but passed out with stool [135 - 139].

Urinary calcium

Hypercalciuria, which is an important risk factor for CaOx stone formation, was defined by Nordin as having 24h urinary concentrations of calcium greater than 6.2 mmol in females and 7.5 mmol in males [140]. These observations were made when individuals were taking their normal daily foods or when calcium intake was reduced. Increased urinary calcium excretion is generated from excessive intake of calcium foods or when there is a change in the metabolic pathways responsible for maintaining calcium balance in the body [141 - 145]. In most stone patients, the source of hypercalciuria is unknown [146 - 148].

High intakes of calcium can lead to more calcium being absorbed in the intestines. When there is more calcium than what is needed by the body, the surplus calcium that has been absorbed will be excreted in the urine, prompting the precipitation of CaOx [149 - 154]. Apart from calcium obtained from the diet, urinary calcium excretion results from processes that occur in the gastrointestinal tract, kidney and bone. Regulation of calcium at these 3 organs occurs through the action of 1,25-dihydroxyvitamin D (1,25$(OH)_2$D), a metabolic product of vitamin D [155 - 159].

Calcium absorption in the intestines

Intestinal calcium absorption is determined by the amount of calcium present in food and by the concentrations of $1,25(OH)_2D$ [160 - 162]. Vitamin D is obtained mainly from the skin after ultraviolet light exposure and when foods rich in vitamin D are consumed. After entering the bloodstream, it is transported to the liver where it undergoes metabolism by the action of the enzyme 25-hydroxylase to form 25-hydroxyvitamin D (25(OH)D) [160 - 165]. 25(OH)D is considered the most predominant form of vitamin D in human populations, and assessment of serum 25(OH)D concentrations are used to determine the vitamin D status of an individual [166 - 168]. From the liver, 25(OH)D is carried to the kidney where it is metabolized by the enzyme 1α-hydroxylase, to form $1,25(OH)_2D$ [160 - 165]. When individuals consume high amounts of calcium, the activity of $1,25(OH)_2D$ is increased and this results in hyperabsorption of calcium from the gut. In addition to this, when too much vitamin D is ingested, and the calcium intake is normal, the concentration of $1,25(OH)_2D$ increases and the amount of calcium the intestines absorbs will also rise [169 - 174]. In both cases, increased serum calcium concentration will be accompanied by a corresponding elevated urinary calcium concentration [155 - 159].

Regulation of calcium in the kidney

The intestinally absorbed calcium enters the bloodstream and gets transported to the kidney where it undergoes filtration at the glomerulus. Once filtered, about 98% of serum calcium is reabsorbed along the renal tubules to maintain serum calcium balance. The remainder of the filtered calcium is excreted with urine. When the mechanisms responsible for filtering and reabsorbing calcium are disrupted, the renal tubules lose the ability to regulate the reabsorption of calcium. This disruption results in more calcium released into urine [175 - 180].

Release of calcium by bone and the role of parathyroid hormone

Decreased serum calcium concentrations have been shown to occur in certain individuals because of reduced absorption of calcium from the diet [160 - 162]. 25(OH)D concentrations that are below the acceptable level decrease intestinal calcium absorption, which lowers serum calcium concentrations concentrations [181 - 183]. Another scenario for low serum calcium concentrations is when there is decreased reabsorption of filtered calcium from the kidney [175 - 180].

Under these circumstances, the parathyroid gland is triggered and it secretes the parathyroid hormone (PTH). The function of the PTH is to restore serum calcium concentrations to acceptable levels and it does this in 3 ways. In the kidney, PTH decreases the amounts of calcium concentrations lost into the urine by increasing the reabsorption of calcium by the renal tubules. Another way is to enhance the activity if 1α-hydroxylase to produce $1,25(OH)_2D$ from 25(OH)D. The third way PTH influences serum calcium concentrations is through the release of calcium from bone [184 - 187].

Bone is composed primarily of calcium, magnesium, phosphorus and type I collagen which serves as the organic matrix. Throughout the lifetime of an individual, the bone continually undergoes remodeling. Bone remodeling involves the degradation of old bone by osteoclasts cells in a process called resorption, and the formation of new bone by osteoblast cells [188 – 191]. When low serum calcium concentrations are observed, the PTH hormone stimulates the formation of new osteoclasts cells [192 - 196]. During the break down of bone, calcium, magnesium, phosphate and the products of collagen degradation are released from bone into the bloodstream [197 - 200].

When serum calcium concentrations are returned to normal levels, the parathyroid gland stops secreting PTH and the kidneys begin to excrete excess calcium into the urine [201 - 204]. Along with calcium, hydroxyproline is also released by bone during resorption. Hydroxyproline (HYP) is an amino acid and is produced from the breakdown of collagen. Since more than 50% of urinary HYP is obtained from the breakdown of collagen, urinary HYP is used as a marker of bone resorption [205 - 209].

Urinary citrate

Daily urinary citrate concentrations less that 1.70 mmol in men and 1.90 mmol in females have been shown to be a risk factor for the development of CaOx stones [210, 211]. About 19 to 63 % of stone formers have been reported to excrete low urinary citrate [212 - 217]. The excretion of citrate is dependent on the pH balance of the renal tubular cells where citrate is reabsorbed and excreted. When the cells are in an acidic state, almost all of the reabsorbed citrate is retained by the cells for neutralization, leading to reduced urinary citrate excretion. In contrast, reabsorption of citrate is significantly reduced when a basic pH is observed and almost half of the filtered citrate is excreted in urine, thus increasing urinary citrate excretion [218 - 223].

High protein intakes and certain conditions such as renal tubular acidosis, diarrhea, hypokalemia and urinary tract infections are the main causes of hypocitraturia [224 - 228].

Through various mechanisms, citrate inhibits calcium oxalate stone formation [229 – 234]. Thus low urinary citrate concentrations are often associated with increased risk for stone formation. Oral administration of citrate supplements has been shown to increase urinary citrate concentrations [235 – 240].

Urinary uric acid

Hyperuricosuria results from having uric acid concentrations above 4.8 mmol/24h in men and 4.5 mmol/24h in women [241, 242]. Uric acid stone formers tend to have acidic urine, low urine volume and high urate excretion. The source of uric acid in urine is mainly through the metabolism of dietary meats that contain purines [243 – 247]. Acidic urine can occur as a result of high intakes of foods that contain proteins or due to increased acid production within the body because of a defect in ammonium excretion [248 - 250]. At low urinary pH, precipitation of uric acid begins, and the subsequent crystal formation that follows provides surfaces for the nucleation and growth of CaOx crystals [251 - 253]. Low urinary volume accompanied by acidic urine has been shown to increase the urinary supersaturation of uric acid [254, 255].

Urinary calcium excretion is also affected by an increase in net acid load. Firstly, the increased acid load is neutralization by buffers from the skeleton, thereby increasing bone resorption. The released calcium eventually contributes to urinary calcium which may result in hypercalciuria.

Secondly, the reabsorption of sulfates by the renal tubules is minimal. These compounds form complexes with calcium, preventing its tubular reabsorption and further exacerbating any existing hypercalciuria [256 - 260]. In addition to the above effects, uric acid also influences stone formation by interfering with the compounds that inhibit crystallization. The generated extra acid reduces urinary citrate excretion by enhancing citrate reabsorption in the proximal renal tubule [261 - 264]. It has been observed that uric acid interacts with macromolecular substances in urine such as glycosaminoglycans, thus reducing their concentrations and thereby lowering their inhibitory effect on CaOx crystallization [265, 266]. Hyperuricosuria can be treated by reducing meat and purine intakes or by the administration of citrate to raise urinary pH to safe levels (between 6.0 and 6.5) [267 – 269]. Allopurinol treatment has been shown to block uric acid production [270 - 273].

Urinary pH

Changes in urinary pH can affect stone formation directly by altering the concentrations of substances that contribute to crystal formation or indirectly by favoring conditions that can promote stone formation [274 - 277]. At pH less than 5.5, the solubility of uric acid in urine decreases, leading to crystallization of this salt [278 - 283]. On the other hand, urinary pH above 6 has a tendency to form CaP crystals which may develop into apatite and brushite stones [284 - 288]. The formation of CaOx crystals has not been linked to urinary pH because the solubility of this salt remains unchanged at normal physiological pH [289, 290]. Adjusting urinary pH to values greater than 5.5 and less than 6.0 is recommended for minimizing the crystallization of both UA and CaP in urine.

Urinary magnesium

Hypomagnesiuria is defined as having urinary magnesium concentrations that are less than 50 mg/24h [291, 292]. Chronic diarrhea, low dietary intake and malabsorption are the main causes of low urinary magnesium excretions [293, 295]. Several animal studies have demonstrated that low urinary magnesium excretion increases the risk of stone formation. Dietary restriction of magnesium in hyperoxaluric rats resulted in an increase in CaOx crystal deposition in the renal tubes and reversing hypomagnesiuria prevented CaOx crystallization [296, 297]. In humans, hypomagnesium may be a risk factor because magnesium competes with calcium for combining with oxalate to form a salt that is more soluble than CaOx [298].

1.4 CaOx STONE FORMATION AND CO-MORBIDITIES

Certain diseases such as diabetes mellitus type 2 are associated with the increasing trends in kidney stone disease [299 - 301]. Type 2 diabetes mellitus is a condition that affects the metabolism of sugar in the body. The movement of sugar across cell membranes is regulated by the insulin hormone. Diabetes occurs when there are insufficient amounts of insulin or when the effects of this hormone are resisted by the body [302 - 304]. In either case, the disturbances in the sugar metabolism may change the urinary composition of an individual by increasing the production of ammonium [305 - 307]. The urinary pH of diabetic patients who form kidney stones has been reported to be very acidic [308 - 311]. As discussed earlier, a low urinary pH is a risk factor for uric acid stone formation and lowers urinary citrate concentrations [251 - 253].

More importantly, studies have demonstrated that the ingestion of fructose may decrease the reabsorption of filtered calcium along the renal tubules and as a result, increase the urinary excretion of calcium [312].

Considering the close relationship between sugar consumption and diabetes mellitus, these observations suggest that excessive carbohydrate consumption may be a risk factor for CaOx stone formation. In both diabetic and stone patients, dietary intake of fructose affects the concentrations of serum triglycerides [313, 314]. Consumption of diets high in carbohydrates may be a risk factor for diabetes and kidney stone formation and increased serum triglycerides concentrations may be a biomarker that links these two diseases [315, 316].

1.5 INHIBITORS OF CaOx STONE FORMATION

Urinary stone inhibitors can be divided into two broad classes – those that affect the thermodynamic factors and those that affect the kinetic factors. The saturation of urinary salts falls into the first group while crystallization mechanisms such as crystal nucleation, growth and aggregation fall into the second group.

Supersaturation of stone-forming salts occurs very often in urine. It has been observed that even healthy individuals who do not form stones excrete microscopic crystals quite regularly. This implies that every individual fulfills the initial step of CaOx crystallization. Despite this saturation of stone salts, most humans do not form stones. The ability of urine to resist crystallization has been largely credited to the presence of molecules such as citrate, magnesium, glycoproteins and glycosaminoglycans (GAGs) that are capable of retarding and inhibiting the steps involved in stone formation [317 - 322]. In particular, their chemical nature allows them to alter or modify the processes that lead to stone formation by various mechanisms [323 - 328]. These compounds have been found in very low concentrations in the majority of stone formers and also their protective functions in urine have been somehow altered [329 - 334]. The role of glycosaminoglycans will be discussed in section 1.9.

Citrate

The source of citrate in the body is normally obtained to a large extent as a by-product of the Krebs cycle, while the rest is derived from food intakes [335, 336]. In the liver, citrate is metabolized to bicarbonate which has alkaline properties. The unabsorbed citrate is excreted by the kidney where it contributes to the inhibitory effect of urine against crystallization [337, 338].

In urine, citrate reduces the probability of CaOx formation by binding to the free calcium ion and forming a calcium-citrate complex that is more soluble than CaOx [339 - 342]. In addition to reducing the supersaturation of CaOx, citrate has been shown to retard crystallization and growth of CaOx crystals [343 - 347].

Due to its alkalinization properties, the presence of citrate in urine will raise the urinary pH and dissolve any existing uric acid crystals, thus preventing heterogenous nucleation of CaOx on uric acid crystals [267 - 269].

Magnesium

Several studies have shown that excretion of urinary magnesium may be effective in preventing kidney stone formation. In the gut, magnesium combines with oxalate to form magnesium oxalate (MgOx), a more soluble complex compared to CaOx [348, 349]. The MgOx salt reduces the amount of free oxalate that can be absorbed in the gut, thereby successfully reduces urinary supersaturation of CaOx. It has been shown that oral administration of magnesium oxide salt effectly reduced oxalate absorption from 13.5 to 7.6 % [350]. In another study, Berg et al observed that urinary excretion of oxalate becomes significantly reduced after magnesium supplementation when healthy subjects ate a diet that contained high amounts of spinach [351]. *In vitro* crystallization studies showed that magnesium can reduce both nucleation and growth of crystals [352 - 354]. Furthermore, increasing dietary magnesium intake has been shown to lower urinary oxalate excretion [355, 356]. On the other hand, supplementation with magnesium in healthy subjects resulted in an increased urinary excretion of citrate [357].

Glycoproteins

Various urinary glycoproteins are associated with the development of kidney stone disease. These compounds are mainly composed of proteins which carry covalently bound carbohydrates [358, 359]. Glycoproteins are mostly produced within the kidney and are excreted in urine. For example Tamm Horsfall protein (THP), inter-alpha-trypsin and osteopontin occur on cell surfaces of renal tubules in the ascending loops of Henle [360 - 362]. Their presence in the renal tubules prevents stone retention by inhibiting crystal attachment to renal cells [363 - 368].

In urine, glycoproteins have been found to modify the processes involved in crystallization of CaOx. The inhibitory effects of glycoproteins against stone formation include preventing nucleation of CaOx crystals, slowing down or inhibiting the rate of crystal growth and aggregation [369 - 373]. The individual effects of the glycoprotein inhibitors are presented on Table 1.1.

The protection effect of these inhibitors against stone formation also depends on the physicochemical conditions of the urine. For example, THP can acts as both an inhibitor and a promoter of kidney stone formation. In solutions with high pH, low ionic strengths and low concentrations of divalent ions and THP, this glycoprotein inhibits stone formation. However, THP becomes a promoter of crystal aggregation in the presence of low urine pH and low THP levels, accompanied by high concentrations of calcium, sodium, and hydrogen ions [374].

Table 1.1
Types of inhibitors and their mode of action on CaOx crystallization

Name of inhibitor	Effect on crystallization
Osteopontin	Inhibitor of nucleation, growth and aggregation
Urinary Prothrombin Fragment 1 (UPTF1)	Inhibitor of growth, aggregation and adhesion
Tamm-Horsfall protein (THP)	Promoter of nucleation and growth, inhibitor of aggregation
Renal lithostathine (RL)	Inhibitor of growth
Nephrocalcin (NC)	Inhibitor of growth and aggregation

Source: Gupta M et al, 2011. IJRPC 1 (4): 793-8 [319]

1.6 PROCESSES INVOLVED IN CaOx STONE FORMATION

Normally, urine contains numerous ions including calcium, oxalate, urate, phosphate [375 - 378]. These ions interact with each other and form salts such as CaOx, UA, brushite, etc. When the amounts of these salts reaches a maximum point where they are no longer able to dissolve in urine, the urine is described as being saturated [379 - 381]. As the concentration of these salts is increased further in the saturated urine, crystals begin to precipitate out of the urine.

Supersaturation is defined as the state where the concentration of stone-forming salts exceeds the saturation concentration and is the fundamental driving force for crystallization. It is often expressed as a ratio of salt concentrations over their solubilities [379 - 384].

In general, supersaturation is higher in stone formers than in individuals who do not form stones [385, 386]. At supersaturation ratios above 1, crystals tend to nucleate and grow, promoting stone formation whereas they dissolve at ratios below 1 [379- 384].

Crystal formation, known as nucleation, is the process by which free ions in solution are transformed into loose particles [384]. There are two types of nucleation. The formation of crystals in the absence of surfaces is described as homogeneous nucleation. Homogeneous nucleation rarely occurs in urine due to the presence of dissolved impurities. Heterogenous nucleation is the formation of crystals on the surfaces of substances found in urine [379 - 384]. Surfaces of epithelial cells, urinary casts, red blood cells, and other crystals are known to promote nucleation and to also reduce the amounts of energy required for nucleation [385 - 390].

Secondary nucleation results from the presence of crystals in the supersaturated urine which act as catalysts for nucleation [379 - 384]. Both heterogeneous and secondary nucleation requires lower supersaturation of salts in urine compared to homogeneous nucleation [391].

Crystal growth is the next stage of crystallization where the formed nuclei grow larger by the addition of ions from the supersaturated urine onto the crystal [392, 393]. Since the rate of crystal growth is quite slow and about 5 to 10 minutes are required for the tubular fluid to pass through the kidney, the process of crystal growth alone is not sufficient to give rise to a crystal large enough to be retained inside the kidney [394]. These small crystals are usually passed out of the body with urine without forming a stone [395].

One way of forming large crystal particles is through a process called aggregation. Aggregation is accomplished when crystals come into close contact and bind to each other [396]. Clustered particles can be formed when crystal particles use an organic matrix as the binding agent to attach to other crystals of the same chemical nature [397 – 404]. The organic matrix has been reported to contain proteins, lipids, polysaccharides and other cell derived materials. Pure CaOx stones are suggested to be formed via this process [405]. Alternatively, aggregation may be achieved when pre-formed crystals settle and nucleate on the surfaces of other crystals. For example, CaOx crystals can aggregate on surfaces of both CaP [406] and UA crystals [407, 408] resulting in a mixed CaOx-CaP or CaOx-UA stone, respectively. Compared to crystal growth, aggregation is a rapid process and crystals grow into appreciable sizes during the transit time in the urine [394].

Free- vs fixed-particle theory

Stone development occurs when the formed particles are retained inside the kidney. This process is suggested to occur via 3 mechanisms. In cases where the crystals have aggregated to a substantial size and the urine flow is poor, crystals may become trapped inside the renal tubules. Further crystal growth and aggregation may lead to the formation of large particles that may block the renal tubules. Alternatively, retention of particles inside the kidney can be achieved when particles attach to tubular cell surfaces [409 – 414].

When the particles are confined inside the kidneys due to their large size, the retention process is defined as the free particle mechanism [415]. This mechanism is not favored because the crystal particles can never grow or aggregate into appreciable sizes while travelling through the renal tubules for them to become trapped inside the kidney. These observations were based on calculations made by Finlayson and Reid who calculated the rates of crystal precipitation growth under favorable CaOx supersaturation conditions, while taking into account the kidney structures and nephron dimensions [416].

However, when the major processes involved in stone are dominated by the attachment of crystals to cell surface, the mechanism of stone retention is known as the fixed particle mechanism [417]. Oxidative stress, contact with crystals and bacteria usually causes damage to epithelial cells lining the renal tubules [418]. The exposed cell fragments provide a suitable surface for the attachment of crystals [417]. Proteins and glycosaminoglycans that are found at the renal cell surfaces have been implicated as the sites at which CaOx attach to renal cells.

Once on the surface of the cells, the crystals can either be endocytosed [418] or they may increase in size leading to further crystal aggregation [419]. The crystals that are engulfed by the cells slowly disintegrate without increase in growth or forming a stone [420]. Meanwhile, further crystal growth can occur for long periods as long as the urine is supersaturated and there is aggregation of new crystals. Although debris from the damaged cells usually forms the surface upon which crystals attach to renal tubules, crystal attachment may also occur on calcium deposits found on the renal papilla [421]. Both brushite and apatite crystallization mainly occurs in the loop of Henle, resulting into deposits that may grow extensively to form a plaque known as Randall's plaque [422]. This theory was supported by a study conducted by Aspin et al [423] where he found out that the loop of Henle is supersaturated with respect to calcium-phosphate salts. Stones formed in these plaques are largely composed of CaOx and CaP [424].

1.7 DIETARY FACTORS

The types of foods that an individual consume can modify the composition of urine. As a result, certain foods are suggested to affect the saturation of stone-forming salts and are associated kidney stone development [425 - 430]. On the other hand, the consumption of other types of foods is recommended for people who form stone because they are capable of minimizing or preventing the risk of stone formation [431 - 436].

Fluid intake

Several studies have demonstrated that drinking large amounts of fluids reduces the concentrations of calcium, oxalate, and phosphate in urine, leading to a reduction in the supersaturations of salts such as CaOx and CaP and urate [437 - 440]. In addition, it has been shown that the likelihood of forming another stone can be reduced by 12 % after increasing the amounts of fluid that an individual drinks. However, stone patients who maintain their normal fluid intake are reported to have an average recurrence rate of 27 % [441 - 443].

A high urine volume can be achieved by consuming adequate amounts of various fluids. Water is the most preferred type of fluid to drink because it is easily accessible [443 - 448]. In addition to water, drinking adequate amounts of green teas, coffee, beer and wine [449 - 453] has been found to be useful in increasing urinary volume. An added benefit may be obtained from other types of fluids because of ingredients that they contain which protect against stone formation.

Drinking lemon and orange juices increases urinary concentrations of citrate [454 - 456], an inhibitor of stone formation. Cola, grape-, cranberry and blackcurrant juice consumption have a negative impact on stone formation due to the high levels of oxalate found in these drinks [457 - 459].

Oxalate

Ingestion of foods that have high oxalate content can increase the concentrations of oxalate in urine [460 - 463]. Intestinal hyperabsorption of oxalate from green leafy vegetables (spinach and rhubarb), nuts, tea, chocolate, etc increases the risk of stone formation [464, 465]. It has been shown that as much as 10 - 20 % of oxalate excretion per day is derived from dietary oxalate [466]. However, in a study conducted by Holmes, it was found that almost 50 % of urinary oxalate originates from dietary intakes of foods that contain oxalate [467].

When vitamin C, xylitol and amino acids such as hydroxyproline and glycine are ingested in great amounts, a corresponding increase in urinary oxalate is observed [468 - 473]. This is because the metabolism of these nutrients produces glyoxylate, which is converted to oxalate and excreted as a waste product. CaOx stone formers are normally advised to restrict their daily intakes of foods rich in oxalate since oxalate has a more powerful effect on CaOx saturation than calcium [474, 475].

Calcium

In most hypercalciuric stone patients, consumption of foods that contain high amounts of calcium is directly associated with increased calcium excretion [476 - 479]. Van Unruh et al [480] demonstrated that urinary calcium increased by 8 % and 20 % in healthy controls and stone formers, respectively after ingesting similar calcium intakes.

Normally stone formers will try to minimize the risk of stone formation by decreasing the amounts of calcium in the diet [481 - 484]. This route is not advisable because dietary calcium binds to oxalate in the gut to form a non-absorbable salt [136, 485 - 486]. The benefit of this is that oxalate obtained from foods is eliminated from the body with faeces without increasing the risk of stone formation. In addition, reduced calcium intake is connected to the low calcium content of bone, and can lead to diseases such as osteoporosis [487, 488].

Sodium

Increased salt consumption may promote stone risk by reducing calcium reabsorption, leading to increased urinary calcium excretion [489 - 492]. In the kidney, sodium is reabsorbed along the renal tubules at the same sites responsible for calcium reabsorption [493, 494]. As a result, excretion of one ion may interfere with the excretion of the other [143].

Dietary intake of salts, particularly sodium chloride (NaCl), provides the necessary sodium needed by the body. Nordin et al [495] estimated that about 2 g of NaCl intake will result in 1 mmol increase of urinary calcium excretion in healthy subjects whereas if the stone formers consumed the same amount, 2 mmol of calcium will be lost in urine. Also, high sodium intakes have been associated with a significant decrease in urinary citrate [496].

Since reducing calcium intake may promote the stone incidence, limiting dietary sodium is the most preferred way to lower stone risk [497 - 499] in hypercalciuric stone patients.

Protein

Ingestion of high amounts of foods that contain proteins is considered to increase the risk of developing kidney stones. Animal protein foods are a rich source of sulfur-containing amino acids. Metabolism of these amino acids generates sulfuric acid, which influences the overall acid excretion [248 - 250]. Low urinary pH favors the formation of uric acid stones [251 – 253]. Also, the increased excretion of acid affects the renal reabsorption of citrate and results in decreased urinary citrate excretion [500, 501].

With regards to calcium excretion, high intakes of animal proteins enhance bone resoprtion which may lead to hypercalciuria [500, 502 – 503]. In a study conducted in healthy subjects, Kerstell et al [504] demonstrated that 25 g of protein intake will raise urinary calcium excretion by 0.8 mmol. In addition, Robertson et al observed a 23 % significant increase in urinary calcium excretion after 34 g intakes of proteins per day [505]. Low intakes of dietary proteins are normally recommended for hypercalciuric stone patients [506, 507].

1.8 ROLE OF FATTY ACIDS

Nomenclature

Fatty acids (FAs) are straight chain hydrocarbons possessing a carboxyl group at one end [508, 509]. The most common dietary fatty acids are divided into three classes, namely: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) [510 - 514]. SFAs contain no carbon double bonds between the carbons in the main carbon chain. MUFAs have one carbon double bond while PUFAs have two or more double bonds. According to the International Union of Pure and Applied Chemistry system, FAs are named on the basis of the number of carbon atoms, and the number and position of unsaturated fatty acids relative to the carboxyl group [515].

For example, the chemical formula C18:4 n-3 or C18:4 n-6 indicates that there are 18 carbons in the chain with 4 double carbon bonds. The number 3 or 6 preceded by n, indicates the distance of first double bond, in carbon atoms, from the methyl end (n-end) of the chain. The most encountered dietary fatty acids are shown in Table 1.2.

Action and metabolism

Stone formers have been found to consume higher intakes of dietary fats compared to normal subjects [516, 517]. In two separate studies, evaluation of food questionnaires demonstrated that fat intake correlates positively with urinary oxalate excretion [518, 519]. In animal models, rats fed a fat-rich diet, which was primarily composed of excess cholesterol and neutral fat, resulted in an increase in urinary saturation of CaOx [520].

Recently, fatty acids have been shown to influence the urinary excretion of calcium and oxalate. After analyzing dietary fatty acid intakes of hypercalciuric stone patients, Naya et al reported that arachidonic acid was directly correlated with urinary oxalate excretion in these patients [521]. However, in a study conducted by Taylor et al, there was no association of dietary fatty acid intake with increased risk of stone formation [522].

Table 1.2
Abbreviated chemical formulae and common names of major fatty acids

Chemical formulae	Common name	Chemical name
C18:3 n-3 ALA	Alpha-linolenic acid	9,12,15-octadecatrienoic acid
C20:5 n-3 EPA	Eicosapentaenoic acid	5,8,11,14,17-eicosapentaenoic acid
C22:5 n-3 DPA	Docosapentaenoic acid	7,10,13,16,19-docosapentaenoic acid
C22:6 n-3 DHA	Docosahexaenoic acid	4,7,10,13,16,19-docosahexaenoic acid
C18:2 n-6 LA	Linoleic acid	9,12-octadecadienoic acid
C18:3 n-6 GLA	Gamma-linolenic acid	6,9,12-octadecatrienoic acid
C20:2 n-6 EDA	Eicosadienoic acid	11,14-eicosadienoic acid
C20:3 n-6 DGLA	Dihomo-gamma-linolenic acid	8,11,14-eicosatrienoic acid
C20:4 n-6 AA	Arachidonic acid	5,8,11,14-eicosatetraenoic acid
C22:4 n-6 ADRA	Adrenic acid	7,10,13,16-docosatetraenoic acid
C22:5 n-6 DPA	Docosapentaenoic acid	4,7,10,13,16-docosapentaenoic acid
C16:1 n-7 PTA	Palmitoleic acid	9-Hexadecenoic acid
C18:1 n-7 VA	Vaccenic acid	11-Octadecenoic acid
C18:1 n-9 OA	Oleic acid	9-octadecenoic acid
C20:1 n-9 GA	Gadoleic acid	11-eicosenoic acid
C24:1 n-9 NA	Nervonic acid	15-tetracosenoic acid
C14:0 MA	Myristic acid	Tetradecanoic acid
C16:0 PA	Palmitic acid	Hexadecanoic acid
C18:0 SA	Stearic acid	Octadecanoic acid
C20:0 ARA	Arachidic acid	Icosanoic acid
C22:0 BA	Behenic acid	Docosanoic acid
C24:0 LGA	Lignoceric acid	Tetracosanoic acid

(Source: Hon GM et al, 2012. http://www.intechopen.com/books/gas-chromatography-biochemicals [515])

Other investigators have proposed that fatty acids may influence stone formation via their composition in phospholipids. In hypercalciuric stone patients, Baggio et al observed a discrepancy in the composition of n-6 fatty acids in both the plasma and red blood cells (RBC) membrane phospholipids [523, 524]. Abnormally increased phospholipid concentrations of arachidonic acid (AA, 20:4 n-6) compared to the lower content of linoleic acid (LA, 18:2 n-6), its precursor, have been shown to create anomalies in the transport mechanisms that regulate calcium and oxalate balance in the body. The other fatty acid composition in plasma and RBC were found to be within the normal ranges.

The oxalate transport mechanism across the RBC membranes in the gut and kidney are regulated by AA [525]. Band-3 and band-3-related proteins are the major carriers of oxalate at these sites and through anion exchange mechanisms, are able to move oxalate across different cell membranes [526]. Baggio et al reported that the band-3 phosphorylation is an important step of the oxalate cell flux [523]. AA acts a second cell messenger and activates casein kinase CK1, the enzyme responsible for band-3 protein phosphorylation. Increased AA concentration may results in enhanced activity of this enzyme, leading to high intestinal absorbance and renal clearance of oxalate [527, 528].

AA has been shown to exert its effect on calcium excretion via prostaglandin E2 (PGE2), its major metabolic product [529]. It has been suggested that PGE2 may promote hypercalciuria in the gut by stimulating the activity of 1α-hydroxylase, which leads to the increased production of 1,25(OH)D [528]. In particular, increased serum PGE2 and $1,25(OH)_2D$ concentrations have been observed in hypercalciuric stone patients [530]. In the kidney, PGE2 is suggested to influence calcium balance by reducing the amounts of calcium that are reabsorbed in the thick ascending loop of Henle [528]. It has been demonstrated that PGE2 is involved in the bone resorption process where PGE2 regulates the activity of osteoclastic cells while inhibiting osteoblastic bone formation [531]. Along with high PGE2 concentrations, Pilbeam et al found increased concentrations of several bone resorption markers in patients with osteoporosis [531]. These observations imply that when PGE2 concentrations are high, increased activity of PGE2 at these organs will be increased, leading to hypercalciuria.

The role of PGE2 in calcium excretion was first investigated by Buck et al in animal models and stone patients [532]. After injecting rats and monkeys with indomethacin, an anti-inflammatory drug known to inhibit the production of PGE2, decreased urinary calcium concentrations were observed. Following PGE2 administration, the decreased calcium concentrations rose and returned to acceptable levels.

Similarly, a reduction in urinary calcium was observed in a group of hypercalciuric patients after they were given indomethacin [532]. These observations were later confirmed in a study conducted in stone patients by Hirayama et al [533].

AA is an n-6 FA that is obtained directly from dietary sources or indirectly from conversion of LA [534]. Hypercalciuric stone patients have been shown to have a significantly higher concentration of AA than normocalciuric stone patients [523]. Baggio et al hypothesized that this abnormality may be as a result of an abnormal change in the metabolism of these fatty acids rather than malabsorption [520].

Ingestion of n-3 FAs such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) have been suggested to reduce the risk of stone formation [535, 536]. It has been observed that the incidence of stone formation in the Inuit group of Greenland is almost absent due to the diet that is mainly comprised of fish, which is a rich source of EPA and DHA [69].

Dietary n-3 FAs lower AA concentrations in plasma and RBC membrane phospholipids and, therefore, can inhibit the synthesis of PGE2 and modify its effects [537]. Several studies have reported decreased urinary calcium and/or oxalate excretion in stone patients with hypercalciuria after supplementation with these n-3 FAs [538 - 540]. EPA and DHA are obtained directly from the dietary intake of fish and seafood or as a product of the metabolism of alpha-linoleic acid (ALA, 18:3 n-3) [508 - 510].

Metabolic pathways of PUFAs

Both ALA and LA are essential fatty acids because they cannot be produced by the human body, and yet are required for proper physiological functioning [508, 509]. ALA is obtained directly from eating nuts and seed whereas plant oils are rich sources of LA [510]. Once ingested, they can undergo elongation and desaturation reactions to form long-chain polyunsaturated fatty acids.

LA is converted in a variety of tissues by Δ-6-desaturase to form γ-linolenic acid (GLA, 18:3 n-6). GLA in turn is rapidly elongated to dihomo-gamma-linolenic acid (DGLA, 20:3 n-6) which is desaturated to AA by a Δ-5-desaturase. In turn, ALA is converted to EPA and DHA by the same set of desaturase and elongation enzymes responsible for synthesizing AA [541 - 543].

Although the metabolic pathways of n-3 and n-6 fatty acids use the same enzymes, the major products of each pathway are structurally unique and have different physiological functions [544 - 547]. For example, AA is a precursor of eicosanoids which are involved in inflammatory responses. In contrast, EPA and DHA give rise to eicosanoids that have anti-inflammatory properties and which are inflammatory resolving [547].

Nonetheless, a competition exists between these two families for metabolism [541 - 543]. An abundance of one family of fatty acids overwhelms the enzymes, causing a significant decrease in the conversion of the other [541 - 543]. The metabolic pathway that converts LA to AA and ALA to DHA occurs very slowly in humans and so these FAs are mainly obtained directly from the diet [508 - 510].

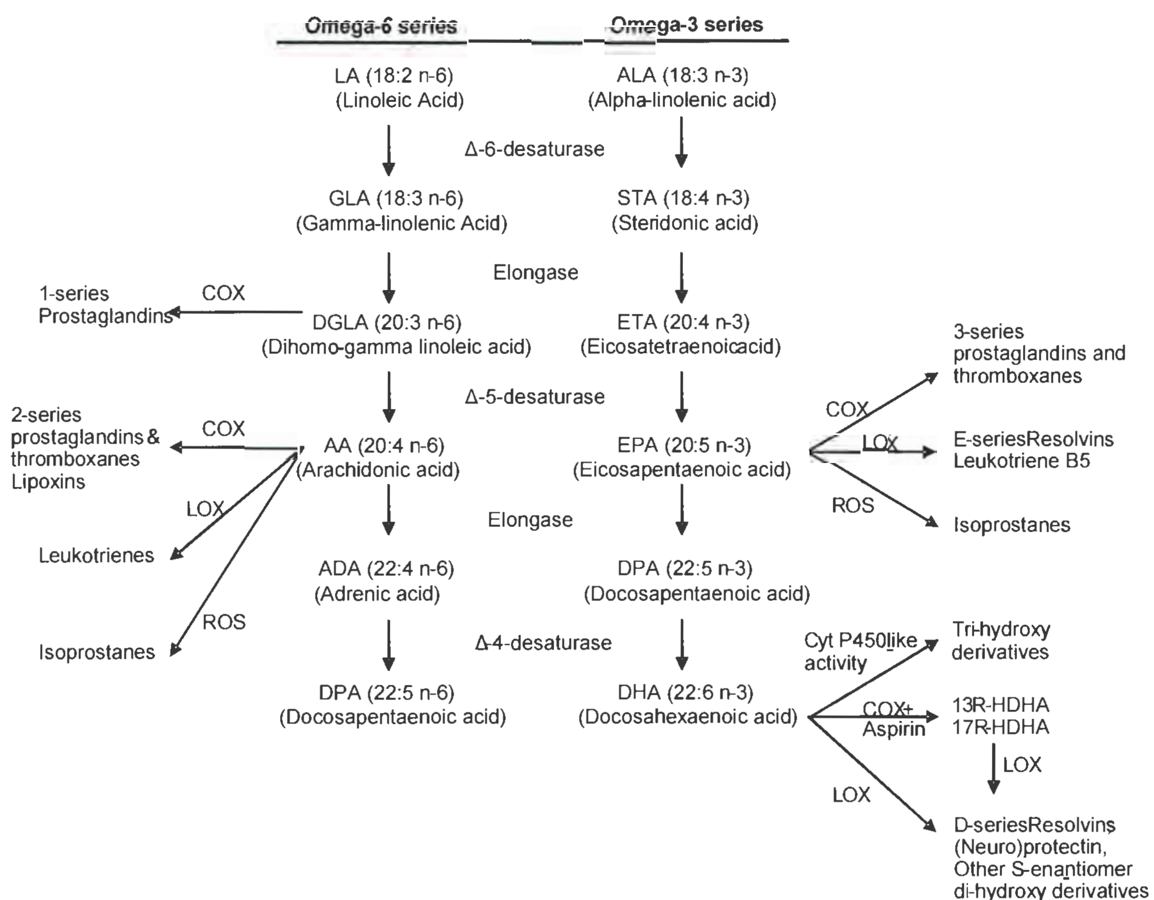

Figure 1.1: Metabolic pathway for polyunsaturated fatty acids

(Source: Poulsen RC et al 2007. Exp Biol Med 232(10): 1275-83 [513])

1.9 ROLE OF MACROMOLECULES

Glycosaminoglycans (GAGs) are large polysaccharides that are found in numerous cells in the human body, often as part of the epithelial cells in renal tubules. They are linear in structure and are built by repeating units of disaccharides containing one uronic acid (D-glucuronic acid or L-iduronic acid) and an amino sugar (N-acetylgalactosamine GalNAc) or N-acetylglucosamine (GlcNAc)). GAGs are negatively charged macromolecules as a result of the carboxylate content of the uronate monomer and the sulfate content of the galactosamine or glucosamine sugar. The number of the repeat units of the uronic acid and sugar monomers varies with each GAG but has been reported to reach an average of about 600 units, which correlates to molecular weights between 10 – 100 kDa. GAGs are not usually found as free-chain polymers. They have been reported to exist as proteoglycans, which are proteins attached to glycosaminoglycan chains by covalent bonds [548 - 553].

There are 7 types of GAGs, namely: heparin, heparan sulfate, chondroitin-4-sulfate (CSA), chondroitin-6-sulfate (CSC), dermatan sulfate, hyaluronic acid, and keratan sulfate [554, 555]. These GAGs differ from each other according to the structure of the disaccharide unit, molecular weight and distribution in the body. In urine, the most abundant GAG is chondroitin sulfate (A and C), 55 %, followed by heparin sulfate (20 %). Heparan sulfate has been reported to contribute about 11 % of all GAGs in urine while the percentage for hyaluronic acid is between 4 – 10 % [556, 557].

Several *in vitro* experiments have demonstrated that urinary GAGs protect individuals from stone formation by interfering with the crystallization processes of stone formation. The proposed mechanisms by which GAGs have been shown to inhibit stone formation include retarding crystallization, preventing stone retention inside the kidney, decreasing urinary oxalate excretion and reducing the availability of free calcium in urine [558 – 563]. The latter mechanism is of interest in the context of the present study.

It has been suggested that due to their overall negative charge, GAGs inhibit stone formation by blocking the growth sites of the crystals and thereby prevent or delay crystal development [564 - 566]. Several *in vitro* studies have shown that chondrotin sulfate, heparin sulfate and heparin inhibit nucleation, crystal growth and aggregation of CaOx crystals [567 - 572]. However, further studies in urine samples and rat models did not show that exogenous GAGs have an inhibitory effect on stone formation.

It has been shown that dosing urine samples of stone patients with chondroitin sulfate [565] and heparan sulfate [573] had no effect on the metastable limit and mass deposition. In another study, it was reported that chondroitin sulfate promoted nucleation in urines of stone formers whereas heparan sulfate enhanced nucleation [574]. In rat models, chondroitin sulfate had no effect on the CaOx deposition in kidneys [575] and instead promoted large particle formation [576].

In the renal tubules, GAGs have been suggested to lower the risk of stone formation by lining the surface of epithelial cells and preventing the confinement of crystals in the kidney [577 - 580]. Experiments conducted in primary cultures of renal epithelial cells demonstrated that the attachment of COM crystals to the surface of epithelial cells was significantly reduced in the presence of hyaluronic acid and other GAGs [581 - 583].

In idiopathic calcium stone patients, the GAG content in red blood cells has been reported to be decreased compared to normal controls [584]. Baggio et al suggested that the indirect relationship observed between the level of phosphorylation and the transmembrane oxalate flow may be due to a link between the erythrocyte deficiency of GAGs and the high concentrations of urinary oxalate associated with idiopathic calcium nephrolithiasis [585, 586]. However, supplementation with GAGs such as heparin, heparin sulfate and CSA was shown to normalize the oxalate self–exchange and the membrane protein phosphorylation mechanisms [586, 587]. In addition, GAG supplementation also significantly reduced urinary oxalate excretion [587].

Another mechanism by which GAGs reduce stone formation has been suggested to be due to the ability of GAGs to directly bind calcium in urine. Since GAGs have an anionic character due to the sulfated disaccharide units, they compete with oxalate ions and form a complex with calcium, thus reducing CaOx precipitation [578 - 590]. Techniques such as equilibrium dialysis [588], ion selective electrodes [589], micropotentiometric titration and ion exchange [590] have been applied to study the mechanisms of calcium-GAG complex formation. For example, the binding constants (K) for the calcium-GAG complex formation have been suggested to increase with the concentration of GAGs when calcium was assumed to bind to one site of the GAG chain [588 – 591]. In addition, the binding between calcium and GAGs has been found to be reduced in the presence of other ions such as sodium or urate, and that binding is also dependent on ionic strength [592, 593].

In principle, these effects can be theoretically investigated by speciation modeling. However, accurate thermodynamic constants for the equilibria between calcium and various GAGs, which are required for such calculations, are not readily available. The same lack of data exists for similar constants involving magnesium. This deficiency in kidney stone literature will be addressed in the present thesis.

With regards to urinary excretion of GAGs, several authors have reported that the urinary excretion of GAGs is significantly lower in stone patients compared to healthy subjects [594 - 598]. However, other investigators found no significant differences in the urinary excretion of GAGs between stone formers and healthy subjects [599 - 602].

The role of GAGs in stone formation therefore remains worthy of further exploration.

1.10 UROLITHIASIS IN SOUTH AFRICA

In South Africa, the incidence of stone formation has been reported to be lower (<1 %) in the black population compared to the white population (15 %) [603]. Over the years, this phenomenon has generated interests in several investigators to determine the cause of this abnormality between these two groups [604 – 606].

In the Kidney Stone Research Laboratory – University of Cape Town, South Africa, differences in CaOx risk factors such as diet [607, 608], genetics [609] and metabolic disorders [610, 611] between blacks and whites have been investigated to further gain insights as to the explanations for the racial differences in the incidence of kidney stone disease between these two groups.

Intervention studies have shown that these two groups have different renal handling mechanisms when it comes to dietary supplement challenges [607 – 612]. In a study conducted by Lewandowski et a [610], it was shown that supplementation with calcium, vitamin B6, L-glutamine and L-cystein had no effect on the urinary risk factors in the black group whereas in whites favourable results were observed. For example, calcium supplementation reduced the Tiselius risk index (TRI) while vitamin B6 significantly decreased the saturation of calcium oxalate and brushite salts. Meanwhile, L-glutamine lowered the relative saturation of calcium oxalate and L-cysteine decreased urinary calcium and the TRI.

In another study, supplementation with a high oxalate and low calcium diet had no effect on urinary risk factors in blacks whereas in whites urinary oxalate was increased [607]. Further studies showed that low calcium and lacto vegetarian diets and vitamin C supplementation were not associated with changes in urinary risk factors in the white population [608]. In the black group several responses were reported. In the low oxalate diet protocol, urinary oxalate and the relative supersaturation (RS) of brushite were increased while RS CaOx was decreased. However, an increase in urinary pH and TRI were observed in the lacto vegetarian and vitamin C studies, respectively. Ingestion of high oxalate diets, however, increased urinary citrate in blacks whereas urinary pH, potassium and RS brushite were increased in whites [608]. Lastly, glucose and xylitol supplementation in the black population decreased urinary phosphate and increased urinary oxalate, respectively [612]. In contrast, these supplements had no effect on the urinary risk factors in the white group.

The differences in responses to dietary interventions suggest that the renal handling mechanisms of supplement challenges may account for the black and white dissimilarities in the incidence of kidney stone disease. Thus, insights gained from studies examining the effects of supplement challenges in urinary risk factors of black and white subjects are of particular importance to research efforts aimed at reducing the prevalence of kidney stone disease.

1.11 MOTIVATION FOR THE PRESENT PROJECT

Despite much research, no single cause for kidney stone disease has been identified. As such, it is not surprising that effective conservative therapeutic protocols are still being explored. Among these are polyunsaturated fatty acids and GAGs. There is a need to further interrogate their potential roles in treating stone disease by investigating the basic science mechanisms by which they may exert their respective influences.

In addition, the anomaly of the different stone prevalence rates in South Africa's black and white population groups remains unresolved. It is well established that the two groups respond differently to different dietary and supplemental interventions. The possibility exists that n-6 and n-3 FAs and GAGs also may elicit different responses in the two groups. The present thesis will attempt to integrate these themes in the hope of casting light on stone pathogenesis on the one hand and stone formation on the other.

1.12 AIMS AND OBJECTIVES

There are two main aims of the studies undertaken for this thesis.

The primary aim of the project is to investigate the potential beneficial effects of supplemental polyunsaturated fatty acids (PUFAs) and supplemental chondroitin sulfate (CS) on the risk factors for calcium oxalate kidney stone formation using theoretical, experimental and human models wherever it is feasible and meaningful to do so.

The secondary aim of the project is to investigate whether black and white South Africans respond differently to these supplements and if so, whether such differences provide insights into understanding the anomaly of the different stone incidence rates in the two race groups, and whether these in turn, provide insights into understanding stone pathogenesis and management in general.

In order to achieve these aims, the following objectives were established:

- to administer and analyze 24h dietary recall questionnaires in healthy black and white subjects
- to administer n-6 FA (GLA) and n-3 FAs (EPA and DHA), and a combination thereof, in both groups of subjects
- to administer chondroitin sulfate in another cohort of healthy black and white South African subjects
- to determine urinary risk factors for CaOx stone formation in both cohorts, at baseline and at various times during the test period
- to determine blood risk factors at baseline and at various times in the PUFA cohort
- to determine the total concentration of urinary GAGs (and other urinary components) in both groups of subjects, at baseline and at various times during the test period in the CS cohort

- to test the effect of chondroitin sulfate on calcium oxalate crystallization processes in synthetic, 24h and pooled urine samples
- to experimentally determine the thermodynamic binding constants for the formation of calcium and magnesium complexes with the most commonly occurring urinary GAG, chondroitin sulfate
- to use these constants to calculate the chemical speciation and the urinary supersaturation of various calcium salts in 24h urines of healthy subjects, and to model the effect of varying the concentration of chondroitin sulfate
- to calculate and compare the chemical speciation and the relative supersaturation of various calcium salts in 24h urines obtained at baseline with those collected post supplementation using physiological concentrations of chondroitin sulfate

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Chapter 2

Investigation of the effects of n-6 fatty acids (GLA) on blood and urinary risk factors for CaOx urolithiasis

2.1 INTRODUCTION

As previously described in Chapter 1, the abnormal composition of FAs in plasma phospholipids of idiopathic calcium stone patients has been directly linked to CaOx stone formation. Compared to other n-6 FAs, AA has been reported to be the most abundant one in plasma phospholipids of such patients [1]. High AA concentrations have been shown to lead to increased production of PGE2, a major metabolite of AA which is associated with excess calcium and oxalate excretion [2]. The unbalanced FA content in phospholipids can be corrected by administering different essential fatty acids that are precursors to AA. For example, dietary intakes enriched with LA have the potential to normalize the concentrations of n-6 FAs [3]. This route is unfavorable because the conversion of LA to GLA and DGLA occurs very slowly in humans [4]. On the other hand, dietary supplementation with GLA has been reported to change the FA profile in various lipid fractions and the subsequent metabolite production in humans [5].

The present study was designed to examine the effects of GLA supplementation on the composition of FAs in plasma total phospholipids of healthy black and white South African male subjects. A second objective was to assess whether the risk factors for CaOx stone formation would be affected by GLA supplementation. This was achieved by measuring the changes in both serum biomarkers and urinary physicochemical parameters before and after supplementation. In view of the reported differences between black and white subjects in terms of their renal handling of different lithogenic agents [6], the study was also undertaken to compare the responses of the two groups to GLA supplementation. Finally, the present study aimed to investigate the effect of GLA supplementation on the crystallization kinetics of CaOx in urine.

2.2 STUDY DESIGN

Before the commencement of the study, approval (**HREC REF**: **366/2011**) was obtained from the Human Research Ethics Committee at the University of Cape Town, South Africa (Appendix 2.1). Volunteers were told about the research goals and all aspects of the study; if they were interested in participating, they signed informed consent forms (Appendix 2.2). Subjects provided information on their general background characteristics and health history by means of a questionnaire (Appendix 2.3).

Age, weight and height were obtained from the questionnaire and the body mass index (BMI) was calculated. Subjects also provided information on their use of medication; none were taking any form of medication or supplements during the course of the study.

A total of 20 healthy males (10 blacks (*B*) and 10 whites (*W*)), aged between 19 - 60 years old, participated in the study. This sample size was calculated using the statistical package *GraphPad InStat 2*. In order to compute the sample size required to achieve statistical power, the package requires that a key parameter be identified and that the expected standard deviation for this parameter be provided. In the present study, urinary oxalate and urinary calcium are both key parameters that are likely to be influenced by the ingestion of GLA supplements. Expected standard deviations for both calcium and oxalate were obtained from the Kidney Stone Research Laboratory's database (University of Cape Town – South Africa, where the present study was undertaken) of over 700 urines collected from healthy and stone-forming males. At the desired statistical power of 80 %, calcium and oxalate each yielded a required sample size of n = 10. The sample size was assessed at a significance level of p-value = 0.05, two-tailed.

At baseline, subjects collected blood and 24h urine samples that were used as control samples. Instructions on how to collect 24h urine samples were provided (Appendix 2.4). During the 24h urine collection period, they recorded in a food diary the types and amounts of food that they consumed (Appendix 2.5). Subjects consumed their normal diet but were instructed to avoid foods with high oxalate content (Appendix 2.6). GLA supplements were obtained from Solgar, UK (Solgar One-A-Day GLA 150 mg). Each softgel capsule contained 225 mg LA, 150 mg GLA and 250 mg of other fatty acids. Subjects took 2 capsules per day, one capsule with breakfast and one with lunch, for 30 days.

In a previous study conducted at the Kidney Stone Research Laboratory (University of Cape Town, South Africa) supplementation with 80 mg GLA (Natrodale, Kuils River, South Africa) in healthy black and white South African male subjects for 20 days changed some of the urinary risk factors associated with CaOx stone formation favourably. In both groups, it was shown that urinary calcium excretion was significantly reduced after supplementation. Although urinary oxalate also decreased, it did not reach statistical significance at day 20 [7]. The present study, which was conducted at the same laboratory, was undertaken to further elucidate the effects of GLA supplementation on these and other risk factors for CaOx stone formation when a higher dose of GLA was given to the same group of subjects for a longer period.

Several studies have demonstrated that there are no health risks associated with GLA supplementation when doses ranging from 30 to 640 mg are given to healthy subjects [8 – 10].

24h urine samples were collected during the supplementation period on day 15 and on the last day of supplementation at day 30. Blood samples were also obtained on day 30. A washout period of 5 days was observed, after which additional 24h urine samples were collected (day 35) to determine possible carry over effects after supplementation. Copies of the food diaries recorded at baseline were given to subjects prior to sample collection during the supplementation and washout periods so that they could follow the same dietary intake on those days when blood and urine samples were collected.

2.3 EXPERIMENTAL ANALYSIS

2.3.1 Nutrient intake assessment

The recorded food data were translated into nutrient intake values using the Foodfinder II program based on South African Medical Research Council (Cape Town, South Africa) Food Consumption Tables [11].

2.3.2 Blood analysis

Sample collection

Before donating blood samples, subjects observed a 12-hour fast the night before which consisted of not eating any kinds of foods or drinking any fluids after 8 pm. At 8 am the next morning, blood samples were collected into 10 mL evacuated tubes (Vacutainer, USA) by a trained nurse. Serum separating tubes (SST) were used for collecting blood samples that were going to be used for the determination of serum 25(OH)D and serum TAGs. For plasma and RBC membrane total phospholipid analysis, blood samples were collected into tubes containing ethylene diamine tetraacetic acid (EDTA) as an anticoagulant. Tubes were continually mixed by gentle inversion and were separated into different fractions within an hour of blood collection.

Analysis of serum samples

The SST tubes were centrifuged at 3000 rpm on a Heraeus Labofuge 200 centrifuge with Sepatech 3760 rotor (Heraeus, Germany) for 10 minutes at room temperature. The serum obtained was separated into 2 aliquots and immediately frozen at -20 °C for the analysis of 25(OH)D and TAGs.

Serum 25-hydroxyvitamin D3 analysis

Serum 25(OH)D samples were analyzed at Pathcare Laboratories (Cape Town, South Africa). The measurements were performed with a DiaSorin® chemiluminescent immunoassay kit (DiaSorin, USA) using an automated chemistry analyzer (Modular E170 - Roche Diagnostics®, Germany) [12]. The first step in the assay involved the incubation of samples for 10 minutes at 25 °C where 25(OH)D was dissociated from its binding protein and became bound to the specific antibody on the solid phase. The tracer, vitamin D linked to an isoluminol derivative, was added to the samples. This was followed by incubation of the samples at 25 °C for 10 minutes. The unbound material was removed with a wash cycle. The starter reagents were then added to initiate a flash chemiluminescent reaction. The light signal, which is inversely proportional to 25(OH)D concentrations in the serum samples, controls and calibrators, was measured by the automated analyzer.

Serum 25(OH)D concentrations above 30 ng/mL are usually regarded as normal levels in healthy populations [13]. In addition, when individuals have 25(OH)D concentrations between 21-30 ng/mL, they are regarded as vitamin D insufficient, whereas populations with concentrations below 20 ng/mL are considered to be vitamin D deficient.

Serum triglycerides

Fasting serum samples were taken to the South African Medical Research Council Laboratory (Tygerberg Hospital – Parow, Cape Town) for the analysis of TAGs. The concentration of TAGs in serum samples was determined using the Liquicolor triglyceride assay (Human, Germany) [14]. According to the principle of the assay, TAGs are first incubated with lipoprotein lipase to release glycerol and free fatty acids. This is followed by the conversion of glycerol by glycerol kinase (GK) and adenosine triphosphate (ATP) to form glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP).

G3P is then oxidized by glycerol phosphate dehydrogenase (GPO) to dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). The hydrogen peroxide further reacts with 4-aminoantipyrine in the presence of peroxidase (POD) to give a red colored dye. The intensity of the color formed is proportional to the triglyceride concentration in the sample.

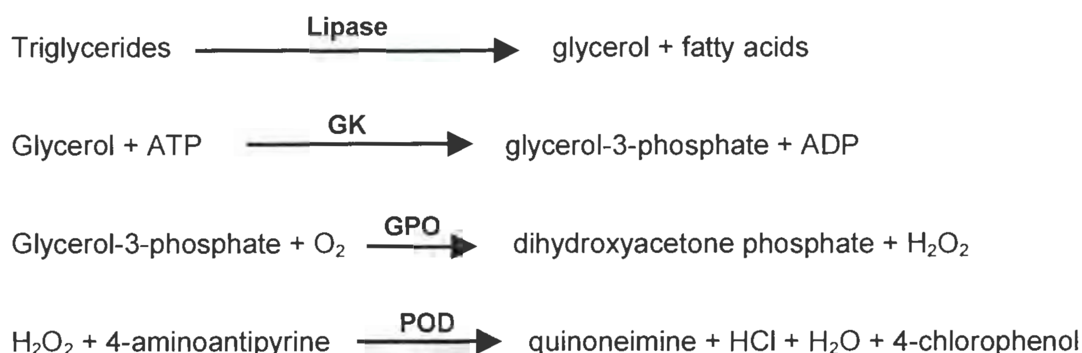

Figure 2.1: Schematic representation for serum TAG analysis

For the analysis of TAGs in serum, samples and blank were mixed with RGT (kit supplied reagent). The resultant mixture was left for 10 minutes at 20 °C. The absorbance of the red complex was read at 500 nm. The concentration of serum triglycerides was calculated as:

$$\boldsymbol{Concentration\ (mmol/L)} = \frac{sample\ absorbance}{standard\ absorbance}\ x\ standard\ concentration$$

Total phospholipid fatty acid analysis

Sample preparation

Red blood cells (RBCs) were separated from the plasma by centrifugation of the EDTA tubes at 2500 rpm for 10 minutes at room temperature. The plasma (upper phase) was removed and transferred into 1.5 mL safe-lock tubes (Eppendorf, Germany) and stored at -80 °C. The buffy coat (small white layer between the plasma and RBC) was aspirated and discarded. The RBCs were washed three times with saline solution (0.9 % NaCl in distilled water) by resuspension and centrifugation at 2500 rpm for 10 minutes; supernatant and remaining buffy coat were discarded after each centrifugation step. After the last wash, the packed RBCs were transferred into 1.5 mL safe-lock Eppendorf tubes and stored at -80 °C [15].

Extraction of lipids from plasma and RBC membranes

On the days of analysis, the stored plasma and RBC samples were thawed at room temperature. Total lipids were extracted from the plasma and RBC samples according to a modified method described by Folch et al [16].

Plasma (200 µL) and red blood cells (300 µL + 300 µL distilled water) were placed in 15 mL glass tubes with screw caps (KiMax, SA) and mixed with 3 mL of methanol by shaking with a vortex (BOECO Vortex Mixer V1 Plus - Optima Scientific, South Africa) for 30 s. Then 6 mL of chloroform was added and mixed for another 30 s on the vortex. Both methanol and chloroform solvents contained 0.01 % butylated hydroxytoluene as an antioxidant. The red blood cell lipid extract was filtered (glass funnel lined with 110 mm filter paper (Advantec, Japan)) and the clear filtrate collected into a clean glass tube. To the plasma and red blood cell lipid extracts, 1/5 volume (1.8 mL) of saline saturated with CMS (chloroform:methanol:saline, 86:14:1, v/v/v) was added and the contents mixed for 20 s using a vortex and then centrifuged (Beckman ® J-6B Centrifuge – Soma Technology Inc, USA) at 2500 rpm for 10 min at 4 °C. The top layer was aspirated and discarded and the bottom layer transferred into a clean glass tube and sealed under nitrogen gas for storage at 4 °C until the next day or to proceed the same day. The extracts were dried under nitrogen in a water bath (Ecobath 207 - Labotec, South Africa) at 37 °C until dry. The dried lipid sample was concentrated by rinsing the inside surface of the tubes with 1 mL CMS three times, evaporating the CMS under nitrogen in the water bath after each addition. The tubes containing the completely dried lipid samples were put onto ice before continuing with thin-layer chromatography.

Isolation of the total phospholipid fraction

The total phospholipid (TPL) fraction was separated from neutral lipids using the thin-layer chromatography (TLC) technique [15]. A solution containing petroleum ether (boiling point 40-60 °C), diethyl ether (peroxide free) and acetic acid (90:30:1 - by volume) was prepared as the developing solvent. TLC pre-coated silica gel 60 plates (10 x 10 cm) without a fluorescent indicator (No. 1.05721; Merck, SA) were activated by drying in an oven (Heraeus, Thermo Scientific, South Africa) at 100 °C for 30 min and then allowed to cool to room temperature. The dried lipid extracts were redissolved in 1 mL ice-cold CMS, an aliquot was applied to the TLC plate and developed in a chromatography tank containing 30 mL of the developing solvent.

The developed plates were dried in a closed tank under nitrogen gas and then sprayed with chloroform:methanol (1:1, by volume) containing 2,5-bis-(5'-tert.-butylbenzoxazolyl-[2'])thiophene (5 mg/100 mL; Sigma-Aldrich). The separated lipid bands (total phospholipid remaining at the origin of application) were visualized under long-wave ultraviolet light (Ultra-violet products Inc, UK). The total phospholipid bands (origin) were scraped off into a glass tube, mixed with internal standard (C17:0, 54 mg /50 mL; 20 µL for plasma and RBC samples) and 2 mL of transmethylating reagent (TMR, 5 % sulphuric acid-methanol). The tubes were heated at 70 °C for 2 hours to produce FA methyl esters (FAMEs), which were recovered for gas chromatography (GC) analysis after cooling.

The FAMEs were extracted by adding 4 mL *n*-hexane and 1 mL distilled water and shaking on a vortex-mixer for 30 s. The upper hexane phase was recovered and evaporated to dryness under a stream of nitrogen gas in a water bath at 37 °C. The FAMEs were redissolved in carbon disulfide (CS_2, Riedel-de Haen - BDH Chemicals, South Africa), 20 µL for the plasma and 15 µL for the RBC TPL-FAMEs; 1 µL was injected in the GC for separation of the individual FAMEs [15].

Analysis of fatty acid methyl esters

Gas chromatography was performed on a Finnigan Focus GC equipped with a flame ionization detector (Thermo Electron Corporation, TX, USA), using a 30 m x 0.32 mm internal diameter BPX 70 0.25 µm capillary column (SGE International Pty Ltd, Victoria, Australia). Gas flow rates were: N_2 (make up gas), 25 mL/min; air, 250 mL/min; H_2 (carrier gas), 25 mL/min, with a split ratio of 20:1. Temperature programming was linear at 5 °C/min, initial temperature 140 °C, final temperature 220 °C, injector temperature 220 °C, and detector temperature 250 °C. The separated FAMEs were identified by comparing the peak retention times with those of a known standard FAME mixture (Nu-Check Prep Inc. MN, USA).

For the relative total phospholipid fatty acid composition profiles the amount of each fatty acid was calculated as a percentage of the total sum of all identified fatty acids. Individual fatty acids were quantified against the internal standard peak area (C17:0) and expressed as µg/mL plasma and µg/mL packed RBC. When plasma or RBC TPL-FA were below the limits of detection (no peak integration), they were reported as "trace amount" and as nondetectable when no corresponding peak was observed [15].

2.3.3 Urine analyses

Urinary risk factors

24h urine samples were collected in 2.5 L polyethylene plastic bottles without any preservatives. The total volume of each collected urine sample was measured using a plastic measuring cylinder. After measuring the volume, the urine samples were tested for bacterial infection. This was done by placing approximately 50 mL of the urine sample in a 100 mL glass beaker (United Scientific, South Africa). Medi-test Combi 10 nitrite test strips (Macherey-Nagel, Germany) were dipped in the urine sample. The color changes of the test strips were compared to the color scale on the container of the test strips after dipping in the urine sample. A pink color was credited to the presence of increased bacterial concentration. None of the collected urine samples tested positive for bacterial infection.

The pH of each 24h urine sample was measured using a pH meter (model pH 221 - Hanna, UK) at room temperature. On each day of measurement, the pH meter was calibrated with pH 7.00 and 4.00 buffer solutions (Merck, SA). Calcium, magnesium, potassium and sodium concentrations in urine were determined using a flame atomic absorption spectrophotometer (Model 1275 - Varian Techtron AA-5, Australia) [17 - 19]. Chloride measurements were determined using an ion selective electrode (Metrohm, USA).

Creatinine concentrations are measured to assess the completeness of collection in 24h urine samples [20]. Creatinine was estimated by the Jaffe rate method using the Synchron LX assay kits (Beckman Coulter Inc., UK) [21]. The assay is based on the reaction where creatinine combines with picrate in an alkaline solution to form a red color creatinine-picrate complex. Absorbance readings were taken at 520 nm and were proportional to the concentration of creatinine in urine. In healthy individuals, urinary creatinine concentrations are normally between 9 - 19 mmol/24h [20].

Urinary oxalate was determined using a commercial oxalate kit (Sigma Diagnostics, USA). The principle of the assay is based on the enzymatic conversion of oxalate by oxalate oxidase to carbon dioxide and hydrogen peroxide. The hydrogen peroxide reacts with 3-methyl-2-benzothiazolinine acid and 3-(dimethylamino)benzoic acid in the presence of peroxidase to yield an indamine dye which has an absorbance at 590 nm. The intensity of the color produced is directly proportional to the concentration of oxalate in the sample.

Citrate was analyzed using assay kits from Boehringer Mannheim, Germany. During the first reaction, conversion of citric acid to oxaloacetate and acetate is catalyzed by the citrate lyase enzyme. In the presence of the enzymes L-malate dehydrogenase and L-lactate dehydrogenase, oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively, by nicotinamide-adenine dinucleotide (NADH). The amount of NADH oxidized in the above reactions is stoichiometric to the amounts of citrate. The optical density of NADH was determined spectrophotometrically at 340 nm.

The concentrations of urate and phosphorus in 24h urine samples were determined using commercially Synchron LX® System kits (Beckman Coulter Inc., UK). The uric acid reagent was used to measure the uric acid concentration by a timed-endpoint method. Uric acid was oxidized by uricase to produce allantoin and hydrogen peroxide. The hydrogen peroxide reacted with 4-aminoantipyrine and 3,5-dichloro-2-hydrobenzene sulfonate in a reaction catalyzed by peroxidase to produce a coloured product which was read at 520 nm. For the analysis of phosphate, the phosphorus reagent was used to measure phosphorus concentrations by a timed rate method. In the reaction, inorganic phosphorus reacted with ammonium molybdate in an acidic solution to form a coloured phosphomolybdate complex that had an optical density at 365 nm.

Ionized calcium measurements

The concentration of unbound calcium in unfiltered urine samples was measured using a calcium ion-selective electrode (Metrohm 794 Basic Titrino, Germany) with KCl as a reference electrode [22]. The electrode was calibrated by measuring the electrode potentials of calcium standard solutions; and these were used to plot a calibration curve against known standard concentrations. The concentration of ionized calcium in urine was measured in millivolts, and the true value extrapolated from the calibration curve.

Urinary PGE2 assay

The concentration of PGE2 was determined using an enzyme-linked immunoassay (ELISA) kit (R&D System, USA) [23]. The assay is based on the forward sequential competitive binding technique in which PGE2 present in a sample competes with horseradish peroxidise (HRP)-labeled PGE2 for a limited number of binding sites on a mouse monoclonal antibody. PGE2 in the sample is allowed to bind to the antibody in the first incubation.

During the second incubation, HRP-labeled PGE2 binds to the remaining antibody sites. Following a wash to remove unbound materials, a substrate solution is added to the wells to determine the bound enzyme activity. After color development is stopped, the absorbance is read at 450 nm. The intensity of the color produced is inversely proportional to the concentration of PGE2 in the sample.

All the standards and samples for the assay were prepared according to the kit manufactures' instructions. The urine samples were prepared with a 3-fold dilution (150 µL sample + 300 µL of calibrator diluent RD5-56). A series of standard solutions (2 500, 1 250, 625, 313, 156, 78 and 39 pg/mL) were prepared by diluting the PGE2 standard stock solution (25,000 pg/mL) with calibrator diluent RD5-56. These were used to generate a standard curve for the assay with GraphPad Prism 6 as shown in Figure 2.2. A 500 mL wash buffer solution was prepared by diluting 20 mL of wash buffer concentrate with distilled water. The substrate solution was prepared by mixing together the color reagents A and B in equal volumes 15 minutes before use.

A 96-well plate coated with a goat anti-mouse polyclonal antibody was included in the kit supplied. The diluted urine samples, blanks and standard solutions (150 µL) were first added to the microplate. For the assay, each plate contained 2 blanks, 2 non-specific binding wells (NSB), 2 maximum binding wells (Bo) and an 8 point standard curve run in duplicate. Each urine sample was run once.

The plate was incubated with 50 µL of the antibody for 1 hour at room temperature on a horizontal orbital shaker (Genie 2 vortex, Scientific Industries Inc., South Africa). 50 µL of the PGE2 conjugate was added to all the wells, and the plate was further incubated for 2 hours at room temperature on the shaker. All the unbound materials were removed by aspirating and washing the wells with the wash buffer, and this was done four times. After the last wash, the plate was dried by blotting with a clean paper towel. Color development was generated by adding 200 µL of the substrate solution to the wells, followed by 30 minute incubation at room temperature. The reaction was stopped by adding 100 µL of the stop solution to the wells. The intensity of the generated color was read on a microplate reader (SpectraMax 340 PC 384, USA) at 450 nm and 540nm. To correct for optical imperfections in the plate, absorbance readings at 540 nm were subtracted from the readings at 450 nm. After making corrections for the dilution of the samples (dividing by the dilution factor), the concentrations of the PGE2 in the samples were extrapolated from the prepared calibration curve.

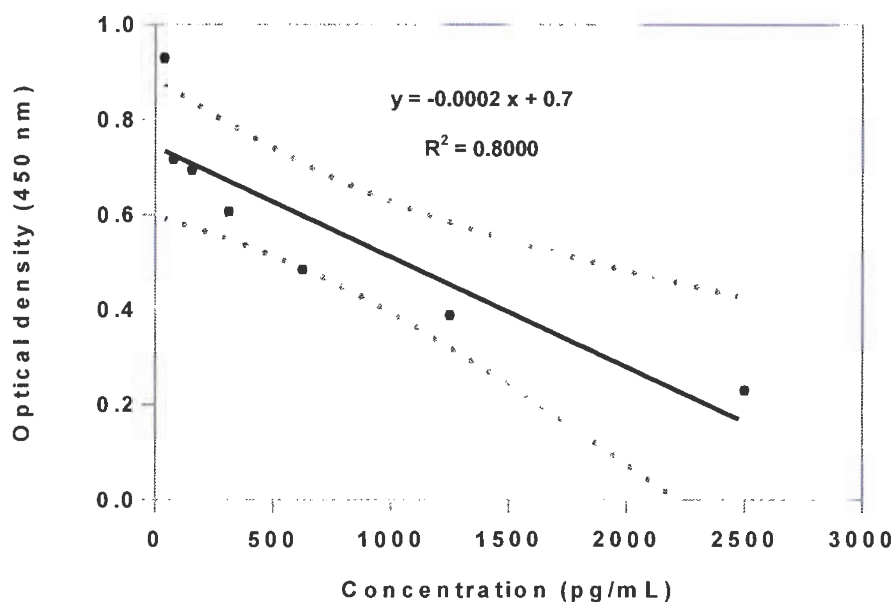

Figure 2.2: PGE2 calibration curve prepared using standard solutions

Urinary hydroxyproline assay

Aliquots of urine samples from randomly selected subjects (5 blacks and 5 whites) that were collected at baseline and day 30 were sent to National Health Laboratories (Red Cross Hospital, Cape Town) for the analysis of hydroxyproline (HYP). HYP was quantified in urine samples using the gas chromatography mass spectrometry (GCMS) technique. Amino acids were extracted from 100 µL of acidified urine samples by solid phase extraction onto a cation exchange column before being washed, eluted and derivatised to their corresponding alkyl chloroformates using a commercial kit (EZ:faast, Phenomenex, Torrance CA, USA) [24]. After derivatisation, the amino acids were extracted into solvent and 2 µL injected into an Agilent 7890A/5975C GCMS system fitted with a Zebron ZB AAA 10 m x 0.25 mm capillary GC column. The samples were injected at 1:10 split and 250 °C. Helium was used as the carrier gas at 1.65 mL/min, and the column heated from 110 °C to 320 °C at 30 °C per minute.

The amino acids were quantified using a norvaline internal standard against a standard curve generated from a traceable calibrator using the following ions and (qualifier ions): hydroxyproline – mz 172 (86.1), and norvaline – mz 158 (72, 116). Urine creatinine concentrations were used to adjust for variation in bone mass within groups and urinary HYP concentrations are reported as a ratio with creatinine (HYP:Creatinine – nmol/mg creatinine) [25].

2.3.4 Crystallization experiments

CaOx metastable limit determination

The CaOx metastable limit (MSL) of each urine sample was determined according to the method described by Ryall et al [26]. MSL is defined as the lowest oxalate concentration required to produce microscopically detectable crystals in urine. Na_2Ox standard solutions needed for the experiment were prepared according to the procedure described in the paper [26]. For each collected 24h urine sample, a 200 mL aliquot was filtered through 0.75 mm filter paper (Macherey-Nigel, Germany) followed by 0.45 µm cellulose nitrate filters (Sartorius Stedim Biotech, Germany). 10 mLs of the filtered urine were transferred into 13 coulter cups and warmed at 37 °C in an oven (Memmert, Germany) for 10 minutes.

To initiate CaOx precipitation, 100 µL of the Na_2Ox standard solutions were added to each cup 1 minute apart, at increasing concentrations from 15 to 195 mM (in increments of 15 mM) to give final concentrations of 0.15 to 1.95 mM. The samples were further warmed at 37 °C. After 30 minutes, absorbance was measured with an ultraviolet/visible (UV) spectrophotometer (Specord 40 Analytik Jena, UK) at a wavelength of 620 nm. The measured absorbance was plotted against the final concentration of the dosed urine samples, and the metastable limit was defined as lowest Na_2Ox concentration that gave a sharp increase in optical density [Figure 2.3].

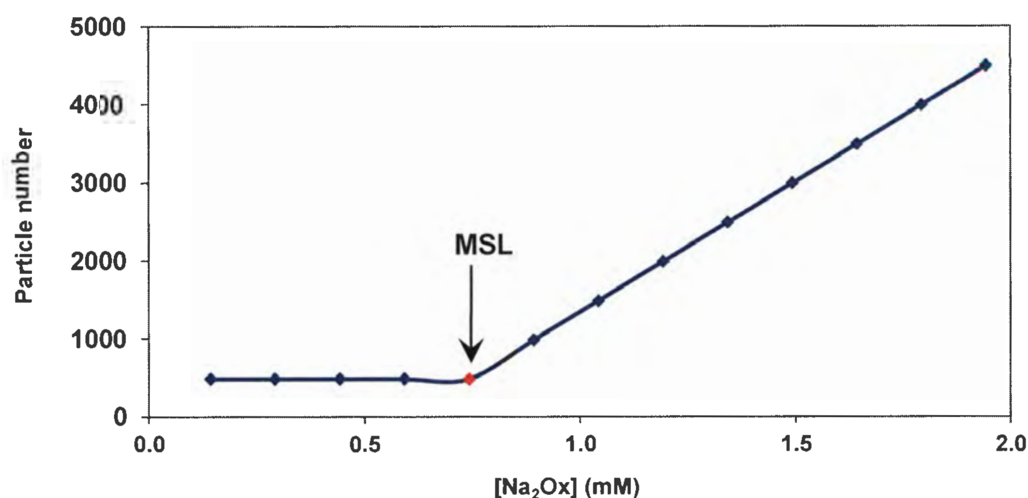

Figure 2.3: A typical CaOx metastable limit plot

CaOx crystallization kinetics

Once the metastable limit was determined, the rate of CaOx crystallization in the urine samples was studied. Approximately 50 mL of the filtered urines were transferred into a 150 mL soda lime bottle (United Scientific, South Africa). The urine samples were then dosed with 0.50 mL Na_2Ox using a concentration of 15 mM above the previously determined MSL, and incubated at 37 °C in a shaking water bath (Labex, South Africa) at 100 rpm for 2 hours. In the black subjects, particle number was measured by a Coulter Multisizer (Coulter Electronic Ltd – serial no 030288, UK) using a 100 µM orifice aperture every 30 minutes. However, this instrument broke down irreparably after measurements in the black group were completed. As such, it was not possible to use it to measure the rate of CaOx crystallization in the white group. Thus, the formation rate of particles in the white group was determined by measuring absorbance at 620 nm every 30 minutes using a spectrophotometer (Specord 40 Analytik Jena, UK). Plots of absorbance, and where appropriate particle number, versus time were constructed; and the slope of the linear portion in these plots was taken as the measure of the rate of crystallization. Thus, although different instruments were used to measure crystallization kinetics in the two groups, the effects (if any) of GLA ingestion, within each group, could be routinely determined.

2.3.5 Risk indices

To evaluate the possible effect of GLA supplementation on urinary supersaturation of stone salts, risk indices, based on lithogenic components, were calculated.

The Tiselius risk index (TRI) predicts the risk of CaOx stone formation [27] and was determined by the mathematical formula:

$$TRI = \frac{(Ca/Cr)^{0.71} \, x \, (Ox/Cr)}{(Mg/Cr)^{0.14} \, x \, (Cit/Cr)^{0.10}}$$

where Ca (calcium), Ox (oxalate), Mg (magnesium) and Cit (citrate) were expressed in mmol and Cr (creatinine) in mo/L.

Urinary concentrations of calcium, oxalate, phosphate, citrate, urate, magnesium, sodium and potassium were used as input for the speciation program EQUIL 2 [28] to calculate the supersaturation (SS) of various salts including calcium oxalate monohydrate (COM), calcium hydrogen phosphate dihydrate (brushite) and uric acid (UA).

2.3.6 Statistical analysis

All data are presented as mean ± SE (standard error). The Instat GraphPad3 software program was used to test for statistical significance. For both study groups, student's paired t-test was applied to assess the outcomes of measurements during the supplementation period (days 15 & 30) and at day 35 compared to baseline values. The differences between groups at each sample collection period were determined using the unpaired t-test. Two sets of data were considered significantly different from each other when the $p\text{-value} \le 0.05$. Microsoft Excel 2010 was used for the preparation of graphs with standard error bars. Urine collections were excluded from statistical analyses if the creatinine concentrations were < 9 mmol/24h [20].

2.4 RESULTS

2.4.1 Subject characteristics

The mean age of black and white subjects at baseline are shown in Table 2.1. Raw data showing the age, height, weight and BMI for each individual are presented in Appendix 2.7. There were no significant differences observed with regards to age and BMI between the groups.

Table 2.1
Subject's characteristics at baseline – n-6 FA study

Parameter	*Blacks*	*Whites*	*p-value*
Age	21.8 ± 1.47	23.6 ± 3.32	0.6257
BMI	23.5 ± 1.15	24.8 ± 0.82	0.3795

2.4.2 Nutrient intakes

Raw data showing the nutrient intakes for each individual are shown in Appendix 2.8. The results for the calculated nutrient intakes of black and white subjects recorded at baseline will be discussed in Chapter 8 because normal dietary intake of the two groups is not the main focus of the present study.

2.4.3 Supplement compliance test

The fatty acid composition of cell membranes has been shown to be dependent on both dietary fatty acid intakes and metabolic pathways [29 - 31]. Since subjects were given capsules that contained GLA, changes in the concentration of GLA in RBCs total phospholipids were used to test for compliance after supplementation. RBCs were chosen because they have a lifespan of 120 days and the FA profile in these cells has been shown to reflect the amounts of dietary fatty acid intakes [32, 33]. Five subjects from each study group were randomly selected for the test. Changes in the concentrations of fatty acids in RBC membranes were calculated as follows [15]:

$$\% \, Change = \frac{final\ concentration - baseline\ concentration}{baseline\ concentration} \, x \, 100$$

Table 2.2 shows the percentage changes in the concentrations of GLA and DGLA after supplementation for the subjects that were selected for the compliance test. Raw data showing the FA composition (as concentration (µg/mL)) in RBC total phospholipids for each individual are presented in Appendix 2.9.

In both study groups, the concentrations of GLA were extremely low for most subjects that were selected and could not be quantified as shown in Table 2.2. In the present study, the concentrations of GLA in RBCs total phospholipids were expected to increase in response to dietary supplementation [32, 33]. These results imply that the observed FA composition in RBC total phospholipids is not due to GLA supplementation. The absence of change in GLA concentrations in plasma total phospholipids after supplementation may be explained by two hypotheses.

Table 2.2
Concentrations (µg/mL) of GLA and DGLA in RBC total phospholipids after n-6 FA supplementation

Subject	*GLA*			*DGLA*		
	Day 0	Day 30	% Change	Day 0	Day 30	% Change
B-1	trace	1.35	ND	14.0	18.3	30.9
B-2	trace	trace	ND	13.1	13.4	1.64
B-3	1.30	1.34	3.65	22.9	19.5	-15.0
B-4	0.71	trace	ND	29.5	31.7	7.51
B-5	trace	trace	ND	20.5	16.3	-20.8
W-1	trace	trace	ND	17.4	15.8	-9.28
W-2	trace	trace	ND	12.4	10.0	-19.4
W-3	trace	trace	ND	14.5	7.52	-48.2
W-4	trace	trace	ND	11.9	15.4	28.9
W-5	trace	trace	ND	11.6	6.10	-47.6

ND = not determined

B = blacks and W = whites

Firstly, it was assumed that the steps involved in the pathways of fatty acid synthesis and metabolism occurred very quickly, and that GLA was rapidly converted into DGLA. Fan et al reported that the step for the conversion of GLA into DGLA occurs very quickly in humans [34].

Therefore, the percentage changes in the concentration of DGLA in RBC total phospholipid were calculated to determine supplement compliance because it was assumed that the DGLA concentrations after supplementation were partly due to the metabolism of GLA to DGLA. The concentrations of DGLA in RBC total phospholipids for each subject that was selected for the compliance test are shown in Table 2.2.

The mean percentage change for DGLA was 0.86 (p-value = 0.9716) in blacks and -19.1 (p-value = 0.2855) in whites. The negative value for the mean percentage change of DGLA in the white group indicates that mean concentrations of DGLA at day 30 were lower than at day 0, hence the non-significant increase in the percentage change that was observed. These results suggest that there was no significant increase in DGLA concentrations after supplementation and that the fatty acid composition of DGLA in RBCs does not reflect the metabolism of GLA. It has been reported that RBCs are not efficient in synthesizing and metabolizing fatty acids [31]; which might explain why there is no significant change in the concentrations of DGLA in the present study. The non-significant changes in both GLA and DGLA concentrations have been observed in RBC phospholipids of older subjects after GLA supplementation for 3 months with a dose similar to the one used in the present study [35].

Therefore, it is suggested that the FA composition of RBC total phospholipids after supplementation in the present study does not reflect the amounts of GLA given during the supplementation period and that GLA was not metabolized to form DGLA. Therefore, supplement compliance could not be determined by measuring the changes in GLA or DGLA concentrations.

The second hypothesis is that instead of being incorporated into phospholipids, GLA becomes incorporated into other lipid fractions. It has been shown that the FA composition of cholesterol ester is closely associated with the amounts of dietary fatty acid intakes [36]. In other studies, van Rooyen et al [37] and Brouwer et al [38] reported a significant increase in GLA concentrations in the FA profile of cholesterol ester and triglycerides after GLA supplements were given to animals and healthy subjects. Unfortunately, due to financial constraints, the changes in GLA concentrations in cholesterol ester and triglycerides could not be determined as a measure of supplement compliance in the present study. Overall, supplement compliance was not confirmed by calculating the changes in the fatty acid composition of GLA in RBCs after supplementation. As a result, supplement compliance was determined using another technique, as described in the paragraph below.

In addition to measuring the changes in FA composition after supplementation, supplement compliance can also be determined by counting the number of capsules returned after the end of a study [39, 40]. Compliance is considered to be complete if more than 85 % of the capsules given to subjects are consumed during the entire supplementation period [41, 42]. In the present study, compliance was monitored by instructing the subjects to come to the laboratory where the study was undertaken every 10 days to collect the next supply of supplements and also to keep all the supplements that they missed taking. At each laboratory visit, they reported on side effects that they experienced while taking the supplements and also answered questions about supplement compliance. None of the participants returned GLA capsules on completing the study and also there were no side effects observed. Since > 85 % of the capsules given to subjects were consumed, it was concluded that the subjects had complied with the supplement protocol.

2.4.4 Serum biomarkers and FA profiles in plasma total phospholipids

It should be noted that in the following discussion, the results for the measurement of fatty acids are presented as both absolute amounts (where concentration is expressed as µg/mL plasma) and as relative percentages (where the amount of each fatty acid was calculated as a percentage of the total sum of all identified fatty acids). In certain diseases, the fatty acid compositions in various lipid fractions of subjects are usually reported as the percentage composition of all identified FAs [29 - 33; 35 - 38]. Hence, when the results of the present study are compared to literature data, only the percentage composition of fatty acids in plasma will be compared. However, absolute amounts are also important because they have been shown to reflect their possible availability for metabolite synthesis [43]. In addition, the fatty acids that are of particular interest to the present study are n-6 FAs such as GLA, DGLA, AA and n-3 FAs such as EPA and DHA. These fatty acids have been directly linked to stone formation [1- 2, 4], and they will be the main focus for this study.

Baseline

Concentrations of serum biomarkers and plasma FA profile in black and white subjects are shown in Table 2.3. Raw data showing the concentrations of serum 25(OH)D and TAGs for each subject are presented in Appendices 2.10 and 2.11, respectively. Raw data showing the concentrations of FAs in plasma phospholipids for each individual are presented in Appendix 2.12.

Table 2.3
Concentrations of serum biomarkers and FAs in plasma total phospholipids after n-6 FA supplementation

Parameter	*Blacks*			*Whites*			*B vs W (p-values)*	
	Day 0	**Day 30**	**p-value**	**Day 0**	**Day 30**	**p-value**	**Day 0**	**Day 30**
Serum biomarkers								
25(OH)D (ng/mL)	13.6 ± 1.45	13.5 ± 1.26	0.9587	33.7 ± 3.71	28.8 ± 2.67	0.2975	<0.0001‡	<0.0001‡
TAGs (mmol/L)	0.71 ± 0.10	0.84 ± 0.09	0.3348	1.14 ± 0.14	1.02 ± 0.13	0.5293	0.0250‡	0.2869
SFAs (µg/mL)								
C14:0 MA	3.36 ± 0.89	2.79 ± 0.59	0.6001	4.23 ± 0.64	4.59 ± 0.87	0.7422	0.4380	0.1032
C16:0 PA	246 ± 13.2	248 ± 10.5	0.9152	262 ± 21.0	282 ± 29.7	0.5947	0.5317	0.2994
C18:0 SA	134 ± 6.56	132 ± 7.80	0.8405	113 ± 9.50	131 ± 11.4	0.2481	0.0881	0.9401
20:0 ARA	5.18 ± 1.07	4.60 ± 0.36	0.6121	3.57 ± 0.33	4.68 ± 0.38	0.0435*	0.1859	0.8832
22:0 BA	13.7 ± 1.16	14.1 ± 0.90	0.7993	7.73 ± 0.88	10.9 ± 1.12	0.0375*	0.0007‡	0.0404‡
24:0 LGA	12.7 ± 1.21	13.3 ± 0.96	0.7110	7.35 ± 1.16	9.76 ± 1.21	0.1659	0.0048‡	0.0335‡
MUFAs (µg/mL)								
C16:1 n-7 PTA	2.64 ± 0.28	3.21 ± 0.71	0.4658	5.15 ± 1.09	4.15 ± 0.74	0.4434	0.0250‡	0.3712
C18:1 n-9 OA	62.8 ± 4.51	73.8 ± 8.90	0.2852	79.3 ± 8.50	93.6 ± 8.30	0.2458	0.1037	0.1220
C18:1 n-7 VA	10.3 ± 0.41	8.86 ± 0.50	0.0426*	8.32 ± 1.03	10.1 ± 1.26	0.2901	0.0962	0.3748
20:1 n-9 GA	1.78 ± 0.38	1.17 ± 0.09	0.0962	ND	ND	ND	ND	0.1908
24:1 n-9	14.9 ± 0.97	16.5 ± 1.17	0.2945	8.07 ± 1.15	12.2 ± 1.56	0.0461*	0.0003‡	0.0404‡
n-6 FAs (µg/mL)								
C18:2 n-6 LA	203 ± 10.6	202 ± 10.7	0.9431	187 ± 12.7	212 ± 12.8	0.1777	0.3342	0.5580
C18:3 n-6 GLA	ND	ND	ND	ND	ND	ND	ND	ND
C20:2 n-6 EDA	3.59 ± 0.18	3.44 ± 0.21	0.5971	2.78 ± 0.42	3.18 ± 0.33	0.4656	0.0605	0.5004
C20:3 n-6 DGLA	29.7 ± 4.50	34.9 ± 3.64	0.3805	22.2 ± 2.36	30.7 ± 2.21	0.0173*	0.1612	0.3410
C20:4 n-6 AA	109 ± 5.62	104 ± 4.71	0.4880	85.1 ± 10.4	105 ± 9.93	0.1908	0.0583	0.9413
C22:4 n-6 ADRA	4.60 ± 0.17	5.23 ± 0.60	0.3210	3.46 ± 0.34	4.37 ± 0.48	0.1649	0.0049‡	0.2795
C22:5 n-6 DPA	3.55 ± 0.30	3.33 ± 0.32	0.6228	2.38 ± 0.18	3.10 ± 0.56	0.3720	0.0316‡	0.7196
n-3 FAs (µg/mL)								
C18:3 n-3 ALA	ND	ND	ND	3.48 ± 1.31	3.80 ± 1.93	0.9046	ND	ND
C20:5 n-3 EPA	3.13 ± 0.42	3.20 ± 0.69	0.9370	5.13 ± 0.86	5.58 ± 1.23	0.7723	0.0479‡	0.1009
22:5 n-3 DPA	6.74 ± 0.56	6.44 ± 0.62	0.7246	5.83 ± 0.73	7.18 ± 0.87	0.2560	0.3331	0.4963
C22:6 n-3 DHA	37.6 ± 3.84	31.0 ± 1.81	0.1353	28.2 ± 3.69	32.5 ± 3.95	0.4355	0.0931	0.7337

** : p-value ≤ 0.05 compared to baseline (intragroup comparison)*

‡ : p-value ≤ 0.05 (intergroup comparison)

Serum concentrations of 25(OH)D and TAGs were found to be significantly lower in the black subjects compared to the white subjects. These results are in agreement with previous studies where lower 25(OH)D concentrations (range 6.00 ± 1.00 to 19.6 ± 10.0, ng/mL) were observed in black subjects compared to white subjects (range 20.0 ± 2.00 to 34.8 ± 13.2, ng/mL) [44 – 49]. Several studies have also reported on the significantly lower serum triglyceride concentrations in the black population compared to the white population [50 – 54]. In these studies, the TAG ranges in the black group were reported as 0.43 ± 0.10 to 1.12 ± 0.64 mmol/L whereas in whites, the TAG concentrations were in the range of 0.79 ± 0.37 to 1.46 ± 1.03 mmol/L.

The differences in the concentrations of FAs in plasma total phospholipids between the groups at baseline are shown in Table 2.3 and are summarized in Table 2.4.

Table 2.4
Summary of significant differences in concentrations of FAs (µg/mL) between groups at day 0 – n-6 FA study

FA class	Type of FA	B vs W
SFA	BA, LGA	B > W‡
MUFA	PTA	B < W‡
	24:1 n-9	B > W‡
PUFA	ADRA, DPA (n-6 FA)	B > W‡
	EPA	B < W‡

‡: *p-value ≤ 0.05 (intergroup comparison)*

The percentage compositions of FAs in plasma total phospholipids between the two groups were compared and are shown in Table 2.5. Raw data showing the percentage composition of FAs in plasma phospholipids for each individual are presented in Appendix 2.13.

Table 2.5
Percentage composition of FAs in plasma total phospholipids after n-6 FA supplementation

Parameter	*Blacks*			*Whites*			*B vs W (p-values)*	
	Day 0	Day 30	p-value	Day 0	Day 30	p-value	Day 0	Day 30
SFAs								
C14:0 MA	0.36 ± 0.09	0.30 ± 0.05	0.5485	0.51 ± 0.07	0.45 ± 0.05	0.4760	0.1976	0.0441‡
C16:0 PA	27.0 ± 0.55	27.2 ± 0.44	0.7118	31.4 ± 0.85	28.8 ± 0.96	0.0602	0.0004‡	0.1511
C18:0 SA	14.7 ± 0.32	14.4 ± 0.29	0.5239	13.5 ± 0.44	13.5 ± 0.40	0.9604	0.0459‡	0.0950
20:0 ARA	0.55 ± 0.08	0.50 ± 0.03	0.6252	0.41 ± 0.03	0.48 ± 0.03	0.1037	0.1538	0.5688
22:0 BA	1.50 ± 0.10	1.56 ± 0.10	0.6509	0.92 ± 0.07	1.12 ± 0.09	0.0895	0.0001‡	0.0057‡
24:0 LGA	1.39 ± 0.10	1.47 ± 0.10	0.5893	0.86 ± 0.09	1.01 ± 0.10	0.2451	0.0009‡	0.0042‡
MUFAs								
C16:1 n-7 PTA	0.29 ± 0.03	0.34 ± 0.06	0.5004	0.56 ± 0.11	0.41 ± 0.05	0.1781	0.0169‡	0.3711
C18:1 n-9 OA	6.88 ± 0.37	7.88 ± 0.61	0.1781	9.28 ± 0.35	9.71 ± 0.35	0.4006	0.0002‡	0.0171‡
C18:1 n-7 VA	1.14 ± 0.06	0.99 ± 0.07	0.1197	0.99 ± 0.09	1.04 ± 0.11	0.7278	0.1980	0.6838
20:1 n-9 GA	0.19 ± 0.04	0.12 ± 0.01	0.1172	0.29 ± 0.02	0.33 ± 0.03	0.3751	ND	0.1286
24:1 n-9	1.64 ± 0.08	1.84 ± 0.15	0.2481	0.95 ± 0.11	1.24 ± 0.10	0.0697	0.0001‡	0.0033‡
n-6 FAs								
C18:2 n-6 LA	22.3 ± 0.84	22.2 ± 0.71	0.9043	22.6 ± 0.79	22.4 ± 1.10	0.9188	0.8163	0.8392
C18:3 n-6 GLA	ND	ND	ND	ND	ND	ND	ND	0.6274
C20:2 n-6 EDA	0.40 ± 0.02	0.38 ± 0.02	0.5222	ND	ND	ND	0.0054‡	0.2401
C20:3 n-6 DGLA	3.26 ± 0.46	3.81 ± 0.37	0.3663	2.63 ± 0.14	3.22 ± 0.16	0.0123*	0.2032	0.1599
C20:4 n-6 AA	12.1 ± 0.74	11.5 ± 0.52	0.4814	9.91 ± 0.60	10.8 ± 0.46	0.2682	0.0310‡	0.3154
C22:4 n-6 ADRA	0.51 ± 0.03	0.57 ± 0.05	0.3945	0.37 ± 0.02	0.45 ± 0.03	0.0724	0.0056‡	0.0667
C22:5 n-6 DPA	0.39 ± 0.03	0.36 ± 0.03	0.4889	0.26 ± 0.03	0.31 ± 0.04	0.4526	0.0146‡	0.2465
n-3 FAs								
C18:3 n-3 ALA	ND	ND	ND	0.53 ± 0.21	0.35 ± 0.14	0.4743	0.8109	ND
C20:5 n-3 EPA	0.35 ± 0.04	0.33 ± 0.05	0.7620	0.56 ± 0.07	0.53 ± 0.07	0.7948	0.0234‡	0.0378‡
22:5 n-3 DPA	0.75 ± 0.06	0.70 ± 0.04	0.5015	0.65 ± 0.04	0.73 ± 0.06	0.2984	0.2109	0.6499
C22:6 n-3 DHA	4.10 ± 0.32	3.49 ± 0.29	0.1780	3.45 ± 0.59	3.33 ± 0.29	0.8575	0.3485	0.7113

* : *p-value ≤ 0.05 compared to baseline (intragroup comparison)*

‡: *p-value ≤ 0.05 (intergroup comparison)*

The differences between groups that were observed at baseline are summarized in Table 2.6.

Table 2.6
Summary of significant differences in the % compositions of FAs between groups at baseline – n-6 FA study

FA class	Type of FA	B vs W
SFA	PA	B < W‡
	SA, BA, LGA	B > W‡
MUFA	PTA, OA, 24:1 n-9	B < W‡
PUFA	EDA, AA, ADRA, DPA (n-6 FA)	B > W‡
	EPA (n-3 FA)	B < W‡

‡: p-value ≤ 0.05 (intergroup comparison)

Post supplementation

The effects of supplementation on the concentrations of serum biomarkers and FAs in plasma phospholipids within groups are shown in Table 2.3. In both study groups, supplementation with GLA did not result in significant changes in the concentrations of serum 25(OH)D and TAGs. Within the black group, VA concentrations decreased significantly after supplementation at day 30. No significant changes in the concentrations of SFAs and FAs were observed. In the white group, GLA supplementation resulted in a significant increase in ARA, BA and 24:1 n-9 concentrations. More importantly, the concentration of DGLA (n-6 FA) was increased significantly compared to baseline concentrations.

The effects of supplementation on the percentage compositions of FAs in plasma phospholipids within groups are shown in Table 2.5. The changes in the percentage composition of FAs relevant to the present study are illustrated in Figures 2.4 and 2.5. The percentage composition of n-3 and n-6 FAs in plasma total phospholipids did not show any significant change in the black group (Figure 2.4) whereas in whites, the percentage composition of DGLA was significantly increased after supplementation as shown in Figure 2.5. There were no significant changes in the percentage composition of the other FAs (Table 2.5).

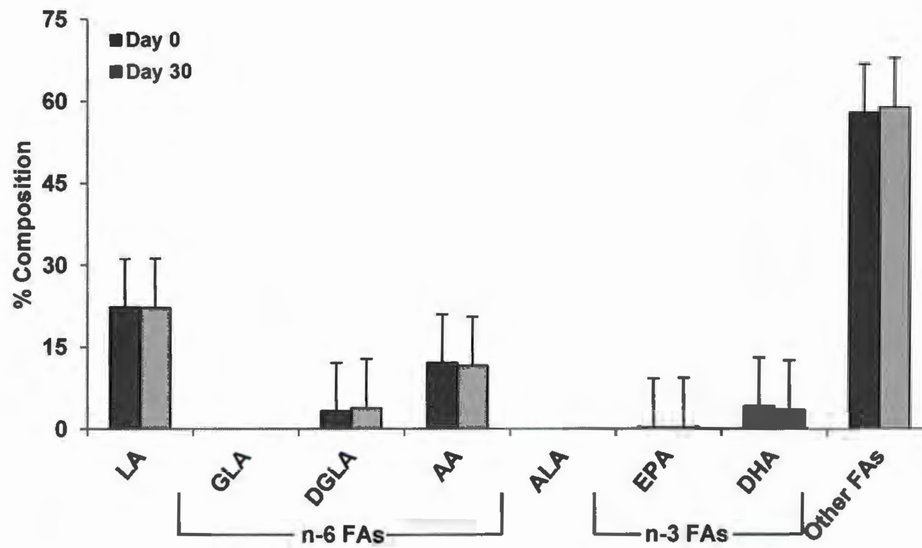

Figure 2.4: Percentage composition of PUFAs in blacks after n-6 FA supplementation

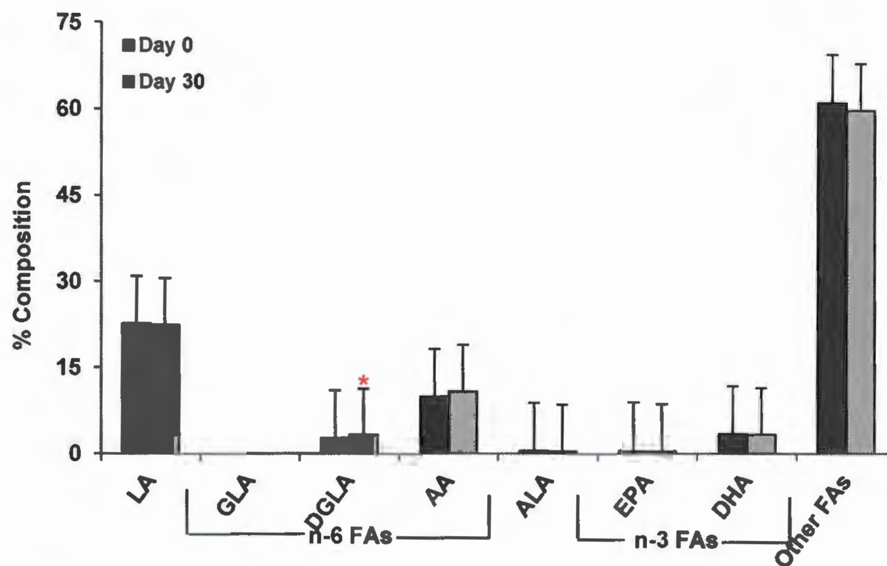

Figure 2.5: Percentage composition of PUFAs in whites after n-6 FA supplementation

After supplementation, there were no significant differences in the concentrations and percentage compositions of FAs in plasma phospholipids between the two groups (Table 2.3 and 2.5). The only major difference was a lower percent composition of MA in blacks compared to whites (Table 2.5). It was noted that although the concentrations of serum 25(OH)D, SFAs (BA and LGA) and MUFAs (24:1 n-9) were significantly different between groups at day 30, these differences were not attributed to the effect of supplementation since they were also present at baseline.

Similarly, the percentage composition of SFAs (BA and LGA), MUFAs (OA and 24:1 n-9) and n-3 FA in the form of EPA remained significantly different between groups at day 30.

2.4.5 Urinary risk factors

Baseline

Baseline urine parameters in blacks and whites are shown in Table 2.7. Raw data showing the urinary composition of each subject are presented in Appendix 2.14. At baseline, oxalate was significantly higher in blacks, whereas phosphate and the SS brushite were lower. The concentrations of phosphate remained significantly lower in blacks throughout the study on days 15, 30 and 35.

Post-supplementation

Urine values in blacks and whites post supplementation are given in Table 2.8. In blacks, the significant increases in the magnesium concentration and in TRI at day 30 relative to baseline are in conflict with each other since the former effect should have caused a decrease in TRI. An explanation for this anomaly is not obvious, but may be due to subtle, non-significant changes in the other urinary parameters which are used to determine TRI, such as the concentrations of calcium, oxalate and citrate.

In whites, different parameters were affected at day 30, but TRI also increased as in blacks. The significant increases in citrate and oxalate might have been expected to cancel with respect to their effects on TRI, but it is clear that the increase in oxalate dominated, together with subtle other changes, as alluded to in the black group. The decrease in the concentration of ionized Ca^{2+} in whites at day 30 is interesting, but surprisingly, this did not affect SS values of the calcium-containing salts, CaOx and brushite.

Changes in urinary parameters appeared to occur earlier (i.e. day 15) in whites than in blacks, but the latter group showed a greater sensitivity towards “carry-over” effects.

Ultimately, when the risk ratios TRI and SS changed significantly, they did so unfavourably, thereby indicating that GLA has a negative effect on this important risk indicator.

Table 2.7

Comparison of urinary risk factors between groups at baseline – n-6 FA study

Parameter	*Blacks*	*Whites*	p-values
pH	6.45 ± 0.14	6.20 ± 0.08	0.1355
Volume (mL/24h)	1370 ± 149	983 ± 118	0.0559
Calcium (mmol/24h)	1.71 ± 0.17	2.03 ± 0.23	0.2839
Chloride (mmol/24h)	133 ± 13.2	113 ± 16.5	0.3577
Citrate (mmol/24h)	2.18 ± 0.33	1.83 ± 0.18	0.3727
Creatinine (mmol/24h)	18.3 ± 1.06	17.2 ± 1.40	0.5282
Magnesium (mmol/24h)	1.36 ± 0.14	1.92 ± 0.28	0.0944
Oxalate (mmol/24h)	0.26 ± 0.02	0.20 ± 0.02	0.0358‡
Phosphate (mmol/24h)	22.6 ± 1.50	32.5 ± 2.77	0.0057‡
Potassium (mmol/24h)	26.9 ± 1.52	27.3 ± 1.58	0.8643
Sodium (mmol/24h)	99.5 ± 7.43	96.0 ± 5.30	0.7081
Urate (mmol/24h)	3.47 ± 0.23	3.31 ± 0.28	0.6607
Ionized Ca^{2+} (mmol/L)	0.37 ± 0.08	0.48 ± 0.09	0.3737
PGE2 (ng/24h)	711 ± 84.2	534 ± 39.0	0.0725
HYP (nmol/mg creatinine)	1.46 ± 0.32	1.11 ± 0.19	0.3789
MSL (mM)	1.03 ± 0.16	0.90 ± 0.14	0.5524
TRI	120 ± 8.05	115 ± 13.4	0.7189
SS CaOx	2.80 ± 0.30	3.64 ± 0.31	0.0686
SS Brushite	0.77 ± 0.17	1.43 ± 0.25	0.0391‡
SS Uric acid	1.04 ± 0.38	1.84 ± 0.34	0.1315

‡ : *p-value ≤ 0.05 (intergroup comparison)*

Table 2.8

Urinary risk factors within groups after n-6 FA supplementation

Parameter	*Blacks*				*Whites*			
	Day 0	**Day 15**	**Day 30**	**Day 35**	**Day 0**	**Day 15**	**Day 30**	**Day 35**
pH	6.45 ± 0.14	6.19 ± 0.15	6.15 ± 0.09	6.23 ± 0.19	6.20 ± 0.08	6.30 ± 0.13	6.31 ± 0.12	6.22 ± 0.12
Volume (mL/24h)	1370 ± 149	1164 ± 234	1299 ± 229	1378 ± 317	983 ± 118	1179 ± 160	1256 ± 200	1295 ± 170
Calcium (mmol//24h)	1.71 ± 0.17	1.95 ± 0.13	1.99 ± 0.15	2.63 ± 0.21*	2.03 ± 0.23	2.17 ± 0.20	1.99 ± 0.11	1.96 ± 0.11
Chloride (mmol//24h)	133 ± 13.2	115 ± 9.20	104 ± 10.5	126 ± 19.4	113 ± 16.5	99.7 ± 14.1	122 ± 12.9	137 ± 12.5
Citrate (mmol/24h)	2.18 ± 0.33	2.43 ± 0.55	2.93 ± 0.48	3.51 ± 0.70	1.83 ± 0.18	2.68 ± 0.41	3.05 ± 0.47*	2.66 ± 0.48
Creatinine (mmol//24h)	18.3 ± 1.06	17.2 ± 1.10	15.0 ± 1.15*	15.5 ± 1.11	17.2 ± 1.40	16.0 ± 1.39	17.3 ± 1.54	17.8 ± 2.17
Magnesium (mmol//24h)	1.36 ± 0.14	1.88 ± 0.13*	1.84 ± 0.15*	2.36 ± 0.24*	1.92 ± 0.28	2.22 ± 0.20	1.95 ± 0.12	2.10 ± 0.16
Oxalate (mmol//24h)	0.26 ± 0.02	0.21 ± 0.04	0.26 ± 0.03	0.35 ± 0.06	0.20 ± 0.02	0.27 ± 0.04	0.32 ± 0.02*	0.31 ± 0.04*
Phosphate (mmol//24h)	22.6 ± 1.50	24.2 ± 1.54	21.0 ± 1.80	24.8 ± 1.29	32.5 ± 2.77	37.0 ± 4.36	32.5 ± 3.13	35.7 ± 4.63
Potassium (mmol/24h)	26.9 ± 1.52	30.1 ± 1.82	31.7 ± 2.28	31.2 ± 2.47	27.3 ± 1.58	33.1 ± 1.93*	33.0 ± 2.17*	30.5 ± 2.57
Sodium (mmol/24h)	99.5 ± 7.43	95.3 ± 5.97	90.7 ± 8.00	100 ± 8.24	96.0 ± 5.30	94.7 ± 6.61	101 ± 8.17	110 ± 8.46
Urate(mmol/24h)	3.47 ± 0.23	3.00 ± 0.21	2.50 ± 0.18*	2.93 ± 0.33	3.31 ± 0.28	3.43 ± 0.41	3.48 ± 0.38	3.48 ± 0.44
Ionized Ca^{2+} (mmol/L)	0.37 ± 0.08	0.43 ± 0.10	0.43 ± 0.11	0.41 ± 0.11	0.48 ± 0.09	0.23 ± 0.05*	0.13 ± 0.03*	0.45 ± 0.27
PGE2 (ng/24h)	711 ± 84.2	ND	721 ± 96.4	ND	534 ± 39.0	ND	570 ± 118	ND
HYP (nmol/mg creatinine)	1.46 ± 0.32	ND	0.82 ± 0.19	ND	1.11 ± 0.19	ND	0.81 ± 0.12	ND
TRI	120 ± 8.05	112 ± 15.1	182 ± 28.0*	241 ± 22.5*	115 ± 13.4	168 ± 19.5*	186 ± 26.0*	166 ± 20.2*
SS CaOx	2.80 ± 0.30	3.35 ± 0.42	3.77 ± 0.66	5.73 ± 0.95*	3.64 ± 0.31	3.72 ± 0.47	4.81 ± 1.18	3.69 ± 0.47
SS Brushite	0.77 ± 0.17	1.30 ± 0.46	0.71 ± 0.18	1.13 ± 0.35	1.43 ± 0.25	1.18 ± 0.13	1.10 ± 0.23	0.92 ± 0.15
SS Uric acid	1.04 ± 0.38	1.97 ± 0.64	1.19 ± 0.19	1.59 ± 0.43	1.84 ± 0.34	2.01 ± 0.66	1.55 ± 0.54	1.70 ± 0.49

* : *p-value ≤ 0.05 compared to baseline*

Intergroup comparisons

Comparison of urine parameters between blacks and whites on each day of the experiment are shown in Table 2.9. Significant differences between the black and white groups for any urinary parameter on any particular day (days 15, 30 or 35) can only be attributed to GLA ingestion if the same difference did not occur in baseline urines. Thus the significantly lower phosphate concentration in blacks relative to whites on days 15, 30 and 35 can be ignored. However, the significantly lower citrate and higher ionized Ca^{2+} in blacks relative to whites on day 30 may be tentatively attributed to the ingestion of GLA, for reasons which are not apparent. Finally, the significantly higher calcium and significantly higher TRI in blacks relative to whites in the carry-over period may be similarly attributed to GLA ingestion. While the explanations for these effects are not obvious, an important point is that black and white subjects have responded differently to GLA, thereby yet again drawing attention to the possibility of different handling mechanisms in the two race groups.

2.4.6 CaOx MSL and crystallization kinetics studies

The average CaOx MSL for black and white subjects observed on days 15, 30 and 35 are shown in Table 2.10. The p-values are shown in Table 2.11. In both groups, there were no significant differences observed in the CaOx MSL values during the supplementation period on days 15 and 30 compared to baseline. At day 35, a significant increase in the CaOx MSL (p-value = 0.0221) was observed in the black group whereas in whites the MSL was not statistically significant (Table 2.11).

The p-values for the CaOx MSL intergroup comparisons on each day of the experiment are shown in Table 2.11. The CaOx MSL between groups was not statistically significant.

The rates of particle formation during the supplementation and washout periods compared to baseline are shown in Figures 2.6 and 2.7 for blacks and whites, respectively. Each curve is the mean of two experiments. The mean values for each group are shown in Table 2.10 and the p-values are shown in Table 2.11. Raw data for each experiment are presented in Appendix 2.16.

Table 2.9

Comparison of urinary risk factors between groups at days 15, 30 and 35 – n-6 FA study

Parameter	DAY 15			DAY 30			DAY 35		
	Blacks	*Whites*	p-value	*Blacks*	*Whites*	p-value	*Blacks*	*Whites*	p-value
pH	6.19 ± 0.15	6.30 ± 0.13	0.6117	6.15 ± 0.09	6.31 ± 0.12	0.3167	6.23 ± 0.19	6.22 ± 0.12	0.9420
Volume (mL/24h)	1164 ± 234	1179 ± 160	0.9583	1299 ± 229	1256 ± 200	0.8903	1378 ± 317	1295 ± 170	0.8143
Calcium (mmol/24h)	1.95 ± 0.13	2.17 ± 0.20	0.3697	1.99 ± 0.15	1.99 ± 0.11	0.9959	2.63 ± 0.21	1.96 ± 0.11	0.0093‡
Chloride (mmol/24h)	115 ± 9.20	99.7 ± 14.1	0.3631	104 ± 10.5	122 ± 12.9	0.3137	126 ± 19.4	137 ± 12.5	0.6382
Citrate (mmol/24h)	2.43 ± 0.55	2.68 ± 0.41	0.7201	2.93 ± 0.48	3.05 ± 0.47	0.8676	3.51 ± 0.70	2.66 ± 0.48	0.3207
Creatinine (mmol/24h)	17.2 ± 1.10	16.0 ± 1.39	0.5098	15.0 ± 1.15	17.3 ± 1.54	0.2327	15.5 ± 1.11	17.8 ± 2.17	0.3679
Magnesium (mmol/24h)	1.88 ± 0.13	2.22 ± 0.20	0.1648	1.84 ± 0.15	1.95 ± 0.12	0.5793	2.36 ± 0.24	2.10 ± 0.16	0.3644
Oxalate (mmol/24h)	0.21 ± 0.04	0.27 ± 0.04	0.2765	0.26 ± 0.03	0.32 ± 0.02	0.1438	0.35 ± 0.06	0.31 ± 0.04	0.5806
Phosphate (mmol/24h)	24.2 ± 1.54	37.0 ± 4.36	0.0129‡	21.0 ± 1.80	32.5 ± 3.13	0.0052‡	24.8 ± 1.29	35.7 ± 4.63	0.0450‡
Potassium (mmol/24h)	30.1 ± 1.82	33.1 ± 1.93	0.2784	31.7 ± 2.28	33.0 ± 2.17	0.6956	31.2 ± 2.47	30.5 ± 2.57	0.8605
Sodium (mmol/24h)	95.3 ± 5.97	94.7 ± 6.61	0.9505	90.7 ± 8.00	101 ± 8.17	0.3966	100 ± 8.24	110 ± 8.46	0.4271
Urate (mmol/24h)	3.00 ± 0.21	3.43 ± 0.41	0.3649	2.50 ± 0.18	3.48 ± 0.38	0.0312‡	2.93 ± 0.33	3.48 ± 0.44	0.3378
Ionized Ca^{2+} (mmol/L)	0.43 ± 0.10	0.23 ± 0.05	0.0981	0.43 ± 0.11	0.13 ± 0.03	0.0154‡	0.41 ± 0.11	0.45 ± 0.27	0.9114
PGE2 (ng/24h)	ND	ND	ND	721 ± 96.4	570 + 118	0.3313	ND	ND	ND
HYP (nmol/mg creatinine)	ND	ND	ND	0.82 ± 0.19	0.81 ± 0.12	0.9653	ND	ND	ND
TRI	112 ± 15.1	168 ± 19.5	0.0347‡	182 ± 28.0	186 ± 26.0	0.9260	241 ± 22.5	166 ± 20.2	0.0235‡
SS CaOx	3.35 ± 0.42	3.72 ± 0.47	0.5655	3.77 ± 0.66	4.81 ± 1.18	0.4523	5.73 ± 0.95	3.69 ± 0.47	0.0628
SS Brushite	1.30 ± 0.46	1.18 ± 0.13	0.8067	0.71 ± 0.18	1.10 ± 0.23	0.2047	1.13 ± 0.35	0.92 ± 0.15	0.5643
SS Uric acid	1.97 ± 0.64	2.01 ± 0.66	0.9645	1.19 ± 0.19	1.55 ± 0.54	0.5332	1.59 ± 0.43	1.70 ± 0.49	0.8719

‡ : *p-value ≤ 0.05 (intergroup comparison)*

Table 2.10
CaOx MSL and average rates of particle formation within groups after n-6 FA supplementation

Parameter	*Blacks*				*Whites*			
	Day 0	Day 15	Day 30	Day 35	Day 0	Day 15	Day 30	Day 35
MSL (mM)	1.03 ± 0.16	1.28 ± 0.13	1.41 ± 0.13	1.52 ± 0.11*	0.90 ± 0.14	1.13 ± 0.16	1.20 ± 0.12	1.25 ± 0.13
Kinetics (particle/min)	270 ± 90.1	313 ± 89.8	284 ± 35.9	413 ± 55.5	6.3e-04 ± 1.9e-04	3.7e-04 ± 8.0e-05	5.1e-04 ± 2.9e-04	2.8e-04 ± 1.1e-04

Table 2.11
P-values for the comparison of CaOx MSL and average rates of particle formation within and between groups after n-6 FA supplementation

Parameter	*Blacks*			*Whites*			*B vs W*		
	Day 15	Day 30	Day 35	Day 15	Day 30	Day 35	Day 15	Day 30	Day 35
MSL (mM)	0.2360	0.0822	0.0221*	0.2884	0.1254	0.0838	0.5097	0.2606	0.1378
Kinetics (particle/min)	0.7364	0.8793	0.1785	0.2433	0.7312	0.1448	ND	ND	ND

* : *p-value ≤0.05 compared to baseline (intragroup comparison)*

In the black group, non-significant increases in particle formation kinetics were observed during the supplementation and washout periods compared to baseline. Within the white group, the rate of particle formation during the supplementation and washout periods was higher than that in baseline samples, but differences were not statistically different. Differences in crystallization rates between blacks and whites were not compared since they were measured with two different instruments, as explained in page 80.

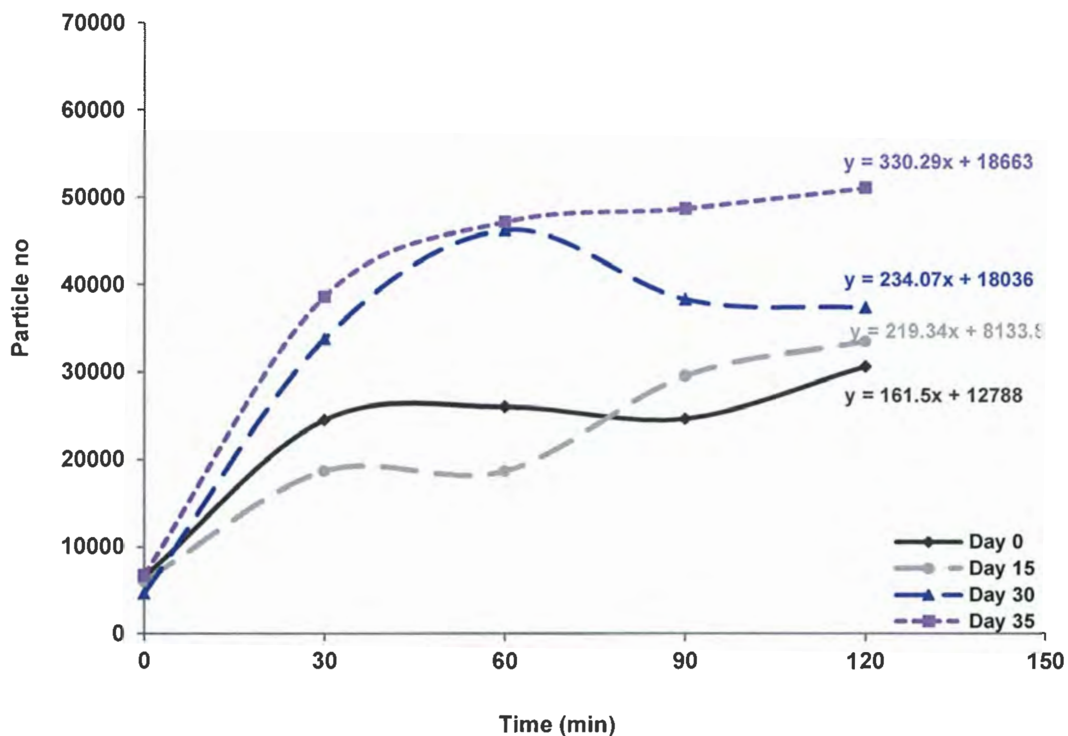

Figure 2.6: Rates of particle formation in blacks after n-6 FA supplementation

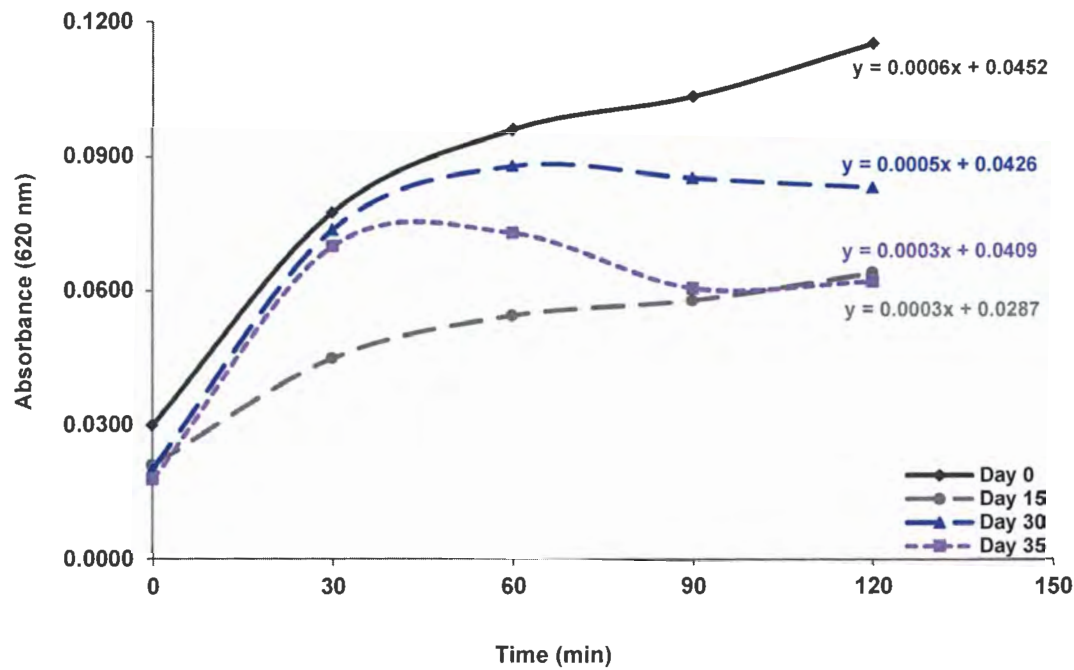

Figure 2.7: Rates of particle formation in whites after n-6 FA supplementation

2.5 DISCUSSION

Risk factors for CaOx stone disease can be broadly divided into two classes – those associated with blood (serum and plasma) biochemistry and those associated with urine physical chemistry. As mentioned in Chapter 1 - page 15, diet influences stone formation by altering urine chemistry, and will be discussed in Chapter 8. In the present study, the effects of dietary supplementation with GLA on serum, plasma and urinary risk factors for CaOx stone formation in healthy subjects were evaluated.

2.5.1 Serum biomarkers

Supplementation did not show any effect on the serum concentrations of 25(OH)D and TAGs in both population groups. Comparison of these results with other studies is not possible because the effect of GLA supplementation on these serum biomarkers has not been previously reported. In the USA, individuals with dark skin have been shown to have significantly lower serum 25(OH)D concentrations compared to individuals who are lighter skinned [44 – 46]. This is mainly attributed to melanin, a pigment on the surface of the skin, which protects the skin against ultraviolet light [44, 49]. As a result, the darker pigmentation of black subjects reduced the ability of the skin to convert vitamin D from the sun to 25(OH)D [49]. As such, the results of the present study are important, albeit that no supplementation effects were observed.

2.5.2 Plasma total phospholipids

Baseline

Comparison of the percentage composition of FAs in plasma phospholipids at baseline showed that there were several differences between the two race groups (Table 2.5). Similar to the present study, Steffen et al found a significantly higher percentage composition of AA in plasma phospholipids of black subjects compared to the white subjects from the USA [55]. Although several studies have investigated the percentage compositions of FAs in plasma phospholipids of both black and white subjects, the differences that were observed in these latter studies [56 - 58] were not confirmed in the present study.

It is suggested that the differences between blacks and whites observed in the present study are caused by the different dietary habits of the two groups since fatty acid composition in plasma total phospholipids reflects dietary intakes of fatty acids [30 – 31, 59]. However, the percentage compositions of FAs in plasma total phospholipids of both groups were found to be in agreement with those reported by other investigators in healthy populations [60 – 64].

Post-supplementation

In both groups, only trace concentrations of GLA in plasma total phospholipids before and after supplementation were detected (Appendices 2.9 and 2.10). These results are similar to observations made by Yoshimoto-Furuiel et al [65] where change in the percentage composition of GLA in plasma total phospholipids was not observed after a protocol in which subjects were given 2 g GLA per day for 6 weeks. In the same study, the percentage composition of GLA in plasma cholesterol ester and triglycerides was reported to have increased significantly compared to baseline concentrations. It is suggested that the trace amounts of GLA detected in the present study are due to the limited incorporation of GLA in plasma phospholipids. Since the compositions of fatty acids in cholesterol ester and triglycerides were not determined because of financial constraints, the changes in GLA after supplementation could not be confirmed. However, the concentrations and percentage compositions of GLA in these lipid fractions are expected to increase since the fatty acid compositions in various lipid fractions reflects dietary intakes of fatty acids [30 - 31].

Despite these results, GLA supplementation increased the concentrations and the percentage composition of DGLA in plasma phospholipids of black and white subjects (Table 2.3 and 2.5). Of particular importance is that this increase became statistically significant in the white group at day 30. Hornych et al. reported a non-significant increase in the percentage composition of DGLA in plasma phospholipids after giving a dose of 320 mg GLA to subjects for 30 days [35]. The direct comparison of the significant increase in the percentage composition of DGLA in plasma phospholipids of white subjects with previous results tended to be difficult because of the varying doses of GLA that were used and also because of the different durations of the supplementation periods.

In a study conducted by Khan et al in healthy subjects, supplementation with 400 mg GLA for 8 months was reported to have significantly increased the percentage composition of DGLA in plasma phospholipids [66]. In addition, significant increases in the percentage composition of DGLA in plasma total phospholipids have been reported when 1 g of GLA was given to subjects for 6 weeks [67]. Yoshimoto-Furuiel et al reported similar results after administering 2 g GLA to subjects for 6 weeks [65]. The findings of the present study demonstrate that GLA supplementation does increase the percentage compositions of DGLA in membrane phospholipids, as was expected.

On the other hand, supplementation appears not to have caused any significant changes in the concentrations and percentage compositions of AA in plasma phospholipids in both groups (Table 2.3 and 2.5). These results are in agreement with the observations made by other investigators [35, 65 and 67]. In humans, DGLA is converted to AA by the Δ-5-desaturase enzyme (Chapter 1 – page 20). However, it has been shown that the conversion of DGLA to AA is particularly slow in humans [4]. Thus, supplementation with GLA is expected to increase the percentage composition of DGLA in plasma phospholipids without significantly changing the percentage composition of AA in plasma total phospholipids, as observed in the present study.

DGLA can also undergo metabolism and produce prostaglandin E1 (PGE1). PGE1 is an inflammatory agent and has functions that are reported to be different from those of PGE2 [68]. Thus, high amounts of DGLA in membrane phospholipids may be beneficial for reducing urinary calcium and oxalate excretion since the formation of PGE1 will be higher than the formation of PGE2. In the present study, urinary PGE1 excretion was not measured. However, it has been reported that supplementation with GLA increases PGE1 synthesis in normal subjects. Therefore, an increase in the production of PGE1 in the present study is possible as well [69].

Intergroup comparison

When comparing the effects of supplementation between the two groups, it was observed that although supplementation resulted in an increase in the concentrations and percentage compositions of DGLA in both groups, this increase was significant in the white subjects only (Table 2.3). However, these amounts were found not to be significantly different when they were compared (Table 2.5). There are no studies which have investigated the changes in fatty acid composition between black and white subjects after GLA supplementation. As a result, the observed changes between these groups could not be compared with literature data. The absence of a significant increase in the black group suggests that the rate of DGLA incorporation in membrane phospholipids in this group may have occurred very slowly compared to the white subjects since the concentration and the percentage composition of DGLA in plasma phospholipids were similar at baseline (Table 2.3 and 2.5).

2.5.3 Urinary risk factors

Baseline

The observed differences in urinary oxalate and phosphate (Table 2.7) excretion between the two groups are similar to those reported for the same population groups by Whalley et al. [70]. High urinary concentrations of phosphate will increase the saturation of brushite, as observed in the white group [71]. Differences in the metabolic characteristics between black and white South African populations have been suggested as contributing to the unequal prevalence of stone formation in these groups [70]. The discussion for these metabolic characteristics will be addressed in Chapter 8.

Post-supplementation

In both study groups, GLA supplementation resulted in a significant increase in the TRI at day 30 (Table 2.8). The comparison of these changes with other published data proved difficult because there is only one study in which the effects of GLA supplementation on urinary CaOx risk factors were investigated [7]. After administering 80 mg GLA for 20 days, Rodgers et al. reported a significant decrease in the TRI in the black population after supplementation whereas in white subjects, the decrease in TRI was not significant [7].

Thus, the findings in the present study are in contrast to those observed by Rodgers et al probably due to the administration of a different dose of GLA and different durations of the supplementation periods. As shown Chapter 2 – page 81, calculation of TRI is based on lithogenic components.

As a result, the changes that occurred in urinary parameters within groups during supplementation may account for the observed significant difference in TRI that was observed. After supplementation, urinary calcium excretion was not statistically different when compared to baseline concentrations in both groups (Table 2.8). These results are in contrast to those reported by Rodgers et al [7] who observed a significant decrease in urinary calcium excretion in black and white South African subjects after supplementation. However, the present results are consistent with the finding in the present study that after supplementation, the composition of AA in plasma phospholipids and the concentrations of urinary PGE2 (Table 2.8) were not affected by the increased intakes of GLA. As such, an increase in urinary calcium was not expected.

With regards to urinary oxalate, supplementation had different effects within groups. In the black group, the concentration of oxalate after supplementation was similar to baseline concentrations, whereas in whites, a significant increase was observed at day 30. The urinary oxalate results observed in the black group are similar to those reported by Rodgers et al [7]. Since the composition of AA in plasma phospholipids and the concentration of PGE2 were unaffected by supplementation, urinary oxalate concentrations were also expected to be unchanged. Thus, other metabolic processes must have been the cause of an increase in urinary oxalate in whites.

It is noted, however, that there were significant differences in the urinary risk factors within groups at day 15 compared to baseline values. In Table 2.8, it is shown that urinary magnesium was significantly higher than baseline in blacks whereas in whites, potassium and the TRI were significantly higher than baseline while ionized Ca^{2+} was significantly lower. Importantly, these changes were also observed at day 30. These results are promising. Supplementation for longer time periods may be required for other effects to occur.

Intergroup comparisons

As previously mentioned in Chapter 1 – page 24, black and white South African populations are suggested to have different renal handling mechanisms in response to different lithogenic agents [6]. This was confirmed in the present study, in which the two groups responded differently with respect to urinary parameters after GLA supplementation (Table 2.8).

The differences in the urinary risk factors after supplementation suggest that the pathways for GLA metabolism are not similar between groups. Thus, the hypothesis that blacks and whites have different renal handling mechanisms with respect to different supplemental challenges was confirmed in the present study.

With regards to the increased urinary citrate in whites, similar observations have been reported when GLA supplements were given to healthy white South African subjects [7]. Rodgers et al. suggested that the increase in urinary citrate may be due to increased filtration of plasma citrate by the glomerulus due to the inhibited lipogenesis which decreases citrate consumption. Because kidney stone disease is a multifactorial disease [72 - 75], other factors such as genetics and environmental factors may be associated with the observed changes in other urinary risk factors after supplementation in the two race groups.

Washout period

A washout period is defined as a period in a trial during which the effect of a treatment given previously is believed to disappear [76]. The main purpose for a washout period is to allow for the entire administered drug or supplement to be eliminated from the body and to determine the length of time for the measured parameters to return to baseline values. These observations may be beneficial for future trials in which these values may be used to assess the absolute effects of the trial medication with other similar trials. In addition, washout periods are necessary to prevent harmful effects from interactions between trial drugs or supplements and previous medications [77].

The duration of the washout period usually depends on the half-life of the drug within the population of interest [78]. Nonetheless, washout periods ranging from 1 day to 8 weeks have been reported in studies where fatty acid supplements were given to healthy subjects [79 – 81]. In the present study, a 5 day washout period was chosen because a washout period of 4 days was found to be insufficient for urinary parameters to return to baseline values after 80 mg of GLA supplements were given to healthy subjects [7].

In the present study, several urinary risk factors were found to be significantly increased during the washout period compared to baseline values in both race groups (Table 2.8). In the black group, magnesium and TRI remained significantly higher than baseline whereas in whites, significantly higher urinary oxalate and the TRI were observed.These findings could not be compared with the study by Rodgers et al [7], since they were not similar during the supplementation period. In addition, a significant increase in urinary calcium, MSL and SS CaOx was observed at day 35 in the black population. This is of particular interest since these observations were not observed during the supplementation period itself. These (mainly unfavourable) findings imply that that the effects of GLA supplementation on urinary risk factors were delayed during the initial stages of supplementation. Thus, it can be concluded that a washout period of 5 days is not sufficient for restoring the amounts of fatty acids in plasma phospholipids during the supplementation period to baseline levels.

Summary

In conclusion, the results of the present study have demonstrated that n-6 FA supplementation did not significantly reduce blood and urinary risk factors associated with CaOx stone formation in healthy subjects. Firstly, the percentage composition of AA in plasma total phospholipids was not affected by GLA supplementation. Secondly, the increase in the TRI that was observed in both population groups during the supplementation period suggests that GLA may promote CaOx stone formation. Lastly, supplementation had no effect on the CaOx MSL and crystallization kinetics of subjects. However, an increase in the percentage composition of DGLA was observed in both population groups, although it was only significant in the white group. This finding is favourable since it implies that n-6 FAs may exert their effects on CaOx stone formation via other pathways. This potential effect of GLA supplementation on CaOx stone formation will be explored in Chapter 4.

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Chapter 3

Investigation of the effects of n-3 fatty acids (EPA and DHA) on blood and urinary risk factors for CaOx urolithiasis

3.1 INTRODUCTION

The incidence of cardiovascular diseases in the Greenland Inuit tribes is reported to be lower than that for Western populations [1-4]. The lower incidence of heart diseases in this tribe is largely credited to their dietary habits. Sinclair reported that the Inuit population consumes diets that are high in fats and rich in n-3 FAs such as EPA and DHA [5]. Based on this information, the potential effects of these fatty acids as alleviating agents for CaOx stone formation have been investigated in several studies. In a study conducted by Buck et al [6], dietary supplementation with 10 g fish oil for 8 weeks decreased urinary calcium and oxalate excretion in hypercalciuric patients. In another study, Baggio et al. [7] reported that urinary calcium and oxalate were significantly reduced in hypercalciuric stone patients after providing them with 2.31 g n-3 FAs (1.313 g EPA and 0.997 g DHA) for 30 days. Recently, Ortiz-Alvarado et al reported a decrease in urinary calcium and oxalate excretion after giving 1.2 g per day of EPA and DHA to hypercalciuric stone patients for 10 months [8]. These results show that supplementation with EPA and DHA may be beneficial for stone formation since the major urinary risk factors for CaOx stone formation are reduced.

However, not all studies have shown that both urinary calcium and oxalate are simultaneously decreased after EPA and DHA supplementation. Rothwell et al reported a significant decrease in urinary calcium after giving 5 g of 2.7 g EPA and 1.8 g of DHA for 4 weeks in hypercalciuric recurrent stone patients but no decrease in urinary oxalate [9]. In another study, Konya et al demonstrated that supplementation with 1.8 g EPA, as ethyl icosapentate, per day for 6 months had no effect on the urinary excretion of calcium and oxalate in hypercalciuric stone patients [10]. After administering 1.8 g EPA, in a pure form, per day for 3 months to hypercalciuric stone patients, Yasui et al reported a significant decrease in the urinary excretion of calcium in these patients but no decrease in the urinary excretion of oxalate [11].

Thus, the outcomes of EPA and DHA supplementation on the urinary risk factors for CaOx stone formation are not consistent. Differences in urinary calcium and oxalate excretion after supplementation are suggested to be due to the type of supplement used, varying doses of EPA and DHA and to the different durations of the supplementation periods.

The research goal of the study described in the present chapter is to investigate the effects of administering n-3 FAs (EPA and DHA) supplements to black and white South African subjects as outlined in Chapter 1 – page 26.

As such, the current study aims to address further the effects of n-3 FA supplementation on the urinary risk factors associated with CaOx stone formation. Since there is only one study that has investigated other CaOx risk factors (such as fatty acid composition in plasma total phospholipids and serum 25(OH)D) besides urinary risk factors [7], the present study also aimed to evaluate the effects of n-3 FA supplementation on serum and plasma risk biomarkers for CaOx stone formation. In addition, the effects of EPA and DHA supplementation on CaOx risk factors in the black and white South African populations were also compared.

3.2 STUDY PROTOCOL

The study was undertaken at the University of Cape Town after approval (**HREC REF: 249/2010**) was obtained from the Human Research Ethics Committee – Cape Town, South Africa (Appendix 3.1). All participants provided written informed consent (Appendix 2.2). Subjects had no family history of renal or kidney stone disease and they were not allowed to participate in the study if they had diseases such as diabetes, bleeding or blood clotting disorders that affect lipid metabolism. None of the participants were taking any medication or supplements during the course of the study.

12 Black and 12 white healthy South African males, aged between 18 - 32 years old, were initially recruited from the University of Cape Town to participate in the study. The chosen number of participants was based on sample size calculations to achieve a statistical power of 80 % as described in Chapter 2 – page 69. One subject from the white group dropped out of the study due to personal commitments. Thus, the total number of white subjects who were enrolled in the study was 11.

A 30 day supplementation period was observed by subjects where they were required to take encapsulated fish oils, Omega-3 “700”, purchased from Solgar – UK. The capsules provided 1.92 g of EPA and DHA per day. Each capsule contained 0.38 g of EPA, 0.26 g of DHA and 0.06 g of other omega-3 fatty acids. Participants were given instructions to take 1 capsule 3 times per day.

The supplements that were used in the present study contained EPA and DHA as the major components. These supplements were chosen because the diet of Inuit is mainly composed of fish and these fatty acids are reported to be natural components of fishoil [5]. Several studies have reported that consumption of 0.4 to 2 g of n-3 FAs per day, as a combination of EPA and DHA, from food or supplements is recommended for healthy individuals to increase the EPA and DHA content in various lipid fractions [12 – 14]. Supplementation with doses greater than 2 g per day is suggested to cause health complications such as increased risk of bleeding, upset stomach and elevated levels of LDL cholesterol ester in healthy individuals [15 - 17]. Thus, the supplement dose of 1.92 g EPA and DHA was chosen because it was regarded as effective in increasing the EPA and DHA content in plasma lipid fractions without imposing health risks. In addition, previous studies have shown that EPA and DHA supplementation for four weeks is sufficient to significantly increase the EPA and DHA content in various lipid fractions [18 – 21]. Thus, 30 days of EPA and DHA supplementation were chosen for the present study because it was expected that moderate changes in the percentage composition of these FAs can be achieve within this time period.

3.3 EXPERIMENTAL ANALYSIS

3.3.1 Sample collection

In the present study, blood and 24h urine samples were collected prior to the administration of n-3 FA supplements at day 0. Additional blood and 24h urine samples were collected during the supplementation period at both days 15 and 30. Final blood and 24h urine samples were collected at day 35 during the washout period after the subjects had stopped taking the supplements. Food intakes were recorded on a food diary (Appendix 2.5) at baseline; and subjects followed the same diet on blood and urine sample collecting days.

3.3.2 Nutrient intake assessment

Dietary assessment was carried out using the Foodfinder II computer program as described in Chapter 2 – page 71. The nutrient intakes were only measured on the food diaries recorded at baseline.

3.3.3 Blood analysis

The collected blood samples were collected and divided into different fractions as previously described in Chapter 2 – pages 71. Serum samples collected at days 0 and 30 were taken to the Pathcare Laboratories and to the South African Medical Research Council Laboratories for the analysis of 25(OH)D and TAGs, respectively. The concentrations of serum 25(OH)D and TAGs were measured according the procedures described in Chapter 2 – pages 72. The FA profiles in plasma and RBC total phospholipids were measured at the South African Medical Research Council using the method described in Chapter 2 – pages 72.

3.3.4 Urine analyses

Urinary risk factors

The collected 24h urine samples were analyzed for urinary risk factors as previously discussed in Chapter 2 – page 76. Ionized calcium was not measured due to technical problems with the calcium electrode.

3.3.5 Crystallization experiments

CaOx metastable limit

The CaOx metastable limit was determined according to the method described in Chapter 2- page 80. For both groups, the particle number was determined using the Coulter Counter.

CaOx crystallization kinetics experiment

In the present study, the results for the rates of particle formation were not included due to instrument malfunction. During the course of the study, the particle sensor inside the Coulter Counter was not functioning properly. This caused a false count of particles while conducting experiments. Furthermore, the instrument broke down before all the experiments could be completed.

3.3.6 Risk indices

The TRI and the SS values of COM, brushite and UA were determined as previously described in Chapter 2 – page 81.

3.3.7 STATISTICAL ANALYSES

Statistical analysis was carried out using *GraphPad Instat* 3 program. All data are expressed as mean ± SE and were considered significant if p-value ≤ 0.05. Graphs were prepared with Microsoft Excel 2010 with standard error bars.

3.4 RESULTS

3.4.1 Subject's characteristics

The mean age and BMI of subjects at baseline are shown in Table 3.1. Raw data showing each subject's age, height, weight and BMI are presented in Appendix 3.2. There were no significant differences observed between the groups with regard to age and BMI at baseline.

Table 3.1
Subject's characteristics at baseline – n-3 FA study

Parameter	*Blacks*	*Whites*	p-value
Age (years)	24.5 ± 1.11	22.2 ± 0.63	0.0912
BMI (kg/m^2)	25.0 ± 1.18	23.3 ± 0.98	0.2886

3.5.2 Nutrient intakes

Raw data showing the calculated nutrient intakes for each subject are presented in Appendix 3.3. The results for the mean intakes of nutrients, minerals and fatty acids of black and white subjects recorded at baseline will be discussed in Chapter 8.

3.4.3 Compliance test

In the present study, supplement compliance was determined using two techniques. Firstly, compliance was determined by counting the number of capsules remaining after the supplementation period, as described in Chapter 2 – page 83. Only two subjects returned capsules after the supplementation period; one black subject returned 3 capsules and one white subject returned 2 capsules. It was then concluded that > 85 % of the capsules given to subjects were consumed during the course of the study.

Secondly, compliance was assessed by comparing the changes in the concentrations of EPA and DHA in RBC total phospholipids during the supplementation period, on days 15 and 30, with the concentrations at baseline. The changes in these FAs were calculated because the capsules that were given to subjects contained EPA and DHA as the major components.

As previously described in Chapter 2 – page 83, RBCs were chosen because of their 120 day lifespan and because they reflect dietary intakes of FAs. In each race group, 5 subjects were randomly selected for the compliance test. The percentage changes in the concentrations of EPA and DHA in RBC total phospholipids for each group were calculated as previously described in Chapter 2 – page 83.

Raw data showing the FA composition (as concentration (µg/mL)) in RBC total phospholipids for each individual are presented in Appendix 3.4. The percentage changes in the concentrations of EPA and DHA for each subject are shown in Table 3.2. At day 15, the black subjects had a 300 % mean percentage change for EPA (p-value < 0.0001) whereas in whites, the mean percent change was 185 % (p-value = 0.0003). The mean percentage changes in EPA were further increased at day 30 (p-value < 0.0001) in both groups and reached mean percentage changes of 482 % and 253 % for blacks and whites, respectively.

On the other hand, at each sampling point, the percentage changes for DHA increased to a smaller extent compared to baseline concentrations in both race groups. In the black group, the mean percentage change for DHA at day 15 was 11 % (p-value = 0.2988). This was further increased to 22 % (p-value = 0.0581) at day 30. The mean percentage changes for DHA in the white subjects were 12 % (p-value = 0.0551) at day 15 and 20 % (p-value = 0.0067) at day 30. It has been reported that the rate of DHA incorporation into membrane phospholipids occurs at a slower rate as compared to that of EPA [22]. In addition, it has been observed that DHA can be converted back to EPA, thus increasing the amounts of EPA in membrane phospholipids [22]. These two findings are suggested to be the cause of the lower DHA percentage composition in RBC membrane phospholipids that was observed after supplementation in the present study.

The changes in EPA and DHA concentrations that were observed at day 30 are in agreement with the results obtained by other investigators after administering fish oil supplements to healthy subjects for 4 weeks with EPA and DHA doses similar to the one used in the present study [23 – 25].

Overall, the results from the capsule counting technique and the changes in the concentrations of EPA and DHA in RBC membrane total phospholipids indicate that the subjects adhered to the supplement protocol.

Table 3.2

Concentrations (µg/mL) of EPA and DHA in RBC total phospholipids after n-3 FA supplementation

Subjects	EPA					DHA				
	Concentration (µg/mL)			% Change		Concentration (µg/mL)			% Change	
	Day 0	Day 15	Day 30	Day 0 vs 15	Day 0 vs 30	Day 0	Day 15	Day 30	Day 0 vs 15	Day 0 vs 30
B-1	0.27	0.86	1.40	219	419	4.93	5.50	6.07	12	23
B-2	0.17	0.87	1.33	412	682	4.08	4.50	4.87	10	19
B-3	0.24	1.28	1.63	433	579	3.44	4.19	4.50	22	31
B-4	0.26	1.09	1.61	319	519	4.48	5.16	5.63	15	26
B-5	0.44	0.96	1.36	118	209	5.21	4.96	5.87	-5	13
W-1	0.53	1.40	1.62	164	206	4.81	5.73	6.03	19	25
W-2	0.28	0.97	1.25	246	346	4.29	5.01	5.32	17	24
W-3	0.23	0.90	1.14	291	396	4.84	5.24	5.53	8	14
W-4	0.45	1.04	1.17	131	160	5.56	5.85	6.17	5	11
W-5	0.51	1.00	1.32	96	159	4.80	5.34	5.93	11	24

B = blacks and W = whites

3.4.4 Serum biomarkers and FA profile in plasma total phospholipids

As mentioned in Chapter 2 – page 86, attention is drawn toward n-6 PUFAs such as GLA, DGLA, AA and n-3 PUFAs such as EPA and DHA since they are of most importance with respect to modulatory risk factors for kidney stone formation. Also, only the percentage compositions of fatty acids in plasma total phospholipids will be compared with other studies.

Baseline

The differences in the concentrations of serum biomarkers and fatty acids in plasma total phospholipids between groups observed at baseline are presented in Table 3.3. Raw data showing the concentrations of serum 25(OH)D and TAGs for each subject are presented in Appendices 3.5 and 3.6, respectively. Raw data showing the concentrations of FAs in plasma phospholipids for each individual are presented in Appendix 3.7. The comparison of serum 25(OH)D concentrations between the two groups showed that the black group had significantly lower 25(OH)D concentrations compared to the white subjects. These differences are similar to those observed in the previous study (Chapter 2 – page 86) and also to those reported by other investigators [26 - 31].

Table 3.3

Concentrations of serum biomarkers and FAs in plasma total phospholipids after n-3 FA supplementation

Parameter	*BLACKS*				*WHITES*				*B vs W (p-values)*			
	Day 0	Day 15	Day 30	Day 35	Day 0	Day 15	Day 30	Day 35	Day 0	Day 15	Day 30	Day 35
Serum biomarkers												
25(OH)D (ng/mL)	14.2 ± 2.65	ND	15.3 ± 2.64	ND	23.2 ± 2.03	ND	24.8 ± 2.74	ND	0.0147‡	ND	0.0217‡	ND
TAGs (mmol/L)	0.87 ± 0.09	ND	0.79 ± 0.11	ND	0.89 ± 0.11	ND	0.77 ± 0.13	ND	0.9053	ND	0.9032	ND
SFAs (µg/mL)												
C14:0 MA	2.26 ± 0.14	2.39 ± 0.28	2.05 ± 0.13	3.64 ± 1.03	3.32 ± 0.41	3.22 ± 0.32	3.03 ± 0.34	4.11 ± 0.45	0.0226‡	0.0695	0.0110‡	0.7013
C16:0 PA	267 ± 14.8	275 ± 13.7	279 ± 12.5	309 ± 16.7	338 ± 20.7	298 ± 16.5	296 ± 18.5	329 ± 24.7	0.0107‡	0.2894	0.4531	0.5017
C18:0 SA	147 ± 7.11	156 ± 7.36	153 ± 5.29	183 ± 22.7	173 ± 10.6	163 ± 9.15	160 ± 10.1	177 ± 12.6	0.0497‡	0.5396	0.5261	0.8272
20:0 ARA	4.26 ± 0.25	4.59 ± 0.22	5.08 ± 0.50	5.00 ± 0.46	5.50 ± 0.55	5.06 ± 0.40	6.09 ± 0.63	5.41 ± 0.26	0.0417‡	0.3059	0.2198	0.4409
22:0 BA	14.9 ± 0.62	15.6 ± 0.68	16.5 ± 1.28	16.6 ± 1.28	15.9 ± 1.37	15.8 ± 1.21	17.2 ± 1.30	17.3 ± 0.64	0.5326	0.8803	0.6824	0.6370
24:0 LGA	15.5 ± 0.58	16.3 ± 0.62	17.0 ± 1.09	17.4 ± 1.19	16.7 ± 1.16	16.2 ± 1.06	17.3 ± 1.12	17.9 ± 0.83	0.3423	0.9340	0.8515	0.7388
MUFAs (µg/mL)												
C16:1 n-7 PTA	3.59 ± 0.33	3.36 ± 0.25	3.25 ± 0.27	4.53 ± 0.41	7.26 ± 0.89	4.77 ± 0.42*	5.11 ± 0.60	5.86 ± 0.52	0.0010‡	0.0084‡	0.0071‡	0.0560
C18:1 n-9 OA	67.4 ± 5.89	68.4 ± 4.20	69.3 ± 3.17	82.5 ± 3.45*	108 ± 9.00	86.8 ± 5.30	79.5 ± 7.54*	95.5 ± 7.13	0.0009‡	0.0119‡	0.2135	0.1073
C18:1 n-7	11.4 ± 0.81	12.4 ± 0.63	11.4 ± 0.81	13.1 ± 1.18	15.9 ± 1.36	14.2 ± 1.32	12.7 ± 1.28	15.3 ± 1.56	0.0084‡	0.2333	0.6560	0.2591
20:1 n-9 GA	1.66 ± 0.13	2.02 ± 0.26	1.46 ± 0.09	1.90 ± 0.17	2.10 ± 0.31	1.88 ± 0.25	1.75 ± 0.21	1.87 ± 0.19	0.2239	0.7319	0.2014	0.9257
24:1 n-9	17.6 ± 0.93	21.3 ± 0.96*	21.8 ± 1.34*	22.0 ± 1.81*	23.2 ± 2.18	23.0 ± 1.79	22.4 ± 1.82	24.5 ± 1.58	0.0242‡	0.3956	0.7792	0.3195
PUFAs (µg/mL)												
C18:2 n-6 LA	224 ± 17.5	222 ± 18.0	212 ± 13.4	258 ± 15.8	266 ± 17.8	227 ± 15.3	210 ± 14.9*	281 ± 24.8	0.1029	0.8303	0.9486	0.4197
C18:3 n-6 GLA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:2 n-6 EDA	3.59 ± 0.21	3.89 ± 0.76	3.37 ± 0.31	4.45 ± 0.90	6.15 ± 2.37	3.17 ± 0.20	3.73 ± 0.68	3.77 ± 0.36	0.2511	0.3687	0.6081	0.5078
C20:3 n-6 DGLA	29.8 ± 2.78	22.6 ± 2.07*	22.7 ± 1.98*	31.6 ± 2.90	41.5 ± 3.23	28.4 ± 2.26*	27.1 ± 3.57*	33.6 ± 3.55	0.0121‡	0.0733	0.2834	0.6735
C20:4 n-6 AA	128 ± 11.4	109 ± 7.13	106 ± 6.34	114 ± 8.08	145 ± 10.8	120 ± 8.65	108 ± 9.86*	122 ± 10.9	0.2951	0.3428	0.8480	0.5795
C22:4 n-6 ADRA	5.39 ± 0.59	3.03 ± 0.21*	2.57 ± 0.20*	3.92 ± 0.56	5.43 ± 0.31	3.15 ± 0.22*	2.67 ± 0.25*	3.27 ± 0.27*	0.9622	0.6895	0.7472	0.3180
C22:5 n-6 DPA	4.37 ± 0.60	2.64 ± 0.40*	2.09 ± 0.35*	2.43 ± 0.23*	5.94 ± 2.22	2.57 ± 0.26	2.59 ± 0.53	2.19 ± 0.18	0.4860	0.8816	0.4576	0.4327
C18:3 n-3 ALA	1.61 ± 0.10	1.41 ± 0.05	1.57 ± 0.06	1.58 ± 0.09	1.44 ± 0.11	1.88 ± 0.31	1.85 ± 0.21	1.91 ± 0.29	0.2825	ND	ND	0.2917
C20:5 n-3 EPA	4.08 ± 0.43	29.6 ± 2.28*	32.8 ± 3.01*	13.7 ± 1.67*	7.21 ± 0.85	34.1 ± 2.90*	34.0 ± 4.05*	14.9 ± 1.61*	0.0037‡	0.2366	0.8132	0.6037
C22:5 n-3 DPA	8.40 ± 0.72	15.7 ± 1.11*	15.9 ± 0.61*	15.7 ± 1.00*	10.8 ± 0.79	15.3 ± 0.96*	16.0 ± 1.72*	14.8 ± 1.09*	0.0321‡	0.7577	0.9683	0.5610
C22:6 n-3 DHA	36.0 ± 2.51	60.1 ± 2.21*	67.5 ± 3.27*	63.8 ± 3.75*	45.3 ± 4.59	61.2 ± 4.37*	62.9 ± 5.11*	62.1 ± 3.47*	0.0810	0.8283	0.3788	0.8556

*: *p-value ≤ 0.05 compared to baseline (intragroup comparison)*
‡: *p-value ≤ 0.05 (intergroup comparison)*

The differences in the concentrations of FAs in plasma total phospholipids between the two groups at baseline are summarized in Table 3.4.

Table 3.4
Summary of significant differences in concentrations of FAs (µg/mL) between groups at day 0 – n-3 FA study

FA class	Type of FA	B vs W
SFAs	MA, PA, SA and ARA	B < W‡
MUFAs	PTA, OA, VA and 24:1 n-9	B < W‡
PUFAs	DGLA, EPA, DPA (n-3)	B < W‡

‡: *p-value ≤ 0.05 (intergroup comparison)*

The comparison of the percentage compositions of FAs in plasma phospholipids between the two groups are shown in Table 3.5. Raw data showing the percentage composition of FAs in plasma phospholipids for each individual are presented in Appendix 3.8.

Table 3.5
Percentage composition of FAs in plasma total phospholipids after n-3 FA supplementation

FAs	*Blacks*				*Whites*				*B vs W (p-values)*			
	Day 0	Day 15	Day 30	Day 35	Day 0	Day 15	Day 30	Day 35	Day 0	Day 15	Day 30	Day 35
SFAs												
C14:0 MA	0.23 ± 0.01	0.22 ± 0.02	0.20 ± 0.01	0.30 ± 0.06	0.27 ± 0.03	0.28 ± 0.03	0.28 ± 0.02	0.33 ± 0.02	0.1924	0.1082	0.0038‡	0.6332
C16:0 PA	27.0 ± 0.61	26.3 ± 0.23	26.7 ± 0.21	26.6 ± 0.28	27.2 ± 0.44	26.4 ± 0.31	27.5 ± 0.54	26.7 ± 0.55	0.8098	0.7756	0.1900	0.9294
C18:0 SA	15.0 ± 0.50	15.0 ± 0.28	14.7 ± 0.22	15.3 ± 0.94	13.9 ± 0.30	14.5 ± 0.16	14.9 ± 0.66	14.4 ± 0.25	0.1053	0.1594	0.7799	0.3545
20:0 ARA	0.44 ± 0.03	0.44 ± 0.02	0.48 ± 0.04	0.44 ± 0.03	0.45 ± 0.03	0.45 ± 0.03	0.56 ± 0.05	0.45 ± 0.02	0.8131	0.8210	0.1994	0.8026
22:0 BA	1.55 ± 0.10	1.51 ± 0.06	1.57 ± 0.09	1.43 ± 0.07	1.28 ± 0.09	1.41 ± 0.08	1.60 ± 0.08*	1.45 ± 0.08	0.0701	0.2959	0.8036	0.8370
24:0 LGA	1.60 ± 0.10	1.59 ± 0.07	1.63 ± 0.08	1.50 ± 0.07	1.36 ± 0.08	1.45 ± 0.07	1.62 ± 0.08*	1.49 ± 0.08	0.0729	0.1731	0.935	0.9013
MUFAs												
C16:1 n-7 PTA	0.35 ± 0.02	0.32 ± 0.02	0.31 ± 0.03	0.39 ± 0.03	0.58 ± 0.05	0.42 ± 0.03*	0.44 ± 0.04	0.48 ± 0.03	0.0006‡	0.0031‡	0.0178‡	0.0504
C18:1 n-9 OA	6.65 ± 0.31	6.52 ± 0.15	6.67 ± 0.21	7.19 ± 0.25	8.70 ± 0.50	7.76 ± 0.37	7.25 ± 0.34*	7.78 ± 0.29	0.0020‡	0.0041‡	0.1572	0.1352
C18:1 n-7 VA	1.13 ± 0.04	1.20 ± 0.05	1.15 ± 0.04	1.11 ± 0.05	1.29 ± 0.10	1.27 ± 0.11	1.19 ± 0.09	1.23 ± 0.08	0.1477	0.5698	0.7063	0.1925
20:1 n-9 GA	0.16 ± 0.01	0.19 ± 0.03	0.13 ± 0.01	0.15 ± 0.01	0.17 ± 0.02	0.19 ± 0.03	0.15 ± 0.01	0.15 ± 0.02	0.9787	0.8593	0.3588	0.9402
24:1 n-9	1.79 ± 0.08	2.07 ± 0.10*	2.10 ± 0.12*	1.91 ± 0.12	1.88 ± 0.14	2.06 ± 0.13	2.10 ± 0.14	2.02 ± 0.10	0.5615	0.9368	0.9915	0.4686
n-6 PUFAs												
C18:2 n-6 LA	22.3 ± 0.88	21.0 ± 0.75	20.2 ± 0.68	22.2 ± 0.56	21.4 ± 0.61	20.1 ± 0.73	19.6 ± 0.82	22.7 ± 0.64	0.4368	0.4101	0.5339	0.5619
C18:3 n-6 GLA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
20:2 n-6 EDA	0.37 ± 0.02	0.37 ± 0.06	0.32 ± 0.03	0.39 ± 0.07	0.48 ± 0.17	0.28 ± 0.01	0.33 ± 0.05	0.29 ± 0.01	0.4616	0.2181	0.8572	0.2038
C20:3 n-6 DGLA	2.99 ± 0.24	2.20 ± 0.21*	2.18 ± 0.18*	2.71 ± 0.20	3.32 ± 0.14	2.51 ± 0.15*	2.43 ± 0.20*	2.75 ± 0.26	0.2524	0.2481	0.3653	0.9205
C20:4 n-6 AA	12.8 ± 0.69	10.5 ± 0.41*	10.1 ± 0.29*	9.89 ± 0.54*	11.7 ± 0.59	10.7 ± 0.57	9.92 ± 0.56*	9.86 ± 0.59*	0.2460	0.7367	0.8411	0.9637
22:4 n-6 ADRA	0.53 ± 0.04	0.29 ± 0.01*	0.24 ± 0.02*	0.33 ± 0.03*	0.44 ± 0.03	0.28 ± 0.02*	0.25 ± 0.01*	0.27 ± 0.01*	0.0959	0.8008	0.9855	0.0665
22:5 n-6 DPA	0.44 ± 0.05	0.24 ± 0.03*	0.19 ± 0.03*	0.21 ± 0.01*	0.47 ± 0.16	0.22 ± 0.01	0.23 ± 0.05	0.18 ± 0.01	0.8470	0.5246	0.549	0.0871
n-3 PUFAs												
C18:3 n-3 ALA	0.15 ± 0.02	0.13 ± 0.01	0.16 ± 0.01	0.14 ± 0.02	0.12 ± 0.01	0.16 ± 0.03	0.13 ± 0.02	0.13 ± 0.01	0.1616	ND	ND	0.4816
C20:5 n-3 EPA	0.40 ± 0.04	2.86 ± 0.19*	3.19 ± 0.29*	1.18 ± 0.11*	0.58 ± 0.07	3.01 ± 0.14*	3.05 ± 0.26*	1.27 ± 0.18*	0.0388‡	0.5496	0.7299	0.6674
C22:5 n-3 DPA	0.84 ± 0.05	1.52 ± 0.09*	1.55 ± 0.08*	1.35 ± 0.05*	0.88 ± 0.05	1.36 ± 0.06*	1.45 ± 0.08*	1.21 ± 0.03*	0.5213	0.1794	0.3899	0.0207‡
C22:6 n-3 DHA	3.65 ± 0.21	5.84 ± 0.22*	6.52 ± 0.27*	5.54 ± 0.24*	3.64 ± 0.28	5.45 ± 0.24*	5.73 ± 0.21*	5.18 ± 0.20*	0.9672	0.2284	0.0358‡	0.2705

**: p-value ≤ 0.05 compared to baseline (intragroup comparison)*

‡: p-value ≤ 0.05 (intergroup comparison

Table 3.6 shows the summary of the differences between groups that were observed at baseline.

Table 3.6
Summary of significant differences in the % compositions of FAs between groups at baseline – n-3 FA study

FA class	Type of FA	B vs W
MUFA	PTA and OA	B < W‡
PUFA	EPA	B < W‡

‡: p-value ≤ 0.05 (intergroup comparison)

Post-supplementation

The effects of supplementation on the concentrations of serum biomarkers and FAs in plasma phospholipids during the supplementation period are summarized in Table 3.3. The p-values are presented in Appendix 3.9. In both groups, there were no significant changes in the concentrations of serum 25(OH)D and TAGs observed at day 30.

With regards to the FA composition in plasma phospholipids, there were several changes that occurred in both groups at day 30. Within the black group, 24:1 n-9 concentrations significantly increased after supplementation. In addition, a significant decrease in the concentrations of n-6 FAs such as DGLA, ADRA and DPA was observed. As expected, supplementation significantly increased the concentrations of n-3 FAs such as EPA, DPA and DHA. In the white subjects, the concentration of OA was significantly decreased compared to baseline. For n-6 FAs; LA, DGLA, AA and ADRA were significantly decreased at day 30. Increases in EPA, DPA and DHA (n-3 FAs) were statistically significant compared to baseline concentrations after supplementation, as expected.

During the supplementation period at day 15, the concentrations of certain fatty acids were significantly different compared to baseline values in both groups. In the black group, the concentration of 24:1 n-9 was significantly increased at day 15. In addition, the concentrations of n-6 FAs such as DGLA, ADRA and DPA were significantly decreased.

On the other hand, the concentrations of n-3 FAs such as EPA, DPA and DHA were significantly increased compared to baseline. Within the white population, PTA concentrations were significantly decreased at day 15. For PUFAs; DGLA and ADRA (n-6 FAs) were significantly decreased compared to baseline whereas EPA, DPA and DHA (n-3 FAs) were significantly increased.

The changes in the percentage composition of FAs during the supplementation periods compared to baseline within groups are shown in Table 3.5. The p-values are presented in Appendix 3.10. The percentage compositions of n-6 and n-3 PUFAs relevant to this study are illustrated in Figures 3.1 and 3.2. Within the black group, the percentage composition of 24:1 n-9 was significantly increased after supplementation at day 30. n-6 FAs such as DGLA, AA, ADRA and DPA were significantly decreased at day 30 whereas the percentage compositions of n-3 FAs such as EPA, DPA and DHA were significantly increased.

In whites, the percentage compositions of SFAs such as BA and LGA were significantly increased after supplementation. With regards to MUFAs, OA was significantly lower at day 30 compared to baseline. The percentage compositions of DGLA, AA and ADRA (n-6 FAs) were significantly decreased at day 30 whereas the percentage compositions of n-3 FAs such as EPA, DPA and DHA were significantly increased.Significant changes in the percentage compositions of certain FAs also occurred at day 15. Within the black group, the percentage composition of 24:1 n-9 was significantly increased. Also at day 15, the percentage compositions of n-6 FAs such as DGLA, AA, ADRA and DPA were significantly decreased whereas the percentage compositions of n-3 FAs such as EPA, DPA and DHA were significantly increased. In whites, the percentage composition of PTA was significantly decreased at day 15. The percentage compositions of DGLA and ADRA (n-6 FAs) were significantly decreased. In addition, the percentage compositions of n-3 FAs such as EPA, DPA and DHA were significantly increased compared to baseline.

Intergroup comparisons

There were no significant differences observed in the concentrations of FAs between groups during the supplementation period at both days 15 and 30 (Table 3.3). The lower MA, PTA and OA concentrations observed in blacks compared to whites at both days 15 and 30 were not attributed to the effect of supplementation since they were present at baseline.

The comparison of the percentage compositions of FAs in plasma total phospholipids between groups are shown in Table 3.5. At day 30, the percentage composition of MA was lower in blacks whereas the percentage composition of DHA (n-3 FA) was higher. The differences in the percentage compositions of PTA and OA observed at days 15 and 30 were also present at baseline.

Washout period

The concentrations of FAs in plasma total phospholipids during the washout period within both groups are shown in Table 3.3. In the black population, the concentrations of OA and 24:1 n-9 were significantly higher than baseline concentrations. In addition, the concentration of DPA (n-6 FA) in plasma phospholipids was significantly lower than baseline. With regards to n-3 FAs; EPA, DPA and DHA concentrations were significantly higher than baseline concentrations. Within the white group, the concentration of ADRA (n-6 FA) was significantly higher compared to baseline concentrations. As for EPA, DHA and DPA (n-3 FAs), the concentrations of these FAs were significantly higher compared to baseline.

For both race groups, the percentage compositions of FAs in plasma total phospholipids during the washout period are shown in Table 3.5. In blacks, the percentage compositions of AA, ADRA and DPA (n-6 FAs) were significantly lower compared to baseline concentrations. On the other hand, the percentage compositions of EPA, DHA and DPA (n-3 FAs) were significantly higher than baseline values. In the white group, n-6 FAs such as AA and ADRA were significantly lower compared to baseline concentrations whereas n-3 FAs such as EPA, DPA and DHA were significantly higher than baseline.

There were no significant differences in the concentrations of FAs between groups during the washout period. On the other hand, the percentage composition of DPA (n-3 FA) in plasma phospholipids was higher in blacks compared to whites.

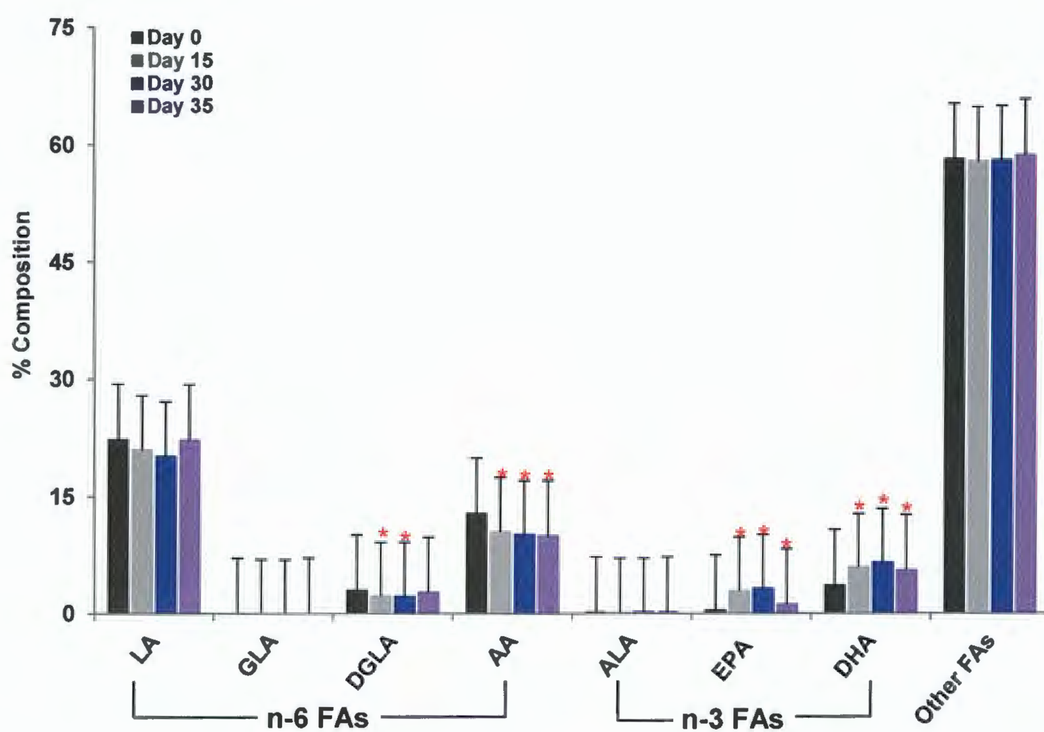

Figure 3.1: Percentage composition of PUFAs in blacks after n-3 FA supplementation

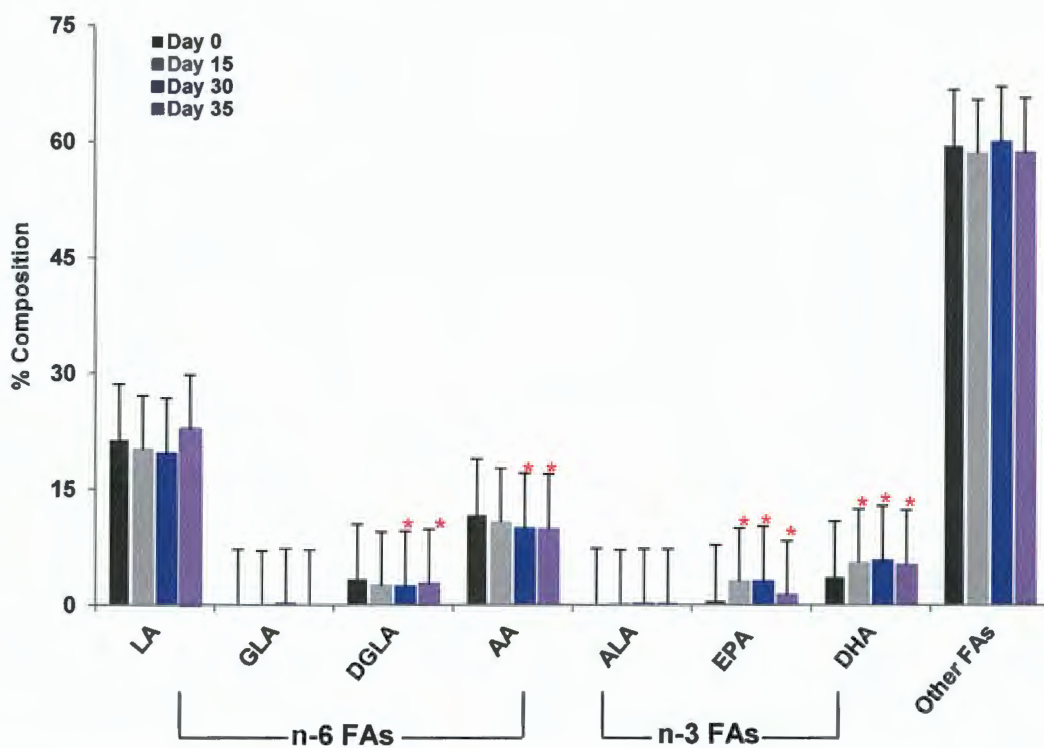

Figure 3.2: Percentage composition of PUFAs in whites after n-3 FA supplementation

3.4.5 Urinary risk factors

Baseline

Comparisons of urinary parameters between groups at baseline are shown in Table 3.7. Raw data showing the urinary composition of each subject at each sampling point are presented in Appendix 3.11. Calcium, magnesium and phosphate were significantly lower in the black group compared to the white subjects at baseline.

Table 3.7
Comparison of urinary risk factors between groups at baseline – n-3 FA study

Parameter	*Blacks*	*Whites*	p-value
pH	6.56 ± 0.15	6.23 ± 0.11	0.0821
Volume (mL/day)	1154 ± 102	1158 ± 184	0.9880
Calcium (mmol/24h)	2.63 ± 0.21	3.23 ± 0.14	0.0278‡
Magnesium (mmol/24h)	2.19 ± 0.26	3.00 ± 0.17	0.0171‡
Phosphate (mmol/24h)	24.6 ± 2.02	32.5 ± 2.23	0.0147‡
Chloride (mmol/24h)	161 ± 14.6	123 ± 21.5	0.1441
Citrate (mmol/24h)	2.12 ± 0.22	2.21 ± 0.35	0.8197
Creatinine (mmol/24h)	17.1 ± 0.97	18.1 ± 1.00	0.4967
Oxalate (mmol/24h)	0.24 ± 0.02	0.24 ± 0.03	0.9743
Potassium (mmol/24h)	47.2 ± 5.16	43.8 ± 5.06	0.6459
Sodium (mmol/24h)	147 ± 13.2	126 ± 14.4	0.2925
Urate (mmol/24h)	3.69 ± 0.29	3.80 ± 0.28	0.7908
PGE2 (ng/24h)	507 ± 83.0	627 ± 71.9	0.2925
HYP (nmol/mg creatinine)	2.85 ± 1.65	0.94 ± 0.28	0.2859
MSL (mM)	0.91 ± 0.07	0.83 ± 0.05	0.3468
TRI	202 ± 29.6	244 ± 81.2	0.6204
SS CaOx	4.13 ± 0.75	5.75 ± 0.83	0.1589
SS Brushite	1.73 ± 0.46	2.44 ± 0.47	0.3014
SS Uric acid	0.94 ± 0.24	1.87 ± 0.42	0.0637

‡ : p-value ≤ 0.05 intergroup comparison

Post-supplementation

The changes in the urinary risk factors within groups during the supplementation period are shown in Table 3.8. In the black group, there were no significant changes observed in the urinary parameters after supplementation at both days 15 and 30. Within the white group, a significant increase in the urinary concentration of magnesium was observed at day 30. In addition, the relative supersaturation of brushite was significantly decreased at day 30 compared to baseline.

Intergroup comparison

The differences in the urinary parameters between groups during the supplementation period are shown in Table 3.9. Comparison between groups showed that the relative supersaturation of brushite was significantly lower in the black group compared to whites at day 15. The other urinary parameters were not statistically different between the two groups at both days 15 and 30. However, it was noted that although the black subjects had lower urinary magnesium and phosphate compared to whites at days 15 and 30 and also lower urinary calcium at day 30; these differences were present at baseline.

Washout period

The urinary parameters of both groups during the washout period are shown in Table 3.8. In the black group, there were no significant differences observed in the urinary risk factors during the washout period compared to baseline. In whites, urinary magnesium and phosphate were significantly higher compared than baseline concentrations. There were no significant differences in the urinary risk factors between groups at day 35 (Table 3.8). The lower urinary calcium, magnesium and phosphate concentrations that were observed in the black group at baseline were also present during the washout period.

3.4.6 CaOx MSL

The average CaOx MSL for black and white subjects observed on days 15, 30 and 35 are shown in Table 3.10. The p-values are shown in Table 3.11. In both groups, there were no significant differences observed in the CaOx MSL values during the supplementation and washout periods.

The p-values for the comparison of the CaOx MSL between groups at days 15, 30 and 35 are shown in Table 3.11. At each day of the experiment, there were no significant differences observed in the CaOx MSL between groups.

Table 3.8

Urinary risk factors within groups after n-3 FA supplementation

Parameter	*Blacks*				*Whites*			
	Day 0	**Day 15**	**Day 30**	**Day 35**	**Day 0**	**Day 15**	**Day 30**	**Day 35**
pH	6.56 ± 0.15	6.23 ± 0.15	6.33 ± 0.12	6.17 ± 0.12	6.23 ± 0.11	6.09 ± 0.09	6.19 ± 0.29	5.98 ± 0.12
Volume (mL/day)	1154 ± 102	1242 ± 122	1082 ± 143	1076 ± 96.0	1158 ± 184	1072 ± 125	1128 ± 122	1214 ± 149
Calcium (mmol/24h)	2.63 ± 0.21	2.76 ± 0.28	2.71 ± 0.12	2.80 ± 0.13	3.23 ± 0.14	3.27 ± 0.18	3.61 ± 0.22	3.30 ± 0.20
Magnesium (mmol/24h)	2.19 ± 0.26	2.32 ± 0.22	2.60 ± 0.18	2.68 ± 0.19	3.00 ± 0.17	3.09 ± 0.14	3.65 ± 0.16*	3.55 ± 0.16*
Phosphate (mmol/24h)	24.6 ± 2.02	23.7 ± 1.88	26.8 ± 2.21	25.6 ± 2.13	32.5 ± 2.23	33.1 ± 3.63	37.8 ± 2.94	38.7 ± 1.26*
Chloride (mmol/24h)	161 ± 14.6	151 ± 8.90	149 ± 15.4	155 ± 11.8	123 ± 21.5	132 ± 12.5	150 ± 15.8	157 ± 18.0
Citrate (mmol/24h)	2.12 ± 0.22	2.66 ± 0.38	2.73 ± 0.43	2.46 ± 0.28	2.21 ± 0.35	2.74 ± 0.34	2.85 ± 0.28	2.74 ± 0.36
Creatinine (mmol/24h)	17.1 ± 0.97	16.5 ± 1.21	17.4 ± 1.32	17.3 ± 1.05	18.1 ± 1.00	16.3 ± 0.89	19.5 ± 1.39	19.6 ± 0.87
Oxalate (mmol/24h)	0.24 ± 0.02	0.23 ± 0.02	0.24 ± 0.02	0.23 ± 0.02	0.24 ± 0.03	0.23 ± 0.02	0.29 ± 0.02	0.27 ± 0.03
Potassium (mmol/24h)	47.2 ± 5.16	49.5 ± 3.85	45.0 ± 3.55	45.0 ± 3.53	43.8 ± 5.06	48.6 ± 4.88	43.4 ± 2.69	41.4 ± 5.44
Sodium (mmol/24h)	147 ± 13.2	126 ± 10.3	129 ± 11.9	127 ± 9.74	126 ± 14.4	131 ± 15.5	131 ± 13.3	128 ± 14.6
Urate (mmol/24h)	3.69 ± 0.29	2.98 ± 0.24	3.56 ± 0.33	3.67 ± 0.37	3.80 ± 0.28	3.50 ± 0.40	4.24 ± 0.38	4.19 ± 0.43
PGE2 (ng/24h)	507 ± 83.0	ND	542 ± 82.3	ND	627 ± 71.9	ND	548 ± 93.3	ND
HYP (nmol/mg creatinine)	2.85 ± 1.65	ND	1.52 ± 0.70	ND	0.94 ± 0.28	ND	0.76 ± 0.09	ND
MSL (mM)	0.91 ± 0.07	1.05 ± 0.08	1.08 ± 0.10	1.09 ± 0.08	0.83 ± 0.05	1.00 ± 0.07	1.02 ± 0.09	0.96 ± 0.07
TRI	202 ± 29.6	277 ± 70.4	181 ± 36.7	179 ± 28.7	244 ± 81.2	198 ± 34.8	244 ± 55.5	223 ± 60.1
SS CaOx	4.13 ± 0.75	3.95 ± 0.55	4.41 ± 0.72	4.70 ± 0.70	5.75 ± 0.83	5.92 ± 1.43	5.86 ± 0.59	5.25 ± 0.69
SS Brushite	1.73 ± 0.46	0.99 ± 0.22	1.82 ± 0.49	1.34 ± 0.26	2.44 ± 0.47	1.87 ± 0.34	1.25 ± 0.24*	1.97 ± 0.62
SS Uric acid	0.94 ± 0.24	1.60 ± 0.55	1.58 ± 0.51	2.07 ± 0.56	1.87 ± 0.42	2.02 ± 0.36	3.31 ± 0.90	2.69 ± 0.47

*: *p-value ≤ 0.05 compared to baseline (intragroup comparison)*

Table 3.9

Comparison of urinary risk factors between groups at days 15, 30 and 35 – n-3 FA study

Parameter	Day 15			Day 30			Day 35		
	Blacks	*Whites*	p-value	*Blacks*	*Whites*	p-value	*Blacks*	*Whites*	p-value
pH	6.23 ± 0.15	6.09 ± 0.09	0.4231	6.33 ± 0.12	6.19 ± 0.29	0.6533	6.17 ± 0.12	5.98 ± 0.12	0.2663
Volume (mL/day)	1242 ± 122	1072 ± 125	0.3415	1082 ± 143	1128 ± 122	0.8126	1076 ± 96.0	1214 ± 149	0.4361
Calcium (mmol/24h)	2.76 ± 0.28	3.27 ± 0.18	0.1435	2.71 ± 0.12	3.61 ± 0.22	0.0013‡	2.80 ± 0.13	3.30 ± 0.20	0.0457‡
Magnesium (mmol/24h)	2.32 ± 0.22	3.09 ± 0.14	0.0090‡	2.60 ± 0.18	3.65 ± 0.16	0.0004‡	2.68 ± 0.19	3.55 ± 0.16	0.0026‡
Phosphate (mmol/24h)	23.7 ± 1.88	33.1 ± 3.63	0.0281‡	26.8 ± 2.21	37.8 ± 2.94	0.0068‡	25.6 ± 2.13	38.7 ± 1.26	<0.0001‡
Chloride (mmol/24h)	151 ± 8.90	132 ± 12.5	0.2348	149 ± 15.4	150 ± 15.8	0.9641	155 ± 11.8	157 ± 18.0	0.9383
Citrate (mmol/24h)	2.66 ± 0.38	2.74 ± 0.34	0.8858	2.73 ± 0.43	2.85 ± 0.28	0.8276	2.46 ± 0.28	2.74 ± 0.36	0.5401
Creatinine (mmol/24h)	16.5 ± 1.21	16.3 ± 0.89	0.9114	17.4 ± 1.32	19.5 ± 1.39	0.2701	17.3 ± 1.05	19.6 ± 0.87	0.1160
Oxalate (mmol/24h)	0.23 ± 0.02	0.23 ± 0.02	0.8979	0.24 ± 0.02	0.29 ± 0.02	0.1160	0.23 ± 0.02	0.27 ± 0.03	0.3331
Potassium (mmol/24h)	49.5 ± 3.85	48.6 ± 4.88	0.8896	45.0 ± 3.55	43.4 ± 2.69	0.7231	45.0 ± 3.53	41.4 ± 5.44	0.5858
Sodium (mmol/24h)	126 ± 10.3	131 ± 15.5	0.7837	129 ± 11.9	131 ± 13.3	0.9225	127 ± 9.74	128 ± 14.6	0.9418
Urate (mmol/24h)	2.98 ± 0.24	3.50 ± 0.40	0.2648	3.56 ± 0.33	4.24 ± 0.38	0.1888	3.67 ± 0.37	4.19 ± 0.43	0.3613
PGE2 (ng/24h)	ND	ND	ND	542 ± 82.3	548 ± 93.3	0.9603	ND	ND	ND
HYP (nmol/mg creatinine)	ND	ND	ND	1.52 ± 0.70	0.76 ± 0.09	0.3134	ND	ND	ND
MSL (mM)	1.05 ± 0.08	1.00 ± 0.07	0.6073	1.08 ± 0.10	1.02 ± 0.09	0.6961	1.09 ± 0.08	0.96 ± 0.07	0.2201
TRI	277 ± 70.4	198 ± 34.8	0.3404	181 ± 36.7	244 ± 55.5	0.3451	179 ± 28.7	223 ± 60.1	0.4991
SS CaOx	3.95 ± 0.55	5.92 ± 1.43	0.1977	4.41 ± 0.72	5.86 ± 0.59	0.1439	4.70 ± 0.70	5.25 ± 0.69	0.5848
SS Brushite	0.99 ± 0.22	1.87 ± 0.34	0.0370‡	1.82 ± 0.49	1.25 ± 0.24	0.3323	1.34 ± 0.26	1.97 ± 0.62	0.3467
SS Uric acid	1.60 ± 0.55	2.02 ± 0.36	0.5362	1.58 ± 0.51	3.31 ± 0.90	0.0998	2.07 ± 0.56	2.69 ± 0.47	0.4108

‡ : *p-value ≤ 0.05 (intergroup comparison)*

Table 3.10

Average CaOx MSL within groups after n-3 FA supplementation

Parameter	*Blacks*				*Whites*			
	Day 0	**Day 15**	**Day 30**	**Day 35**	**Day 0**	**Day 15**	**Day 30**	**Day 35**
MSL (mM)	0.91 ± 0.07	1.05 ± 0.08	1.08 ± 0.10	1.09 ± 0.08	0.83 ± 0.05	1.00 ± 0.07	1.02 ± 0.09	0.96 ± 0.07

Table 3.11

P-values for the comparison of CaOx MSL within and between groups after n-3 FA supplementation

Parameter	Blacks			Whites			*B vs W*		
	Day 15	**Day 30**	**Day 35**	**Day 15**	**Day 30**	**Day 35**	**Day 15**	**Day 30**	**Day 35**
MSL (mM)	0.2047	0.1795	0.1042	0.0541	0.0746	0.1640	0.6073	0.6961	0.2201

3.5 DISCUSSION

In the present study, the effects of EPA and DHA supplementation on serum biomarkers, plasma total phospholipids and urinary risk factors were investigated in healthy black and white South African males. Several significant differences between blacks and whites in parameters related to stone formation were observed at baseline. The differences in the dietary intakes between the two groups will be discussed in Chapter 8.

3.5.1 Serum biomarkers

Baseline

The lower serum 25(OH)D concentrations in blacks subjects compared to whites (Table 3.4) are suggested to be due to the inefficient ability of the darker skin pigment of blacks to absorb UV rays [32 – 34]. This difference has been discussed in Chapter 2 – page 100.

Post supplementation

Supplementation did not show any effect on serum 25(OH)D and TAG concentrations in both population groups (Table 3.3). The unchanged 25(OH)D concentrations after supplementation are in agreement with the results obtained by Baggio et al. [7]. Vitamin D, EPA and DHA are natural constituents of oily fish [35, 36]. An increase in 25(OH)D concentrations was expected in the present study since traces of vitamin D are usually found in EPA and DHA capsules [37]. The amounts of vitamin D in the EPA and DHA capsules used in the present study were not reported by the manufacturer (Solgar, UK). In addition, the vitamin D content in the capsules could not be determined experimentally due to financial constraints. Thus, the amounts of vitamin D in these capsules could not be confirmed.

With regards to serum TAG concentrations (Table 3.3), similar results have been reported by other investigators after supplementation with EPA and DHA in healthy subjects for 4 weeks [38, 39]. Fish oil supplementation has been shown to decrease triglyceride concentrations in hypertriglyceridemic patients [40 - 42].

The most likely reason for not observing a significant change in TAG concentrations in the present study may be due to the difference in the health status of subjects, i.e. healthy subjects in the present study but stone formers in other studies.

3.5.2 Plasma total phospholipids

Baseline

The fatty acid composition in plasma total phospholipids of healthy subjects has been shown to reflect the normal dietary intake of fatty acids [43 – 45]. Thus, the differences in the concentrations and percentage compositions of fatty acids in plasma total phospholipids between the two groups observed at baseline (Table 3.4 and 3.5) are suggested to de due to the differences in the dietary habits of black and white populations. As previously described Chapter 2 – page 100, comparison of the fatty acid composition between blacks and whites with other studies was not possible because of absence of published data. In the present study, the concentrations and the percentage compositions of these fatty acids in plasma total phospholipids were found to be within the normal ranges for healthy individuals [46 – 49].

Post supplementation

In both groups, the concentrations and the percentage compositions of PUFAs in plasma total phospholipids changed in a specific manner for each individual fatty acid during the supplementation period (Table 3.3 and 3.4). A significant decrease in the percentage compositions of DGLA and AA was observed at day 30. On the other hand, the concentrations and the percentage compositions of EPA and DHA were significantly increased after supplementation. The significant changes in the percentage compositions of these fatty acids are consistent with the results observed in other studies after EPA and DHA supplementation in healthy subjects [20, 25 and 50 - 53]. Thus, these findings meet the expectations of the present study and confirm good compliance by the subjects.

At day 15, certain significant changes occurred in the concentrations and percentage compositions of n-6 and n-3 FAs in both groups (Table 3.4 and 3.5). In the black group, the significant changes in the concentrations and percentage compositions of n-6 and n-3 FAs that were observed at day 15 were also significantly different at day 30 compared to baseline. In addition to the significant changes in the concentrations and percentage compositions of n-6 and n-3 FAs that occurred at day 15, a significant increase in the concentration and percentage composition of AA was observed in whites at day 30.

These observations are consistent with the results from other n-3 FA supplementation studies where the percentage compositions of EPA and DHA in plasma total phospholipids were found to reach statistical significance compared to baseline with 1 week [54, 55] and 14 days [56]. However, in all these studies, the amounts of AA in plasma total phospholipids were not significantly reduced. The findings of the present study suggest that EPA and DHA supplementation can significantly affect the fatty acid composition in membrane phospholipids within 15 days, and that the therapeutic effects of these FAs can be achieved within this time period. However, supplementation for longer periods may be required in order to achieve maximum levels of these FAs in total phospholipids and for additional effects in membrane phospholipids to occur.

Supplementation with EPA and DHA has been shown to modulate the formation of PGE2 from AA [57, 58]. After AA is incorporated into membrane phospholipids, the phospholipase A2 enzyme releases AA from the membrane phospholipids so that it can undergo metabolism to form PGE2. EPA has been shown to inhibit the release of AA from the membrane phospholipids, thus reducing the amounts of substrate available for the production of PGE2 [57, 58]. Because EPA and DHA are incorporated into membrane phospholipids just like AA, an increase in the percentage composition of these FAs in membrane phospholipids will result in a decrease in the incorporation of AA [59, 60], further reducing the metabolism of AA into PGE2. Another beneficial effect from increased EPA and DHA percentage compositions in membrane phospholipids is that the eicosanoids produced from the metabolism of these FAs have different biological activities from those of PGE2 [61 – 63]. Thus, increased dietary intakes of EPA and DHA may be of importance as a strategy to alleviate the effects of PGE2 in CaOx stone formation.

Intergroup comparisons

In both groups, a decreasing trend in the concentrations and percentage compositions of n-6 FAs was observed during the supplementation period (Table 3.4 and 3.5). Although the concentrations of AA in plasma total phospholipids decreased during the supplementation period, statistical significance was achieved by the white group only at day 30. With regard to the percentage composition of AA in membrane phospholipids, statistical significance was observed at both days 15 and 30 in the black group, whereas in whites, AA was merely decreased significantly at day 30. In addition, it was observed that the black group had a significantly higher percentage composition of DHA at day 30 compared to whites (Table 3.5). These findings imply that the incorporation rates of certain FAs in membrane phospholipids differed between the two groups. Comparison of these observations with other studies was not possible because of absence of data. Regarding n-3 FAs, the concentrations and percentage compositions of EPA and DHA were found to be increased in a similar manner in both groups after supplementation.

Washout period

Within both groups, the concentrations and percentage compositions of n-6 FAs appeared to increase during the washout period whereas a decreasing trend in n-3 FAs was observed. Nevertheless, certain FAs were still significantly different compared to baseline values after supplementation was discontinued (Table 3.4 and 3.5). In the present study, a period of 5 days was not sufficient for FAs in plasma phospholipids to return to pre-supplementation levels. Similar findings have been reported in other studies after observing washout periods of 7 days [55], 8 weeks [25] and 24 weeks [51]. These results indicate that the effects of n-3 FA supplementation on risk factors for kidney stone formation continue for long periods after cessation of supplementation.

3.5.3 Urinary risk factors

Baseline

Consistent with observations made previously in healthy black and white South African subjects, black subjects were found to have significantly lower urinary calcium, magnesium and phosphate concentrations compared to whites [64]. The discussion for the differences in CaOx risk factors between the two groups will be addressed in Chapter 8.

Post supplementation

In the present study, there were no statistically significant changes in urinary calcium and oxalate excretion in both groups following n-3 FA supplementation (Table 3.8). These findings are contrary to those reported by Baggio et al [7] who observed significant decreases in both parameters after giving hypercalciuric stone patients EPA and DHA capsules with doses similar to the ones used in the present study for the same period of time. The discrepancy between results from the present study and those of Baggio et al [7] might be explained by the different health status of the participants in the respective studies. Healthy subjects were investigated in the present study, while hypercalciuric stone patients were investigated in the other study. This implies that FA metabolism in healthy subjects might not be the same as in stone formers. Of particular interest is that there were no significant changes observed in urinary PGE2 excretion after supplementation in both groups (Table 3.8). These results are comparable to those reported by Siener et al [65] in the normal healthy male population on a similar dose of EPA and DHA supplementation for the same duration. n-3 FA supplementation has been shown to result in decreased production of PGE2 and increased formation of n-3 eicosanoids such as prostaglandin E3 (PGE3) [61, 66 - 68]. In the present study, it is speculated that a decrease in PGE2 might have occurred if the duration of the supplementation had been longer. Cao et al [25] showed that the incorporation of EPA and DHA in phospholipids of healthy subjects is a rapid process while decreases in the percentage compositions of AA proceed more slowly. The changes in the concentrations of AA in plasma phospholipids (Table 3.3) after supplementation demonstrate the slower incorporation of AA in membrane phospholipids very aptly and are suggested to account for the absence of a decrease in PGE2.

Intergroup comparisons

As shown in Table 3.4, the response of the two groups to n-3 FA supplementation was not the same. This suggests that the metabolic pathways of FAs are sensitive to physiological features which are characteristic of each population. Regarding the lower SS brushite in blacks at day 15 (Table 3.9), this difference is suggested to be of little clinical significance since it is speculated that it may have been greatly influenced by the increase in phosphate concentration in the white group.

Washout period

Despite withdrawal of n-3 FA supplements, additional supplementation effects on urinary risk factors were observed within the white group at day 35 (Table 3.8). These observations provide further evidence that a 5 day washout period is not sufficient for reducing the membrane phospholipids levels of EPA and DHA to baseline levels.

Summary

The present study has shown that ingestion of n-3 FAs did not elicit any changes in urinary calcium and oxalate in healthy subjects. This is in contrast to previous studies involving stone-forming patients, in whom n-3 FAs were found to regulate calcium and oxalate balance. Since the present study did not involve kidney stone patients, no conclusions can be drawn regarding the efficacy or mode of action of n-3 FA supplementation in managing stone disease. However, the conflicting results in healthy subjects and stone forming patients might be indicative of different handling mechanisms in the two groups. In addition, the study has demonstrated that although 30 days of supplementation may be adequate for altering the FA composition in membrane phospholipids, supplementation for longer periods may be beneficial for producing positive effects in urinary CaOx risk factors. Lastly, the findings of the present study have again demonstrated that blacks and whites differ in their response to supplemental challenges.

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Chapter 4

Investigation of the effects of n-6 & n-3 fatty acids (GLA, EPA and DHA) in combination on blood and urinary risk factors for CaOx urolithiasis

4.1 INTRODUCTION

Previous studies have shown that supplementation with GLA results in an increase in the percentage composition of DGLA in plasma phospholipids [1 - 5]. High amounts of DGLA in membrane phospholipids may be beneficial for kidney stone formation because of two reasons. Firstly, a high percentage composition of DGLA will result in a decrease in the levels of AA in plasma phospholipids [6 - 8]; which is favourable because it implies a reduction in the production of PGE2. Secondly, a high percentage composition of DGLA in membrane phospholipids is suggested to favour the formation of prostaglandin E1 (PGE1), which has anti-inflammatory properties that suppress those of PGE2 [9, 10].

The main concern with regard to increases in DGLA is that the metabolism of this FA may also lead to the formation of AA [11 – 13]. Johnson et al demonstrated that supplementation with 1.5, 3.0 and 6.0 g GLA per day for 3 weeks significantly increased the concentrations of AA in healthy subjects [14]. Therefore, supplementation with GLA for longer periods may possibly increase PGE2 production with subsequent elevations in the urinary concentrations of calcium and oxalate.

Nonetheless, supplementation with EPA and DHA has been shown to replace the n-6 FAs content, especially AA, in membrane phospholipids with these FAs [15 – 18]. Thus, addition of EPA and DHA capsules to dietary intakes during GLA supplementation may complement the effects of DGLA by blocking the accumulation of AA in membrane phospholipids.

In view of this, the present study was undertaken to investigate the influence of dietary supplementation with a combination of n-6 and n-3 FAs on the fatty acid composition in plasma total phospholipids of healthy black and white South African subjects. The effects of n-6 and n-3 FAs supplementation on serum and urinary risk factors for CaOx stone formation were also studied. Furthermore, the effects of supplementation in black and white South African subjects were investigated to determine whether this might lead to new insights as to why the incidence of stone disease is lower in the black group.

4.2 STUDY PROTOCOL

Participants were recruited from students enrolled at the University of Cape Town – South Africa. The Human Research Ethics Committee of the University of Cape Town - South Africa, approved (**HREC REF: 366/2011**) the study (Appendix 4.1) and all volunteers gave informed consent before taking part in the study. Participants were excluded if they had metabolic disorders or medical conditions such as heart disease, diabetes and urinary tract diseases. During the course of the study, none of the participants were taking any medication including antibiotics, vitamins or fatty acid supplements.

10 Black and 10 white healthy South African males participated in the study. This samples size was chosen because it was sufficient to give a statistical power of 80 %, as described in Chapter 2 – page 69. Seven of the original 10 subjects from the white group and 10 subjects from the black group completed the study. Before the administration of n-6 and n-3 FAs capsules to subjects, blood and 24h urine samples were collected at baseline as control samples. Subjects recorded their food intakes in a food diary (Appendix 2.5); and while they were collecting samples, they were required to avoid foods that have high oxalate content (Appendix 2.6).

Subjects were required to take a combination of n-3 FAs (1.08 g EPA and 0.72 g DHA; Omega-3 Double strength) and n-6 FAs (0.30 g GLA; One-a-day GLA) supplements that were obtained from Solga, UK. Each n-3 FA capsule was reported to contain 0.36 g EPA, 0.24 g DHA and mixed tocopherols as antioxidants. The n-6 FA capsule contained 225 mg LA, 150 mg GLA and 250 mg of other fatty acids. Specifically, subjects were required to consume a total of 3 n-3 FA capsules (i.e. one during breakfast, one during lunch and one during supper) and a total of 2 n-6 FAs capsules (one during breakfast and one during lunch) daily, over a period of 30 days.

The dose for n-6 FA capsules was chosen in accordance with the findings described in Chapter 2. The capsules that contained the dose used in Chapter 3 for n-3 FA supplementation were no longer provided by the manufacturer (Solgar – UK). As a result, a dose (1.80 g EPA and DHA) similar to the one (1.92 g EPA and DHA) used in Chapter 3 was chosen because its effect on the fatty acid composition in plasma total phospholipids was anticipated to be similar to the latter.

Further, the chosen dosages for GLA, EPA and DHA were within the recommended daily intakes for healthy individuals [19, 20].

Additional blood samples were obtained at days 0 and 30 while 24h urine samples were collected during the supplementation period, at days 15 and 30; and at day 35 during the washout period. The food intakes recorded at baseline were consumed again by subjects on blood and 24h urine sample collecting days.

4.3 EXPERIMENTAL ANALYSIS

4.3.1 Nutrient intakes

The energy and nutrient intakes of subjects at baseline were calculated using the Foodfinder II computer program as described in Chapter 2 – page 71.

4.3.2 Blood analysis

The collected fasting blood samples were separated into different fractions as described in Chapter 2 – page 71. Serum 25(OH)D and TAGs concentrations were measured at Pathcare Laboratories and South African Medical Research Council, respectively; using the procedure described in Chapter 2 - page 72. Plasma and RBCs samples were sent to South African Medical Research Council and the FA profile in each sample was determined using the method described in Chapter 2 – page 72.

4.3.3 Urinary risk factors

The urinary composition of each 24h urine samples was evaluated as described in Chapter 2 – page 76.

4.3.4 Crystallization experiments

The CaOx MSL and crystallization kinetics were determined in all urine samples using the spectrophotometer as described in Chapter 2 – page 80.

4.3.5 Risk indices

The TRI and SS values of CaOx, brushite and uric acid were calculated in all 24h urine samples as described in Chapter 2 – page 81.

4.3.6 Statistical analysis

Data were analyzed using *GraphPad Instat* and a p-value ≤ 0.05 was regarded as statistically significant. All the results are reported as average values with standard error (SE). Graphs with standard error bars were prepared with Microsoft Excel 2010.

4.4. RESULTS

4.4.1 Subject's characteristics

The mean age and BMI of subjects at baseline are shown in Table 4.1. Raw data for the subjects' characteristics recorded at baseline are presented in Appendix 4.2. Intergroup comparison showed that the subject's characteristics were not significantly different.

Table 4.1
Subject's characteristics at baseline – n-6 & n-3 FA study

Parameter	*Blacks*	*Whites*	p-value
Age (years)	21.8 ± 0.51	21.9 ± 0.91	0.9539
BMI (kg/m^2)	22.6 ± 1.24	23.1 ± 1.00	0.7881

4.4.2 Nutrient intake assessment

Raw data for each subject's nutrient intake recorded at baseline are presented on Appendix 4.3. The results for the nutrient intakes for the two groups are going to be discussed in Chapter 8.

4.4.3 Supplement compliance test

Subject's adherence to the supplement protocol was determined using the two techniques described in Chapter 2 – page 83. Firstly, the number of capsules remaining after the supplementation period was counted to determine compliance. One participant from the white group missed taking the supplements for 2 days. The other remaining 16 subjects did not have leftover capsules at day 30, showing that they had adhered to the supplement protocol.

Secondly, the changes in the concentration of EPA, DHA and GLA in RBC total phospholipids after supplementation compared to baseline were calculated as a measure of supplement compliance. These FAs were chosen because they were the major components of the n-6 and n-3 FA capsules given to subjects. 5 Black and 5 white subjects were randomly selected from the study participants for the compliance test.

The concentrations of EPA and DHA in RBCs total phospholipids of the selected subjects at days 0 and 30 are shown in Table 4.2. Raw data showing the FA composition (as concentration (µg/mL)) in RBC total phospholipids for each individual are presented in Appendix 4.4. The percentage changes of FAs after supplementation were calculated as shown in Chapter 2 – page 83.

In both groups, supplementation significantly increased the concentrations of EPA in plasma total phospholipids at day 30. In blacks, the mean percentage change for EPA in plasma total phospholipids was 530 % (p-value < 0.0001), whereas in whites, a mean percentage change of 315 % (p-value <0.0001) was observed. The concentrations of DHA in plasma phospholipids increased to a lesser degree in comparison to those of EPA. The mean percentage change for DHA in plasma total phospholipids was 46 % (p-value = 0.0871) in blacks and 39 % (p-value = 0.0305) in whites. These results are in agreement with the changes observed in Chapter 3 – page 120 and with those in other studies where the n-3 FA dose similar to the one used in the present study was given to healthy subjects [15, 21 and 22].

Concentrations of GLA in RBC total phospholipids after supplementation in the majority of subjects were extremely low (Table 4.2). As such, the percentage changes in GLA concentrations in plasma total phospholipids could not be determined as a measure of compliance for the n-6 FA capsules.

As an alternative, the changes in the concentrations of DGLA in RBC phospholipids were calculated to determine whether the absence of an increase in GLA concentrations was due to the metabolism of GLA to DGLA [11 – 13]. The concentrations of DGLA in RBC total phospholipids for each subject at days 0 and 30 are shown in Table 4.2. The mean percentage change for DGLA was -11.2 % (p-value = 0.5924) in blacks and -5.9 % (p-value = 0.4502) in whites. These values show that there was no increase in the concentration of DGLA after supplementation. It was therefore concluded that the concentrations of DGLA in RBC total phospholipids do not reflect the metabolism of GLA to DGLA. The explanations for the low GLA and DGLA concentrations in RBC total phospholipids after GLA supplementation are described in Chapter 2 – page 83. In the present study, the changes in the concentrations of GLA were not determined in other lipid fractions because of financial constraints.

As a result, n-6 FA capsule compliance by subjects could not be confirmed. However, since subjects were instructed to take the n-6 FA capsules together with n-3 FA capsules during meal times, it was reasonably assumed that they had complied.

Overall, it was concluded that subjects had adhered to the supplement protocol since > 85 % of the fatty acid capsules given to subjects were consumed [23, 24]; and because of the changes in the concentrations of EPA and DHA in RBC total phospholipids that were observed.

Table 4.2

Concentrations (µg/mL) of GLA, DGLA, EPA and DHA in RBC total phospholipids after n-6 & n-3 FA supplementation

Subjects	EPA			DHA			GLA			DGLA		
	Day 0	Day 30	% Change	Day 0	Day 30	% Change	Day 0	Day 30	% Change	Day 0	Day 30	% Change
B-1	1.30	7.81	500	18.5	27.4	48	trace	trace	ND	7.86	3.49	-56
B-2	1.81	8.71	383	35.1	47.1	34	trace	trace	ND	8.25	8.40	2
B-3	2.06	13.0	533	38.5	52.6	37	trace	0.9	ND	15.3	13.5	-11
B-4	1.60	12.7	698	32.5	39.7	22	trace	1.9	ND	11.8	9.47	-20
B-5	1.79	11.4	537	18.8	35.8	90	trace	trace	ND	7.85	10.1	29
W-1	2.96	12.0	306	25.7	41.0	59	trace	trace	ND	10.6	10.7	1
W-2	2.44	11.8	386	30.5	49.9	63	trace	trace	ND	12.4	13.2	7
W-3	3.06	12.0	292	36.3	45.9	26	trace	trace	ND	13.8	14.5	5
W-4	5.12	14.3	178	48.0	53.4	11	trace	trace	ND	17.9	13.2	-26
W-5	2.74	14.1	413	30.8	40.8	32	trace	trace	ND	15.2	12.8	-16

ND = not determined

B = blacks and W = whites

4.4.4 Serum biomarkers and FA composition in plasma total phospholipids

Baseline

The differences in the concentrations of serum biomarkers and fatty acids in plasma total phospholipids between the black and white subjects observed at baseline are shown in Table 4.3. Raw data showing the concentrations of 25(OH)D and TAGs for each subject are shown in Appendix 4.5 and 4.6 respectively. Raw data showing the concentrations of FAs in plasma phospholipids for each individual are presented in Appendix 4.7. Serum 25(OH)D concentrations were significantly lower in the black group compared to the white subjects.

The differences in the concentrations of FAs between the two groups at baseline are summarized in Table 4.4. The comparison of the percentage composition of FAs in plasma phospholipids between the two groups at baseline are shown in Table 4.5. Raw data showing the percentage composition of FAs in plasma phospholipids for each individual are presented in Appendix 4.8. There were no significant differences observed in the percentage composition of FAs between the groups observed at baseline.

Table 4.3

Concentrations of serum biomarkers and FAs in plasma total phospholipids after n-6 & n-3 supplementation

Parameter	*Blacks*			*Whites*			*B vs W (p-values)*	
	Day 0	Day 30	p-value	Day 0	Day 30	p-value	Day 0	Day 30
Serum biomarkers								
25(OH)D (ng/mL)	17.1 ± 2.16	18.9 ± 2.00	0.5594	36.3 ± 4.05	34.5 ± 2.62	0.7067	0.0005‡	0.0003‡
TAGs (mmol/L)	0.59 ± 0.10	0.58 ± 0.07	0.9609	0.90 ± 0.16	0.77 ± 0.09	0.4784	0.0976	0.1172
SFAs (µg/mL)								
C14:0 MA	2.97 ± 0.62	1.73 ± 0.12	0.0659	3.20 ± 0.47	3.10 ± 0.46	0.8813	0.7890	0.0041‡
C16:0 PA	231 ± 11.5	211 ± 12.8	0.2723	279 ± 17.5	255 ± 15.8	0.3216	0.0299‡	0.0491‡
C18:0 SA	125 ± 5.94	126 ± 5.85	0.9002	135 ± 8.25	141 ± 8.52	0.6147	0.3493	0.1629
20:0 ARA	4.42 ± 0.28	3.99 ± 0.29	0.3015	5.29 ± 0.47	5.71 ± 0.62	0.5934	0.1147	0.0141‡
22:0 BA	13.9 ± 0.95	12.3 ± 1.10	0.2803	13.8 ± 1.10	15.5 ± 1.64	0.4139	0.9531	0.1121
24:0 LGA	13.5 ± 0.99	11.6 ± 1.01	0.1881	13.8 ± 0.84	15.4 ± 1.53	0.4018	0.8023	0.0464‡
MUFAs (µg/mL)								
C16:1 n-7 PTA	2.77 ± 0.37	2.09 ± 0.28	0.1635	3.76 ± 0.77	2.39 ± 0.34	0.1278	0.2193	0.5079
C18:1 n-9 OA	59.6 ± 4.98	53.5 ± 4.23	0.3665	86.3 ± 7.15	71.2 ± 4.14	0.0919	0.0063‡	0.0116‡
C18:1 n-7 VA	10.5 ± 0.80	9.18 ± 0.68	0.2341	13.1 ± 1.17	11.5 ± 0.63	0.2444	0.0710	0.0321‡
20:1 n-9 GA	1.25 ± 0.09	1.14 ± 0.07	0.3790	1.37 ± 0.09	2.00 ± 0.50	0.2829	0.4528	0.0635
24:1 n-9	15.7 ± 1.40	15.3 ± 1.28	0.8434	18.1 ± 1.02	19.3 ± 2.22	0.6399	0.2164	0.1160
n-6 FAs (µg/mL)								
C18:2 n-6 LA	178 ± 7.26	175 ± 13.3	0.8509	222 ± 9.11	212 ± 9.96	0.4619	0.0015‡	0.0565
C18:3 n-6 GLA	ND	ND	ND	ND	ND	ND	ND	ND
C20:2 n-6 EDA	3.27 ± 0.18	2.71 ± 0.14	0.0241*	3.10 ± 0.13	3.21 ± 0.23	0.6789	0.4886	0.0709
C20:3 n-6 DGLA	24.0 ± 3.30	18.9 ± 2.01	0.1993	29.8 ± 3.81	24.4 ± 2.96	0.2814	0.2710	0.1303
C20:4 n-6 AA	111 ± 7.04	86.2 ± 5.81	0.0140*	112 ± 14.3	87.7 ± 8.17	0.1646	0.9462	0.8786
C22:4 n-6 ADRA	4.46 ± 0.36	2.41 ± 0.24	0.0002*	4.30 ± 0.46	2.66 ± 0.28	0.0096*	0.7847	0.5090
C22:5 n-6 DPA	3.57 ± 0.37	2.02 ± 0.24	0.0095*	3.11 ± 0.60	2.33 ± 0.48	0.3961	0.5054	0.5400
n-3 FAs (µg/mL)								
C18:3 n-3 ALA	ND	ND	ND	ND	ND	ND	ND	ND
C20:5 n-3 EPA	3.14 ± 0.46	26.5 ± 1.68	<0.0001*	5.09 ± 1.16	26.1 ± 4.08	0.0003*	0.0990	0.9176
22:5 n-3 DPA	5.82 ± 0.60	12.8 ± 1.90	0.0027*	7.69 ± 0.69	11.2 ± 0.97	0.0114*	0.0608	0.5400
C22:6 n-3 DHA	30.2 ± 2.09	49.4 ± 3.41	0.0001*	32.1 ± 2.25	48.0 ± 3.61	0.0029*	0.5540	0.7785

* : *p-value ≤ 0.05 compared to baseline (intragroup comparison)*

‡: *p-value ≤ 0.05 (intergroup comparison)*

Table 4.4 Summary of significant differences in concentrations of FAs (µg/mL) between groups at day 0 – n-6 & n-3 FA study		
FA class	Type of FA	B vs W
SFA	PA	B < W‡
MUFA	OA	B < W‡
PUFA	LA (n-6 FA)	B < W‡

‡: p-value ≤ 0.05 (intergroup comparison)

Post-supplementation

The concentrations of serum biomarkers and FAs in plasma phospholipids at day 30 are shown in Table 4.3. In both groups, the concentrations of serum 25(OH)D and TAGs at day 30 were not statistically different to those observed at baseline. In the black group, significant decreases in the concentrations of n-6 FAs such as EDA, AA, ADRA and DPA were observed after supplementation. On the other hand, concentrations of n-3 FAs such as EPA, DPA and DHA were significantly increased at day 30 compared to baseline. Within the white group, the concentration of ADRA (n-6 FA) was significantly reduced at day 30 whereas concentrations of EPA, DPA and DHA (n-3 FAs) were significantly increased. There were no significant changes in the concentrations of SFAs and MUFAs after supplementation in both groups. The differences in the concentrations of FAs in plasma total phospholipids between groups at day 30 are shown in Table 4.3. Comparison of the fatty acid concentrations in plasma total phospholipids between groups at day 30 showed VA (MUFA), and SFAs such as MA, ARA and LGA to be significantly lower in the black group. Similar to the observations at baseline, the concentrations of 25(OH)D, PA (SFA) and OA (MUFA) were found to be significantly lower in the black group at day 30.

The percentage compositions of FAs in plasma phospholipids at day 30 are shown in Table 4.5. The changes in the percentage composition of PUFAs in blacks and whites relevant to the present study are illustrated in Figures 4.1 and 4.2, respectively. Within the black group, the percentage compositions of PA (SFA) and n-6 FAs such as EDA, AA, ADRA and DPA were significantly decreased at day 30. In contrast, significant increases in the percentage compositions of n-3 FAs such as EPA, DPA and DHA were observed after supplementation.

Table 4.5
Percentage composition of FAs in plasma total phospholipids after n-6 & n-3 FA supplementation

Parameter	*Blacks*			*Whites*			*B vs W (p-values)*	
	Day 0	Day 30	p-value	Day 0	Day 30	p-value	Day 0	Day 30
SFAs								
C14:0 MA	0.37 ± 0.09	0.21 ± 0.01	0.0996	0.33 ± 0.05	0.31 ± 0.03	0.7877	0.7494	0.0066‡
C16:0 PA	27.3 ± 0.46	25.6 ± 0.59	0.0340*	28.1 ± 0.54	26.4 ± 0.42	0.0268*	0.2767	0.3253
C18:0 SA	14.9 ± 0.50	15.4 ± 0.38	0.4173	13.6 ± 0.37	14.6 ± 0.33	0.0549	0.0763	0.1749
20:0 ARA	0.52 ± 0.02	0.49 ± 0.03	0.3643	0.54 ± 0.05	0.59 ± 0.05	0.5172	0.7009	0.0765
22:0 BA	1.66 ± 0.11	1.50 ± 0.12	0.3356	1.42 ± 0.12	1.60 ± 0.13	0.3265	0.1671	0.5761
24:0 LGA	1.60 ± 0.11	1.40 ± 0.11	0.2041	1.42 ± 0.11	1.59 ± 0.11	0.3105	0.2639	0.2703
MUFAs								
C16:1 n-7 PTA	0.33 ± 0.04	0.24 ± 0.03	0.0894	0.37 ± 0.06	0.24 ± 0.03	0.0803	0.5368	0.9700
C18:1 n-9 OA	7.03 ± 0.43	6.47 ± 0.33	0.3195	8.64 ± 0.26	7.45 ± 0.42	0.0329*	0.0125	0.0825
C18:1 n-7 VA	1.24 ± 0.06	1.11 ± 0.04	0.0993	1.31 ± 0.06	1.20 ± 0.06	0.204	0.3914	0.1981
20:1 n-9 GA	0.15 ± 0.02	0.13 ± 0.01	0.3816	0.13 ± 0.02	0.22 ± 0.04	0.13	0.6817	0.0304‡
24:1 n-9	1.86 ± 0.15	1.87 ± 0.14	0.9848	1.86 ± 0.13	1.98 ± 0.15	0.549	0.9821	0.5966
n-6 FAs								
C18:2 n-6 LA	21.2 ± 0.91	21.2 ± 1.14	0.9876	22.7 ± 1.16	22.4 ± 1.52	0.8724	0.3327	0.5411
C18:3 n-6 GLA	ND	ND	ND	ND	ND	ND	ND	ND
C20:2 n-6 EDA	0.39 ± 0.02	0.33 ± 0.01	0.0130*	0.32 ± 0.02	0.33 ± 0.02	0.4659	0.0123	0.9083
C20:3 n-6 DGLA	2.76 ± 0.28	2.31 ± 0.23	0.2178	2.96 ± 0.27	2.54 ± 0.27	0.2892	0.6312	0.5276
C20:4 n-6 AA	13.1 ± 0.34	10.5 ± 0.36	<0.0001*	11.1 ± 0.88	9.05 ± 0.54	0.0739	0.0298	0.0390‡
C22:4 n-6 ADRA	0.53 ± 0.03	0.29 ± 0.03	<0.0001*	0.43 ± 0.02	0.27 ± 0.02	0.0003*	0.02	0.7044
C22:5 n-6 DPA	0.42 ± 0.03	0.23 ± 0.02	0.0006*	0.31 ± 0.04	0.22 ± 0.04	0.2207	0.0428	0.8380
n-3 FAs								
C18:3 n-3 ALA	ND	ND	ND	ND	ND	ND	ND	ND
C20:5 n-3 EPA	0.37 ± 0.05	3.26 ± 0.21	<0.0001*	0.49 ± 0.10	2.67 ± 0.34	<0.0001*	0.2273	0.1396
22:5 n-3 DPA	0.68 ± 0.04	1.56 ± 0.23	0.0013*	0.77 ± 0.04	1.17 ± 0.08	0.0011*	0.1871	0.1930
C22:6 n-3 DHA	3.57 ± 0.16	6.07 ± 0.40	<0.0001*	3.24 ± 0.14	5.03 ± 0.36	0.0006*	0.1688	0.0858

** : p-value ≤ 0.05 compared to baseline (intragroup comparison)*

‡ : p-value ≤ 0.05 (intergroup comparison)

In the white group, the percentage composition of ADRA (n-6 FA) was significantly decreased at day 30. On the other hand, the percentage compositions of EPA, DPA and DHA (n-3 FAs) in plasma total phospholipids were significantly increased after supplementation.

The differences in the percentage composition of FAs in plasma TPL between groups at day 30 are shown in Table 4.5. After supplementation, the percentage composition of MA (SFA) and GA (MUFA) were found to be significantly lower in blacks compared to whites, whereas AA (n-6 FA) was significantly higher.

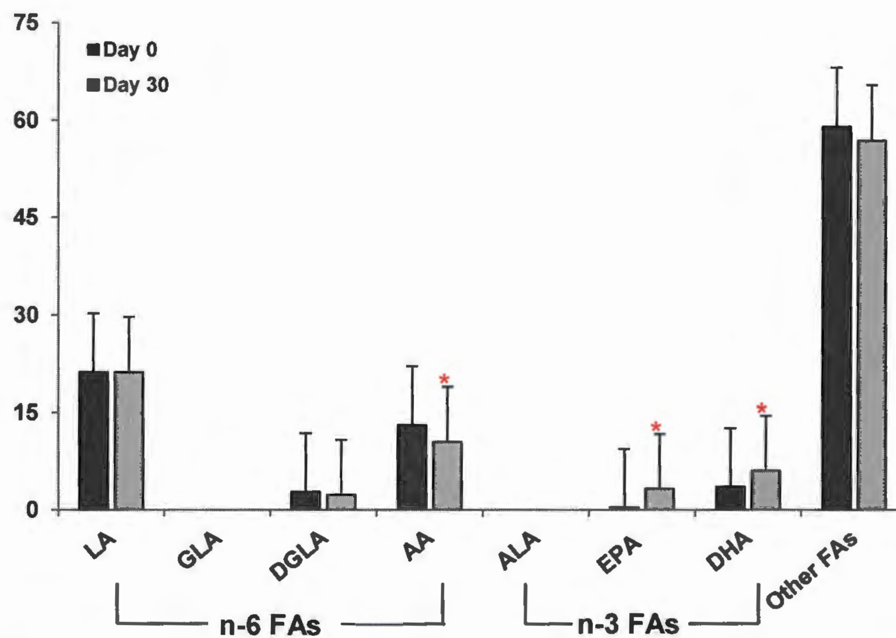

Figure 4.1: Percentage composition of PUFAs in blacks after n-6 & n-3 FA supplementation

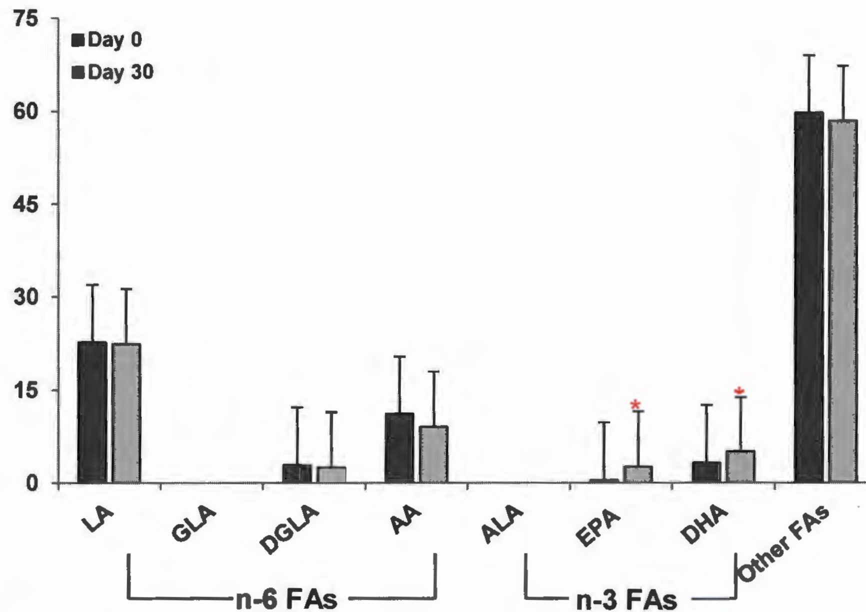

Figure 4.2: Percentage composition of PUFAs in whites after n-6 & n-3 FA supplementation

4.4.5 Urinary risk factors

Baseline

The differences in the urinary parameters between groups at baseline are shown in Table 4.6. Raw data showing the urinary parameters of each subject are presented in Appendix 4.9. The comparison of urinary composition between groups at baseline showed urinary calcium, magnesium and RS brushite to be significantly lower in blacks, whereas urinary sodium was significantly higher. Urinary calcium, magnesium and RS brushite remained significantly lower in blacks during the supplementation and washout periods.

Post-supplementation

The changes in the urinary risk factors within groups during the supplementation period are shown in Table 4.7. The p-values are presented in Appendix 4.10. In the black group at day 30, urinary citrate was significantly increased compared to baseline whereas urinary oxalate and RS CaOx were significantly decreased. In whites, a significant increase in urinary magnesium was observed at day 30.

Table 4.6 Comparison of urinary risk factors between groups at baseline – n-6 & n-3 FA study			
Parameter	***Blacks***	***Whites***	**p-value**
pH	6.34 ± 0.34	6.15 ± 0.16	0.6718
Volume (mL/24h)	1397 ± 210	1361 ± 281	0.9172
Calcium (mmol/24h)	1.77 ± 0.20	2.46 ± 0.22	0.0358‡
Magnesium (mmol/24h)	1.55 ± 0.20	2.29 ± 0.22	0.0260‡
Phosphate (mmol/24h)	24.1 ± 2.56	30.5 ± 1.83	0.0821
Chloride (mmol/24h)	136 ± 12.4	118 ± 20.0	0.4284
Citrate (mmol/24h)	2.03 ± 0.38	1.78 ± 0.25	0.6272
Creatinine (mmol/24h)	16.2 ± 0.96	17.4 ± 0.79	0.4020
Oxalate (mmol/24h)	0.31 ± 0.03	0.32 ± 0.06	0.9184
Potassium (mmol/24h)	33.2 ± 2.17	27.4 ± 3.30	0.1477
Sodium (mmol/24h)	128 ± 9.96	90.0 ± 9.60	0.0193‡
Urate (mmol/24h)	3.07 ± 0.29	3.83 ± 0.22	0.0729
Ionized Ca^{2+} (mmol/L)	0.37 ± 0.07	0.52 ± 0.16	0.3662
PGE2 (ng/24h)	604 ± 88.4	509 ± 70.7	0.4460
HYP (nmol/mg creatinine)	2.14 ± 1.33	0.89 ± 0.24	0.3827
MSL (mM)	1.12 ± 0.14	0.99 ± 0.13	0.5145
TRI	179 ± 19.9	203 ± 43.1	0.5821
SS CaOx	3.78 ± 0.68	5.48 ± 1.08	0.1808
SS Brushite	0.52 ± 0.10	1.32 ± 0.44	0.0497‡
SS Uric acid	1.91 ± 0.54	2.25 ± 0.72	0.7082

‡ : *p-value ≤ 0.05 (intergroup comparison)*

The differences in the urinary parameters between groups during the supplementation period are shown in Table 4.8. The comparison of urinary parameters between groups at day 30 showed phosphate, creatinine, urate and SS CaOx to be significantly lower in the black group. At day 15, it was observed that n-6 & n-3 FA supplementation had an effect on the urinary parameters in both groups. Within the black group, creatinine, oxalate, potassium and urate were significantly decreased compared to baseline. In whites, magnesium was significantly increased at day 15 compared to baseline concentrations.

Similarly to the observations made at day 30, the comparison of urinary parameters between groups at day 15 showed phosphate, creatinine, urate and SS CaOx to be significantly lower in the black group (Table 4.8).

Washout period

The urinary compositions of the two groups during the washout period are shown in Table 4.7. In the black group, urinary oxalate and sodium were significantly lower than baseline concentrations at day 35. In whites, phosphate, creatinine and urate were significantly lower than baseline during washout. There were no significant differences in the urinary parameters between groups during the washout period (Table 4.8).

Table 4.7
Urinary risk factors within groups after n-6 & n-3 FA supplementation

Parameter	*Blacks*				*Whites*			
	Day 0	**Day 15**	**Day 30**	**Day 35**	**Day 0**	**Day 15**	**Day 30**	**Day 35**
pH	6.34 ± 0.34	6.52 ± 0.31	6.51 ± 0.35	6.16 ± 0.27	6.15 ± 0.16	6.28 ± 0.20	6.10 ± 0.20	6.09 ± 0.14
Volume (mL/day)	1397 ± 210	1298 ± 187	1498 ± 224	1369 ± 206	1361 ± 281	1109 ± 119	1342 ± 278	1106 ± 199
Calcium (mmol/24h)	1.77 ± 0.20	1.64 ± 0.20	1.67 ± 0.10	1.75 ± 0.17	2.46 ± 0.22	2.63 ± 0.09	2.73 ± 0.10	2.40 ± 0.11
Magnesium (mmol/24h)	1.55 ± 0.20	1.24 ± 0.17	1.88 ± 0.12	1.77 ± 0.22	2.29 ± 0.22	2.88 ± 0.08*	3.01 ± 0.15*	2.57 ± 0.12
Phosphate (mmol/24h)	24.1 ± 2.56	19.6 ± 2.04	20.8 ± 1.90	22.7 ± 1.79	30.5 ± 1.83	30.5 ± 1.28	29.7 ± 1.60	24.3 ± 1.67*
Chloride (mmol/24h)	136 ± 12.4	132 ± 8.39	143 ± 16.7	115 ± 17.1	118 ± 20.0	126 ± 27.8	108 ± 19.1	85.7 ± 15.3
Citrate (mmol/24h)	2.03 ± 0.38	2.29 ± 0.34	3.92 ± 0.55*	2.91 ± 0.45	1.78 ± 0.25	1.76 ± 0.16	2.42 ± 0.40	1.68 ± 0.31
Creatinine (mmol/24h)	16.2 ± 0.96	13.6 ± 0.65*	14.4 ± 0.84	14.2 ± 0.86	17.4 ± 0.79	16.2 ± 1.00	16.7 ± 0.29	12.1 ± 0.77*
Oxalate (mmol/24h)	0.31 ± 0.03	0.21 ± 0.03*	0.23 ± 0.03*	0.21 ± 0.03*	0.32 ± 0.06	0.27 ± 0.05	0.23 ± 0.04	0.19 ± 0.03
Potassium (mmol/24h)	33.2 ± 2.17	26.4 ± 1.49*	33.6 ± 1.77	27.2 ± 2.21	27.4 ± 3.30	28.8 ± 3.40	30.4 ± 2.79	27.7 ± 2.38
Sodium (mmol/24h)	128 ± 9.96	107 ± 6.08	107 ± 5.22	93.0 ± 8.62*	90.0 ± 9.60	95.3 ± 9.47	93.3 ± 7.30	87.0 ± 10.0
Urate (mmol/24h)	3.07 ± 0.29	2.35 ± 0.18*	2.40 ± 0.19	2.77 ± 0.31	3.83 ± 0.22	3.67 ± 0.29	3.36 ± 0.26	2.59 ± 0.20*
Ionized Ca^{2+} (mmol/L)	0.37 ± 0.07	0.51 ± 0.13	0.31 ± 0.09	0.32 ± 0.08	0.52 ± 0.16	0.39 ± 0.09	0.26 ± 0.08	0.34 ± 0.09
PGE2 (ng/24h)	604 ± 88.4	ND	649 ± 132	ND	509 ± 70.7	ND	851 ± 203	ND
HYP (nmol/mg creatinine)	2.14 ± 1.33	ND	2.04 ± 1.05	ND	0.89 ± 0.24	ND	0.89 ± 0.20	ND
MSL (mM)	1.12 ± 0.14	0.83 ± 0.12	1.16 ± 0.28	1.35 ± 0.13	0.99 ± 0.13	0.94 ± 0.18	0.75 ± 0.11	1.10 ± 0.19
TRI	179 ± 19.9	159 ± 34.0	133 ± 14.5	136 ± 18.6	203 ± 43.1	198 ± 41.5	145 ± 20.6	204 ± 31.1
SS CaOx	3.78 ± 0.68	2.31 ± 0.42	1.63 ± 0.24*	2.82 ± 0.59	5.48 ± 1.08	5.24 ± 0.67	4.07 ± 0.79	4.75 ± 0.78
SS Brushite	0.52 ± 0.10	0.50 ± 0.11	0.39 ± 0.11	0.43 ± 0.09	1.32 ± 0.44	1.72 ± 0.34	1.44 ± 0.50	1.44 ± 0.39
SS Uric acid	1.91 ± 0.54	1.29 ± 0.57	0.88 ± 0.29	1.83 ± 0.34	2.25 ± 0.72	2.18 ± 0.97	2.31 ± 0.91	2.30 ± 0.83

* : *p-value ≤ 0.05 compared to baseline (intragroup comparison)*

Table 4.8

Comparison of urinary risk factors between groups at days 15, 30 and 35 – n-6 & n-3 FA study

Parameter	Day 15			Day 30			Day 35		
	Blacks	***Whites***	**p-value**	***B***	***W***	**p-value**	***B***	***W***	**p-value**
pH	6.52 ± 0.31	6.28 ± 0.20	0.5762	6.51 ± 0.35	6.10 ± 0.20	0.3689	6.16 ± 0.27	6.09 ± 0.14	0.8497
Volume (mL/24h)	1298 ± 187	1109 ± 119	0.4540	1498 ± 224	1342 ± 278	0.6657	1369 ± 206	1106 ± 199	0.3933
Calcium (mmol/24h)	1.64 ± 0.20	2.63 ± 0.09	0.0014‡	1.67 ± 0.10	2.73 ± 0.10	<0.0001‡	1.75 ± 0.17	2.40 ± 0.11	0.0090‡
Magnesium (mmol/24h)	1.24 ± 0.17	2.88 ± 0.08	<0.0001‡	1.88 ± 0.12	3.01 ± 0.15	<0.0001‡	1.77 ± 0.22	2.57 ± 0.12	0.0110‡
Phosphate (mmol/24h)	19.6 ± 2.04	30.5 ± 1.28	0.0009‡	20.8 ± 1.90	29.7 ± 1.60	0.0039‡	22.7 ± 1.79	24.3 ± 1.67	0.5585
Chloride (mmol/24h)	132 ± 8.39	126 ± 27.8	0.8024	143 ± 16.7	108 ± 19.1	0.1887	115 ± 17.1	85.7 ± 15.3	0.2473
Citrate (mmol/24h)	2.29 ± 0.34	1.76 ± 0.16	0.2356	3.92 ± 0.55	2.42 ± 0.40	0.0565	2.91 ± 0.45	1.68 ± 0.31	0.0576
Creatinine (mmol/24h)	13.6 ± 0.65	16.19 ± 1.00	0.0382‡	14.4 ± 0.84	16.7 ± 0.29	0.0339‡	14.2 ± 0.86	12.1 ± 0.77	0.1017
Oxalate (mmol/24h)	0.21 ± 0.03	0.27 ± 0.05	0.3124	0.23 ± 0.03	0.23 ± 0.04	0.9846	0.21 ± 0.03	0.19 ± 0.03	0.6573
Potassium (mmol/24h)	26.4 ± 1.49	28.8 ± 3.40	0.4657	33.6 ± 1.77	30.4 ± 2.79	0.3174	27.2 ± 2.21	27.7 ± 2.38	0.8841
Sodium (mmol/24h)	107 ± 6.08	95.3 ± 9.47	0.3123	107 ± 5.22	93.3 ± 7.30	0.1538	93.0 ± 8.62	87.0 ± 10.0	0.6577
Urate (mmol/24h)	2.35 ± 0.18	3.67 ± 0.29	0.0009‡	2.40 ± 0.19	3.36 ± 0.26	0.0093‡	2.77 ± 0.31	2.59 ± 0.20	0.6556
Ionized Ca^{2+} (mmol/L)	0.51 ± 0.13	0.39 ± 0.09	0.4912	0.31 ± 0.09	0.26 ± 0.08	0.6602	0.32 ± 0.08	0.34 ± 0.09	0.8902
PGE2 (ng/24h)	ND	ND	ND	649 ± 132	851 ± 203	0.3960	ND	ND	ND
HYP (nmol/mg creatinine)	ND	ND	ND	2.04 ± 1.05	0.89 ± 0.20	0.3145	ND	ND	ND
MSL (mM)	0.83 ± 0.12	0.94 ± 0.18	0.5848	1.16 ± 0.28	0.75 ± 0.11	0.1484	1.35 ± 0.13	1.10 ± 0.19	0.2986
TRI	159 ± 34.0	198 ± 41.5	0.4798	133 ± 14.5	145 ± 20.6	0.6379	136 ± 18.6	204 ± 31.1	0.0665
SS CaOx	2.31 ± 0.42	5.24 ± 0.67	0.0013‡	1.63 ± 0.24	4.07 ± 0.79	0.0053‡	2.82 ± 0.59	4.75 ± 0.78	0.0618
SS Brushite	0.50 ± 0.11	1.72 ± 0.34	0.0013‡	0.39 ± 0.11	1.44 ± 0.50	0.0376‡	0.43 ± 0.09	1.44 ± 0.39	0.0100‡
SS Uric acid	1.28 ± 0.57	2.18 ± 0.97	0.4147	0.88 ± 0.29	2.31 ± 0.91	0.1197	1.83 ± 0.34	2.30 ± 0.83	0.5641

‡ : *p-value ≤ 0.05 (intergroup comparison)*

2.4.6 CaOx MSL and crystallization kinetics studies

The average CaOx MSL for black and white subjects observed on days 15, 30 and 35 are shown in Table 4.9. The p-values are shown in Table 4.10. Within groups, there were no significant differences observed in the CaOx MSL during the supplementation and washout periods compared to baseline.

The p-values for the CaOx MSL intergroup comparisons during the supplementation and washout period2 are shown in Table 4.10. The CaOx MSL between groups was not statistically significant on each day of the experiment.

The average values for the rates of particle formation in each group at each sampling point are shown in Table 4.9 and illustrated in Figures 4.3 and 4.4 for blacks and whites, respectively. The p-values are shown in Table 4.10. Raw data for each individual are presented in Appendix 4.11. Within groups, there was no significant change in the rates of particle formation during the supplementation and washout periods compared to baseline. However, it was noted that there was an increasing trend in the rates of particle formation in the black group during the supplementation and washout period compared to baseline values (Figure 4.3). In contrast, a decreasing trend in the rates of CaOx crystallization was observed in whites at days 15, 30 and 35 compared to those observed at baseline (Figure 4.4).

Quantitative comparison of CaOx crystallization kinetics between groups was not determined because different instruments were used, and therefore different values were obtained (Table 4.9).

Table 4.9

CaOx MSL and average rates of particle formation within groups after n-6 & n-3 FA supplementation

Parameter	Blacks				Whites			
	Day 0	Day 15	Day 30	Day 35	Day 0	Day 15	Day 30	Day 35
MSL (mM)	1.12 ± 0.14	0.83 ± 0.12	1.16 ± 0.28	1.35 ± 0.13	0.99 ± 0.13	0.94 ± 0.18	0.75 ± 0.11	1.10 ± 0.19
Kinetics (particle/min)	171 ± 29.0	242 ± 49.0	163 ± 73.0	288 ± 84.0	3.4e-04 ± 6.1e-05	2.9e-04 ± 6.0e-05	3.5e-04 ± 9.6e-05	2.1e-04 ± 6.5e-05

Table 4.10

P-values for the comparison of MSL and rates of particle formation within and between groups after n-6 & n-3 FA supplementation

Parameter	Blacks			Whites			*B vs W*		
	Day 15	Day 30	Day 35	Day 15	Day 30	Day 35	Day 15	Day 30	Day 35
MSL (mM)	0.1387	0.8698	0.2616	0.8495	0.2109	0.6238	0.5848	0.1484	0.2986
Kinetics (particle/min)	0.2175	0.9046	0.1497	0.5157	0.9495	0.1548	ND	ND	ND

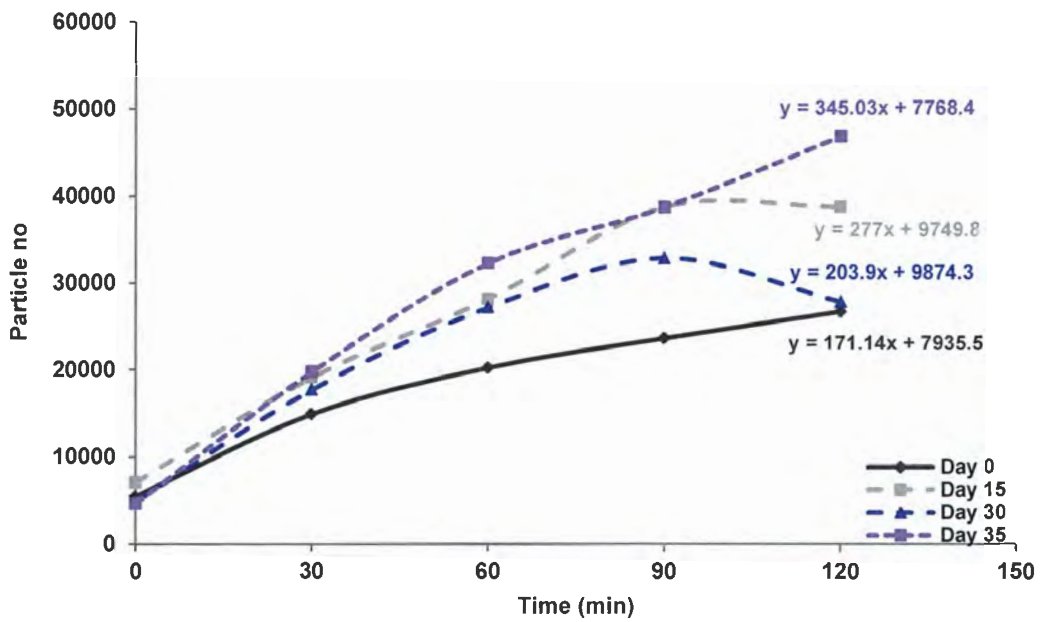

Figure 4.3: Rates of particle formation in blacks after n-6 & n-3 FA supplementation

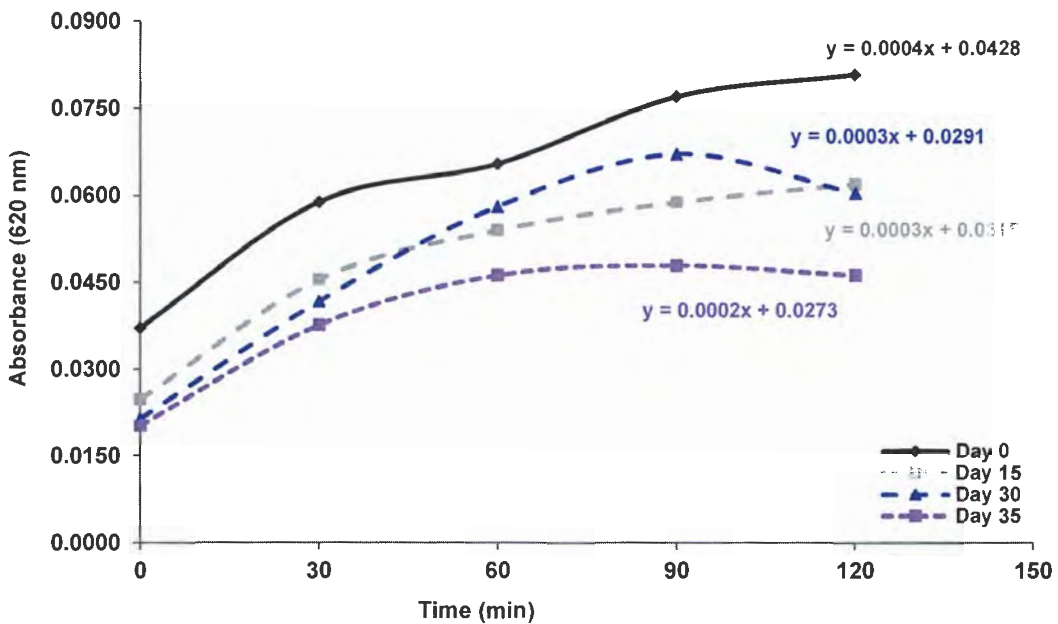

Figure 4.4: Rates of particle formation in whites after n-6 & n-3 FA supplementation

4.5 DISCUSSION

As indicated in the preceding chapters, the discussion in the present study will focus on plasma and urinary risk factors for CaOx stone formation while dietary intakes of subjects recorded at baseline will be discussed in Chapter 8.

4.5.1 Serum biomarkers

Supplementation did not show any effect on the serum concentrations of 25(OH)D and TAGs in both groups (Table 4.3). Comparison of these results with those of other studies was not possible because the latter have not investigated the effects of a combination of n-6 & n-3 FAs on these serum biomarkers as has been done in the present study. The lower serum 25(OH)D concentrations in blacks observed at baseline compared to whites in the present study are consistent with results obtained in Chapters 2 and 3 (Tables 2.3 and 3.3) and are associated with the differences in vitamin D synthesis between the groups.

4.5.2 Plasma total phospholipids

Baseline

As previously described in Chapter 2 – page 100, the differences in the fatty acid composition in plasma total phospholipids between the two race groups at baseline are attributable to the differences in the dietary habits of the two populations.

Post supplementation

In the present study, it was speculated that supplementation with a combination of n-6 and n-3 FAs would result in an increase in the concentrations and percentage compositions of EPA, DHA, GLA and DGLA in plasma total phospholipids. Furthermore, supplementation was not expected to change the concentrations and percentage compositions of AA in plasma total phospholipids.

EPA and DHA – n-3 FAs

Within groups, the concentrations and percentage compositions of EPA and DHA in plasma total phospholipids were significantly increased after supplementation (Table 4.3 and 4.5). Thus, the n-3 FA content of the capsules given to subjects during supplementation period increased the proportions of these FAs in plasma phospholipids, as expected. These results are similar to those reported by other investigators after supplementation with a combination of n-3 and n-6 FAs for 3 to 6 weeks with doses of GLA, EPA and DHA comparable to those used in the present study [25 – 27].

GLA and DGLA – n-6 FAs

In the present study, the amounts of GLA in plasma total phospholipids before and after supplementation were low for the majority of black and white subjects (Appendix 4.7 and 4.8). Considering that GLA supplementation is expected to increase the amounts of GLA in various lipid fractions, an increase in the fatty acid composition of this FA was also expected in this study. Haglund et al [26] reported similar observations with regards to the low GLA amounts in plasma total phospholipids after supplementation with n-6/n-3 FAs for 4 weeks. In contrast, significant increases in the percentage compositions of GLA in other lipid fractions such as serum phospholipids and plasma total lipids has been reported by other studies after n-6/n-3 FA supplementation [25, 27]. As previously described in Chapter 2 – page 90, the low GLA amounts after supplementation in the present study are suggested to be due to the limited incorporation of GLA into plasma phospholipids compared to other lipid fractions. Due to financial constraints, the changes in the concentrations and percentage composition of GLA in these lipid fractions could not be determined.

With regards to DGLA, a decreasing trend in the concentrations and percentage compositions of this FA in plasma total phospholipids was observed in both groups after supplementation (Table 4.3 and 4.5). Similar observations were reported by other studies after supplementation with n-6 & n-3 FAs [25, 26]. Nonetheless, these findings are contrary to those reported by Laidlaw et al. [27] and Geppert et al [28] on the same supplement protocol.

Most importantly, it was shown in Chapter 2 (Table 2.3 and 2.5) that GLA supplementation with the same dose used in the present study results in an increase in the concentrations and percentage compositions of DGLA in plasma total phospholipids. Therefore, the absence of an increase in the amounts of DGLA in total phospholipids in the present study is suggested to be due to the action of EPA and DHA.

In Chapter 3 (Table 3.3 and 3.5), it was demonstrated that EPA and DHA supplementation resulted in a significant decrease in the concentrations and percentage compositions of DGLA in plasma total phospholipids in both groups. The inclusion of EPA and DHA in the current supplement protocol is suggested to have prevented the incorporation of DGLA in membrane phospholipids since there is competition between these n-6 and n-3 FAs for PUFA metabolic synthesis which favours the n-3 FA pathway [28 – 31].

AA – n-6 FA

In the present study, the concentrations and percentage compositions of AA in plasma total phospholipids decreased after supplementation (Table 4.3 and 4.5). Furthermore, this decrease was statistical significant in the black group. These observations are similar to those made by Laidlaw et al. [26] and Haglund et al [27] after supplementation with a combination of n-6 and n-3 FAs for 4 weeks. In Chapter 2 (Table 2.3 and 2.5), it was demonstrated that GLA supplementation with the same dosage used in the present study does not affect the fatty acid composition of AA in plasma total phospholipids. However, in Chapter 3 (Table 3.5), the percentage composition of AA in plasma total phospholipids was significantly decreased in both groups after supplementation. Thus, it can be seen that in the present study, EPA and DHA competed with AA for incorporation into membrane phospholipids which decreased the amounts of AA in this lipid fraction.

Intergroup comparisons

The effects of supplementation with a combination of n-6/n-3 FAs on the fatty acid composition within black and white subject were similar. In both groups, a decreasing trend in the amounts of n-6 FAs was observed, whereas n-3 FAs were significantly increased after supplementation (Table 4.3 and 4.5). However, the significantly reduced AA amounts that were observed in blacks only (Table 4.3 and 4.5) suggests that the incorporation rates of FAs in membrane phospholipids may be uniquely different for each population group.

This study shows that dietary supplementation with a combination of GLA, EPA and DHA in healthy subjects can be used to increase the amounts of EPA and DHA in plasma total phospholipids without increasing those of AA. These results may have favourable implications for CaOx stone formation since they imply a higher formation of anti-inflammatory agents from EPA and DHA as opposed to the pro-inflammatory agents from AA which are known to increase calcium and oxalate excretion.

4.5.3 Urinary risk factors

In the present study, the urinary excretion of PGE2 within groups was not significantly affected by supplementation with n-6 & n-3 FAs. As such, urinary calcium and oxalate were expected to remain unchanged after supplementation. The unchanged concentrations of urinary calcium in both groups and urinary oxalate in whites are in line with the expectations of the study (Table 4.7). The changes in urinary oxalate that were observed in the black group are not considered to be due to the significantly decreased amounts of AA in plasma total phospholipids since urinary PGE2 was not decreased after supplementation. However, it is speculated that the lower urinary oxalate and therefore, SS CaOx in blacks, may be due to the effects of the metabolic products of EPA and DHA on the urinary CaOx risk factors. An increased production of PGE3 has been demonstrated after increasing the fatty acid composition of EPA and DHA in membrane phospholipids; whereas lower PGE2 formation is associated with decreases in AA [32 - 34]. Although the n-3 FA metabolites were not determined in the present study, an increase was expected since significant increases in the fatty acid composition of EPA and DHA were observed. The comparison of the effects of the current supplement protocol on the CaOx urinary risk factors with literature could not be determined because of lack of data.

Moreover, the increase in urinary citrate and magnesium that was observed in blacks and whites, respectively, is suggested to be due to the effects of both n-3 and n-6 FAs since these changes were also observed after supplementation with these fatty acids separately (Chapter 2 – Table 2.8 and Chapter 3 – Table 3.8). Thus, the combination of n-6 & n-3 FA supplement protocol may be more effective than n-3 or n-6 FAs alone in reducing the risk factors for kidney stone disease.

Additionally, the current supplement protocol elicited some changes on the urinary risk factors in both groups at day 15 (Table 4.7). These findings suggest that supplementation for this period with these FAs may be sufficient for generating beneficial changes in urinary CaOx risk factors. With regards to the effects of supplementation within groups, the study has shown that the two groups responded differently to the supplement protocol as observed in the urinary risk factors after supplementation (Table 4.7). These observations support the views of other investigators where these two groups have been observed to have different renal handling mechanisms with respect to dietary supplementation agents [35].

Despite supplement withdrawal, additional changes in the urinary risk factors within both were observed at day 35 (Table 4.7). These findings support the hypothesis that a washout period of 5 days is insufficient for the fatty acid composition in plasma total phospholipids to return to baseline values in healthy subjects. Due to financial constraints, these observations could not be confirmed by determining the fatty acid composition in membrane phospholipids at day 35.

Summary

Combined supplementation produced conflicting favorable results within the 2 race groups. In the black group, a significant decrease in AA was observed after n-6 & n-3 FA supplementation. In addition, a significant decrease in urinary oxalate and SS CaOx was observed at day 30 while urinary citrate was significantly increased. Meanwhile, a significant increase in urinary magnesium was observed in whites after n-6 & n-3 FA supplementation. These results confirm the findings from other studies where blacks and whites responded differently to dietary supplement challenges.

In plasma total phospholipids, common favorable changes were observed in both groups after supplementation. A significant increase in the percentage compositions of EPA and DHA were observed post-supplementation compared to baseline. This increase may be beneficial to CaOx stone formation since it implies that the EPA and DHA metabolites may be formed to a greater extent compared to PGE2. Overall, there were no significant decreases in AA concentrations and urinary calcium and oxalate after supplementation in both groups. Thus, n-6 & n-3 FA supplementation was not effective in reducing the blood and urinary risk factors associated with CaOx stone formation.

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Chapter 5

Investigation of the effects of chondroitin sulfate on blood and urinary risk factors for CaOx urolithiasis

5.1 INTRODUCTION

Urinary excretion of glycosaminoglycans (GAGs) in stone patients has been shown to be considerably lower than in healthy individuals [1 - 4]. As a result, the role of GAGs in kidney stone disease has been investigated in numerous studies [5 - 8]. When tested in *in vitro* experiments, various GAGs have been shown to have protective effects against stone formation by retarding the kinetic processes involved in crystal formation. For example, chondroitin sulfate (CS) [9-11], heparan sulfate [12, 13] and dermatan sulfate [14] have been found to inhibit nucleation, crystal growth and aggregation in synthetic and/or real urine samples.

In view of this, it has been suggested that increasing the levels of urinary GAGs may be of great benefit to stone formers by preventing or reducing the risk of kidney stone formation [7, 15]. The possibility of achieving the required levels of GAGs by ingesting GAG supplements needs to be investigated.

The present study was therefore undertaken to investigate the role of GAG supplementation in kidney stone formation. The particular GAG selected was CS because it is the major GAG found in urine, and therefore its role in kidney stone disease is of great interest. The objectives of the study were to evaluate the urinary excretion of total GAGs in healthy black and white South African subjects before and after supplementation with CS. In addition, the effect of CS supplementation upon biochemical urinary risk factors in these subjects was also determined. Moreover, the effect of CS on CaOx crystallization in urine samples of subjects before and after supplementation was investigated to determine whether supplementation increases the inhibitory activity of urinary CS against crystallization.

5.2 STUDY PROTOCOL

The study protocol was approved (**HREC Ref: 381/2013**) by the University of Cape Town Human Research Ethics Committee (Appendix 5.1) and informed consent was obtained from all the subjects before the commencement of the study. Subjects had no history of renal disease, blood clotting disorders or bone diseases. 6 Black and 6 white healthy male subjects from the University of Cape Town were enrolled in the study. Prior to the supplementation protocol, subjects collected 24h urine samples that were used as control samples

Food intakes were recorded in a food diary (Appendix 2.5). Subjects were given a list of foods that have high oxalate content (Appendix 2.6), and they were required to avoid these foods while collecting urine samples.

CS supplements were purchased from Biogen, SA (Glucosamine Chondroitin 1500/1200, Biogen Platinum Series). Each capsule contained 1500 mg glucosamine sulfate and 1200 mg chondroitin sulfate. Subjects were instructed to ingest 1 capsule per day, during breakfast, for 7 days.

CS in combination with glucosamine sulfate is often used as a health supplement for the treatment of arthritis [16 – 18]. Glucosamine sulfate and chondroitin sulfate (GLU-CS) supplementation has been reported to be beneficial for patients with arthritis because glucosamine sulfate is a substrate for GAG synthesis in humans [19, 20].

The GLU-CS supplement that was used in the present study was chosen because it contained the lowest number of other components compared to other such products. Other supplement capsules contained components such as magnesium, vitamin D and calcium which were deemed as being potentially conflicting.

The chosen amounts of glucosamine sulfate and CS were within the recommended daily intakes in healthy subjects [21]. Dietary supplementation with 1500 mg glucosamine sulfate in combination with 1200 mg chondroitin sulfate per day, for periods ranging from 1 week to 3 years, has not been associated with any side effects [16, 22 - 24]. Furthermore, it has been shown that supplementation with GLU-CS can increase the concentrations of the glucosamine sulfate and CS sulfate in serum samples of healthy subjects within 5 hours [25].

At day 7, subjects collected additional 24h urine samples while consuming the foods that were recorded at baseline.

5.3 EXPERIMENTAL ANALYSIS

5.3.1 Nutrient intake analysis

The nutrient intakes of the two groups at baseline were calculated using the Foodfinder II computer program as described in Chapter 2 – page 71.

5.3.2 Urinary risk factors

Pooled urine sample preparation

A 250 mL aliquot of the 24h urine sample from each subject was decanted to form a pooled urine sample for each race group. The pooled urine sample was prepared by mixing together six 250 mL of 24h urine samples from each group. Thus, each group had a pooled urine sample (1.5 L) on days 0 and 7. Another 1.5 L pooled urine sample was prepared using the same procedure described above and was reserved for the total GAG assay. The pooling of urine samples was implemented to eliminate interracial urinary differences such as urine chemistry and crystallization parameters.

Urine analyses

24h and pooled urine samples were analyzed for biochemical urinary risk factors as described in Chapter 2 – page 76.

5.3.3 Risk indices

The urinary composition data of 24h and pooled urine samples were used to determine the TRI and the SS values of CaOx, brushite and uric acid as described in Chapter 2 – page 81.

5.3.4 CaOx crystallization experiments

24h urine samples were filtered and prepared for crystallization experiments as described in Chapter 2 – page 80.

CaOx metastable limit

The CaOx metastable limit of each 24h urine sample was determined using the procedure described in Chapter 2 – page 80. The optical density of was measured at 620 nm using a spectrophotometer.

CaOx crystallization kinetics

The rates of CaOx particle formation in each 24h urine sample were determined according to the method described in Chapter 2 - page 81.

5.3.5 Effect of CS on CaOx crystallization experiments

In the present study, the effects of exogenous CS upon CaOx crystallization experiments were investigated in synthetic, 24h and pooled urine samples. CS was chosen because it is the major GAG found in human urine [26], and therefore, its contribution to kidney stone disease is of greater importance compared to other urinary GAGs. The concentration of CS in urine of healthy subjects has been reported to be 0.16 µg/mL of urine [27]. CS normally exists either as chondroitin-4-sulfate (CSA) or chondroitin-6-sulfate (CSC), depending on the position of the ester sulfate group on the galactose ring of the disaccharide unit [28, 29]. When the ester sulfate group is in the 4th position, CS exists as CSA and as CSC when the sulfate group is in the 6th position. Thus, in the present study, the effects of the two isomers will be investigated on CaOx crystallization to determine whether they will be different from each other. The chemical structures of CSA and CSC are shown in Figure 5.1.

Synthetic urine preparation

In an attempt to find the effect of CS *in vitro*, crystallization experiments were conducted in synthetic urine samples containing specific amounts of ions that are usually found in real urine. Synthetic urine was prepared using the procedure described by Walton et al. [30]. Various salts, NaCl (18.7 g), NaH_2PO_4 (6.90 g), $MgCl_2$ (1.33 g), KCl (12.2 g), $K_3C_6H_5O_7$ (1.41 g), $(NH_4)_2SO_4$ (5.79 g) and NH_4Cl (0.37 g), were dissolved in 1 L distilled water. Care was taken to ensure that each salt was dissolved completely before mixing the next one. The pH of the solution was adjusted to 6.0 by the addition of 5 M NaOH.

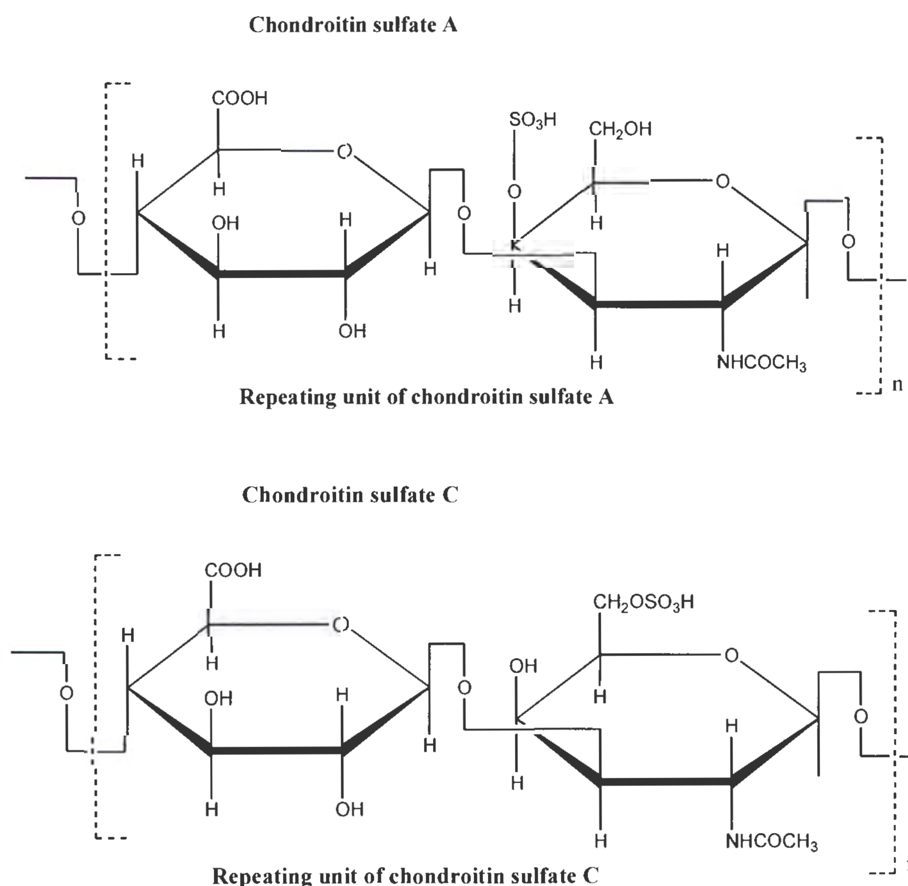


Figure 5.1: Chemical structures of chondroitin sulfate A and C

Thereafter, the synthetic urine was kept in a refrigerator at 4 °C and was used within 4 days. 0.12 M $CaCl_2$ and 0.05 M Na_2Ox were prepared separately and stored at 4 °C. On the day of analysis, the stored solutions were warmed in a waterbath at 37 °C and filtered through a 0.22 µm filter paper (Whatman No 1, Germany) just before use.

500 mL distilled water, 50 mL $CaCl_2$ and 20 mL Na_2Ox solutions were added to 430 mL of the synthetic urine to give a molar calcium and oxalate ratio of 6:1.

Preparation of CS solutions

Chondroitin sulfate sodium salts, CSA from bovine trachea (C-8529, Lot # 84H0127) and CSC from shark cartilage (C-4384, Lot # 1426300V), were obtained from Sigma Aldrich, South Africa. CSA and CSC standard solutions of 150 µg/mL were prepared separately in distilled water.

Effect of CS on CaOx MSL

Seven 100 mL aliquots of synthetic urine samples were used to determine the effect of CS on the CaOx metastable limit [31]. One of the aliquots was treated as a control sample in which no CSA or CSC was added. The other six aliquots were dosed with different volumes of CSA or CSC standard solutions to give various final concentrations as shown in Table 5.1. Thus, CS was tested in synthetic urine at physiological concentrations and at 2, 10 and 100 times higher than physiological concentrations.

Table 5.1
Concentrations of CS in urine samples

Sample	Amounts added (mL)	Concentration (µg/mL)
CSA/C-0	0	0
CSA-1	0.1	0.15
CSA-2	0.2	0.30
CSA-10	1.0	1.50
CSC-1	0.1	0.15
CSC-2	0.2	0.30
CSC-10	1.0	1.50

5 mLs of each synthetic urine sample were transferred into 13 coulter cups and warmed at 37 °C in an oven for 10 minutes. CaOx precipitation was instigated by adding 50 µL Na_2Ox standard solutions to each cup at increasing concentrations from 15 to 195 mM (in increments of 15 mM) to give final concentrations of 0.15 to 1.95 mM. The samples were further warmed at 37 °C. After 30 minutes, optical density was measured with an ultraviolet/visible (UV) spectrophotometer (Specord 40 Analytik Jena, UK) at a wavelength of 620 nm. The measured absorbance was plotted against the final concentrations of the dosed urine samples, and the metastable limit was defined as lowest Na_2Ox concentration that gave a sharp increase in optical density.

24h and pooled urine samples were filtered as described in Chapter 2 – page 80. The effect of CS on the CaOx MSL was determined in each 24 and pooled urine sample after dosing with CSA or CSC using the procedure described for synthetic urine.

Effect of CS on CaOx crystallization kinetics

The effect of CS on CaOx crystallization kinetics was determined according to the method described by Ryall et al [31]. For each sample, 25 mL aliquot of the dosed artificial, 24h and pooled urine sample was transferred into a soda lime bottle and dosed with 25 µL Na_2Ox at a concentration 15 mM above the previously determined MSL. The samples were then incubated for 90 minutes in a shaking waterbath at 37°C. Absorbance at 620 nm for each sample was measured at 15 min intervals using a spectrophotometer. The CaOx crystallization experiment was repeated twice for each urine sample. Plots of absorbance versus time were constructed; and the slope of the linear portion was taken as the rate of CaOx crystal growth.

5.3.6 Total GAG assay

Total GAGs were extracted from the pooled urine samples according to a method described by Gohel et al. [32]. A 1.5 L aliquot of the pooled 24h urine samples was filtered on a plastic collapsible strainer to remove sediments. 4.5 L of 0.025 M acetate buffer (pH 5.8) and 150 mL of cetylpyridium chloride (5 % in acetate buffer) were added to the filtered pooled urine samples. After mixing, the mixture was refrigerated overnight at 4 °C. The supernatant was decanted and the precipitate was centrifuged at 10 000 rpm for 20 minutes at 4 °C using a Model J2-21

Centrifuge (Beckman Coulter, USA) with a J14 rotor. The formed pellet was washed three times by adding cooled distilled water (4 °C), centrifugating for 10 minutes at 4 °C and afterwards discarding the supernatant. The pellet was then dissolved in 1 mL propan-1-ol/water (2:1, v/v). 4 mL of sodium-acetate-saturated ethanol was added to the dissolved pellet mixture and refrigerated overnight at 4 °C to re-precipitate the pellet as sodium salt.

Thereafter, the precipitate was washed twice with cooled ethanol (4 °C) by centrifuging at 10 000 rpm for 10 min and discarding the supernatant. The final pellet, which represents total urinary GAGs, was dried at 60 °C and weighed.

5.3.7 Statistical analysis

Statistical comparison of data was carried out using *GraphPad Instat 3* program. All data are reported as mean±SE. The results were considered statistically significant if p-value ≤ 0.05.

5.4 RESULTS

5.4.1 Subject's characteristics

The man age and BMI of subjects at baseline are shown in Table 5.2. Raw data for each individual's age, height, weight and BMI are presented in Appendix 5.2. There were no significant differences observed with regards to age and BMI between the two groups.

Table 5.2
Subject's characteristics at baseline - CS study

Parameter	*Blacks*	*Whites*	p-value
Age (years)	20.8 ± 0.70	20.7 ± 0.92	0.8883
BMI (kg/m^2)	23.6 ± 1.49	24.4 ± 0.76	0.6222

5.4.2 Nutrient intakes

Raw data showing the dietary intakes of subjects recorded at baseline are presented in Appendix 5.3. Results for the comparison of nutrient intakes between groups are going to be discussed in Chapter 8.

5.4.3 Compliance test

In the present study, supplement compliance was determined by counting the number of capsules remaining at the end of the supplementation period [33, 34]. None of the subjects returned GLU-CS supplements at day 7. As a result, it was concluded that the subjects had adhered to the supplement protocol.

5.4.4 Urinary risk factors

24h urine samples

Mean urinary parameters of the two groups at baseline and on day 7 are shown in Table 5.3. Raw data showing the urinary composition of each subject at days 0 and 7 are presented in Appendix 5.4. Urinary creatinine, oxalate and phosphate were observed to be significantly lower in the black group compared to the white subjects at baseline.

Within groups, there were no significant differences in any of the measured parameters at day 7 compared to baseline.

Comparison of urinary parameters between groups at day 7 showed that the RS brushite was significantly lower in the black group compared to whites. The differences in urinary oxalate and phosphate between groups that were observed at day 7 were not attributed to the effect of supplementation since they were present at baseline.

Table 5.3
Urinary risk factors in 24h urine samples of blacks and whte subjects - CS study

Parameter	*Blacks*		*Whites*		*B vs W (p-values)*	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
pH	6.41 ± 0.21	6.03 ± 0.09	6.20 ± 0.11	6.21 ± 0.12	0.3832	0.2599
Volume (mL/24h)	1213 ± 167	1408 ± 151	1372 ± 124	1383 ± 207	0.4649	0.9241
Calcium (mmol/24h)	4.03 ± 0.29	3.21 ± 0.27	3.38 ± 0.56	4.08 ± 0.94	0.3229	0.3944
Chloride (mmol/24h)	170 ± 15.1	161 ± 20.6	174 ± 27.1	159 ± 24.1	0.9083	0.9428
Citrate (mmol/24h)	2.45 ± 0.25	2.29 ± 0.24	3.26 ± 0.53	2.22 ± 0.31	0.1970	0.8546
Creatinine (mmol/24h)	14.8 ± 0.95	14.7 ± 1.17	20.0 ± 1.55	18.5 ± 1.92	0.0165‡	0.1167
Magnesium (mmol/24h)	3.00 ± 0.42	2.76 ± 0.28	3.83 ± 0.55	3.80 ± 0.55	0.2568	0.1210
Oxalate (mmol/24h)	0.21 ± 0.02	0.22 ± 0.01	0.36 ± 0.04	0.28 ± 0.02	0.0097‡	0.0413‡
Phosphate (mmol/24h)	23.0 ± 1.55	21.7 ± 2.25	41.6 ± 6.00	37.5 ± 5.41	0.0131‡	0.0225‡
Potassium (mmol/24h)	35.4 ± 3.72	33.8 ± 4.29	38.2 ± 5.84	34.7 ± 5.54	0.6962	0.8949
Sodium (mmol/24h)	144 ± 11.5	131 ± 21.4	154 ± 20.8	137 ± 17.1	0.6902	0.8326
Urate (mmol/24h)	3.03 ± 0.27	3.27 ± 0.32	3.92 ± 0.33	4.08 ± 0.41	0.0657	0.1494
TRI	221 ± 19.0	180 ± 15.7	192 ± 20.5	210 ± 28.2	0.3161	0.3772
SS CaOx	5.04 ± 0.76	3.62 ± 0.48	5.23 ± 1.03	6.08 ± 1.58	0.8860	0.1675
SS Brushite	1.51 ± 0.43	0.61 ± 0.09	1.44 ± 0.30	2.03 ± 0.60	0.8932	0.0411‡
SS Uric acid	1.31 ± 0.74	1.73 ± 0.41	1.55 ± 0.40	1.81 ± 0.59	0.7809	0.9099

‡ : *p-value ≤ 0.05 (intergroup comparison)*

Pooled urine samples

The urinary composition of the pooled urine samples for blacks and whites at days 0 and 7 are shown in Table 5.4. Intra- and inter-group statistical comparison of the urinary compositions in pooled urine samples at days 0 and 7 could not be determined because of insufficient data. For each group, there was one pooled urine sample at each day of the experiment. A minimum of three sets of data are required for statistical analysis. As a result, statistical analysis in pooled urine samples was not performed in the present study.

Table 5.4
Urinary risk factors in pooled urine samples of black and white subjects – CS study

Parameter	*Blacks (n=6)*		*Whites (n=6)*	
	Day 0	Day 7	Day 0	Day 7
pH	6.33	6.1	6.18	6.13
Volume (L)	1.50	1.50	1.50	1.50
Calcium (mmol/L)	3.37	3.53	2.87	4.47
Chloride (mmol/L)	182	125	121	120
Citrate (mmol/L)	1.44	1.31	1.37	2.47
Creatinine (mmol/L)	14.7	11.1	15.1	14.7
Magnesium (mmol/L)	2.65	1.85	2.90	3.10
Oxalate (mmol/L)	0.19	0.17	0.29	0.24
Phosphate (mmol/L)	24.2	14.2	31.8	30.2
Potassium (mmol/L)	42.1	13.3	25.2	26.4
Sodium (mmol/L)	146	102	103	96
Urate (mmol/L)	3.07	2.40	3.07	3.40
TRI	190	282	245	267
SS CaOx	5.04	6.67	7.42	8.25
SS Brushite	2.23	1.26	2.01	2.54
SS Uric acid	0.98	1.33	1.42	1.76

5.4.5 CaOx MSL in 24h and pooled urine samples

Table 5.5 shows the values for CaOx MSL in 24h and pooled urine samples of black and white subjects at days 0 and 7. Raw data for the experiments are presented in Appendix 5.5. In 24h urine samples, the average CaOx MSL in each group was not significantly different at day 7 compared to baseline. Intergroup comparisons at both days 0 and 7 showed that the CaOx MSL between groups were not statistically significant.The changes in CaOx MSL of pooled urine samples (Table 5.5) could not be determined because of insufficient data (Explanation in Chapter 5 – page 190).

Table 5.5
CaOx MSL in 24h and pooled urine samples at days 0 and 7 - CS study

Urine sample	*Blacks*		*Whites*		*B vs W (p-values)*	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
24h	1.13 ± 0.08	1.32 ± 0.06	0.95 ± 0.09	1.17 ± 0.06	0.1918	0.0955
Pooled[Φ]	1.20	0.90	0.75	0.60	ND	ND

Φ: No statistical analysis in pooled urine samples

5.4.6 Effect of CS on the CaOx MSL

Synthetic urine

The concentrations of Na_2Ox required to induce crystallization in synthetic urine after dosing with increasing concentrations of CSA and CSC are shown in Table 5.6. Raw data for each experiment are presented in Appendix 5.6. In both experiments, the CaOx MSL was not significantly affected by the addition of CSA or CSC.

Table 5.6 Effect of CS on CaOx MSL in synthetic urine		
Sample	**Expt$^{\Phi}$ 1**	**Expt$^{\Phi}$ 2**
CSA/C-0	1.20	1.20
CSA-1	1.20	1.20
CSA-2	1.20	1.20
CSA-10	1.20	1.05
CSC-1	1.05	1.20
CSC-2	1.20	1.20
CSC-10	1.20	1.20

$^{\Phi}$: *Expt - experiment*

24h urine samples

Table 5.7 shows the CaOx MSL in 24h urine samples of black and white subjects at days 0 and 7. Raw data for the experiments are presented in Appendix 5.7. In each group, the effect of CS dosing was investigated in 3 subjects because the total urine volume in other subjects was not sufficient to conduct a greater number of determinations. Within groups, the CaOx MSL was found to be unaltered in all the urine samples after the addition of CSA and CSC. The shift in the CaOx MSL in subject 2 at day 0 in the black group was not considered to be statistically significant.

Table 5.7

Effect of CS on CaOx MSL in 24h urine samples

Sample	Day 0						Day 7					
	Blacks (n=3)			*Whites (n=3)*			*Blacks (n=3)*			*Whites (n=3)*		
	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3
CSA/C-0	1.20	1.05	1.50	1.05	1.20	0.60	1.05	1.20	0.75	0.90	0.90	0.75
CSA-1	1.20	1.05	1.50	1.05	1.20	0.60	1.05	1.20	0.75	0.90	0.90	0.75
CSA-2	1.20	1.05	1.50	1.05	1.20	0.60	1.05	1.20	0.75	0.90	0.90	0.75
CSA-10	1.20	0.90	1.50	1.05	1.20	0.60	1.05	1.20	0.75	0.90	0.90	0.75
CSC-1	1.20	0.90	1.50	1.05	1.20	0.60	1.05	1.20	0.75	0.90	0.90	0.75
CSC-2	1.20	0.90	1.50	1.05	1.20	0.60	1.05	1.20	0.75	0.90	0.90	0.75
CSC-10	1.20	0.90	1.50	1.05	1.20	0.60	1.05	1.20	0.75	0.90	0.90	0.75

Pooled urine samples

The CaOx MSL for the pooled urine samples in each group at days 0 and 7 are shown in Table 5.8. Raw data for the experiments are presented in Appendix 5.8. In both groups, the CaOx MSL was not affected by the addition of CSA or CSC at both days 0 and 7. The shift in the MSL that was observed after dosing with CSA-1 at day 0 and CSC-1 and CSC-10 at day 7 in blacks; and CSA-2 and CSC-10 at day 7 in whites, was not attributed to the effect of CS dosing since these shifts were not statistically significant.

Table 5.8
Effect of CS on CaOx MSL in pooled urine samples

Sample	*Blacks*		*Whites*	
	Day 0	Day 7	Day 0	Day 7
CSA/C-0	1.20	0.90	0.75	0.60
CSA-1	1.05	0.90	0.75	0.60
CSA-2	1.20	0.90	0.75	0.75
CSA-10	1.20	0.90	0.75	0.60
CSC-1	1.20	0.75	0.75	0.60
CSC-2	1.20	0.90	0.75	0.60
CSC-10	1.20	0.75	0.75	0.45

5.4.7 CaOx crystallization kinetics in 24h and pooled urine samples – days 0 and 7

The average rates of CaOx crystallization kinetics in 24h urine samples at days 0 and day 7 within groups are shown in Table 5.9 and illustrated in Figures 5.2 and 5.3 for blacks and whites, respectively. Raw data for the experiment are presented in Appendix 5.9. There were no significant differences observed within groups at day 7 compared to baseline in both two groups.

Intergroup comparison showed that there were no significant differences in the rates of particle formation between groups at days 0 and 7. The p-values at day 0 and 7 were 0.6348 and 0.0988, respectively.

Table 5.9 Average rates of particle formation (particle/min) in 24h urine samples of blacks and whites at days 0 and 7			
Subjects	**Day 0**	**Day 7**	**p-value**
Blacks	0.0003 ± 0.0001	0.0003 ± 0.0001	0.9853
Whites	0.0004 ± 0.0001	0.0007 ± 0.0002	0.1641

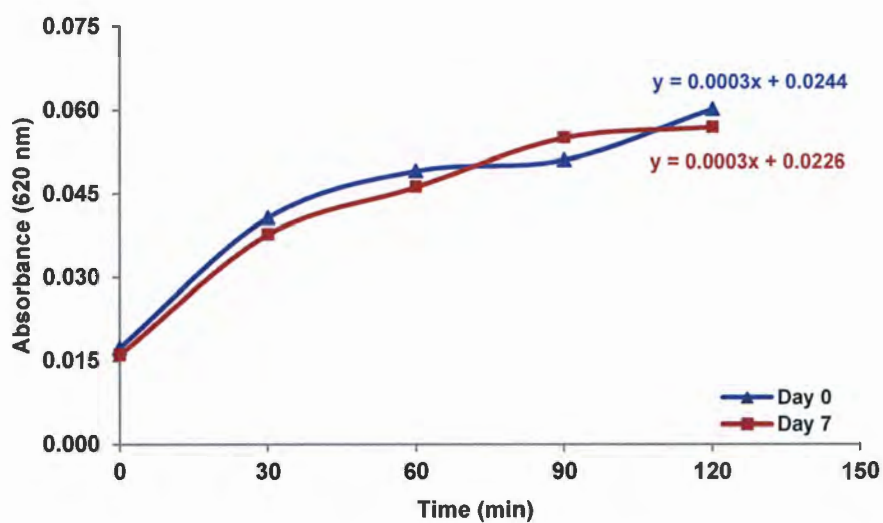


Figure 5.2: Rates of particle formation in 24h urine samples of black subjects

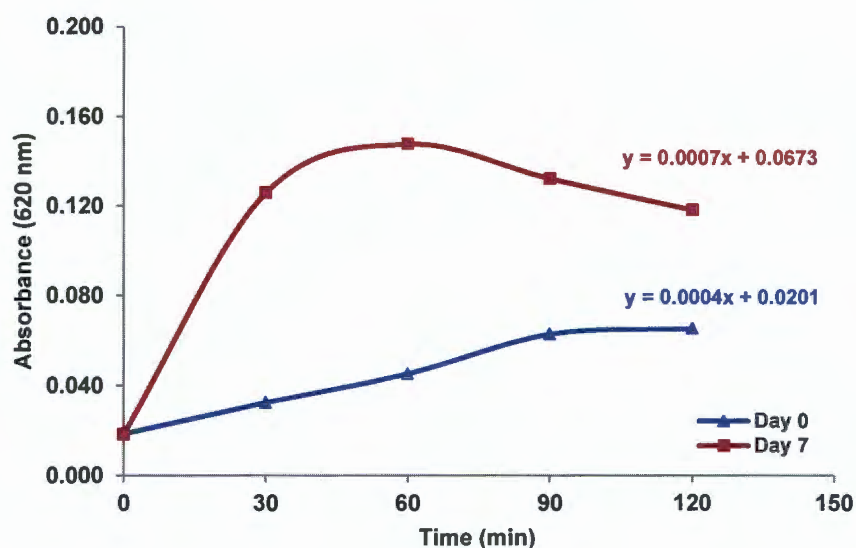


Figure 5.3: Rates of particle formation in 24h urine samples of white subjects

The rates of CaOx crystallization kinetics in pooled urine samples of black and white subjects at days 0 and day 7 are shown in Table 5.10 and illustrated in Figures 5.4 and 5.5 for blacks and whites, respectively. Raw data for the experiment are presented in Appendix 5.10. Statistical analysis on the rates of kinetics was not done as explained in Chapter – page 190.

Table 5.10
Average rates of particle formation (particle/min) in pooled urines of blacks and whites at days 0 & 7

Subjects	Day 0	Day 7	p-value
Blacks	0.0004	0.0002	0.9853
Whites	0.0002	0.0009	0.1641

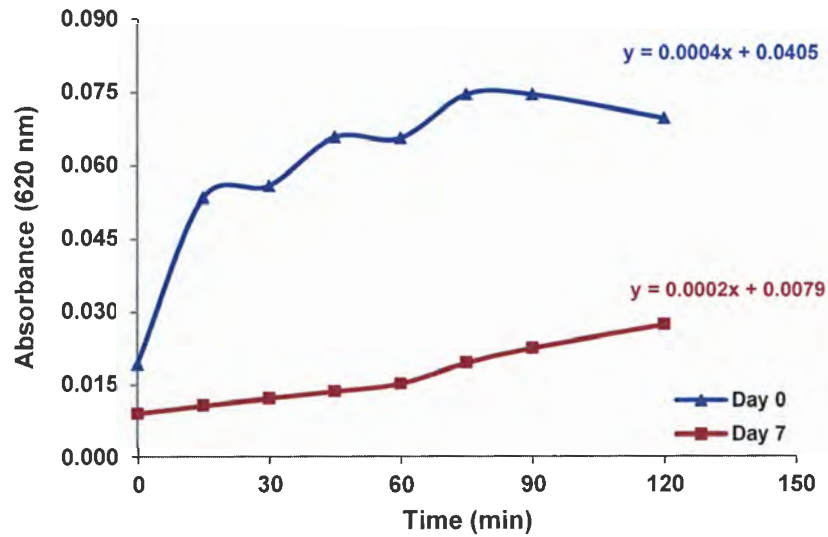


Figure 5.4: Rates of particle formation in pooled urine samples of black subjects

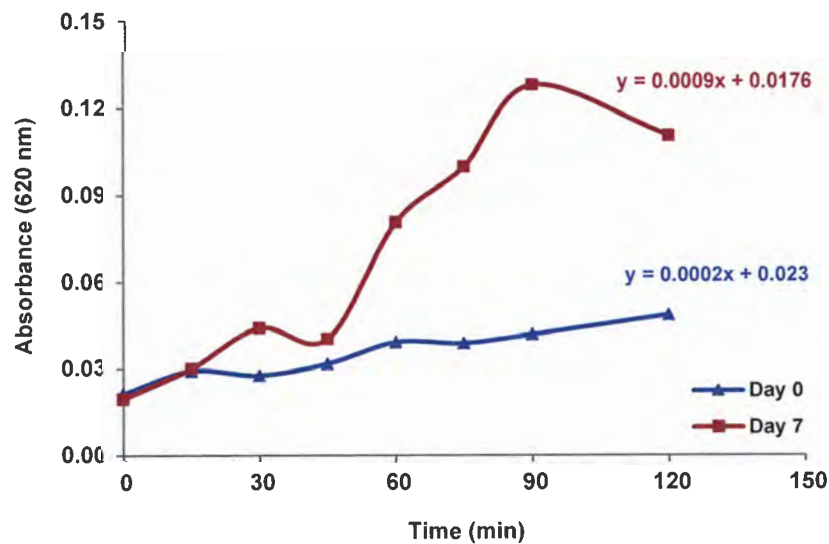


Figure 5.5: Rates of particle formation in pooled urine samples of white subjects

5.4.8 Effect of CS on the CaOx crystallization kinetics

Synthetic urine

The rates of CaOx particle formation in synthetic urine after CS dosing are shown in Table 5.11. The effects of CS on the CaOx crystallization kinetics in synthetic urine are illustrated in Figure 5.6. Raw data for the experiment is presented in Appendix 5.11.

The average rates of particle formation in synthetic urine were found to be CSA/C-0 = CSA-1 = CSA-2 = CSC-2 = CSC-10 > CSA-10 = CSC-1, but there were no significant differences observed in the rates of CaOx particle formation in urines dosed with CSA or CSC compared to the control synthetic urine sample.

Table 5.11
Rates of CaOx particle formation in synthetic urines

Sample	CaOx rates (particle/min)
CSA/C-0	0.0004
CSA-1	0.0004
CSA-2	0.0004
CSA-10	0.0003
CSC-1	0.0003
CSC-2	0.0004
CSC-10	0.0004

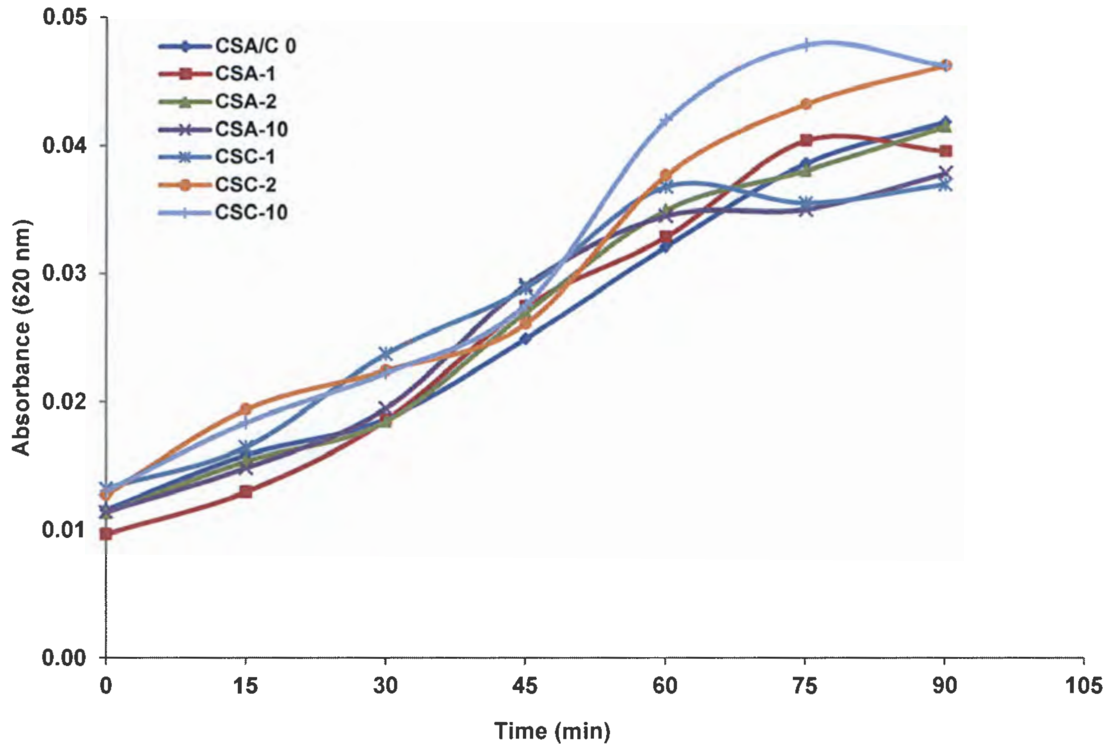


Figure 5.6 – Rates of CaOx crystallization in synthetic urine after CS dosing

24h urine samples

The average rates of particle formation in each group at days 0 and 7 are shown in Table 5.12. The effects of CS on the rates of CaOx particle formation in 24h urine samples of black and white subjects are illustrated in Figure 5.5 and 5.6, respectively. Raw data for each experiment is presented in Appendix 5.12.

Table 5.12 Rates of CaOx crystallization in 24h urine samples				
Sample	***Blacks***		***Whites***	
	Day 0	**Day 7**	**Day 0**	**Day 7**
CSA/C-0	0.0026	0.0003	0.001	0.0004
CSA-1	0.0021	0.0003	0.001	0.0003
CSA-2	0.0027	0.0002	0.001	0.0003
CSA-10	0.0019	0.0002	0.0017	0.0004
CSC-1	0.0028	0.0002	0.0016	0.0004
CSC-2	0.0021	0.0003	0.001	0.0004
CSC-10	0.0024	0.0002	0.001	0.0005

The trends in the rates of particle formation in both groups at days 0 and 7 are summarized below.

Blacks

Day 0: CSC-1 > CSA-2 > CSA/C-0 > CSC-10 > CSA-1 = CSC-2 > CSA-10

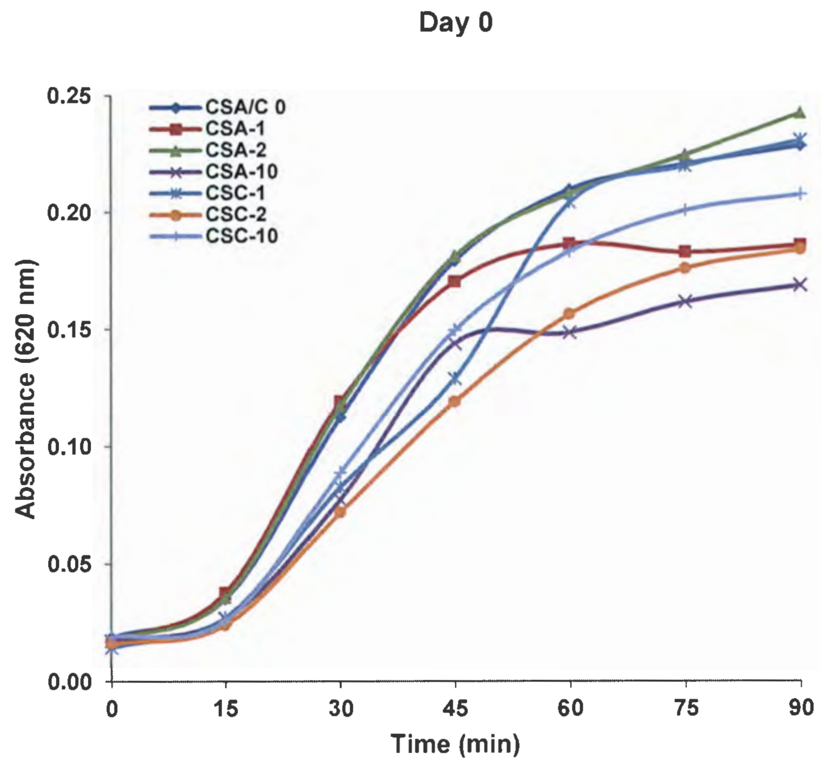
Day 7: CSA/C-0 = CSA-1 = CSC-2 > CSA-2 = CSA=10 = CSC-1 = CSC-10

Whites

Day 0: CSA-10 > CSC-1 > CSA/C-0 = CSA-1 = CSA-2 = CSC-2 = CSC-10

Day 7: CSC-10 > CSA/C-0 = CSA-10 = CSC-1 = CSC-2 > CSA-1 = CSA-2

In both groups, the average rates of particle formation in dosed urine samples were not significantly different from those in which CSA or CSC was not added.



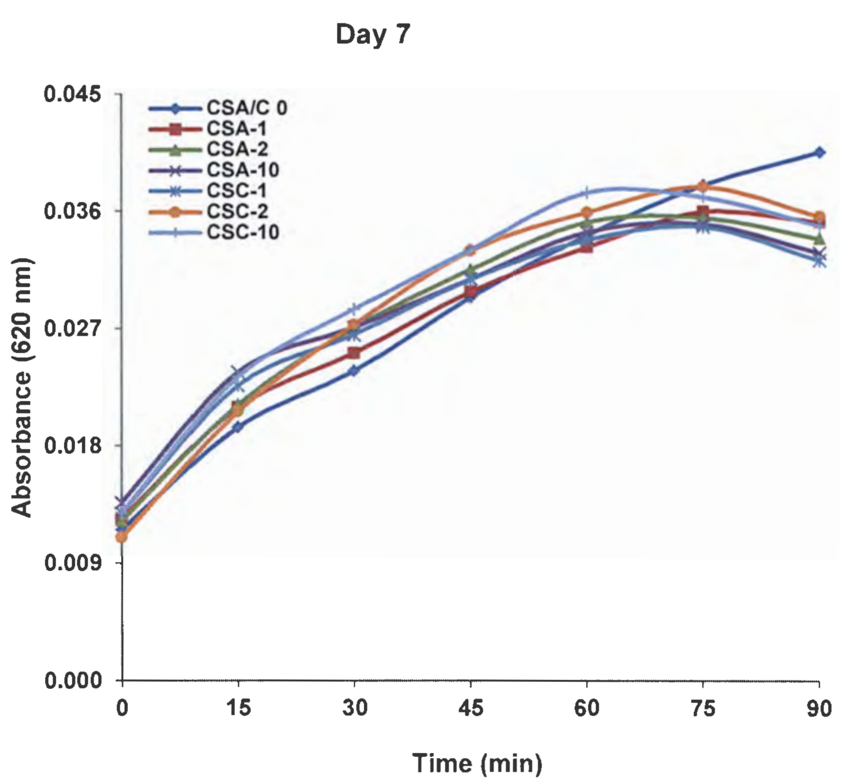
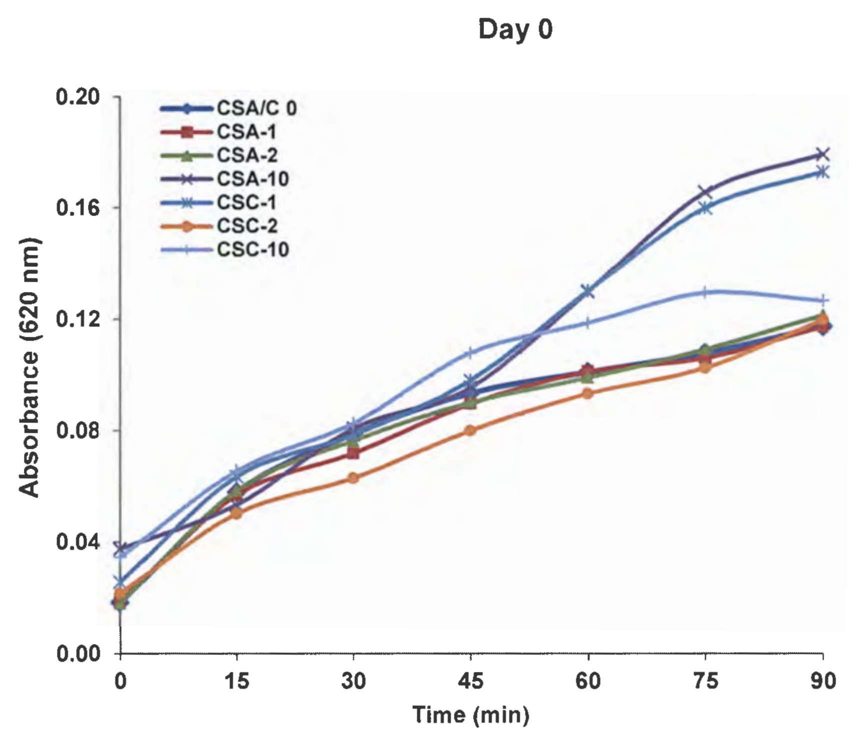


Figure 5.7 – Rates of CaOx crystallization in 24h urine samples from black subjects at days 0 and 7



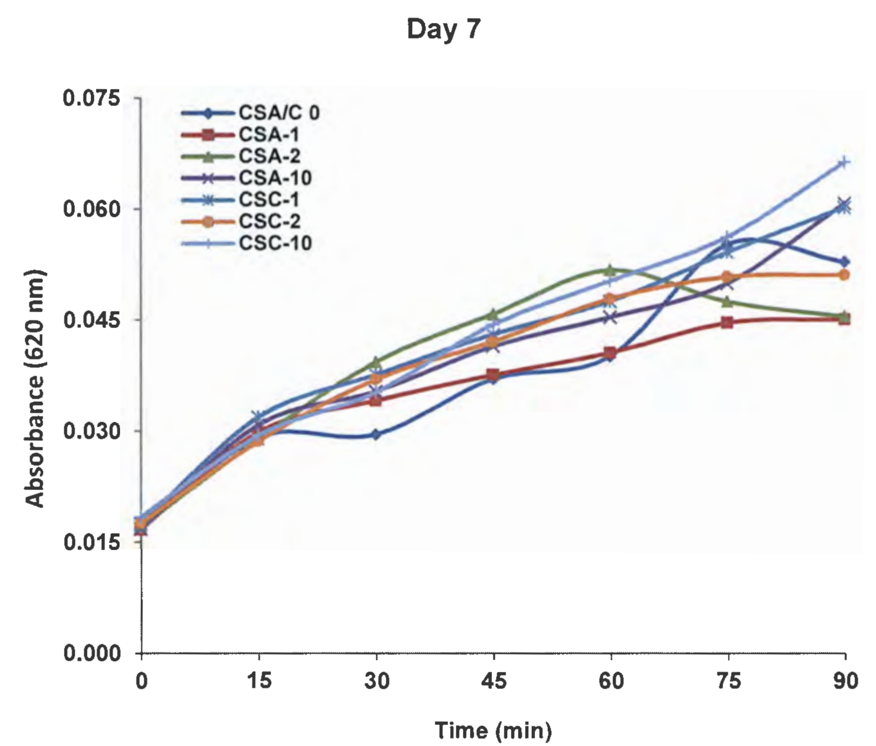


Figure 5.8 – Rates of CaOx crystallization in 24h urine samples from white subjects at days 0 and

Pooled urine samples

The rates of particle formation in pooled urine samples at days 0 and 7 are shown in Table 5.13. The effects of CS on the CaOx crystallization kinetics in pooled urine samples of black and white subjects are illustrated in Figures 5.7 and 5.8, respectively. Raw data for the experiment is presented in Appendix 5.13.

Table 5.13
Rates of CaOx crystallization in pooled urine samples

Sample	*Blacks*		*Whites*	
	Day 0	Day 7	Day 0	Day 7
CSA/C-0	0.0015	0.0001	0.0002	0.0012
CSA-1	0.0012	0.0002	0.0003	0.0012
CSA-2	0.0011	0.0002	0.0004	0.0015
CSA-10	0.0018	0.0001	0.0004	0.0018
CSC-1	0.0014	0.0001	0.0004	0.0015
CSC-2	0.002	0.0001	0.0002	0.0013
CSC-10	0.0015	0.0001	0.0005	0.0015

The trends in the rates of particle formation in pooled urine samples of both groups are summarized below. In both groups, the rates in particle formation within groups were not significantly different between dosed and un-dosed urine samples.

Blacks

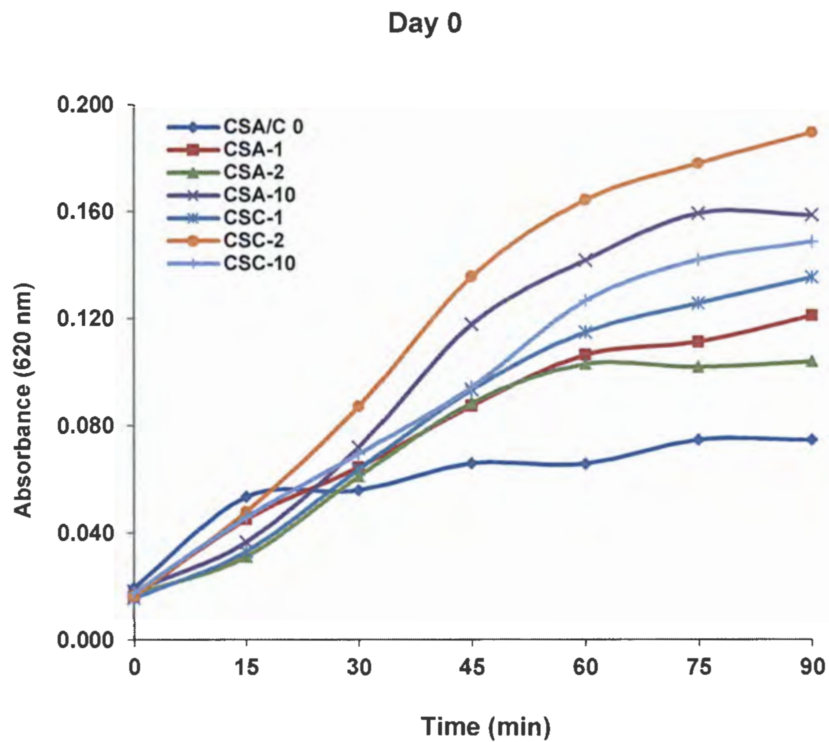
Day 0: CSC-2 > CSA-10 > CSC-10 > CSC-1 > > CSA-1 > CSA-2 > CSA/C-0

Day 7: CSA-1 > CSA-2 > CSA/C-0 = CSA=10 = CSC-1 = CSC-2 = CSC-10

Whites

Day 0: CSC-10 > CSA-2 = CSA10 = CSC-1 > CSA-1 > CSA/C-0 = CSC-2

Day 7: CSA-10 > CSA-2 = CSC-1 = CSC-10 > CSC-2 > CSA/C-0 = CSA-1



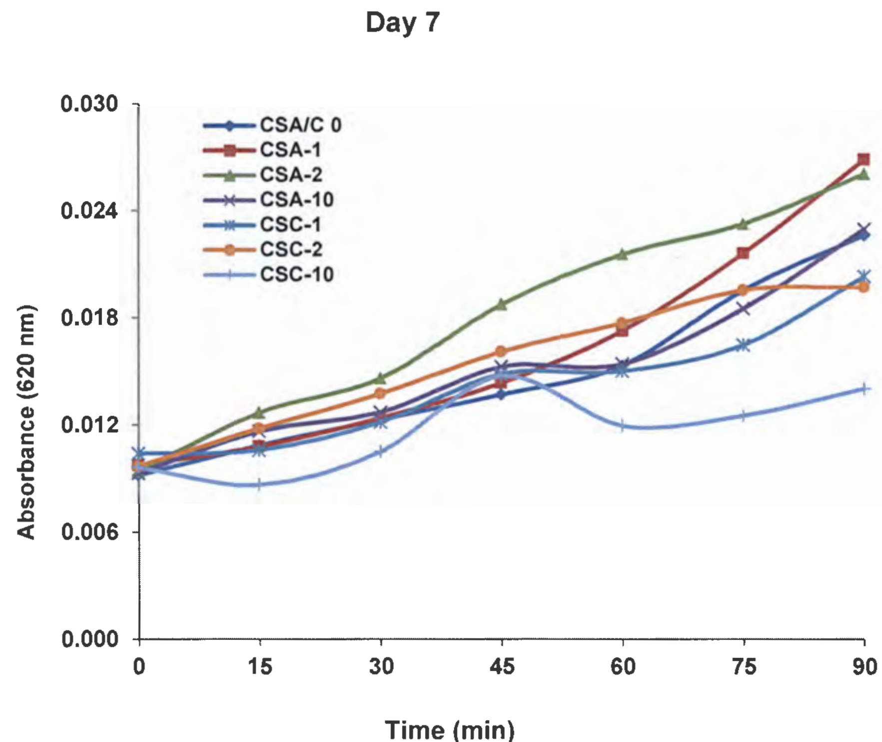
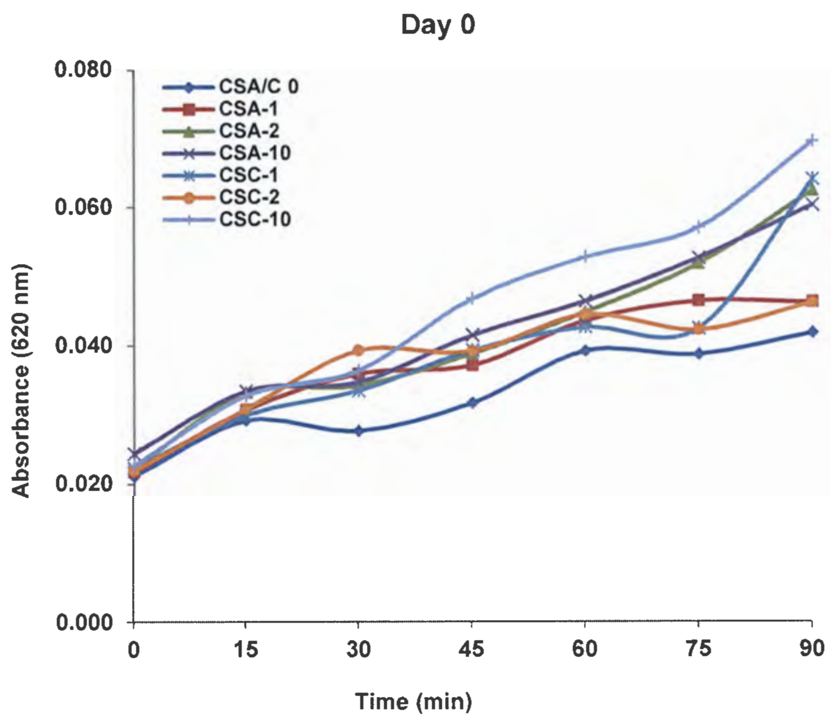


Figure 5.9: Rates of particle formation in pooled urines from black subjects



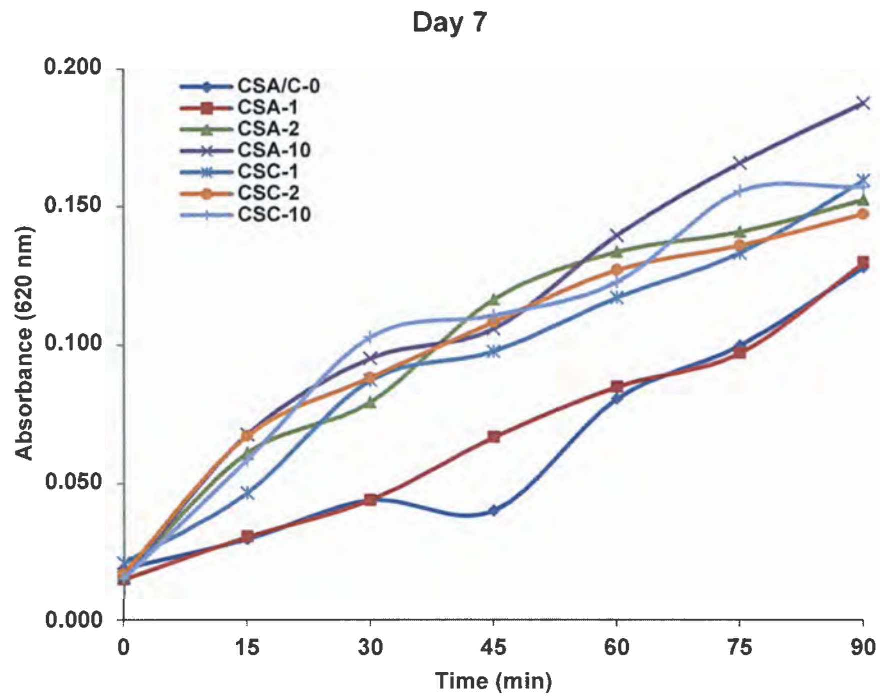


Figure 5.10: Rates of particle formation in pooled urines from white subjects

5.4.9 Total GAG assay

The amounts of total GAGs extracted in the pooled urine samples from before and after supplementation in each group are shown in Table 5.12. The percentage difference in the amounts of total GAGs at day 7 compared to baseline was 11 % in blacks and 3 % in whites. Since total urinary GAGs were extracted from pooled urine samples, statistical analysis on the differences in the excretion of total urinary GAGs could not be performed.

Table 5.14
Total GAGs (mg/1500 mL pooled urines) from black and white subjects

Subjects	Day 0	Day 7
Blacks	0.140	0.155
Whites	0.129	0.133

5.5 DISCUSSION

Intergroup comparisons at baseline showed that there were several significant differences in the urinary composition of the two groups. These differences will be discussed in Chapter 8.

There was no significant increase in the amounts of total GAGs extracted in pooled urine samples after supplementation compared to baseline in both groups. This finding does not reflect the urinary concentrations of CS, which was one of the major components in the supplement capsules given to subjects. As mentioned earlier, due to financial constraints, the isolation of CS from the total GAGs was not feasible. Nonetheless, it has been shown that more than 50 % of the CS administered to healthy subjects is excreted in urine as high and low molecular weight derivatives of CS [35]. Thus, an increase in CS derivatives was expected in the present study as well.

The present study demonstrated that the CaOx MSL and the rates of particle formation in synthetic, 24h and pooled urine samples dosed with different concentrations of CSA or CSC (0.15, 0.30 and 1.5 μg/mL) were not significantly different from control samples in which no CSA or CSC were added. These findings are similar to the observations made by Ryall et al [36] where the physiological concentrations of CSA had no significant effect on the CaOx MSL and crystal growth in 24h urine samples. Since CSA or CSC concentrations 10 times greater than physiological concentrations were used in the present study with no effect, previously reported growth inhibition may be due to *total* GAGs. Other studies have reported that GAGs, heparan sulfate and dermatan sulfate [37], CSC and hyaluronic acid [38] and chondroitin sulfate [39] inhibit crystal growth and aggregation by adsorbing to the surface of crystals, preventing the development of large crystals.

However, it should be noted that in the present study, a low molecular weight CS sodium salt (503.34 g/mol, Sigma Aldrich – South Africa) with one disaccharide unit was used in these crystallization experiments; whereas in urine CS exists as a polymer consisting of about 50 disaccharide units with a molecular weight of about 20-25 kDa and 50-80 kDa for CSA and CSC, respectively [43]. Thus, the mode of action of commercial CS salts on CaOx crystallization may be different from that of CS extracted from urine samples. The extraction of CS from real urine samples could not be undertaken in the present study due to financial constraints; as such, the effect of human CS on CaOx crystallization could not be investigated.

Supplementation with CS in healthy black and white South African subjects for 7 days did not significantly change any of the urinary risk factors associated with CaOx stone formation which were investigated. These findings are in contrast to those made by Baggio et al [44] who observed a significant decrease in urinary oxalate after supplementation with a mixture of GAGs such as heparin and dermatan sulfate. Thus, it is suggested that CS did not interfere with any of the mechanisms responsible for calcium and oxalate excretion in the present study. Furthermore, there were no significant changes observed in CaOx MSL (Table 5. 3) and crystallization kinetics (Table 5.4) after supplementation compared to baseline in both groups.

Summary

Since the excretion of urinary GAGs in stone formers is lower than in healthy subjects, the present study was undertaken to determine the effects of higher urinary CS concentrations on the urinary risk factors for CaOx stone formation and on CaOx crystallization kinetics. Firstly, the effects of exogeneous CS concentrations on the CaOx MSL and crystallization kinetics were investigated in synthetic, 24h and pooled urine samples. The effects of CS at physiological concentrations on the MSL and crystallization kinetics were compared to those above physiological concentrations (2, 10- and 100-fold). The CaOx MSL and the rates of particle formation at higher CS concentrations were not significantly different from those at physiological concentrations. Secondly, the effect of oral administration of CS supplements was investigated in healthy black and white subjects to determine whether the urinary CaOx risk factors and crystallization kinetics will be affected. There were no significant changes on the urinary risk factors and crystallization kinetics experiments post-supplementation compared to baseline in both groups.

Thus, increasing the concentrations of CS by dosing or by supplementation provided no new insights as to the mechanisms by which higher urinary CS concentrations may influence CaOx crystallization. Therefore, the role of CS in kidney stone formation may be further investigated by studying other mechanisms by which GAGs have been reported to inhibit stone formation [40 – 42]. It has been suggested that CS inhibits CaOx crystallizations through mechanisms other than preventing the growth of crystals such as complexation with ions such as Ca^{2+} [40 – 42].

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Chapter 6

Determination of the thermodynamic binding constants for the formation of calcium and magnesium complexes with chondroitin sulfate

6.1 INTRODUCTION

Urinary GAGs such as CS are negatively charged [1 - 4]. As such, they have the ability to bind and form complexes with cations such as calcium. This implies that GAGs can reduce the saturation of CaOx salts in urine by reducing the amounts of ionized calcium available to combine with oxalate.

The binding of CS with calcium has been previously investigated using different techniques such as equilibrium dialysis [5, 6], frog heart perfusion, murexide, ultrafiltration, and calcium selective electrodes [7]. These studies have shown that calcium binds to a disaccharide unit of CSA or CSC to form a Ca-CSA or Ca-CSC complex. In all previous studies, the equilibrium binding constant for the formation of Ca-CSA or Ca-CSC complex was reported to be between 14.9 – 43.4 M^{-1} [5 – 7].

However, in all these studies, the mechanisms by which CSA or CSC bind calcium have not been fully investigated. Since these GAGs are potential inhibitors of kidney stone disease, it is of great interest to investigate the manner in which they bind calcium.

The purpose of the present study, therefore, is to investigate the mechanisms by which CSA and CSC bind calcium using isothermal titration calorimetry (ITC) [8 – 13]. The advantage of this technique over the previously mentioned techniques is that, in addition to measuring the binding constant, ITC also provides information on the stoichiometry (n) and the binding enthalpy (ΔH) of each experiment. Furthermore, the Gibb's free energy (ΔG) and entropy (ΔS) can be calculated from the determined binding constant (K). With ITC, for a given reaction, these thermodynamic parameters are all obtained from a single experiment.

Because urine contains other cations that might compete with calcium for binding to CSA and CSC, it is the aim of the present study to measure the thermodynamic parameters associated with the binding of CSA and CSC to magnesium, and to compare them with those determined for calcium. The role of magnesium in kidney stone disease has been previously described in Chapter 1 – pages 9 and 11.

Lastly, it was shown in the study described in Chapter 1 – page 9 that an increase in the precipitation of CaOx salts is observed at urinary pH values < 5.5 and > 6.4. Therefore, the effect of pH on the binding of CSA or CSC with calcium and magnesium was investigated as well.

6.2 ITC PRINCIPLE

When two components in solution are mixed together at constant temperature and pressure, there is a change in heat energy [8, 9]. ITC is a technique that measures and records the amounts of heat released or absorbed during the interaction of two reactants [10 – 13]. A typical ITC instrument consists of two identical cells made from Hastelloy™ alloy. ITC monitors these heat changes by measuring the differential power, applied to the cell heaters, required to maintain zero temperature difference between the reference and sample cells as the reactants are mixed [14 – 16]. A schematic diagram of an ITC calorimeter is shown in Figure 6.1 [17].

The sample cell in which the titration occurs, usually contains one of the reactants, known as the ligand (L). The reference cell acts as a thermal reference and normally contains distilled water or a buffer solution. The syringe, which also acts as a stirrer to ensure proper mixing of the sample cell components, is usually filled with the metal solution (M), the second reactant. The M and L solutions are always prepared in the same buffer. Situated between the two cells is a calorimeter which monitors the temperature of the sample and reference cells. This calorimeter is controlled by a power feedback system whose purpose is to maintain the temperature difference between the two cells as low as possible. In the absence of any reaction, this feedback power is taken as the baseline level.

During an ITC experiment, the M solution is injected into the sample cell in small quantities using a syringe. The resulting chemical reaction caused by the interaction of the ligand and the metal in the sample cell either releases or absorbs a certain amount of heat. This heat is proportional to the amount of metal bound to the ligand. Thus, the power feedback system will adjust the power applied to the sample cell, depending on the type of reaction that is taking place in the sample cell. If heat is released (exothermic reaction), the power system will decrease the amounts of power supplied to the sample cell. Alternatively, the power applied to the sample cell will increase during an endothermic reaction where heat is absorbed. Thus, the temperature of the two cells is controlled in such a way that they will remain the same during the entire experiment.

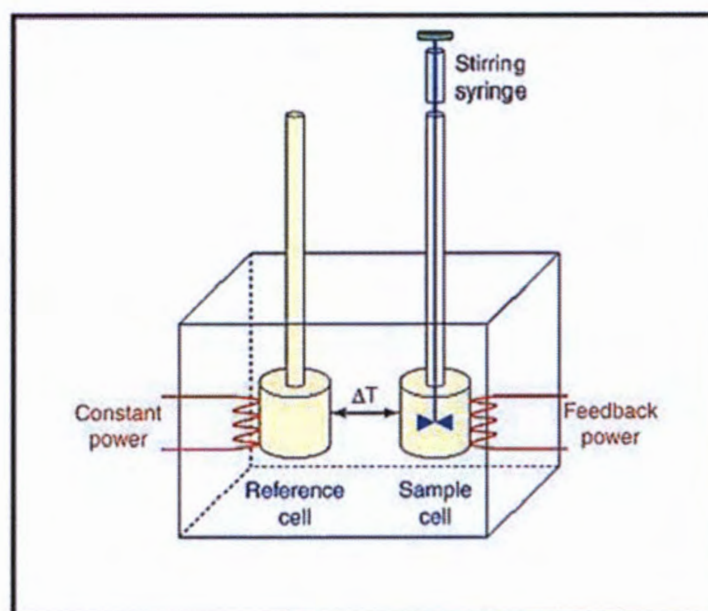

Figure 6.1: A schematic diagram of an isothermal calorimeter [17]

The difference in power (μcal/sec) that is supplied to each cell is recorded by a computer linked to the calorimeter as a function of time and corresponds to the signal seen in the form of a peak. The area under each peak corresponds to the heat released or absorbed during the reaction after each injection. The heat evolved or absorbed by the reaction is then obtained by integration of these deflections from baseline, with respect to time.

At the beginning of the titration, large amounts of heat are released or absorbed on each addition of the metal solution, reflecting substantial increases in complex formation at each step. Further additions of the metal solution will result in decreased peak signals. When the ligand in the sample cell becomes saturated with the metal, the heat signals remain constant. The heat changes registered by the calorimeter after saturation (called heats of dilution) are caused by mixing the contents of the cell and by the dilution of the ligand. These heats of dilution are subtracted from each peak during data analysis.

A binding curve is then obtained from the plots of heats against the ratio of metal and ligand inside the ITC cell from each titration. The binding curve is analysed with an appropriate binding model to determine the stoichiometry (n), the binding enthalpy (ΔH) and the binding constant (K) [10 – 16].

Determination of the heats of dilution

The changes in heat energy due to mixing are usually small; therefore they are ignored during data analysis [17, 18]. The heats resulting from the dilution of the ligand are determined in two ways [17 - 20]. Firstly, control experiments are performed separately to determine the amounts of heat produced by the titration of the ligand into solvent and also the solvent into the ligand. These experiments are conducted in a similar manner as when performing the actual M-L titration experiments. The observed heats of dilution are then subtracted from the overall heat effects measured during the M-L titration. Secondly, the heat effects are automatically subtracted from the overall heat effects when correcting the baseline level during data analysis [17 - 20].

6.3 EXPERIMENTAL ANALYSIS

6.3.1 Solution preparation

Chondroitin sulphate sodium salts (CSA from bovine trachea and CSC from shark cartilage), molecular weight of 503 g/mol were obtained from Sigma Aldrich, South Africa. NaCl, $CaCl_2$ and $MgCl_2.6H_2O$ were purchased from Merck, South Africa. A solution of 0.137 M NaCl was prepared with deionized water from MilliQ Millipore system (Millipore Direct-Q3, France) with a resistivity of 18 Ω cm which had been boiled to remove CO_2 and kept in a container protected by a CO_2 trap. Solutions of 335.05 mM calcium and 67.05 mM magnesium were used as metal solutions and were prepared by dissolving $CaCl_2$ and $MgCl_2.6H_2O$ salts, respectively, in NaCl. Three solutions were prepared for each metal, and the pH was adjusted to 5.8, 6.4 and 7.0, respectively, by the addition of NaOH and HCl. Three ligand solutions of 18.425 mM CSA or CSC were prepared separately by dissolving CSA or CSC sodium salts in NaCl. For each ligand, the pH of each solution was adjusted to 5.8, 6.4 and 7.0 with NaOH or HCl.

6.3.2 ITC titration experiments

Titration experiments were conducted using an isothermal titration calorimeter (ITC_{200} Microcal, GE Healthcare - UK). For each titration, 300 µL of the ligand sample (18.425 mM CSA or CSC solution) was loaded into the sample cell after filling the reference cell with deionized water. The metal solution (calcium or magnesium) was placed in the injection-stirrer syringe. Experiments were carried out at 37 °C.

The metal solution was injected stepwise into the sample cell using a syringe. Each titration consisted of 19 injections with an interval of 120 seconds between injections to ensure that the titration peak had returned to baseline before the next injection. The time taken for each injection was 5s. Because the needle of the syringe is usually not full before the first injection due to the long equilibration time, the first volume of the injection is made to be less than the other injections. In the present study, the volume of the first injection was 1 μL. The other 18 ligand injection volumes were 2 μL. The contents of sample cell were stirred continuously at a rate of 1000 rpm. The heat of each injection was recorded by a calorimeter that was part of the ITC instrument. Each experiment was done three times.

6.3.3 Heats of dilution experiments

Blank titration experiments were conducted to measure the effects of dilution of the ligand. These were done by titrating CSA or CSC (in the syringe) into deionized water (in the sample cell). Secondly, deionized water was placed in the syringe, and it was titrated into the CSA or CSC solution which was placed in the sample cells. The procedure for these heats of dilution experiments was similar to the one described in section 6.3.2 – page 217.

There were no heat signals detected during each heat of dilution titration experiment, indicating that the heat signals observed during the actual M-L titration were only due to the binding of the metal to the ligand. Therefore, the heats of dilution were neglected during data analysis.

6.3.4 Data analysis

The heat signal corresponding to the first injection of the metal (1 μL) was removed before the analysis of data. Origin 7.0 software program (MicroCal, GE Healthcare, UK) was used to analyze the ITC data from each titration using a one-site binding model. A one-site model was chosen because it is the simplest binding model and it assumes that the metal binds to the ligand at a single site [18].

n, *K* and ΔH were calculated by integration of the thermograms. The free energy change (*ΔG*) associated with calcium and magnesium binding to a single site in CSA or CSC was calculated from the *K* values according to the following "*Gibbs Free Energy*" equation:

$$\Delta G = -RT \ln K$$

where ***R*** is the gas constant (1.9872 cal/mol/K) and ***T*** is the absolute temperature (*Kelvin*).

The entropy change (Δ*S*) was then calculated from the *ΔG and ΔH* and T *(K)* according to the following equation:

$$\Delta G = \Delta H - T\Delta S$$

where rearrangement of the above equation gave entropy change as:

$$\Delta S = \frac{\Delta H - \Delta G}{T}$$

6.4 RESULTS

6.4.1 ITC data for the titration experiments

Raw data obtained for the titration of CSA or CSC repeat units with calcium or magnesium at pH values 5.8, 6.4 and 7.0 are illustrated in Figures 6.2 – 6.13

The upper part of the left panel in the ITC output in each figure represents the individual heat signals plotted against time (µcal/sec) for each titration. The lower part represents the ITC binding isotherm obtained by plotting heat versus the molar ratio. The right panel shows the thermodynamic data for each experiment as calculated by the ORIGIN 7.0 software.

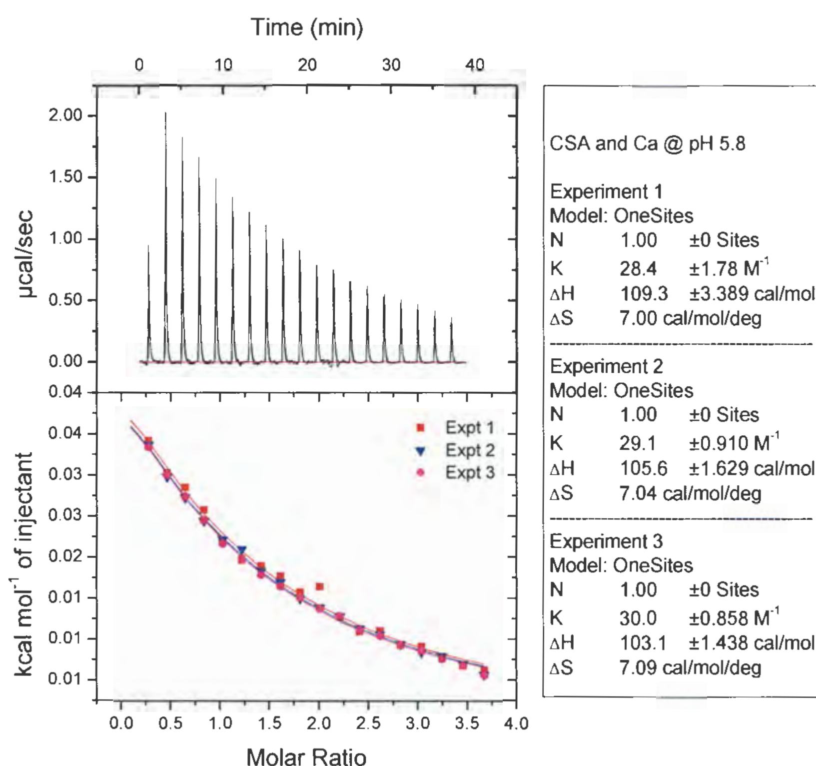

Figure 6.2: ITC output for the titration of CSA with calcium at pH 5.8

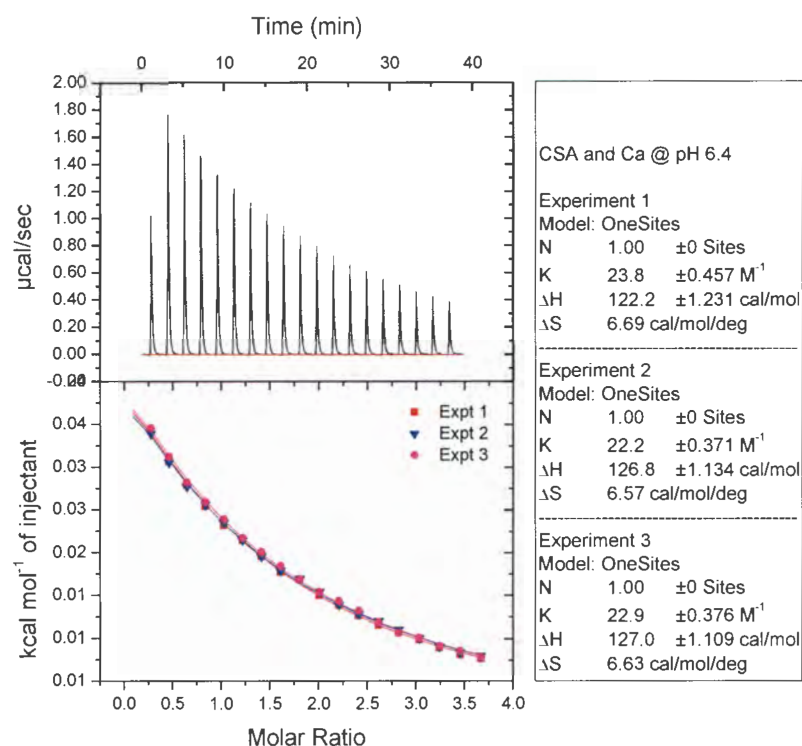

Figure 6.3: ITC output for the titration of CSA with calcium at pH 6.4

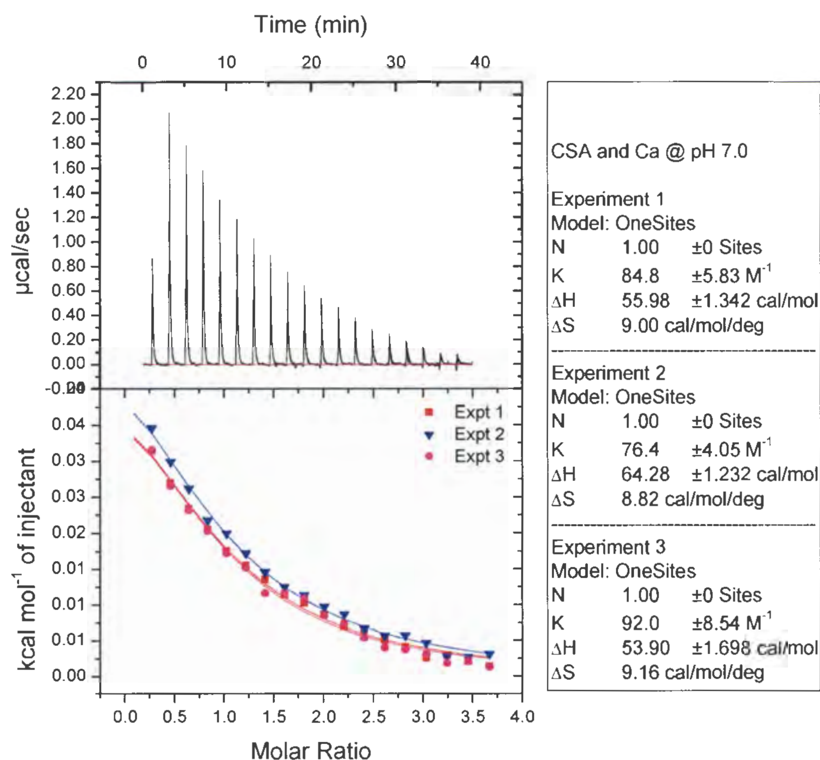

Figure 6.4: ITC output for the titration of CSA with calcium at pH 7.0

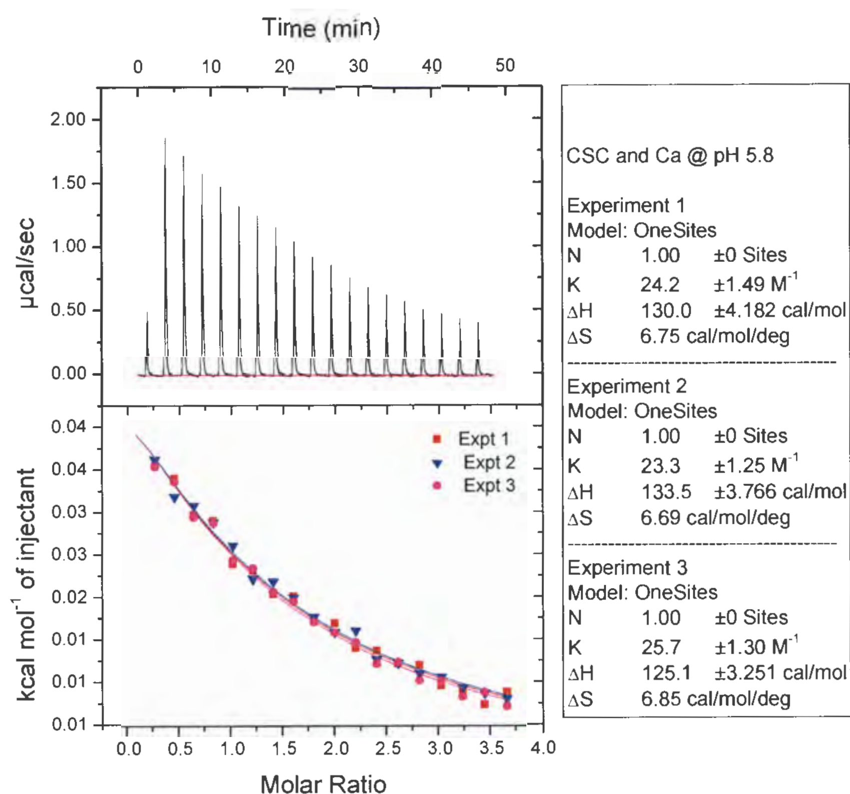

Figure 6.5: ITC output for the titration of CSC with calcium at pH 5.8

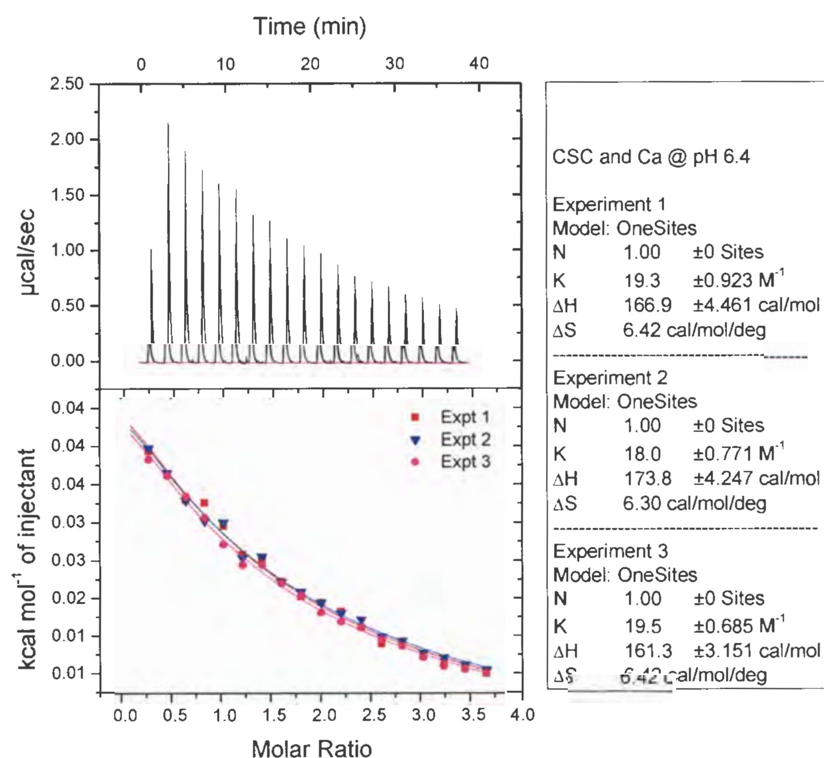

Figure 6.6: ITC output for the titration of CSC with calcium at pH 6.4

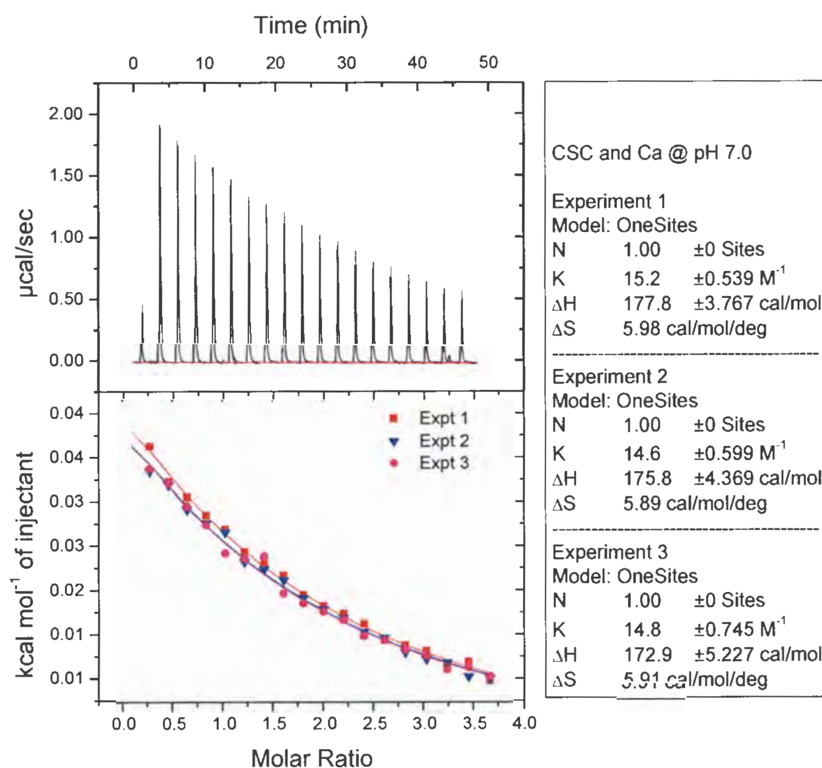

Figure 6.7: ITC output for the titration of CSC with calcium at pH 7.0

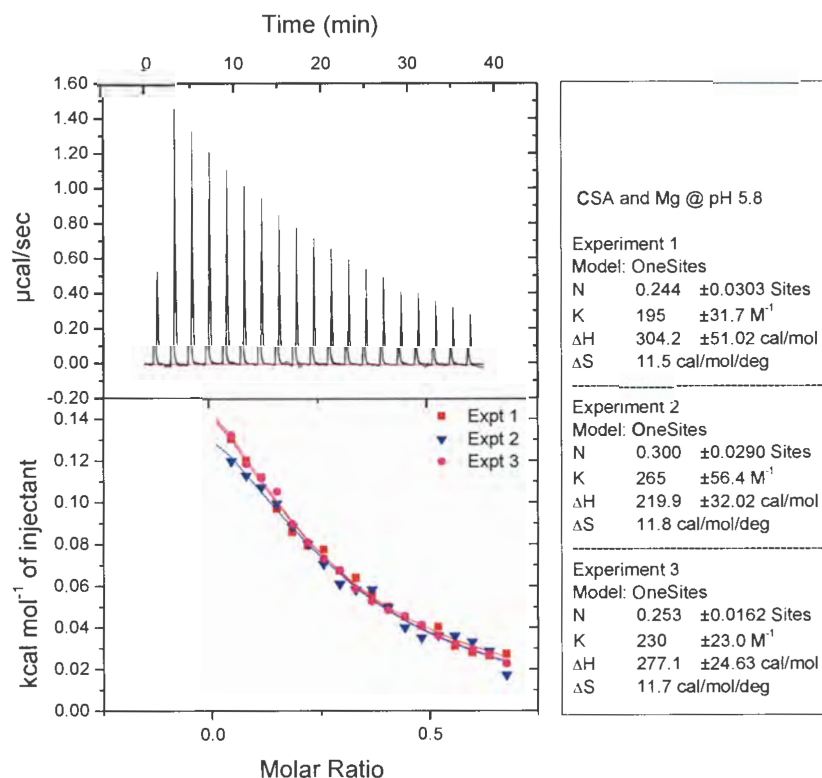

Figure 6.8: ITC output for the titration of CSA with magnesium at pH 5.8

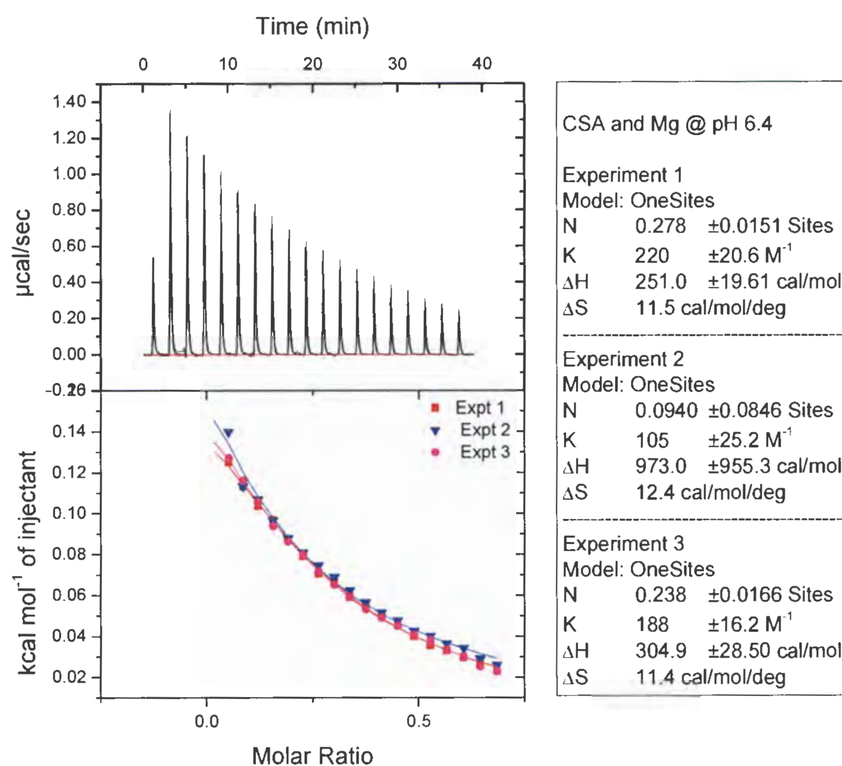

Figure 6.9: ITC output for the titration of CSA with magnesium at pH 6.4

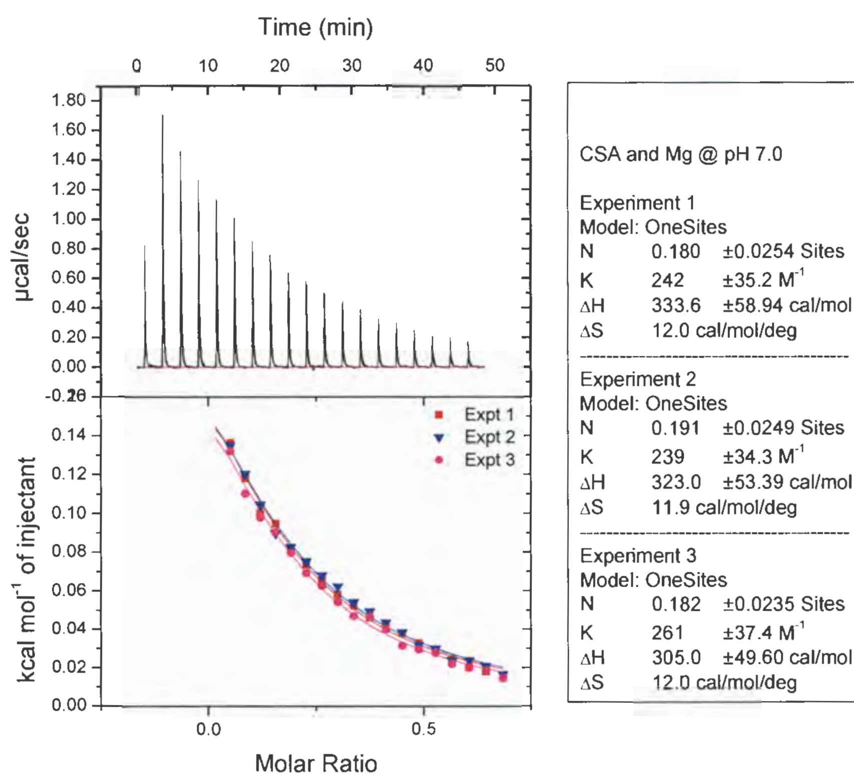

Figure 6.10: ITC output for the titration of CSA with magnesium at pH 7.0

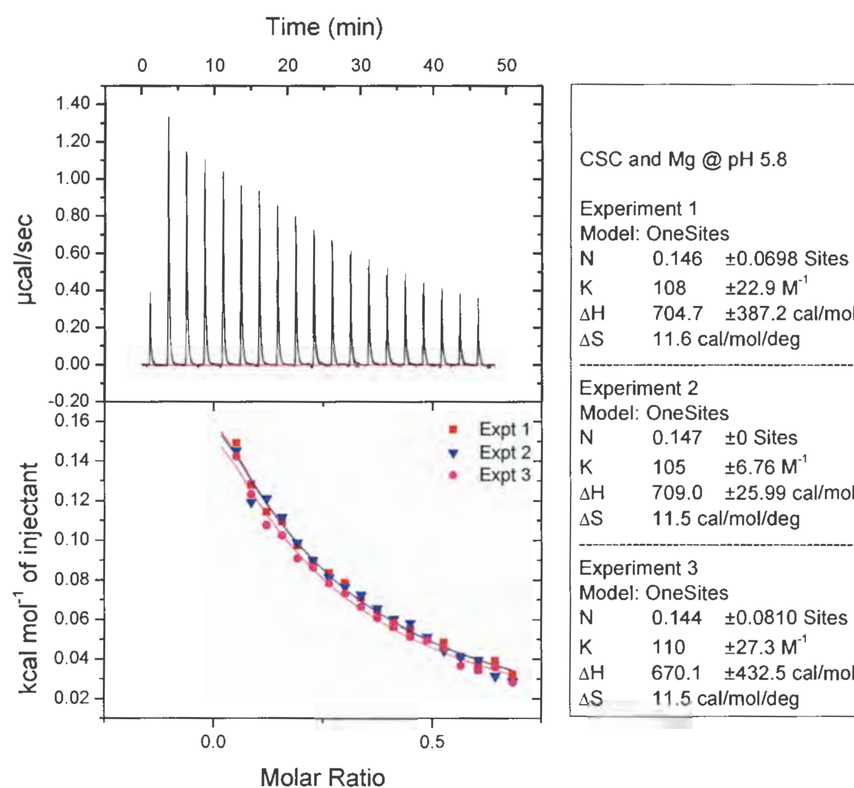

Figure 6.11: ITC output for the titration of CSC with magnesium at pH 5.8

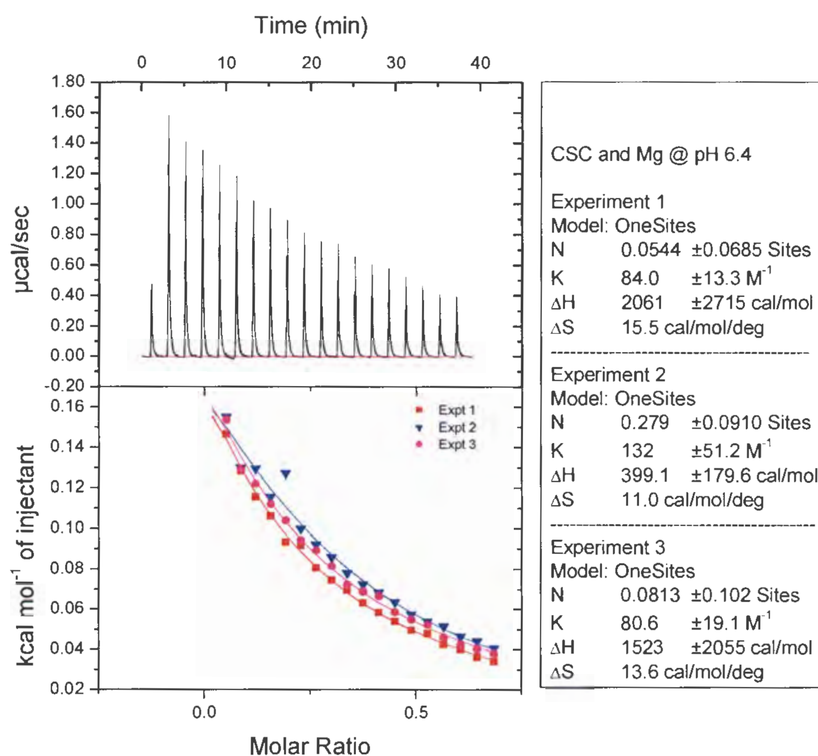

Figure 6.12: ITC output for the titration of CSC with magnesium at pH 6.4

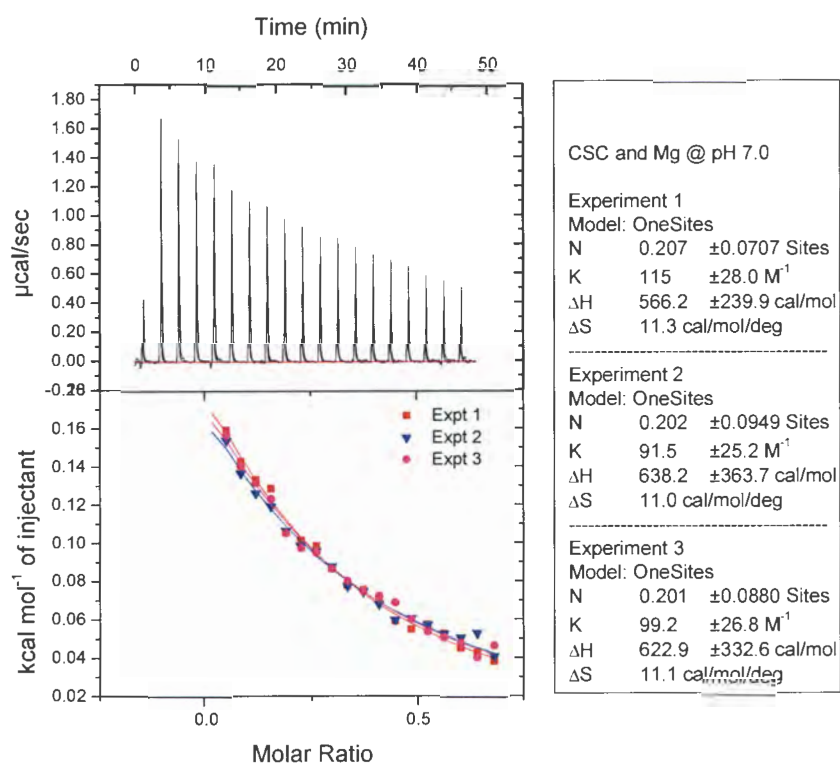

Figure 6.13: ITC output for the titration of CSC with magnesium at pH 7.0

Summary of ITC results

The thermodynamic parameters obtained from the titration of CSA or CSC with calcium and magnesium at different pH values are summarized in Table 6.1. All data are reported as an average of 3 experiments.

Table 6.1
Thermodynamic parameters for the binding of CSA or CSC with calcium and magnesium

Complex	pH	*n*	*K* (M^{-1})	ΔH (cal/mol)	ΔG (cal/mol)	ΔS (cal/mol/K)
Ca-CSA	5.8	1	29.2 ± 3.08	106 ± 5.69	-2078 ± 8.95	7.05 ± 0.02
	6.4	1	23.0 ± 0.99	125 ± 2.84	-1930 ± 12.5	6.63 ± 0.04
	7.0	1	84.4 ± 15.7	58 ± 3.52	-2731 ± 32.1	8.99 ± 0.09
Ca-CSC	5.8	1	24.4 ± 3.31	131 ± 9.19	-1969 ± 17.9	6.77 ± 0.05
	6.4	1	18.9 ± 1.96	167 ± 9.79	-1811 ± 15.5	6.37 ± 0.04
	7.0	1	14.9 ± 1.96	176 ± 11.0	-1663 ± 7.11	5.95 ± 0.03
Mg-CSA	5.8	0.26	230 ± 97.1	267 ± 92.0	-3345 ± 55.2	11.7 ± 0.10
	6.4	0.20	171 ± 51.4	510 ± 1352	-3137 ± 139	11.9 ± 0.31
	7.0	0.18	247 ± 87.3	321 ± 133	-3396 ± 17.5	12.0 ± 0.03
Mg-CSC	5.8	0.15	108 ± 51.3	695 ± 822	-2881 ± 8.95	11.6 ± 0.02
	6.4	0.14	99 ± 79.5	1328 ± 4822	-2813 ± 96.8	13.4 ± 1.30
	7.0	0.20	102 ± 65.4	609 ± 775	-2848 ± 41.5	11.2 ± 0.06

6.4.2 Effect of pH on the thermodynamic parameters for the formation of M-L complexes

Ca-CSA complex formation

Comparison of the thermodynamic parameters at various pH showed that the values of *K*, ΔH, ΔG and ΔS were not quantitatively different at pH 5.8 and 6.4. The values of *K*, ΔG and ΔS at pH 7.0 were observed to be higher than those at pH 5.8 and 6.4 whereas the value for ΔH was lower.

Ca-CSC complex formation

Comparison of the data at various pH values showed that K, ΔG and ΔS values decreased with increasing pH while an increasing trend in the ΔH values was observed.

Mg-CSA complex formation

The n-values were found to decrease with increasing pH. The K value at pH 7.0 was higher than at pH 5.8 and 6.4, with the lowest K value observed at pH 6.4. The ΔH value at pH 6.4 was greater than at pH 7.0, with the least ΔH value observed at pH 5.8. At all pH values, the ΔG and ΔS values were not quantitatively different.

Mg-CSC complex formation

Comparison of thermodynamic parameters at various pH values showed the n value to be higher at pH 7.0. At pH 5.8 and 6.4, the n values were similar. With regards to K and ΔG values, no differences were observed at various pH values. The ΔH and ΔS values were higher at pH 6.4 than pH 5.8; with the least ΔH and ΔS values observed at pH 6.4.

6.5 DISCUSSION

The formation of complexes by the binding of M and L is represented by the following reaction:

$$M + L \leftrightarrow ML,$$

where M is calcium or magnesium, L is CSA or CSC and ML is the Ca-CSA or Ca-CSC complex or the Mg-CSA or Mg-CSC complex.

The binding constant (K) for the calcium and magnesium complexes is described by the following equation:

$$K_{eq} = \frac{[ML]}{[M][L]}$$

where K_{eq} is the equilibrium constant for the reaction, [ML] is the equilibrium concentration of the metal-ligand complex, [M] is the equilibrium concentration of the metal and [L] is the concentration of the ligand. The K value is a measure of the extent to which reactants are converted to products in a reaction [7 - 9].

In the present study, the *K vaues* for the binding of CSA and CSC with calcium were found to be between 14.9 – 84.4 M^{-1} and between 99 – 247 M^{-1} for magnesium. The effect of pH on the binding of calcium and magnesium was small as depicted by the agreement of K values for the formation of each complex by each isomer (Table 6.1). The K values for the calcium complexes obtained in the present study are comparable to those reported by Urist et al [7] who found log K values between 1.04 and 1.64 for CSC. This lends a measure of credibility to the present results.

The binding constants for the calcium complexes were found to be lower than those observed for the magnesium complexes (Table 6.1). These results imply that magnesium binds to both CSA and CSC to a greater extent than calcium.

In the present study, it was expected that calcium and magnesium would bind CSA and CSC in a 1:1 ratio (one-single site model). The n values (Table 6.1) show that this expectation was realized for the calcium complexes.

Non-integer values for n are normally attributed to experimental errors such as high experimental uncertainty of the data set, unspecific binding and degradation of ligand [12, 21]. Therefore, the observed n values for magnesium are suggested to be due to such errors.

In the present study, data analysis for the binding of calcium and magnesium with CSA and CSC was evaluated adopting a single binding site model (section 6.3.4 – page 218). As shown in Chapter 5 – Figure 1, the isomers of CS contain both sulfate and carboxyl groups, which are potential sites for complex formation with calcium and magnesium. It has been suggested however, that calcium preferentially binds with the sulfate group [6].

In the present study, the average log *K* values for calcium complexes ranged between 1.17 and 1.93 for both Ca-CSA and Ca-CSC complexes over the pH range studied. These log *K* values are lower than those reported for the formation of the calcium sulfate complex at 25 °C (log *K* value 2.31) [22]. Log *K* values for calcium acetate (carboxyl group) are not available in the equilibrium constant database (SC-Database [22]) used in the present study. Thus, it was not possible to deduce whether calcium was bound to the sulfate or carboxyl group in the present study.

The general behaviour for the binding of calcium and magnesium with CSA and CSC was observed to be qualitatively similar as shown by the ITC outputs (Figures 6.1 to 6.13). The ΔG values were all negative, indicating that the binding of calcium and magnesium with CSA and CSC occurs spontaneously [8, 9]. In addition, the calculated ΔS values were positive for all the reactions and were observed to be higher for the magnesium complexes compared to the calcium complexes (Table 6.1). ΔS is associated with the disorder of a system [8, 9]. A positive ΔS is favourable and is an indication that the reaction is driven towards the products (calcium and magnesium complexes). For all the complex formation reactions, the ΔH values were positive, indicating that the reactions were endothermic. Endothermic reactions are unfavourable because the energy required for the reaction to occur is obtained from the surroundings [8, 9].

These results are typical for hard metals like calcium (II) and magnesium (II) where the reactions are entropy driven rather than enthalpy driven. In these reactions the entropy change of the solvent, H_2O, is critical and results from solvent reorganization upon charge neutralization which occurs during complexation.

Since CSA and CSC are negatively charged and the metal positively charged, these are heavily solvated. This solvation is released upon complexation and hence the increase in entropy.

Summary

In conclusion, the present study has demonstrated that calcium binds both CSA and CSC to form a calcium complex, as expected (see section 6.1 – page 214). In addition, it was shown that the binding capacity of this cation was lower in magnitude compared to that of magnesium. Since magnesium plays a role in kidney stone disease, these results imply that the magnesium complexes might influence the formation of calcium complexes in real urine samples by competing for binding with CSA and CSC. Furthermore, pH was shown to have minimal effects on calcium and magnesium complex formation. Thus, the formation of these complexes were not pH dependent in the range studied.

The binding of calcium and magnesium to CSA and CSC was shown to be spontaneous, enthalphy unfavourable and entropically favourable.

Determination of these thermodynamic parameters allowed the next phase of the project to be conducted. This involved modelling the effects of the formation of these complexes on urinary ionized calcium and the saturation of calcium salts, followed by a mini-trial in which CS was ingested by human subjects and the effects of complexation in urine was investigated. These experiments are described in the following chapter.

While the results for Mg binding are not as good as the results obtained for Ca the use of the derived binding constant in the speciation calculations in JESS is justified by the obtained standard deviations. In addition, the Mg binding constants are as expected given the Ca results and the normal linear free energy relationship between Ca and Mg binding.

Despite the non integer values, use of the derived binding constant in the speciation calculations in JESS is justified by the obtained standard deviations.

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Chapter 7

Investigation of the effect of calcium and magnesium complexation with chondroitin sulfate on the saturation of urinary CaOx

7.1 INTRODUCTION

In kidney stone formation, the precipitation of CaOx salt is largely influenced by the amount of ionized calcium available to bind with oxalate [1 - 4]. Thus, CaOx stone formation preventative measures are mostly directed at reducing ionized calcium in urine.

In Chapter 6, it was demonstrated that CSA and CSC effectively bind calcium. As a result, there exists the possibility that the formed Ca-CSA and Ca-CSC complexes may function as inhibitors of stone formation by reducing the supersaturation of CaOx in urine.

The present study was undertaken in order to determine theoretically the extent to which CSA and CSC bind with calcium and magnesium in urines of healthy subjects, using chemical speciation computer modelling. In particular, the effect of varying the concentrations of both these GAGs on the chemical speciation of calcium, oxalate, phosphate and magnesium was modelled. In addition, the effectiveness of CS supplementation (described in Chapter 5) on calcium species and on saturation levels was investigated in the urines of black and white subjects.

7.2 CHEMICAL SPECIATION COMPUTER MODELING

Experimental determination of speciation

Chemical speciation analysis of an element in a biological or environmental system is defined as the determination of the concentrations of the different physico-chemical forms of the element which together make up its total concentration in a sample [5 - 8]. Chemical speciation of elements can be determined experimentally by measuring the individual concentrations of species in a particular sample.

Analytical techniques such as gas chromatography [9], reversed phase liquid chromatography-inductively coupled plasma mass spectrometry [10], anodic stripping voltammetry [11] and atomic absorption spectrometry [12] can be used to measure speciation in various samples. Due to the number of species that might be present in a single sample, using these techniques tends to be complicated and time consuming.

Theoretical determination of speciation

Computer modeling offers an alternative approach for determining chemical speciation in a variety of samples. It involves the application of theoretical chemical concepts to predict the distribution and transformations of chemical species in various systems by calculating the concentrations of species in equilibrium [13 -16]. These computer models use the Laws of Mass Action and Mass Balance and thermodynamic formation constants for all of the possible metal-ligand species, in conjunction with defined parameters such as pH and total metal and total ligand concentrations [13 - 16].

7.3 JESS DATABASE COMPUTER PROGRAM

In the present study, chemical speciation in 24h urine samples was evaluated using the Joint Expert Speciation System (JESS) database computer program. The JESS database system provides a powerful and versatile means of storing and retrieving the thermodynamic data associated with chemical reactions [17, 18]. Currently, it contains over 202 000 thermodynamic constants associated with more than 76 000 chemical reactions [19]. Furthermore, it has been shown to be capable of modelling chemical speciation of various elements in blood [20] and urine [21 – 23]. The JESS computer program uses the equilibrium binding constants (log K values) for known chemical reactions, pH and concentrations of the reactants to calculate the chemical speciation of an element in a sample, and therefore the saturation state of dissolved species.

7.4 EXPERIMENTAL ANALYSIS

7.4.1 Inputs for JESS modeling

The JESS database does not contain the equilibrium constants for the formation of the Ca or Mg complexes with either CSA or CSC. Therefore, the binding constants for these complexes that were experimentally measured at pH 6.4 in Chapter 6 were incorporated into the JESS database. The chemical reaction and log K value for each complex as determined in the previous study is shown in Table 7.1. These values were chosen because the pH at which they were measured is relatively close to the pH values observed in the urinary composition of subjects used in the present study (Table 7.2).

Table 7.1 Chemical equations and log *K* values of CSA and CSC complexes	
Chemical equation	**log *K* value**
Ca^{2+} + $[CSA]^-$ ↔ Ca-CSA	1.36
Ca^{2+} + $[CSC]^-$ ↔ Ca-CSC	1.28
Mg^{2+} + $[CSA]^-$ ↔ Mg-CSA	2.23
Mg^{2+} + $[CSA]^-$ ↔ Mg-CSA	2.00

Five different urine models were used for the JESS calculations. These are given in Table 7.2 Urine data from a study by Hesse et al [24] constituted Model 1. This model was included to compare speciation results with those obtained from black and white subjects who participated in the CS study described in Chapter 5.

Table 7.2
Urinary composition of healthy subjects

Parameter	*Hesse* (Model 1)	Day 0		Day 7	
		Blacks (Model 2)	*Whites* (Model 3)	*Blacks* (Model 4)	*Whites* (Model 5)
pH	6.10	6.41	6.20	6.03	6.21
Calcium (mol/L)	3.11E-03	3.32E-03	2.46E-03	2.28E-03	2.95E-03
Magnesium (mol/L)	3.37E-03	2.47E-03	2.79E-03	1.96E-03	2.75E-03
Potassium (mol/L)	4.47E-02	2.92E-02	2.78E-02	2.40E-02	2.51E-02
Sodium (mol/L)	1.26E-01	1.19E-01	1.12E-01	9.30E-02	9.91E-02
Chloride (mol/L)	9.94E-02	1.40E-01	1.27E-01	1.14E-01	1.15E-01
Phosphate (mol/L)	1.61E-02	1.90E-02	3.03E-02	1.54E-02	2.71E-02
Sulfate (mol/L)	1.39E-02	1.00E-15	1.00E-15	1.00E-15	1.00E-15
Urate (mol/L)	2.38E-03	2.50E-03	2.86E-03	2.32E-03	2.95E-03
Oxalate (mol/L)	2.99E-04	1.73E-04	2.62E-04	1.56E-04	2.02E-04
Citrate (mol/L)	9.22E-04	2.02E-03	2.38E-03	1.63E-03	1.61E-03
Ammonium (mol/L)	3.04E-02	1.00E-15	1.00E-15	1.00E-15	1.00E-15
CSA (mol/L)	8.60E-05	8.60E-05	8.60E-05	1.72E-04$^{\phi}$	1.72E-04$^{\phi}$
CSC (mol/L)	8.60E-05	8.60E-05	8.60E-05	1.72E-04$^{\phi}$	1.72E-04$^{\phi}$

Note: intra-group comparision showed no statistical difference at days 0 and 7 for any of these parameters
$^{\phi}$: *See section 7.4.3 – page 239 for explanation of these concentration values*

The concentration of the disaccharide unit of CS in urines of healthy subjects has been reported to be 0.15 µg uronic acid/mL [25]. However, in urine, CS exists as a polymer with a molecular weight of about 100 000 g/mol [26]. Therefore, the concentration of CS was calculated to be 0.086 mM as shown below:

$$[CS]disaccharide = \frac{\mathbf{0.15\ \mu g/mL}}{\mathbf{175\ g/mol}}$$

$$= \mathbf{0.86 \times 10^{-7}\ M}$$

where 175 g/mol is the molecular weight of uronic acid.

Therefore, the concentration of CS was calculated as

$$= \mathbf{(0.86 \times 10^{-6}\ M) \times 100}$$

$$= \mathbf{0.86 \times 10^{-5}\ M}$$

where 100 is the average number of CS repeat units in one chain of CS [26].

Thus, in the present study, the physiological CSA concentration in urines of healthy subjects was estimated to be 0.086 mM. The CSA isomer was chosen because the binding constant for both CSA and CSC were comparable (Chapter 6 – page 227).

7.4.2 Effect of CS on the chemical speciation of calcium, oxalate, magnesium and phosphate

The urinary compositions of healthy subjects (Models 1, 2 & 3 - Table 7.2) were used as inputs in the JESS modeling to determine the chemical speciation of calcium, oxalate, magnesium and phosphate as a function of CS concentrations. For each isomer, a 2-, 10- and 100-fold increase of the physiological concentration of CS (0.086 mM) was modelled. In addition, the effect of CSA and CSC concentrations on the supersaturation (SS) of calcium salts was investigated (page 241).

7.4.3 Effect of CS supplementation on speciation

The actual *in vivo* effect of CS supplementation on calcium speciation and SS of calcium salts in 24 urine samples was calculated using the urinary composition of black and white subjects on days 0 and 7 (Table 7.2).

As described in Chapter 5 – page 207, the urinary concentrations of CS before and after supplementation were not measured in the present study due to financial constraints.

Thus, the concentration of CS that was used as input for speciation modelling at day 0 was that which has been previously reported elsewhere as physiological CS concentration (0.086 mM) [25]. At day 7, the concentration of CS that was used for speciation calculations was 2x physiological CS concentration to ensure that any increase in the concentration of urinary CS which might have occurred as a consequence of supplementation would be adequately covered in the speciation calculations.

7.4.4 JESS CALCULATIONS

After the pH and urinary composition of subjects had been entered into the JESS database, concentrations and percentages of calcium, oxalate, phosphate and magnesium species in urine were computed. The supersaturations of calcium salts were also calculated as part of the output from the speciation modelling.

7.4.5 DATA ANALYSIS

For the modeling calculations, statistical significance was not determined for the different species, as this would have required an extremely labour-intesive exercise for Models 2 and 3. Mean urinary compositions (as opposed to individual compositions) were used in these calculations. For Model 1, only mean values were available anyway, so these were used too. When these results were interrogated, a change in SS of ≥ 10 % was arbitrarily selected as a threshold for potential clinical significance.

7.5 RESULTS AND COMMENTS

The names and symbols for the common calcium salts species given as outputs from JESS calculations are shown in Table 7.3. In the following discussion, the description of calcium phosphate salts refers to a combination of OCP, HAP, triCaP and Brushite whereas CaOx is the same as COM (Chapter 1 – page 2).

Table 7.3
Names of calcium salts and their abbreviations

Calcium salts	Abbreviation
Calcium oxalate monohydrate	COM
Brushite	Brushite
Calcium phosphate, tribasic	triCaP
Hydroxylapatite	HAP
Octacalcium phosphate	OCP

It should be noted that the amounts of calcium, oxalate, magnesium and phosphate species in the following discussion are reported as concentration (mol/L). The amounts of these species as percentage composition of the total species are presented in appendices together with raw data for the concentrations of species. Furthermore, it should be noted that 1x [CSA] and 1x [CSC] refers to the physiological concentration of CSA and CSC in healthy subjects. Thus, 2x, 10x and 100x represents multiples of the physiological concentration.

7.5.1 Theoretical modelling

Effect of CS concentration on the supersaturation (SS) of calcium salts

The SS values for calcium salts after varying the concentration of CSA and CSC in the urinary composition of healthy subjects (Models 1, 2 and 3) are shown in Tables 7.4 and 7.5, respectively.

Table 7.4
Urinary supersaturation of calcium salts in healthy subjects – CSA concentrations

Salt	*[CSA] in Model 1*				*[CSA] in Model 2*				*[CSA] in Model 3*			
	1x	2x	10x	100x	1x	2x	10x	100x	1x	2x	10x	100x
COM	4.55	4.55	4.56	4.56	3.20	3.20	3.21	3.26	3.40	3.40	3.40	3.44
Brushite	1.53	1.53	1.53	1.45	1.95	1.95	1.95	1.86	1.60	1.60	1.60	1.52
triCaP	1.85	1.85	1.82	1.53	8.04[ϕ]	8.02	7.91	6.84	1.36	1.36	1.34	1.16
HAP (x 10^6)	1.60	1.60	1.56	1.17	24.2[ϕ]	24.1	23.5	18.4	0.85	0.85	0.82	0.64
OCP	41.4	41.3	40.6	32.5	226[ϕ]	225	221	183	31.3	31.2	30.5	25.2

Table 7.5
Urinary supersaturation of calcium salts in healthy subjects – CSC concentrations

Salt	*[CSC] in Model 1*				*[CSC] in Model 2*				*[CSC] in Model 3*			
	1x	2x	10x	100x	1x	2x	10x	100x	1x	2x	10x	100x
COM	4.55	4.55	4.55	4.52	3.20	3.20	3.21	3.23	3.40	3.40	3.40	3.41
Brushite	1.53	1.53	1.53	1.46	1.95	1.95	1.95	1.88	1.60	1.60	1.60	1.54
triCaP	1.85	1.85	1.82	1.58	8.04[ϕ]	8.02	7.94	7.06	1.36	1.36	1.34	1.20
HAP (x 10^6)	1.61	1.60	1.57	1.23	24.2[ϕ]	24.0	23.7	19.4	0.85	0.85	0.83	0.68
OCP	41.5	41.3	40.6	33.7	226[ϕ]	225	222	191	31.3	31.2	30.8	26.4

[ϕ]***Footnote:*** *SS values for triCaP, HAP and OCP at 1x [CSA] in Model 2 were considerably higher compared to those in Models 1 and 3. The speciation of calcium phosphate salts has been shown to be pH dependent [22]. Since the pH in urinary composition of Model 2 was higher than that in the other models (Table 7.2), these SS values are attributed to the effect of pH on speciation calculations.*

It is noted that subjects in all 3 models responded qualitatively and quantitatively in the same way to theoretical increases in CSA and CSC thereby predicting that there would not be any difference in their respective effects on urinary saturation levels in clinical situations in which they are independently administered. This is not surprising given that the binding constants for calcium as well as for magnesium have the same order of magnitude (Table 7.1). Thus, both CSA and CSC concentrations achieved the same effects.

Regarding the effects themselves, decreases in SS values >10 % occurred only at 100x physiological values, and only for OCP, HAP and triCaP. SS values for brushite and COM were unchanged. Irrespective of whether these decreases are clinically significant or not, the modelling in the present study has predicted that they will occur only if the urinary concentrations of CSA and CSC are raised to levels which are 100x greater than physiological concentrations. Since concentrations such as these cannot be achieved with dietary or pharmacological interventions at this time, investigators need to make strategic decisions about pursuing this line of research. The key question would be whether it is worth attempting to develop pharmaceutical preparations designed to raize the urinary concentrations of CSA and CSC, given that SS COM and SS brushite would be unaffected anyway.

From the inter-race perspective (Model 2 vs Model 3), there was no difference in the response of the groups with respect of varying CSA and CSC. This is not regarded as being clinically important, but is noted with interest.

Effect of CS concentration on speciation

Although the absolute concentrations of the different species were different in Models 1 – 3 (because component concentrations at baseline were different), the same qualitative trends were observed for all of the models. As such, it is not necessary to present speciation plots for all three models here. Only those for Model 2 are presented. Furthermore, only plots for the speciation calculations using CSA concentrations are reported since similar results were also observed for CSC concentrations.

Calcium, oxalate, magnesium and phosphate speciation plots for Model 2 are given in Figures 7.1 to 7.4, respectively. Identical trends were observed in Models 1 and 3 (Appendices 7.1 and 7.2, respectively). Raw data for these calculations are given in Appendices 7.3, 7.4 and 7.5 for Models 1, 2 and 3, respectively.

The concentration of $[Ca]^{+2}$ in all 3 models decreased only when [CS] was 100x its physiological value (Figure 7.1). This effect can be clearly attributed to the formation of the [Ca-CSA] complex which utilizes $[Ca]^{+2}$ as shown in calcium speciation plots.

The speciation of oxalate showed an interesting feature in all 3 models, namely an increase in $[Ox]^{-2}$ at 100x physiological concentration of CSA (Figure 7.2). This can be attributed to the decrease in the concentration of the [MgOx] complex which releases Ox^{-2}, thereby increasing the concentration of the latter. This is demonstrated in the plot of Ox speciation (Figure 7.2). The decrease in the concentration of [MgOx] itself can be accounted for by the formation of the complex [Mg-CSA] which utilizes Mg^{2+} thereby decreasing its availability for binding with Ox^{-2} (Figure 7.3). Despite this increase in $[Ox]^{-2}$, the supersaturation of COM was unaffected (Tables 7.4 and 7.5), possibly because of the opposite effect due to the accompanying decrease in $[Ca]^{+2}$.

The phosphate speciation (Figure 7.4) does not show any special features which might explain the decreases in the supersaturation of OCP, HAP and triCaP at 100x physiological concentrations (Tables 7.4 and 7.5). This can be attributed to the stoichiometry of the different compounds with respect to the molar content of calcium [27]. In calcium oxalate (CaC_2O_4) and brushite ($CaHPO_4.2H_2O$) there is only one mole of calcium. However, in triCaP ($Ca_3(PO_4)_2$), HAP ($Ca_5OH(PO_4)_3$) and OCP ($Ca_8H(PO_4)_3$ there are 3. 5 and 8 moles of calcium, respectively [27].

Since SS is dependent on the ionic products of these salts (Chapter 1 – page 12), small changes in the concentration of $[Ca]^{+2}$, brought about by complexation with CS at 100x physiological values, will affect the SS of triCaP, HAP and OCP far more than those for COM and brushite. Thus, the decreases in SS of triCaP, HAP and OCP that were observed at 100x physiological concentrations of CS are attributed to the decrease in $[Ca]^{+2}$ (Figure 7.1) at this concentration.

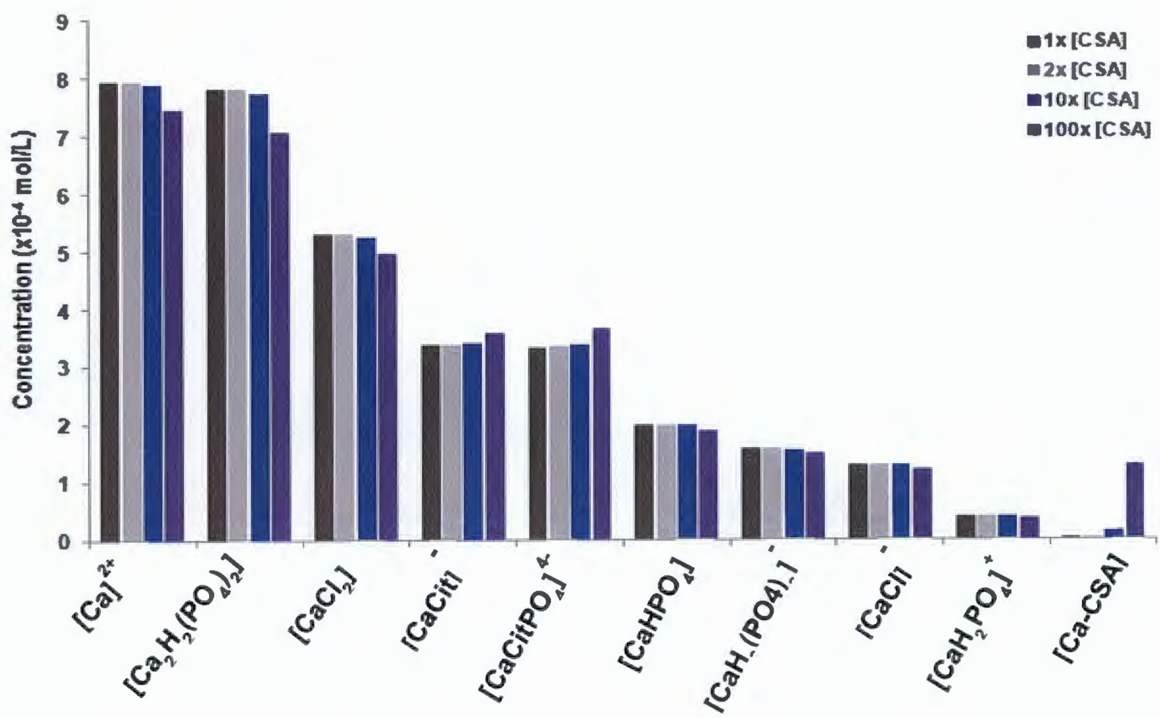

Figure 7.1: Effect of CSA concentrations on calcium speciation in Model 2

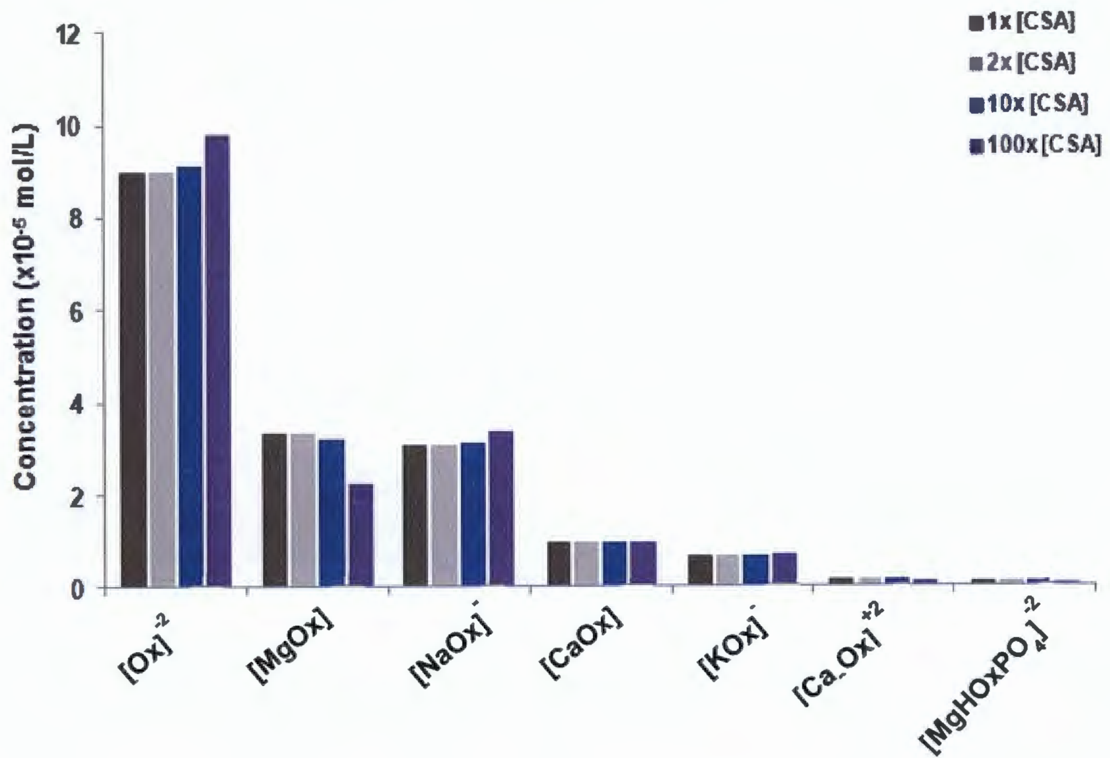

Figure 7.2: Effect of CSA concentrations on oxalate speciation in Model 2

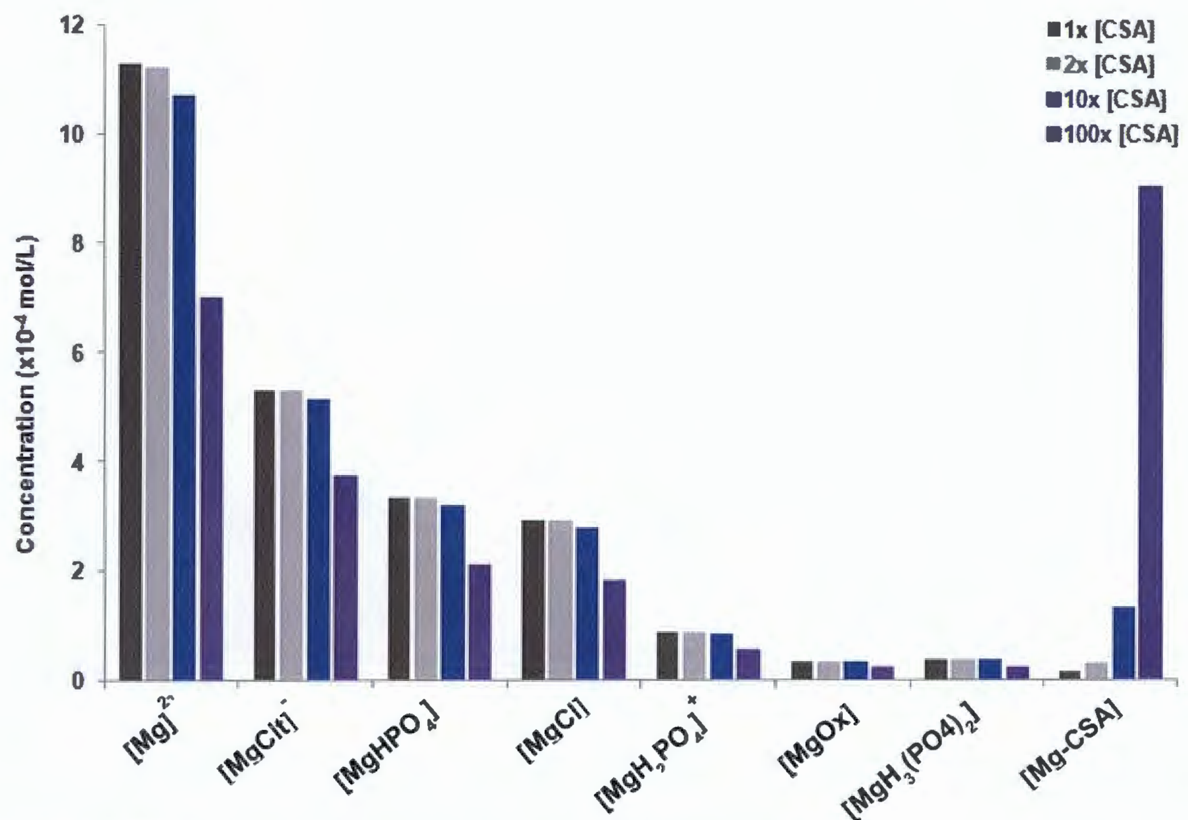

Figure 7.3: Effect of CSA concentrations on magnesium speciation in Model 2

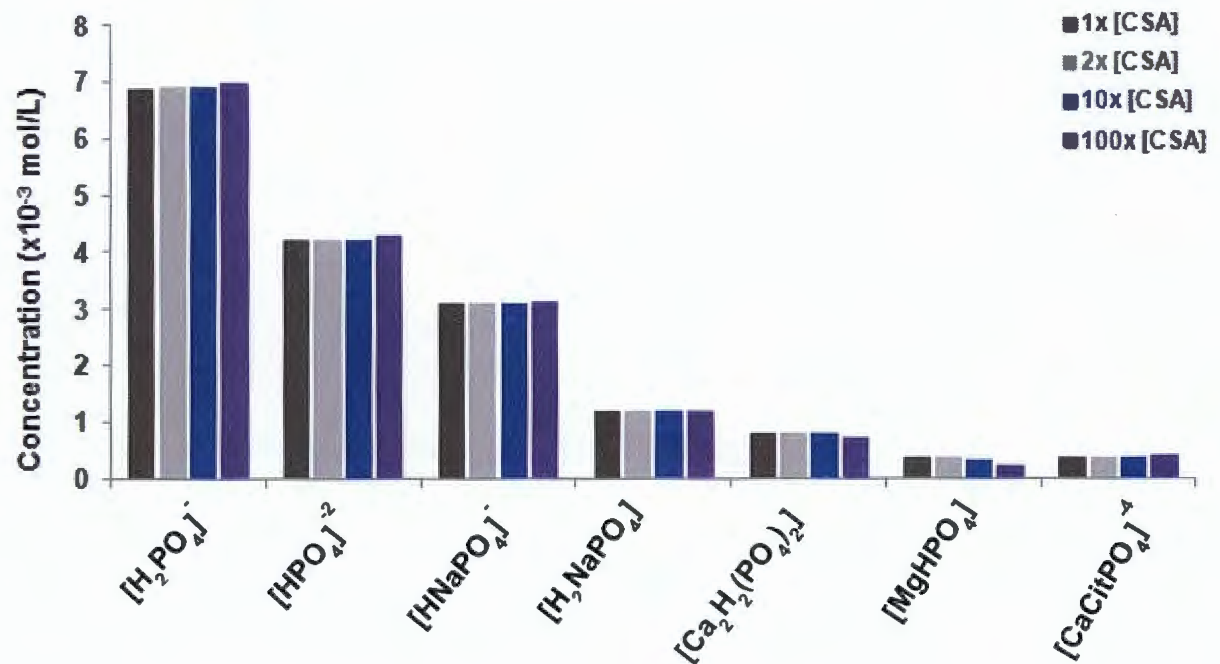

Figure 7.4: Effect of CSA concentrations on phosphate speciation in Model 2

Summary of speciation concentrations

Summaries of the speciation concentrations corresponding to the formation of calcium and magnesium complexes with CSA and CSC are given in Tables 7.6 and 7.7, respectively.

It is seen that substantial changes occurred only at 100x physiological concentration of CS, and that these changes were not always >10 %. It is also noted that the concentrations of the Mg-CS complexes are approximately 10x greater than those of the Ca-CS complexes. This is consistent with the binding constant values (Table 6.1 – page 227) which are approximately 10x greater for magnesium complexes.

Table 7.6

Speciation concentrations as a function of CSA concentrations in healthy subjects

Parameter	*[CSA] in Model 1*				*[CSA] in Model 2*				*[CSA] in Model 3*			
	1x	**2x**	**10x**	**100x**	**1x**	**2x**	**10x**	**100x**	**1x**	**2x**	**10x**	**100x**
$[Ca]^{+2}$ ($x10^{-4}$ M)	12.2	12.2	12.1	11.4	7.92	7.92	7.87	7.45	5.26	5.26	5.23	4.96
[Ca-CSA] ($x10^{-4}$ M)	0.02	0.04	0.18	1.85	0.01	0.03	0.13	1.29	0.01	0.02	0.09	0.86
$[Mg]^{+2}$ ($x10^{-4}$ M)	18.2	18.0	17.2	10.9	11.3	11.2	10.7	6.99	10.8	10.8	10.3	7.06
[Mg-CSA] ($x10^{-4}$ M)	0.20	0.39	1.90	13.2	0.14	0.27	1.30	8.99	0.13	0.26	1.27	9.11

Table 7.7

Speciation concentrations as a function of CSC concentrations in healthy subjects

Parameter	*[CSC] in Model 1*				*[CSC] in Model 2*				*[CSC] in Model 3*			
	1x	**2x**	**10x**	**100x**	**1x**	**2x**	**10x**	**100x**	**1x**	**2x**	**10x**	**100x**
$[Ca]^{+2}$ ($x10^{-4}$ M)	12.2	12.2	12.1	11.5	7.93	7.92	7.89	7.54	5.26	5.26	5.24	5.02
[Ca-CSC] ($x10^{-4}$ M)	0.01	0.03	0.17	1.64	0.01	0.02	0.12	1.13	0.01	0.02	0.08	0.75
$[Mg]^{+2}$ ($x10^{-4}$ M)	18.3	18.1	17.6	12.9	11.3	11.3	10.9	8.20	10.8	10.8	10.5	8.15
[Mg-CSC] ($x10^{-4}$ M)	0.13	0.26	1.26	9.61	0.09	0.17	0.84	6.43	0.08	0.17	0.81	6.43

7.5.2 CS supplementation

Urine composition

Urine parameters before and after supplementation in both race groups have been previously presented (Table 7.2). There were no statistically significant intra-group changes in any of the parameters following CS supplementation.

Effect of CS supplementation on the saturation of calcium salts

The SS values for calcium salts in black and white subjects at days 0 and 7 are shown in Table 7.8. Raw data for the speciation calculations are shown in Appendices 7.6 and 7.7 for blacks and whites, respectively.

Within groups, CS supplementation had no effect on the SS values of COM. However, in the black group, supplementation significantly decreased the SS values of calcium phosphate salts whereas in whites, the SS HAP value was significantly higher at day 7 than baseline. With respect to intragroup comparisons, SS brushite values were significantly lower in black subjects compared to white subjects on day 7. Other significantly different values at day 7 were also present at baseline; as such they can be ignored.

Table 7.8
Effect of CS supplementation on the SS values for calcium salts in black and white subjects

Parameter	*Blacks*			*Whites*			*B vs W*	
	Day 0	Day 7	p-values	Day 0	Day 7	p-values	Day 0	Day 7
COM	3.20 ± 0.84	3.51 ± 0.50	0.7677	3.40 ± 0.71	3.54 ± 0.84	0.8096	0.9054	0.4884
Brushite	1.95 ± 0.23	1.09 ± 0.10	0.0094*	1.60 ± 0.23	1.99 ± 0.39	0.4567	0.6085	0.0331‡
triCaP	8.04 ± 0.93	0.43 ± 0.08	0.0142*	1.36 ± 0.39	2.83 ± 0.43	0.0719	0.0476‡	0.0324‡
HAP (x 10^6)	24.2 ± 1.73	0.13 ± 0.04	0.0214*	0.85 ± 0.09	3.01 ± 0.47	0.0351*	0.0269‡	0.0436‡
OCP	226 ± 6.87	6.60 ± 1.21	0.0354*	31.3 ± 4.72	79.6 ± 5.82	0.0547	0.0397‡	0.0438‡

*: *p-value ≤ 0.05 comapred to baseline (intragroup comparison)*
‡: *p-value ≤ 0.05 (intergroup comparison)*

Effect of CS supplementation on speciation

The distribution of calcium species in the black and white groups at days 0 and 7 are presented in Figures 7.5 and 7.6 and for phosphate speciation in Figures 7.7 and 7.8, respectively. Raw data for the speciation calculations are shown in Appendix 7.6 and 7.7 for blacks and whites, respectively. The concentrations of species before and after supplementation are summarized in Table 7.9.

The decrease in the SS values of CaP salts in black subjects can be attributed to the decrease in the concentration of $[Ca]^{+2}$ and $[HPO_4^{2-}]$ (Table 7.9) albeit that neither of these was statistically significant. These effects can probably be attributed to the decrease in pH which occurred post CS supplementation. Since there were no statistically significant differences in dietary intake at baseline and at day 7, these differences are not diet related. It is not possible to deduce whether the decrease in pH itself can be attributed to the ingestion of CS.

In white subjects, the increase in the SS of HAP can be attributed to the increase in the concentration of $[Ca]^{+2}$ at day 7, despite the decrease in $[HPO_4^{2-}]$ (Table7.9). As noted in the white group, these changes were not statistically significant. Unlike the black group, pH did not change.

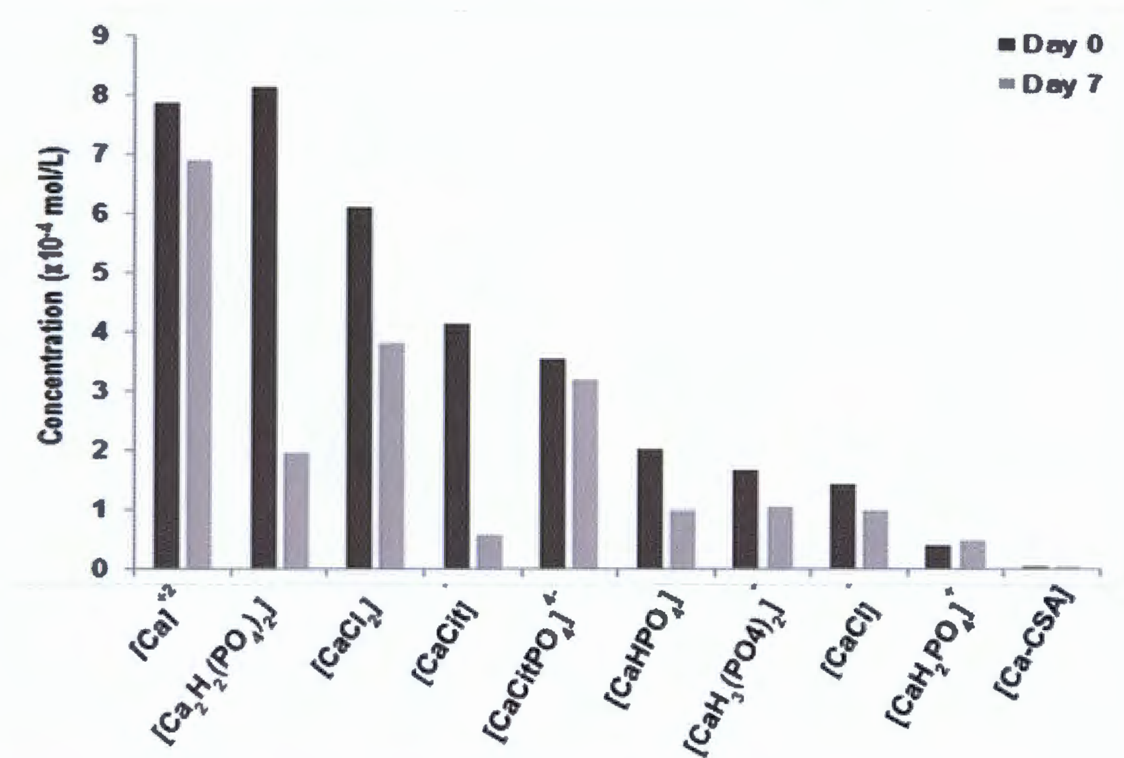

Figure 7.5: Calcium speciation in black subjects pre- and post- supplementation with CS

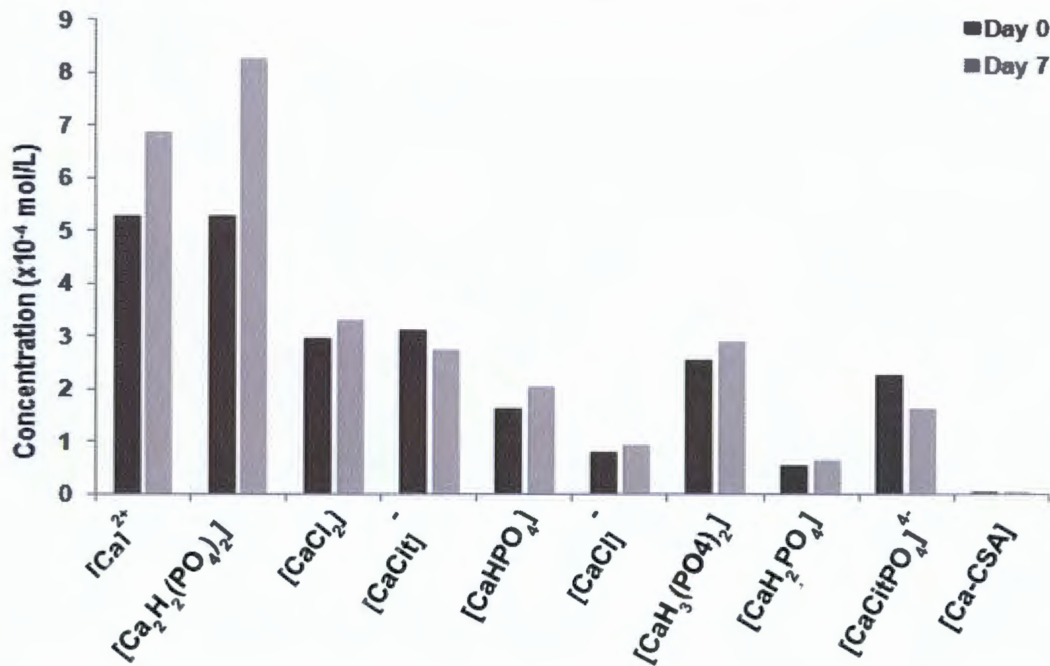

Figure 7.6: Calcium speciation in white subjects pre- and post- supplementation with CS

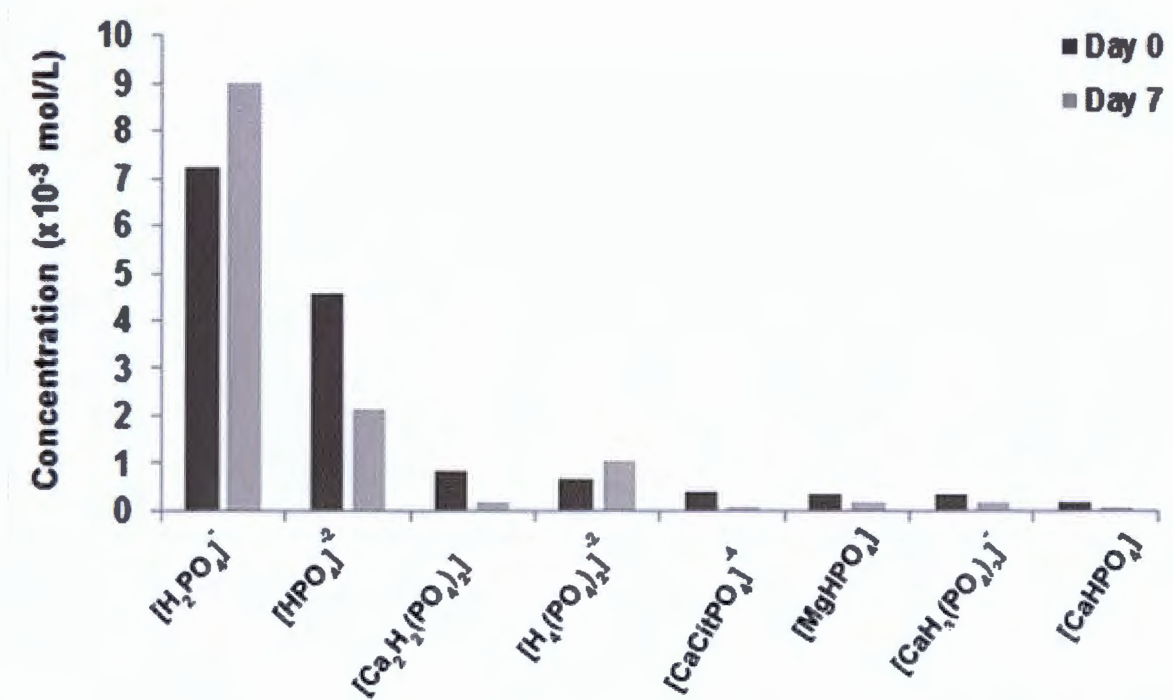

Figure 7.7: Phosphate speciation in black subjects pre- and post- supplementation with CS

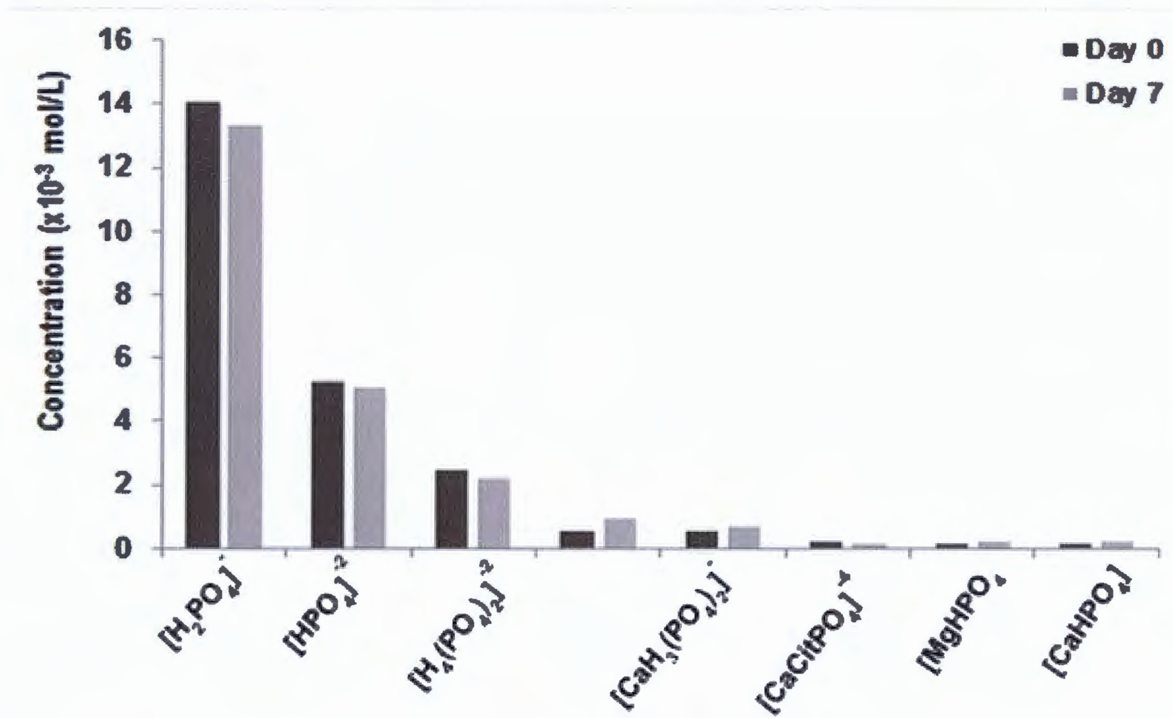

Figure 7.8: Phosphate speciation in white subjects pre- and post- supplementation with CS

Table 7.9

Effect of CS supplementation on the concentration of species in black and white subjects

Parameter	*Blacks*			*Whites*			*B vs W*	
	Day 0	Day 7	p-value	Day 0	Day 7	p-value	Day 0	Day 7
pH	6.41 ± 0.21	6.03 ± 0.09	0.1241	6.20 ± 0.11	6.21 ± 0.12	0.9520	0.3832	0.2599
[Ca] (tot) (x10^4 moles/L)	35.6 ± 4.10	30.1 ± 2.94	0.4120	25.1 ± 3.98	32.5 ± 8.59	0.4502	0.0950	0.2033
$[Ca]^{+2}$ (x10^4 moles/L)	8.29 ± 1.54	6.78 ± 0.77	0.4003	5.37 ± 0.83	7.07 ± 1.50	0.3455	0.1261	0.8642
[Ox] (tot) (x10^4 moles/L)	1.82 ± 0.16	1.63 ± 0.13	0.3763	2.64 ± 0.30	2.27 ± 0.34	0.4231	0.0370‡	0.1067
$[Ox]^{-2}$ (x10^4 moles/L)	0.92 ± 0.07	0.88 ± 0.05	0.6612	1.40 ± 0.12	1.12 ± 0.11	0.1183	0.0050‡	0.0702
[Phos] (tot) (x10^4 moles/L)	200 ± 30.1	163 ± 25.9	0.2962	313 ± 46.7	303 ± 57.2	0.8938	0.0829	0.0508
$[HPO_4]^{-2}$ (x10^4 moles/L)	44.6 ± 10.3	20.6 ± 3.03	0.0566	49.3 ± 5.39	43.8 ± 4.80	0.4638	0.7025	0.0017‡
[Mg] (tot) (x10^4 moles/L)	24.9 ± 1.70	20.4 ± 2.69	0.1956	28.3 ± 3.93	31.2 ± 6.01	0.6933	0.4388	0.1327
$[Mg]^{+2}$ (x10^4 moles/L)	11.6 ± 0.49	9.59 ± 1.20	0.1622	11.0 ± 1.44	13.5 ± 2.88	0.4610	0.7435	0.2383
[Ca-CSA] (x10^4 moles/L)	0.01 ± 0.003	0.02 ± 0.002	0.0217*	0.01 ± 0.001	0.02 ± 0.004	0.0086*	0.1218	0.8807
[Mg-CSA] (x10^4 moles/L)	0.14 ± 0.01	0.24 ± 0.02	0.0029*	0.13 ± 0.01	0.31 ± 0.06	0.0131*	0.7147	0.2733

*: *p-value ≤ 0.05 compared to baseline (intragroup comparison)*

‡: *p-value ≤ 0.05 (intergroup comparison)*

7.6 DISCUSSION

In the present study, the theoretical calculations showed that the reduction in the concentration of ionized calcium, and therefore risk in CaOx stone formation, is largely dependent on the concentrations of CSA and CSC. Although favourable results were observed at 100x for both CSA and CSC, the required CSA and CSC concentrations are unrealistic in humans since they are not clinically feasible. In addition, the binding capacity of CSA and CSC with magnesium in real urines was shown to be higher than that for calcium. These findings are potentially unfavourable for CaOx stone formation in two ways. Firstly, urinary magnesium may compete successfully with calcium for binding CSA and CSC in urine, and thereby compromising the capacity of CSA and CSC to reduce the saturation of CaOx. Secondly, a reduction in the concentration of $[Mg]^{+2}$ (caused by complexation with CS) is unfavourable since this will reduce the concentration of [MgOx], thereby releasing more Ox^{-2} which can potentially bind with calcium and form CaOx crystals.

Although the concentration of the [Ca-CSA] complex was increased in both groups at day 7 compared to baseline, the concentrations of ionized calcium were not significantly different to those at baseline (Table 7.9). Furthermore, the SS values of COM were unaffected by supplementation (Table 7.8). Although SS values of the CaP salts decreased in the black group, this effect could not be attributed to the ingestion of CS *per se*. The increase in SS of HAP in whites is also an anomaly.

It is recognized that an important limitation of this study is that urinary CS concentrations were not measured on days 0 and 7 (Chapter 5 – page 208). Nevertheless, the CS concentrations that were used for speciation calculations were considered appropriate to reflect the CS concentrations that might be expected in healthy subjects before and after supplementation.

Summary

The results of this study show that the formation of the Ca-CSA and Ca-CSC complexes was not effective in reducing the saturation levels of urinary CaOx in healthy subjects. Moreover, the presence of competing cations with calcium such as magnesium might have an influence on the role of these GAGs in real urine samples. The results also demonstrate that black and white subjects respond differently to CS supplementation.

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Chapter 8

Summary of baseline differences between black and white South African healthy subjects

8.1 INTRODUCTION

As previously described in Chapter 1 – page 24, the incidence of kidney stone disease in South Africa is lower in the black population than in whites. The influences of factors such as nutrition, metabolic disorders and genetics on the ethnic difference in the prevalence of kidney stone disease in these populations have been investigated [1 - 5].

In Chapters 2 – 4, several differences in the dietary and urinary risk factors were observed between the two groups at baseline. These differences are not part of the objectives of this thesis, but since they are contributing to the motivation for conducting the present investigation, they need to be discussed.

8.2 DIETARY RISK FACTORS

Mean nutrient intakes in black and white subjects observed at baseline in studies described in Chapter 2 to 5 are shown in Table 8.1. Compared with whites, black subjects had a lower intake total sugars and calcium.

8.3 URINARY RISK FACTORS

The differences in the urinary parameters associated with CaOx stone formation between blacks and whites observed at baseline during studies described in Chapters 2 - 5 are shown in Table 8.2. Urinary pH was significantly higher in blacks compared to the white subjects while magnesium, phosphate, SS CaOx and SS brushite were significantly higher in whites.

Table 8.1
Mean nutrient intake in black and white subjects

Nutrient	***Blacks*** (n= 38)	***Whites*** (n=34)	p-values
Moisture [g]	1929 ± 139	1886 ± 147	0.8342
Energy [kJ]	10101 ± 654	10494 ± 577	0.6564
Total protein [g]	97.9 ± 8.26	99.6 ± 5.63	0.8653
Animal protein [g]	60.4 ± 7.27	59.3 ± 5.05	0.9019
Total fat [g]	82.4 ± 7.90	91.4 ± 8.14	0.4306
Carbohydrate [g]	294 ± 18.9	286 ± 16.1	0.7744
Total sugars [g]	43.0 ± 6.02	67.1 ± 9.59	0.0333‡
Total dietary fibre [g]	23.4 ± 1.91	30.9 ± 3.85	0.0754
Ca [mg]	439 ± 57.2	759 ± 73.3	0.0009‡
Mg [mg]	316 ± 22.8	365 ± 28.4	0.1841
P [mg]	1255 ± 89.6	1446 ± 85.9	0.1307
K [mg]	2935 ± 222	3067 ± 200	0.6622
Na [mg]	2527 ± 262	2788 ± 273	0.4928
Cl [mg]	1875 ± 302	1937 ± 330	0.8892
Phytate [mg]	287 ± 47.1	194 ± 32.7	0.1155
Malic acid [mg]	469 ± 86.4	973 ± 253	0.0526
Citric acid [mg]	969 ± 201	1645 ± 304	0.0629
Oxalic acid [mg]	27.5 ± 5.70	64.0 ± 21.0	0.0816
Vitamin B6 [mg]	1.89 ± 0.17	2.26 ± 0.20	0.1602
Vitamin C [mg]	207 ± 44.6	117 ± 20.7	0.0800
Vitamin D [µg]	3.05 ± 0.61	2.73 ± 0.56	0.7073
Vitamin E [mg]	10.1 ± 1.97	14.6 ± 2.06	0.1182
Hydroxyproline [g]	0.49 ± 0.10	0.33 ± 0.07	0.2190
C18:2 n-6 LA (g)	24.4 ± 2.94	20.3 ± 2.31	0.2831
18:3 n-3 ALA (g)	0.53 ± 0.06	0.66 ± 0.05	0.1108
C18:3 n-6 GLA (g)	0.53 ± 0.06	0.66 ± 0.05	1.1108
20:3 n-6 DGLA (g)	0.02 ± 0.004	0.02 ± 0.01	0.3786
C20:4 n-6 AA (g)	0.13 ± 0.02	0.11 ± 0.02	0.3842
C20:5 n-3 EPA (g)	0.05 ± 0.01	0.07 ± 0.02	0.4609
C22:6 n-3 DHA (g)	0.11 ± 0.03	0.12 ± 0.03	0.8541

‡ : *p-value ≤ 0.05 (intergroup comparison)*

Table 8.2
Mean urine parameters in black and white subjects

Parameter	***Blacks*** (n=38)	***Whites*** (n=34)	p-value
pH	6.45 ± 0.11	6.20 ± 0.05	0.0478‡
Volume (mL/24h)	1284 ± 78.2	1186 ± 92.4	0.4149
Calcium (mmol/24h)	2.38 ± 0.17	2.74 ± 0.16	0.1269
Chloride (mmol/24h)	149 ± 7.22	128 ± 10.8	0.1071
Citrate (mmol/24h)	2.16 ± 0.15	2.20 ± 0.18	0.8895
Creatinine (mmol/24h)	16.8 ± 0.53	18.0 ± 0.61	0.1445
Magnesium (mmol/24h)	1.93 ± 0.15	2.68 ± 0.18	0.0020‡
Oxalate (mmol/24h)	0.26 ± 0.01	0.27 ± 0.02	0.7716
Phosphate (mmol/24h)	23.7 ± 1.01	33.7 ± 1.61	<0.0001‡
Potassium (mmol/24h)	36.3 ± 2.23	34.6 ± 2.39	0.5994
Sodium (mmol/24h)	129 ± 6.24	115 ± 7.34	0.1400
Urate (mmol/24h)	3.37 ± 0.14	3.68 ± 0.14	0.1212
Ionized Ca^{2+} (mmol/L)	0.72 ± 0.12	0.50 ± 0.08	0.1269
PGE2 (ng/24h)	601 ± 50.0	582 ± 42.3	0.7936
HYP (nmol/mg creatinine)	1.26 ± 0.45	0.98 ± 0.13	0.6035
MSL (MmM)	1.19 ± 0.22	0.90 ± 0.05	0.1960
TRI	178 ± 12.5	188 ± 28.7	0.7213
SS CaOx	3.83 ± 0.34	4.98 ± 0.41	0.0331‡
SS Brushite	1.12 ± 0.18	1.73 ± 0.21	0.0304‡
SS Uric acid	1.28 ± 0.22	1.88 ± 0.23	0.0618

‡ : *p-value ≤ 0.05 (intergroup comparison)*

8.4 COMMENTS

The higher dietary intake of calcium and sugar in the white subjects who participated in the various studies described in this thesis might have been expected to cause significantly higher excretion of urinary calcium in this group [1 - 4]. However, this did not occur (Table 8.2).

The significantly lower excretion of magnesium in black subjects is counterintuitive as lower concentrations of this component have been attributed to a higher risk of stone formation by some workers [1, 9].

The most important difference in the urinary composition of the two groups is the significantly higher pH in black subjects which manifests itself in lower values for the supersaturation of CaOx and brushite.

The dietary and urinary similarities, differences and anomalies between blacks and white subjects which were observed in the present study have been reported in numerous previous investigations [1-9]. The findings in this regard are therefore not new. Of importance is whether the mean diets and urine chemistries observed in the present study might be conflicting factors in interpreting the results which have been recorded. Thus, the lower supersaturation values for CaOx and brushite in blacks need to be taken into account in the present studies. Since these parameters did not undergo any changes as a result of FA or CS supplementation, it can be concluded that there was no conflict.

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8.5 REFERENCES

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Concluding remarks

Concluding remarks

In drawing conclusions about the various studies described in this thesis, it is helpful to consider the extent to which the objectives were met and what they revealed.

The first main aim was to investigate potential beneficial effects of supplemental PUFAs and CS on the risk of CaOx stone formation. Although several changes in various blood and urine parameters were observed, ingestion of n-6 and n-3 fatty acids independently and in combination did not significantly reduce blood and urinary risk factors for CaOx urolithiasis. Taken together, these findings indicate that at the tested doses, FA supplementation in healthy subjects were not effective in reducing the risk factors associated with CaOx stone formation. It is not yet possible to draw conclusions on the role of FAs as therapeutic agents for CaOx stone disease since only healthy subjects were studied. Whether the reduction of urinary calcium and oxalate will be associated with FA supplementation still needs to be investigated, perhaps in a follow up study with stone formers. Nevertheless, the findings of the present studies are important since they form a foundation for evaluating the effects of both n-6 and n-3 FAs on CaOx risk factors in human subjects.

Theoretical modeling and *in vitro* crystallization experiments demonstrated that chondroitin sulfates A and C would not have an effect on the supersaturation of urinary calcium salts when present at normal physiological concentrations. This was confirmed by an *in vivo* study in which subjects ingested CS supplements. Overall, findings from these studies suggest that CS does not reduce supersaturation of CaOx stone formation in healthy subjects. Therefore, the use of CS as a therapeutic agent for CaOx stone disease remains doubtful.

It is acknowledged that there were limitations to these studies. The use of commercial salts instead of CS extracted from human urine might have influenced the outcome. The mode of action of commercial CS and human GAGs could not be compared in the present work. Further studies, involving CS obtained from humans, are needed to confirm the present findings. It could also be intriguing to investigate the effect of other urinary GAGs (besides CSA and CSC) binding to calcium as well. The protocol outlined in the present thesis could be used as a template for studies aimed at investigating the role of these GAGs in CaOx stone formation. Another limitation of the present study was the use of estimated urinary CS concentrations as opposed to empirically measured values in human subjects.

Concluding remarks

Thus, the results of the studies in this thesis do not support the hypothesis that supplemental fatty acids or chondroitin sulfate have beneficial effects for reducing blood and urinary risk factors for CaOx stone formation.

The second main aim of the project was to investigate whether black and white South Africans respond differently to FA and CS supplements and if so, whether such differences provide insights into understanding the anomaly of the different stone incidence rates in the two race groups, and whether these in turn, help us understand stone pathogenesis, and thereby leading to insights for improved therapeutic management.

In the present study, the blood (FA studies) and urinary outcomes after FA and CS supplementation were found to be different between the two groups. Thus, supplementation yielded different results within groups. Other studies have also reported different renal handling mechanisms in blacks and whites after various supplement challenges. However, the present (and previous) studies have not been able to provide new information to explain the difference in the incidence of CaOx stone disease in the two population groups.

Thus, the inter-group results of the studies in this thesis do not support the hypothesis that different renal handling of fatty acids and chondroitin sulfate will shed light on the different stone occurrences in the two groups. However, the observation that such differences exist is likely to be important in the eventual resolution of this phenomenon.