Effects of kidney function and dietary vitamin C intake on ascorbate concentrations in plasma, spot urine and 24-h urine and effects of time and initial concentrations on ascorbate losses in 24-h urine samples compared to fresh urine samples.

Thesis

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By

Theopisti Maimari

Registr. Nr: 1030781

Human Nutrition & Metabolism Research and Training Center
Institute of Molecular Biosciences
Karl-Franzens University of Graz
Universitätsplatz 2, 8010 Graz, Austria

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INTRODUCTION

The term vitamin C comprises the reduced form ascorbic acid (or ascorbate) and its oxidized form, dehydroascorbic acid (DHA) *(Fig. 1* (Food and Nutrition Board, 2000)*)*. Ascorbic acid is the enolic form of an α-ketolactone. Due to the two enolic hydrogen atoms the molecule contains, it can provide electrons and thus function as a reductant and antioxidant. An asymmetric carbon atom allows the formation of two enantiomers of which only the L-form occurs naturally (Tolbert et al., 1975). DHA can be reduced back to ascorbic acid *in vivo*. However, it can also be hydrolyzed irreversibly to 2,3-diketogulonic acid (Davis et al., 1991).

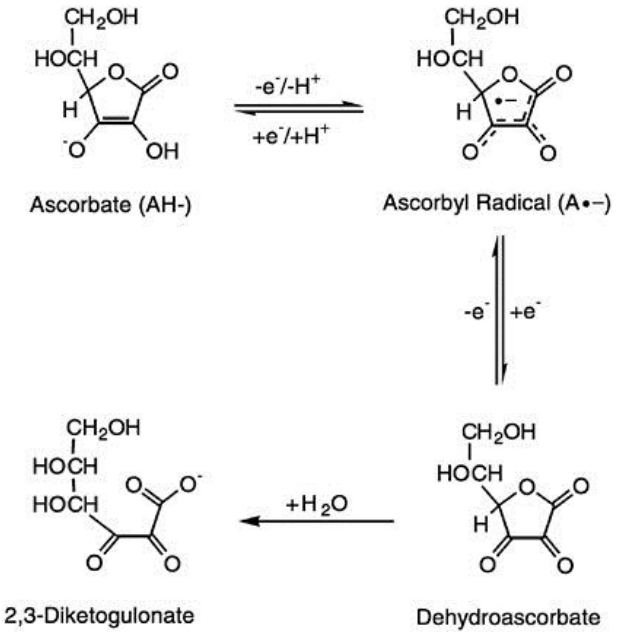

Figure 1: *Oxidation of ascorbic acid to dehydroascorbic acid and hydrolysis to 2, 3-diketogulonic acid.*

Ascorbate is an essential vitamin for humans, because it cannot be synthesized by humans due to the absence of the enzyme, gulonolactone oxidase (GLO), which catalyzes the last enzymatic step in ascorbate synthesis (Mandl, et al., 2009). This vitamin is usually ingested through food, often through supplements and, in rare cases, when necessary, it can be administered intravenously. It is water-soluble and therefore it is excreted through urine.

The World Health Organisation (WHO) recommends a daily intake of 45 mg for healthy adults (WHO International, 2011). The Recommended Daily Allowance (RDA) is higher for

smokers compared to non-smokers and higher for males compared to females: the RDA for male smokers is 90 mg/day and for female smokers 75 mg/day (Food and Nutrition Board, 2000). Increased oxidative stress caused by the variety of oxidizing agents in tobacco smoke leads to an increased consumption of antioxidants. Therefore, an increased intake of vitamin C is recommended in order to inhibit cigarette smoke-induced free radical damage, which might enhance the risk of developing several severe diseases (Bui, et Al., 1992) (Lykkesfeldt, et al., 2007).

Vitamin C functions as an antioxidant, protecting against oxidative stress by scavenging reactive oxygen and nitrogen species, singlet oxygen and hypochlorite (Carr & Frei, 1999). Furthermore, it functions as a co-factor in several enzymatic reactions due to its ability to act as an electron donor (Higdon, 2011). It has been suggested that ascorbate influences collagen gene expression, the secretion of cellular procollagen, as well as the biosynthesis of other connective tissue components (Ronchetti et al., 1996). It has been shown that ascorbate is involved in the accumulation of iron through the divalent metal transporter (DMT1) in astrocytes (Lane et.al, 2010). Vitamin C catalyzes the hydroxylation of the side chain of dopamine to form norepinephrine and xx α-amidating monooxygenase enzymes, which are important for the biosynthesis of neuropeptides (Katsuki 1996). Furthermore, vitamin C has been shown to stimulate the production and function of leukocytes (Levy, et al., 1997) (Prinz, et al., 1977). Functions of neutrophils such as chemotaxis, phagocytosis or superoxide generation showed improvement when treated with ascorbic acid (Levy, et al., 1997). In addition, it has been shown that neutrophils fail to undergo morphological changes associated with apoptosis in the presence of ascorbate deficiency (Vissers & Wilkie, 2007). Apoptosis is of great importance for neutrophils as an injury-limiting mode of neutrophil disposal (Savill, 1997). Vitamin C has also been shown to inhibit stimulated leukocyte adhesion and aggregation (Vitamin C in Health and Disease, 1997).

It has been suggested that very large amounts of vitamin C may exert adverse effects, including cancer, atherosclerosis, kidney stones, excess iron absorption, vitamin B_{12} deficiency, etc. The results of the studies reported are conflicting and no final conclusions could be drawn regarding the adverse effects of vitamin C (Higdon, 2011).

Ascorbate´s biological half-life varies widely from 8 to 40 days and is inversely related to the ascorbate body pool (Mandl, 2009). It has been shown that in a depleted state about 3 % of the total existing pool of ascorbate was degraded daily (Kallner, et. al, 1979). Furthermore, when the administered dose was higher than approximately 500 mg, then all the excess ascorbate was excreted through urine (Levine, et al., 1996). As a result of dietary intake of vitamin C, the plasma concentrations achieved do not exceed 100 µmol/l. The concentrations are always <250 µmol/l and frequently even <150 µmol/l when supplements are administered (Levine, et al., 2011). By intravenous administration of >200 mg/d, tight control is bypassed until excess ascorbate is eliminated by glomerular filtration and renal excretion (Levine et al., 2011).

It has been suggested that the bioavailability of vitamin C in humans is affected by some food components. A recent study showed that the consumption of mashed potatoes and potato chips increased the plasma concentrations of ascorbate to a higher extend as compared to intake of similar amounts of ascorbate present in water, and less ascorbate tended to be excreted in urine than was consumed with potatoes (Kondo, et al., 2012). Another study showed that acerola juice tended to promote the absorption of vitamin C in plasma concentrations and minimized its excretion via urine (Uchida, et al., 2011).

Impaired dietary intake of vitamin C may lead to scurvy, the vitamin C deficiency disease. Vitamin C intake below 7.5 mg/d over a period of less than 90 days increases the risk of symptomatic vitamin C deficiency (Hodges, 1971). Scurvy usually appears at plasma concentrations of less than 11 µmol/l(Food and Nutrition Board, 2000). Characteristic symptoms are follicular hyperkeratosis, petechiae, ecchymosis, coiled hair, inflamed and bleeding gums, hemorrhagic phenomena, joint effusions, muscular aches, arthralgia and impaired wound healing (Hodges, et. al, 1971).

Recent studies claimed that vitamin C deficiency is not only a nutritional disorder but also a genetic disease due to its relation to haptoglobin (Hp). When the antioxidant capacity of Hp is severely impaired, its role is taken over by hemopexin, a heme-binding protein and by vitamin C. (Delanghe, et al., 2011). Haptoglobin is an antioxidant which binds hemoglobin. It shows a genetic polymorphism with two alleles and three types: Hp 1-1, Hp 2-1, Hp 2-2. It

has been shown that subjects with type Hp 2-2 had the lowest serum concentrations of vitamin C, while no differences among the three types were observed for other antioxidants (Langlois, Delanghe, et al., 1997) (Cahill & El-Sohemy, 2010). The renal threshold and urinary excretion of ascorbate did not differ between the Hp genotypes, which suggests that differences in the oxidation rate? state of ascorbic acid in the blood are a more likely explanation of differences between Hp genotypes than differences in vitamin C elimination (Cahill & El-Sohemy, 2010). Further investigations are required in order to verify if haptoglobin indeed plays an important role in the metabolism of vitamin C and to determine how haptoglobin influences the vitamin C requirements. This could allow for the development of polymorphism-targeted treatments for diseases, in which the redox state of the patients is involved.

Recently, the *latent scurvy theory* has been established, based on experimental and clinical observations and on the facts that hypoascorbinaemia shares several clinical symptoms with diabetes mellitus and that vitamin C shares structural similarities with glucose (Fig. 2 Roger, et al.) (Mandl et al., 2009). The hypothesis reported was that hyperglycemia competitively inhibits the cellular uptake of ascorbate, inducing *intracellular* vitamin C deficiency.

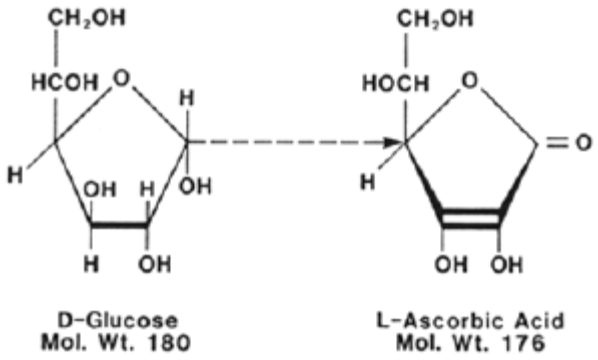

Figure 2: *Molecular structures of D-glucose and L-ascorbic acid.*

Ascorbate concentrations in humans are determined through three mechanisms: intestinal absorption, tissue accumulation and renal reabsorption and secretion (Corper, et al., 2010). In the intestine ascorbate is absorbed from the lumen by enterocytes and in the kidneys, after glomerular filtration, it can be reabsorbed from the renal tubules by renal epithelial cells (Wilson, 2005). The tight control of the ascorbate concentrations is explained by ascorbate transport and tissue accumulation. The transport of ascorbate is known to be

mediated through two sodium-dependent ascorbate transporters (SVCT1, SVCT2), which are expressed by the following genes: *Slc23a1* and *Slc23a2,* respectively (Corper, et al., 2010) (Wilson, 2005) (Daruwala et al., 1999). A recent study showed that *Slc23a1-/-* mice lost their ability to reabsorb ascorbate filtered by the kidneys. Their plasma concentrations were dramatically reduced and a 3-fold increase in urinary ascorbate losses was observed, which led to the conclusion that *Slc23a1* is essential for the tubular reabsorption of ascorbate and the tight control of ascorbate plasma concentrations (Corper, et al., 2010). A study investigating the activity of SVCT in Madin-Darby canine kidney cells transfected with the human MDR1 gene (MDCK-MDR1) and the mechanism of ascorbate uptake showed that the uptake of ascorbate is dependent on the presence of sodium, on temperature and on ascorbate concentrations, with saturation occurring in the micromolar range. The authors also showed that ascorbate uptake was enhanced when the pH in the medium increased (Luo, et al., 2008) (Liang, et al., 2001). In some cell types such as neutrophils, the accumulation of ascorbate could also be achieved via oxidation (Washko, et al., 1993). When neutrophils were activated, the vitamin C located in the extracellular space was oxidized to dehydroascorbate, which was preferentially transported and reduced intracellularly to ascorbate. As the intake of vitamin C increased, the concentrations of ascorbate in the body did not increase proportionally. Instead, it tended to reach saturation. With increasing plasma concentrations of ascorbate, the renal clearance increased due to saturable tubular reabsorption (Blanchard, et al., 1997). Thus, the intake of megadoses of vitamin C does not have apparent effects on vitamin C status.

Since the kidneys play an important role for both tubular re-absorption and elimination of ascorbate, it can be assumed that any kind and stage of renal dysfunction could potentially affect vitamin C status. A study on patients suffering from diabetic nephropathy showed that the patients with clinical nephropathy (with macroalbuminuria) had lower plasma concentrations of ascorbate and higher renal clearance of ascorbate than patients with microalbuminuria (Hirsch, et al., 1998). Another study showed that vitamin C deficiency was prevalent in patients on either continuous ambulatory peritoneal dialysis or maintenance hemodialysis. A possible protective role of vitamin C in the increased inflammatory state of dialysis patients has been suggested (Zhang, et al., 2011). Patients on peritoneal dialysis or hemodialysis who were not supplemented with vitamin C showed significantly lower plasma

ascorbate concentrations compared to control subjects (Sundl, et al., 2009) (Roob, et al., 2000). Low plasma concentrations of vitamin C were also observed in children on peritoneal dialysis in comparison with healthy individuals and losses of 4.476±0.543 µmol/l and 4.659±0.445 µmol/l vitamin C in ultrafiltrated dialysate with 1.36% glucose and 2.27% glucose concentrations in the dialysate, respectively, was documented (Zwolinska, et al., 2009).

Regarding the metabolism of ascorbate in patients with impaired renal function, it is still unclear whether it is consumed as an antioxidant in the circulation due to high oxidative stress caused by impaired renal function (Oberg, et al., 2004) or it is excreted through urine due to impairment or inability of reabsorption (The Nephron Information Center, 2012). Under normal circumstances, when vitamin C deficiency appears, it can be treated with supplementation. Nevertheless, a recent study on patients receiving hemodialysis or having an estimated glomerular filtration rate (GFR) <20 ml/min/1.73 m^2, indicated that supplementing ascorbate does not improve symptoms, but it tends to worsen nausea (Singer,2011). Gastrointestinal disturbances such as nausea, abdominal cramps, and diarrhea are the most common adverse effects of vitamin C intake (Hoffer, 1971).

A study in an Australian peritoneal dialysis population suggested that severe ascorbate deficiency may take several months to develop, but that correction can easily be achieved with ascorbate doses that are unlikely to cause oxalate overload (Singer, etal., 2008). Oxalate is a metabolite of ascorbate. Some studies have demonstrated that high dose intake of ascorbate influenced the urinary excretion of oxalate (Taxer, et al., 2003), (Schmidt et. al, 1981), whereas other studies reported that supplemental vitamin C did not increase the urinary oxalate levels (Auer et. al, 1998), (Wandzilak, et al., 1994). Therefore, it can be concluded that the results regarding the influence of vitamin C on the formation of oxalate kidney stones are conflicting.

A further disease by which the vitamin C status is affected is diabetes mellitus. Type II diabetes (T2DM) is a complex metabolic disease characterized by high blood glucose levels (hyperglycemia), inflammation, high oxidative stress and insulin resistance (Mesallamy, et al., 2007). Several studies have shown that the plasma concentrations of ascorbate in

patients suffering from T2DM are decreased in comparison to healthy individuals (Cunnungham, et al., 1991) (Will & Byers, 1996) (Shim, et al., 2010). A recent study reported an interesting finding: smoking modified the effects of diabetes mellitus on serum concentrations of ascorbate. Among the smokers who participated in the study, diabetes status did not make a difference in serum concentrations of ascorbate. Mean serum concentrations of ascorbate of smoking patients with diabetes mellitus and controls were 19.4±15.7 and 21.2±16.0 µmol/l, respectively, and this difference was not statistically significant (Shim, et al., 2010), suggesting that smoking could have a stronger effect on vitamin C status than diabetes. However, this finding warrants further investigation.

Another study showed that the supplementation of diabetic patients with antioxidant vitamins including ascorbate significantly decreased the *ex vivo* susceptibility of low density lipoprotein (LDL) to oxidation by Cu++ ions (Anderson, et al., 1999). The oxidation of LDL is believed to play an important role in the pathogenesis of atherosclerosis, a disease showing a high prevalence in patients with diabetes mellitus (Anderson, et al., 1999). A further study showed that oral supplementation of vitamin C in combination with metformin, an antidiabetic drug, reduced the blood glucose concentrations and improved glycosylated hemoglobin concentrations in patients with diabetes mellitus (Dakhale, et al., 2011). These findings, along with additional investigations, could potentially open a new treatment option for maintaining good glycemic control in patients with diabetes mellitus.

STUDY AIMS

Part I:

The study aim of the first part of this study was the comparison and statistical analysis of dietary vitamin C intake and ascorbate concentrations in spontaneous (spot) urine samples, 24-h urine collections and plasma of four groups of study subjects with different stages of renal function: a group with GFR <30 ml/min/1.73 m^2), a second group with GFR 30-60 ml/min/1.73 m^2, a third group with GFR 60-90 ml/min/1.73 m^2, and a fourth group with GFR >90 ml/min/1.73 m^2. GFR was determined using the "4-variable MDRD" formula for calculating the GFR developed by the *Modification of Diet in Renal Disease Study Group* (Levey, et al., 1999) (MDRD), which estimates GFR using four variables: serum creatinine, age, race, and gender (National Kidney Foundation).

GFR is an important indicator for the severity of impairment of renal function. A moderately decreased GFR (30-59 ml/min/1.73 m^2) indicates a moderate impairment, a severely decreased GFR (15-29 ml/min/1.73 m^2) indicates severe impairment, and a GFR <15 ml/min/1.73 m^2 indicates kidney failure. Kidney damage is to be observed in mildly impaired (60-89 ml/min/1.73 m^2) or normal or increased GFR ($\geq$ 90 ml/min/1.73 m^2) (Bauer et. al, 2008). Kidney impairment is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies (Bauer, Melamed, & Hostetter, 2008).

Table 2: *Stages of Chronic Kidney Disease (CKD), Bauer et. al (2008)*

CKD stages	***GFR levels***
CKD1 (Normal)	90mL/min/1.73m^2 and no proteinuria
CKD2 (Mild)	60-89 mL/min/1.73m^2 with evidence of kidney damage
CKD3 (Moderate)	30-59 mL/min/1.73m^2
CKD4 (Severe)	15-29 mL/min/1.73m^2
CKD5 (Kidney failure)	< 15 mL/min/1.

Part II:

As a second part of this study, the differences in the concentrations and calculated amounts of ascorbate in spontaneous urine (spot urine) and 24-hour urine collections (24h urine) were investigated in young adults in order to estimate the losses of vitamin C over a period of 24 hours and to show whether these losses are significant or not. This could potentially be of great importance because of the fact that 24-h urine samples are often used, for instance for the estimation of excretion of the total daily amounts and calculations of the ascorbate clearance. The question of whether or not significant losses of ascorbate occur over time has been considered critical for the design of future studies.

STUDY SUBJECTS

Part I:

For the first part of this study, the study subjects (patients and healthy volunteers) were selected from a large study population recruited in the BIOCLAIMS project, entitled ''Biomarkers of robustness of metabolic homeostasis for nutrigenomics-derived health claims made on food'', a project of the 7^{th} Framework Programme of the European Commission. The study protocol was approved by the Ethics Committees of the Medical University of Graz and the Karl-Franzens University of Graz, and informed consent was obtained from the study participants prior to the investigation.

Selection of the study participants has been based on their current renal function. GFR was used for estimation of renal function and the proposed thresholds recommended by the National Kidney Foundation for normal renal function and slightly, mildly and moderately impaired renal functions were applied. The study subjects were assigned to one of 4 groups: groups I-IV with moderately impaired, mildly impaired, slightly impaired, and normal renal function (GFR <30, 30-60, 60-90 and >90 ml/min/1.73 m^2, respectively). Eligible study subjects were identified in the BIOCLAIMS database based on GFR, age, and gender. There were only 17 subjects participating in the BIOCLAIMS project with GFR <30 ml/min/1.73 m^2, of whom 5 were female. They were used as the reference group for matching the other groups for age. They were between 58 and 82 years old and the study subjects in the other groups were matched for age as closely as possible to exclude age effects on the variables

studied as far as possible. In total, all the subjects were between 53 and 82 years old. In addition, 50 % had to be males and 50 % females. Furthermore, they all had to be non-smokers and were not allowed to suffer from diabetes mellitus. Figure 3 shows the four groups with details on age and gender of the individual study subjects

Group I.

Gender	Age	GFR<30
m	76	29.24
m	70	27.17
m	77	27.56
m	70	25.29
m	69	26.60
f	76	26.01
f	68	19.33
f	58	26.63
f	82	26.75
f	78	25.08

Group II.

Gender	Age	GFR 30-60
m	76	42.88
m	70	51.73
m	77	40.39
m	71	48.77
m	70	32.49
f	76	36.85
f	68	32.75
f	59	54.29
f	82	57.41
f	78	46.59

Group III.

Gender	Age	GFR 60-90
m	74	63.12
m	70	80.37
m	79	61.27
m	70	76.54
m	69	63.58
f	74	70.20
f	68	70.23
f	58	78.20
f	62	74.81
f	71	83.80

Group IV.

Gender	Age	GFR >90
m	76	92.60
m	53	99.66
m	62	92.77
m	61	114.80
m	64	95.96
f	74	91.82
f	67	92.49
f	63	107.30
f	72	102.84
f	63	99.20

Figure 3: *The four GFR groups with details on gender and age of the individual study subjects selected. Group I: GFR < 30, Group II: GFR 30-60, Group III: 60-90, Group IV: GFR>90 ml/min/1.73 m^2; m: male, f: female.*

Part II:

The subjects of the second part were 10 healthy non-smoking volunteers, aged 25 to 34 years, 3 females and 5 males, and with no known renal impairment or diabetes.

STUDY DESIGN

Part I:

This was a cross-sectional observational study, using plasma and urine samples as well as dietary protocols that have been collected in the BIOCLAIMS project by the study subjects fulfilling the inclusion/exclusion criteria described above. The samples collected and specifically prepared for vitamin C analysis (addition of metaphosphoric acid) on the day of the investigation were retrieved from the BIOCLAIMS sample collection and the ascorbate concentrations in plasma, spot urine samples and aliquots of 24 h urine collections were determined as part of the present work. In addition, the 5-day prospective dietary records were analysed, using the nutritional software package "nut.s - nutritional.software" (Data Denkwerkzeuge, Vienna, Austria).

Part II:

This was a prospective observational study, using urine samples collected over a period of 24 hours from the study subjects. During the 24-hour study period, the subjects had to collect and immediately deliver every void to the laboratory. During the study period, the subjects had to stay at the investigation unit or at least in close proximity of the laboratory in order to be able to collect and forward every void immediately.

The individual study subjects were eating according to same protocol and were voiding at approximately the same time. Similar meals were provided to all the subjects and the aim was to reproduce usual everyday meals without excluding vitamin C intake. Breakfast consisted of brown bread, butter, cheese, raspberry and apricot jam, and chocolate chip cookies. For lunch the study subjects had cabbage strudel with yoghurt dressing and green salad with herbs dressing. Baguette and kornspitz with ham, smoked ham, cheese, butter, mayonnaise and peppers were offered together with marble cake for dinner. During the day the subjects could drink coffee, milk, juice and tea.

As soon as the spot urine samples were collected, the volume was determined and the samples were further processed in the laboratory.

METHODS

The methods section was divided into 4 parts: preanalytical procedures, analytical methods, analysis of dietary records, and statistical analysis.

1. Preanalytical procedures

Part I:

Two urine samples were collected by each study participant (healthy volunteer or patient): the spontaneous sample (spot urine sample) and the 24-hour urine sample (24-h-urine collection). The spontaneous sample was collected as soon as the fasted subjects arrived at the site of investigation and the 24-hour samples were collected over the last 24 hours by the subjects themselves. Furthermore, blood was taken from the fasting subjects in the morning. As soon as the urine samples were collected, they were processed. Spot urine samples were centrifuged at 3000 rpm for 10 minutes, while the 24-h urine collections were weighed, the volume measured and two 50 ml falcon tubes were filled with aliquots of a given study participant. The samples were centrifuged at 3000 rpm for 10 minutes. From each of the two urine samples 500 µl were taken and added into separate Eppendorf tubes, in which 500 µl of 10% metaphosphoric acid had been already added. Metaphosphoric acid was used for deproteinisation and stabilisation of the samples. The tubes were then strongly shaked to thoroughly mix the content and stored in the -80°C freezer. This procedure was done within a maximum of 30 minutes after urine collection.

Part II:

First, prior to the centrifugation, a small aliquot of the spot urine samples was used for performing a Combur^{10}Test® M urine stripe-test (Roche Diagnostics, Mannheim, Germany). With the specific semi-quantitative stripe-test, the specific gravity (SG) of urine, pH, leukocytes, nitrates, proteins, glucose, ketones, urobilinogen, bilirubin, erythrocytes and hemoglobin were determined. This test was performed in order to predict the presence of bacteria and investigate whether a correlation with the concentrations of vitamin C exist. Next, the spot urine samples were centrifuged at 3000 rpm for 10 minutes and two aliquots were collected. The remaining samples were left standing at the bench in the laboratory for

the next 24 hours. The temperature in the laboratory was 20-21°C during the whole experiment. When the 24-hour period was over, two 500 µl aliquots were again collected from each void, which were left standing at the bench during the 24-hour experimental period. The different samples collected from a given volunteer were combined and mixed in a urine collection bottle. The volume collected over the 24 hours was determined and 500 µl aliquots were collected and added in separate Eppendorf tubes: one was used later for the analysis and the other one was kept as reserve. In these tubes 500 µl 10% metaphosphoric acid had been already added. The tubes were then strongly shaked to thoroughly mix the content and were initially stored in the freezer at -20°C and after a few hours transferred to a -80°C freezer. Finally, all the collected aliquots were analyzed to determine the ascorbate concentrations per ml and calculate the actual amount of vitamin C present in each urine samples collected over the 24-h period (Figure 4).

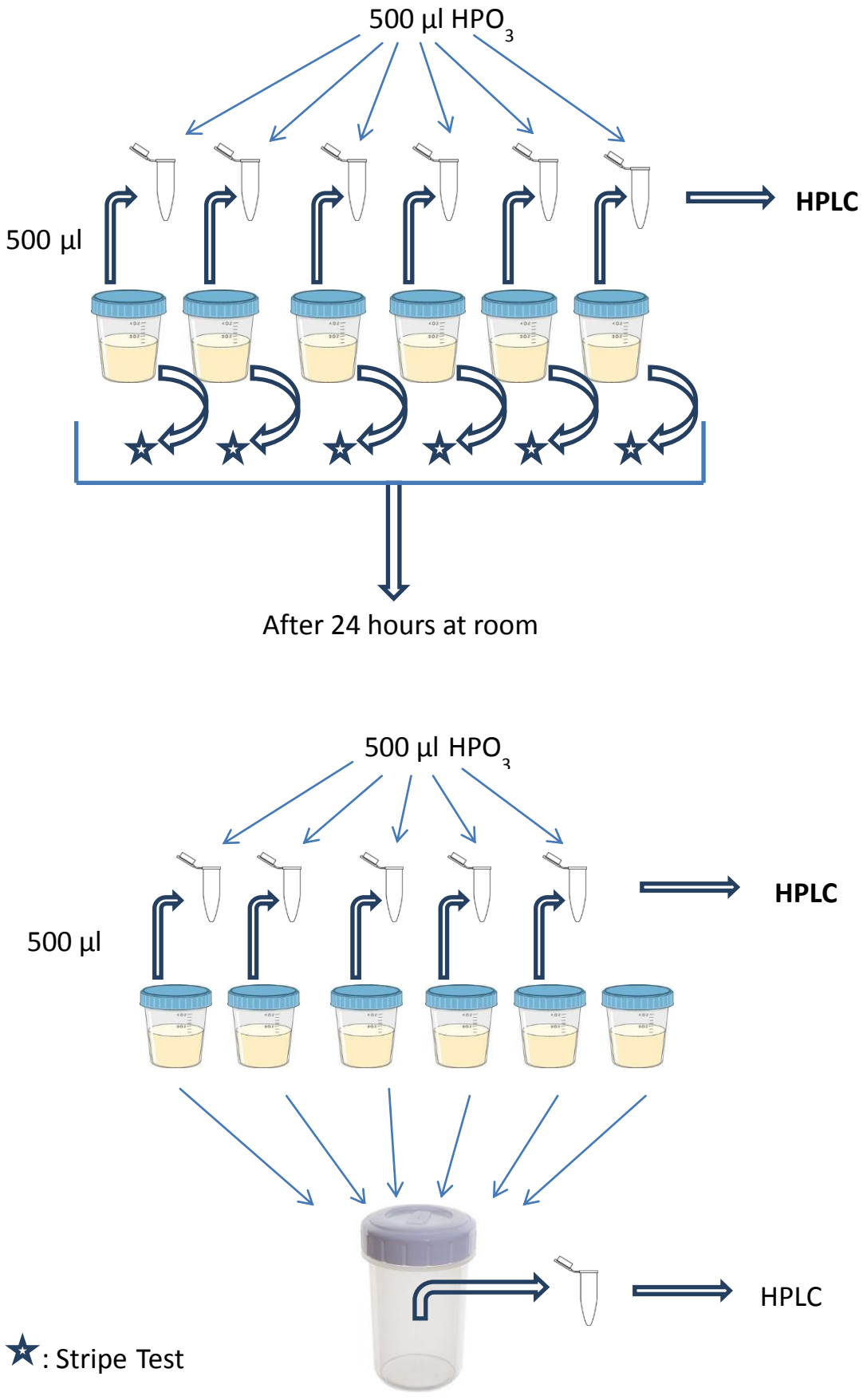

Figure 4: Schematic description of the preanalytical procedure followed in part II.

2. Analytical methods

Analytical procedures were similar in part I and II. The urine samples were thawed, centrifuged and 300 µl of them were placed into HPLC autosampler vials (300 µmol/l, Chromacol). The determination of the ascorbate concentrations was performed using HPLC with electrochemical detection. Due to difficulties with the separation of the ascorbate and the urate peak in the urine samples (Fig.5), different HPLC columns and mobile phases were tested in order to achieve the best possible separation. The column presenting the best separation (Fig.6) was a reversed phase C-18 column (ODS 5µm NC 250x4.6mm). The mobile phase was a mixture of methanol, 0.05M sodium phosphate monobasic, 0.05 M sodium acetate anhydrous, 189 µM dodecyltrimethylammonium bromide and 36.6 µM tetraoctylammonium bromide and pH adjusted to 4.2. Flow rate was at 0.8 ml per minute.

Quality control was performed. In each run, one standard, one control and 6 urine samples were processed. The standard was derived from a stock solution (1mg/ml) ascorbic acid in aqua purificata. The control samples were derived from a control plasma pool. Control plasma was mixed with metaphosphoric acid (10%) and stored at -80°C until analysis. Figure 7 shows the concentrations of the plasma ascorbate concentrations determined in the control plasma samples during the whole analytical study period. The concentrations determined were (mean±SD) 12.75±0.55 µmol/l, and the coefficient of variation was 4.35%. Fig. 7 shows the control sheet which was used as quality control tool. When the discrepancy between target and measured concentrations was ≥15%, a new calibration was performed.

Characteristics of the method, as documented in the standard operation procedure of the laboratory, showed a detection limit of 2 µg/ml (1.136 µmol/l) ascorbate when 4 µl were injected (Winklhofer-Roob, 2011).

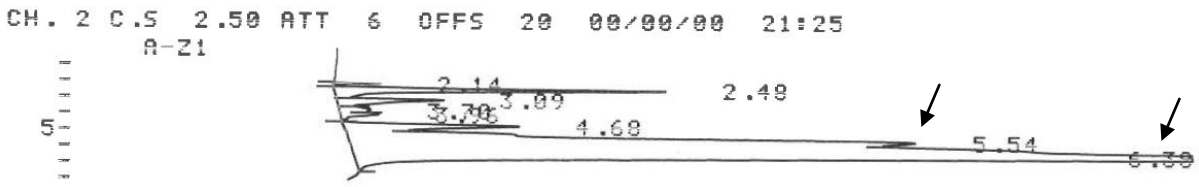

Figure 5: *Interference of ascorbate and uric acid peak observed with the reversed phase C18 7µm-Lichrosorb analytical column.*

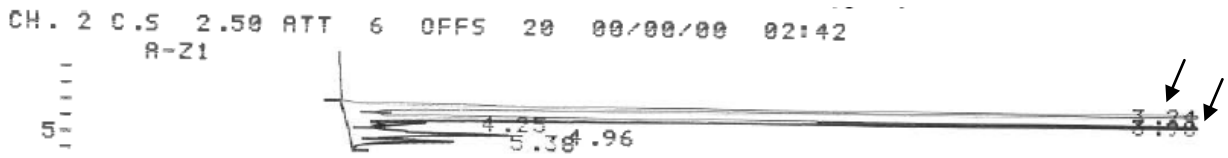

Figure 6: *Ascorbate and uric acid peaks separated with the reversed phase C-18 column ODS 5µm NC 250 x 4.6 mm analytical column.*

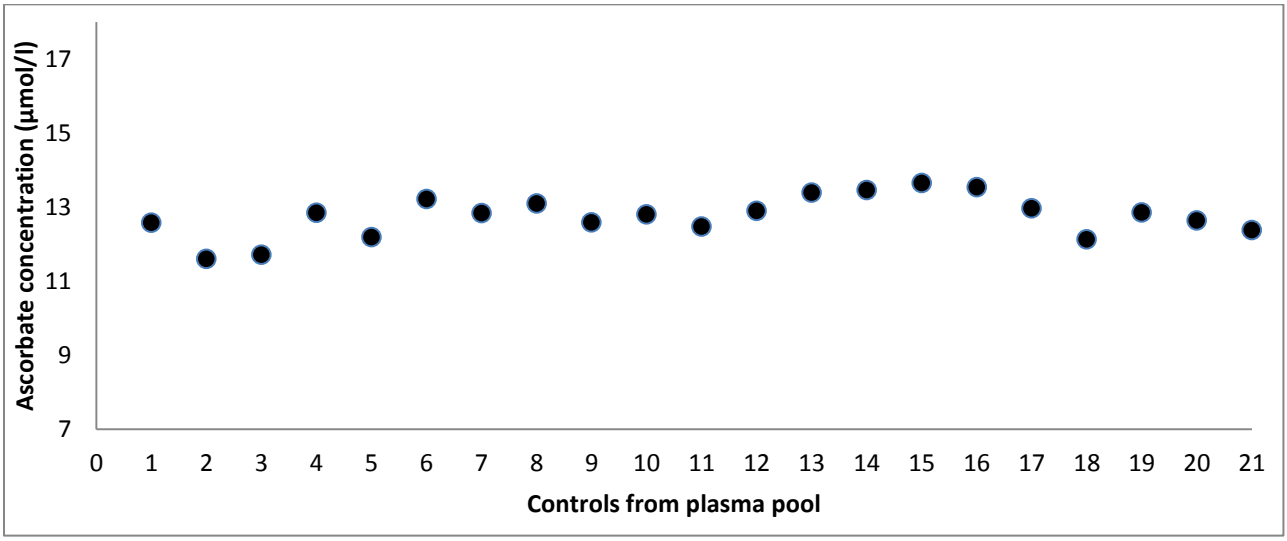

Figure 7: *Control sheet including the concentrations of all plasma ascorbate concentrations determined in the control samples processed along with the samples of the study subjects.*

3. Analysis of dietary records

This analysis was performed only in Part I. Prospective weighed 5-day diet diaries had been completed by the study subjects of the BIOCLAIMS project. These protocols were analysed in order to estimate the amounts of vitamin C consumed daily and to calculate the average (mean) daily intakes of the study subjects. For the estimation of the vitamin C intake, the vitamin C content of each food and drink item in the given amount consumed had to be calculated. This was done using "nut.s nutritional.software" (Data Denkwerkzeuge, Vienna, Austria), a food composition software package based on the German Bundeslebensmittel-schlüssel with addition of specific Austrian food items. Each food and drink item recorded in the diet diary of a given subject was identified in the database and the vitamin contents of the foods and drinks consumed were calculated based on the amounts of the food and drink

items consumed (expressed as grams or milliliter, respectively) (Table 2, Fig. 8). Where indicated, dietary intake of micronutrients from supplements was taken into account; however, none of the study subjects took vitamin C supplements.

Table 2: Steps for calculating the dietary vitamin C intake

Step 1:	Checking the 5-day diet diary for plausibility of the entered foods and drinks.
Step 2:	Identification of the food and drink item consumed in the database.
Step 3:	Entering the amount consumed for each of the food and drink items.
Step 4:	Calculation of the content of vitamin C (and various other nutrients) in all food and drink items consumed in the given amount on a given day.
Step 5:	Calculation of the average daily intake of vitamin C (along with various other nutrients) during the 5-day recording period.

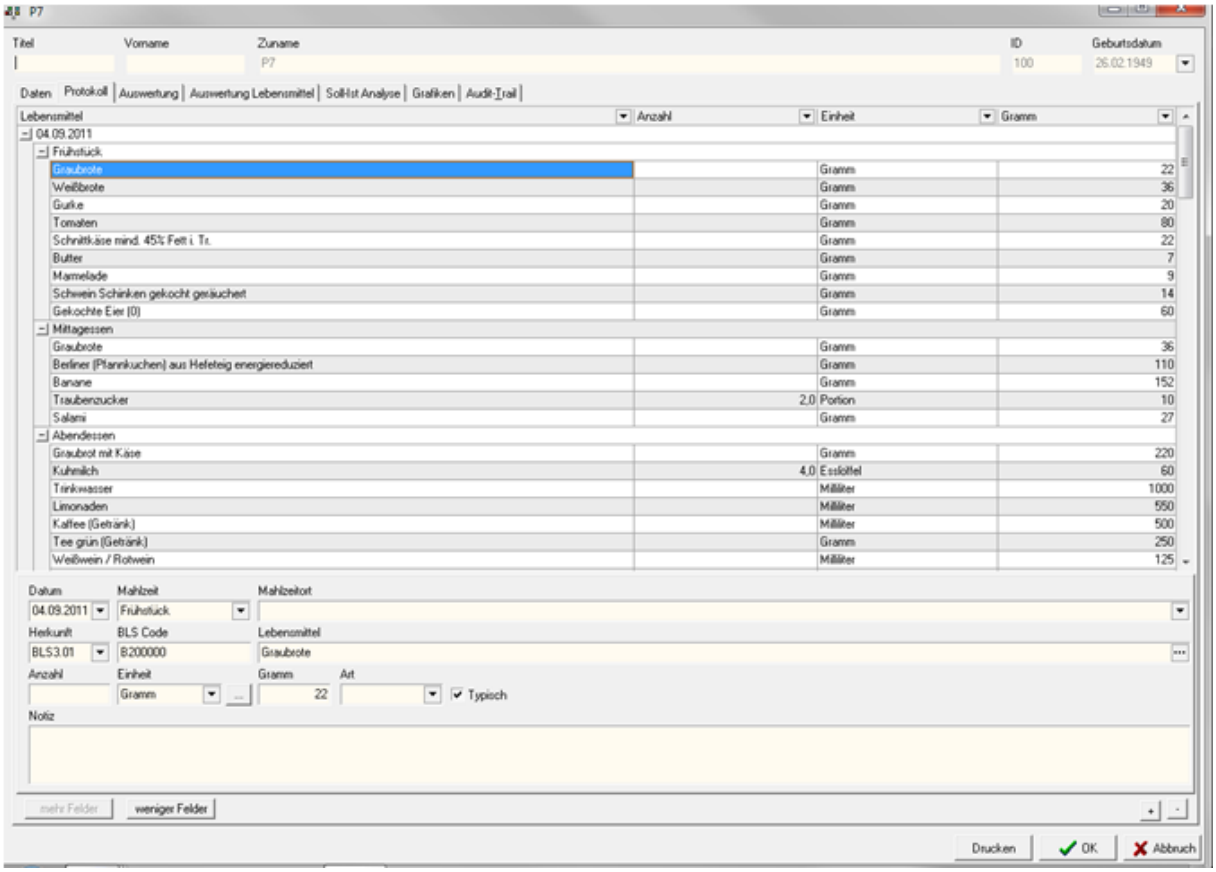

Figure 8: A screenshot of the nut.s nutritional software display, while entering the data (food and drinks intake).

4. Statistical analysis

Statistical analysis procedures were similar in Part I and II. Several statistical tests, such as *t*-tests, linear regression and multiple regression analysis, correlations, normality tests and constant variance tests, were performed in order to identify differences between groups (*t*-tests) and relations between different variables (regression analysis). Variables that did not show normal distribution were transformed, using addition of 100 and log10 transformations. A $P < 0.05$ value was considered statistically significant. The software used was Sigma Plot Version 11.0 from Systat Software Inc, Erkrath, Germany.

RESULTS

Part I:

GFR was used for the estimation of renal function and the proposed thresholds recommended by the National Kidney Foundation for normal, slightly, mildly and moderately impaired renal function were applied (Table 2). The study subjects were selected for the present study based on the GFR results and assigned to one of 4 groups with moderately, mildly and slightly impaired and normal renal function (GFR <30, 30-60, 60-90 and >90 ml/min/1.73 m^2, respectively). Demographic data, kidney function and vitamin C status and ascorbate excretion are presented in Table 3. In addition, creatinine clearance and cystatin C were determined in all study subjects as additional indicators of renal function (Table 3).

Table 3: *Demographic data, kidney function and vitamin C status of study subjects of Part I.*

	GFR < 30 n = 10 (5M, 5F)[1]	**GFR 30-60** n = 10 (5M, 5F)	**GFR 60-90** n = 10 (5M, 5F)	**GFR > 90** n = 10 (5M, 5F)	***P* values** ANOVA[2]
Age, years	72,40 ± 6,8	72,52 ± 6,51	69,63 ± 6,09	65, 41 ± 7,00	0,072
GFR, ml/min/1.73 m^2	25,97 ± 2,61	44,42 ±8,80	72,21 ± 7,08	98,94 ± 7,58	0,001
Creatinine clearance, ml/min	147,73 ± 138,59	59,98 ± 59,18	69,83 ± 40,74	93,21 ± 57,74	0,598
Cystatin C, mg/l	1,86 ± 0,30	1,33 ± 0,30	0,87 ± 0,22	0,71 ± 0,09	<0,001
Dietary vitamin C intake, mg/d	98,11 ± 33,6	91,18 ± 39,50	138,16 ± 60,96	129,31 ± 30,35	0,073
Plasma ascorbate, µmol/l	47,04 ± 27,61	55,24 ±21,97	73,88 ± 16,67	68,06 ± 24,46	0,054
Spot urine ascorbate, µmol/l	85,07 ± 90,89	95,36 ± 92,41	237,77 ± 307,29	72,34 ± 67,53	0,739
24-h urine ascorbate, µmol/l	60,96 ± 120	88,11 ±131,49	233,94 ± 325,35	97,74 ± 89,25	0,095
24-h urine volume, ml	2301,00 ± 887,56	1826,43 ± 591,12	2053,00 ± 1336,04	2415,00 ± 656,46	0,572
24-h ascorbate losses,mg/24h	18,25 ± 29,13	25,09 ± 51,75	89,34 ± 108,45	37,18 ± 36,45	0,132
Ascorbate clearance, ml/min	2,11 ± 2,11	2,69 ± 3,69	7,55 ± 8,98	3,13 ± 2,60	0,330

[1]M: Male, F: Female; [2]ANOVA: Analysis of Variance

Significant differences between the four groups were observed for GFR (the criterion for assignment of the study subjects to the four groups) and cystatin C, but not for creatinine clearance. Differences in plasma ascorbate concentrations and dietary vitamin C intake did not reach statistical significance. Ascorbate excretion, as estimated by ascrobate concentrations in spot urine, 24-h urine and ascorbate clearance, did not show any significant differences between the groups.

GFR and ascorbate concentrations in plasma, spot urine and 24-h urine

For studying the effects of the amounts of dietary vitamin C intake on the concentrations of ascorbate in plasma and urine at different GFR levels, data of 40 study subjects were analysed (*Table 2)*. There were 10 subjects in each GFR group; five of them were female and five male. The subjects were matched for age as closely as possible to eliminate possible effects of age as a confounding variable. First, it was investigated whether there are significant relations between the GFR and the concentrations of ascorbate in plasma, spot urine or 24-h urine. Regression analysis revealed that there was no significant relation between GFR and ascorbate concentrations, neither in spot urine (r = 0.103, P > 0.05) nor in 24-h urine (r = 0.159, P > 0.05). The only significant relation observed was between ascorbate plasma concentrations and GFR (r = 0.389, P = 0.013), indicating that the higher the GFR, the higher the ascorbate plasma concentrations, or, vice versa, the lower the GFR, the lower the ascorbate plasma concentrations (Fig. 9).

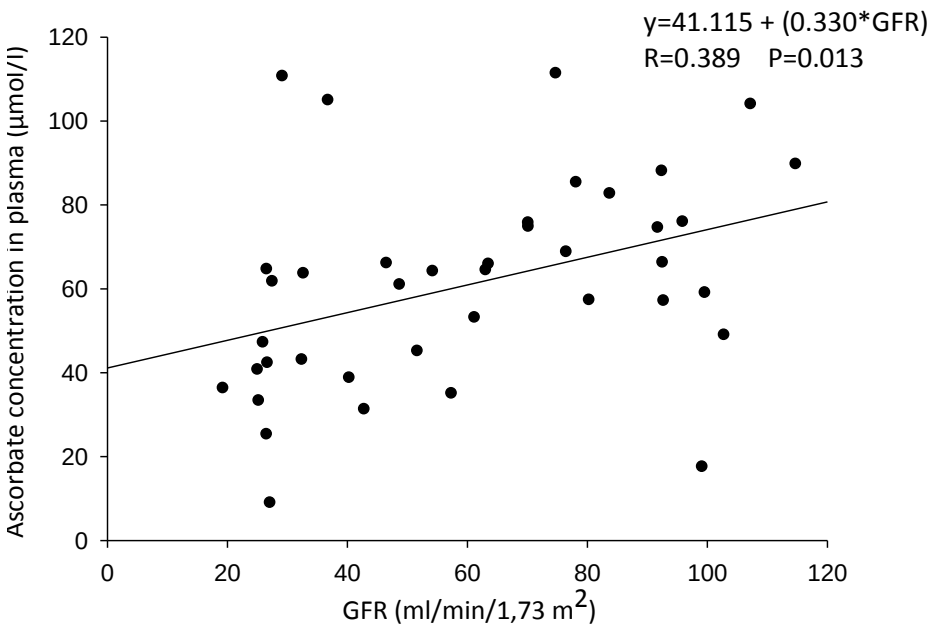

Figure 9: *Plasma ascorbate concentrations were plotted against GFR. There was a significant dependence of plasma ascorbate concentrations on GFR.*

Table 4: *Data of the 40 subjects matched for gender and age.*

Gender	Age	GFR (ml/min/1.73m^2)	Daily intake (mg)[1]	Ascorbate concentration in plasma (µmol/l)	Ascorbate concentration in spot urine (µmol/l)	Ascorbate concentration in 24h urine (µmol/l)
m	76	29.24	89.64	110.59	296.29	393.56
m	70	27.17	85.98	8.90	0.17	2.21
m	77	27.56	107.6	61.67	33.50	51.13
m	70	25.29	100.44	33.23	125.57	24.21
m	69	26.60	106.8	25.20	18.84	29.95
f	76	26.01	75.22	47.13	25.07	1.02
f	68	19.33	63.88	36.22	119.10	5.85
f	58	26.63	163.7	64.57	136.42	0.74
f	82	26.75	50.7	42.26	5.01	2.76
f	78	25.08	137.18	40.65	90.73	95.41
m	76	42.88	129.84	31.19	99.32	173.80
m	70	51.73	97.4	45.07	197.36	87.42
m	77	40.39	165.48	38.70	88.65	1.39
m	71	48.77	91.22	60.91	40.51	29.02
m	70	32.49	106.98	43.01	166.93	87.72
f	76	36.85	93.76	104.85	282.20	428.94
f	68	32.75	48.62	63.57	31.94	50.65
f	59	54.29	39.88	64.08	25.63	16.47
f	82	57.41	95.08	34.95	4.26	0.40
f	78	46.59	43.52	66.03	16.79	5.24
m	74	63.12	134.02	64.38	164.95	213.72
m	70	80.37	74.4	57.23	8.06	7.16
m	79	61.27	136.96	53.07	113.92	42.64
m	70	76.54	80.78	68.73	31.66	131.69
m	69	63.58	73.92	65.82	22.68	89.60
f	74	70.20	158.08	75.65	248.94	163.91
f	68	70.23	108.56	74.73	0.17	18.17
f	58	78.20	199.02	85.29	704.97	1113.53
f	62	74.81	147.8	111.28	202.99	322.34
f	71	83.80	268.06	82.63	879.36	236.59
m	76	92.60	127.68	66.18	78.73	97.04
m	53	99.66	104.28	59.00	12.19	6.73
m	62	92.77	124.64	57.07	179.59	180.10
m	61	114.80	121.56	89.63	63.56	88.09
m	64	95.96	154.36	75.89	49.46	110.42
f	74	91.82	113.3	74.49	68.20	149.30
f	67	92.49	86.76	87.99	62.32	63.68
f	63	107.30	199	103.93	188.93	281.12
f	72	102.84	134.32	48.92	6.39	0.80
f	63	99.20	127.2	17.47	20.40	0.14

[1]*Daily intake indicates the mean amount (mg) of dietary vitamin C intake in a 5 days period*

Dietary vitamin C intake and ascorbate concentrations in plasma, spot urine and 24-h urine

Next, it was tested whether there was a significant relation between the individual dietary vitamin C intake and the ascorbate concentrations measured in plasma, spot urine and 24-h urine. There was no significant relation (r = 0.262, P > 0.05) between plasma ascorbate concentrations and the average amount of dietary vitamin C ingested over the past 5 days prior to the investigation (Fig.10).

However, significant correlations were observed between both spot urine (r = 0.429, P = 0.006) and 24-h urine (r = 0.473, P = 0.002) and the amounts of vitamin C ingested, indicating that the higher the amount of dietary vitamin C intake, the more ascorbate is excreted in spot urine (Fig. 11), as well as in 24-h urine (Fig. 12).

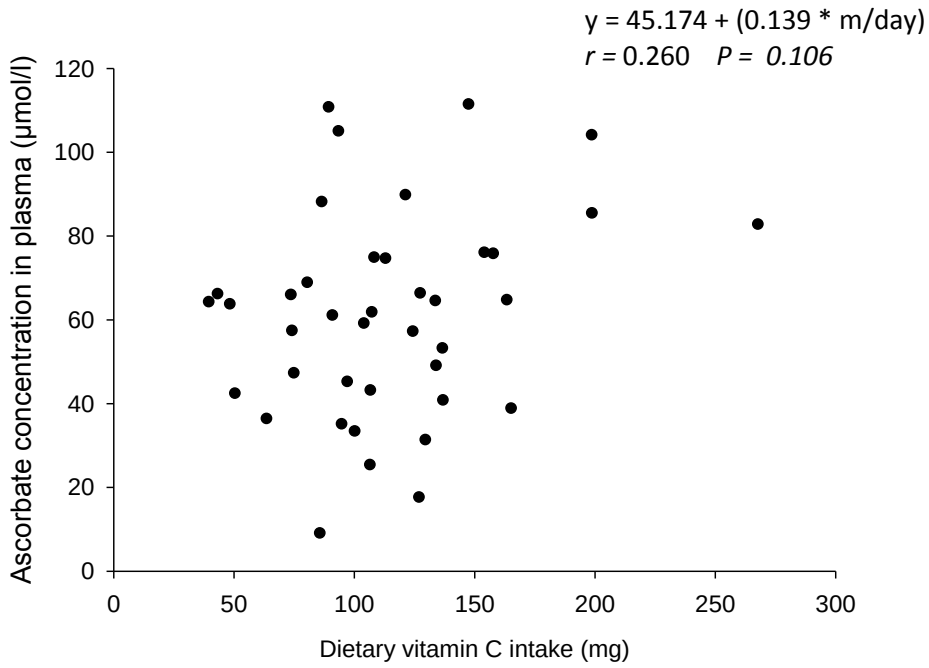

Figure 10: *The ascorbate concentrations in plasma were plotted against the dietary vitamin C intake. There was no significant dependence of ascorbate concentrations in plasma on dietary vitamin C intake.*

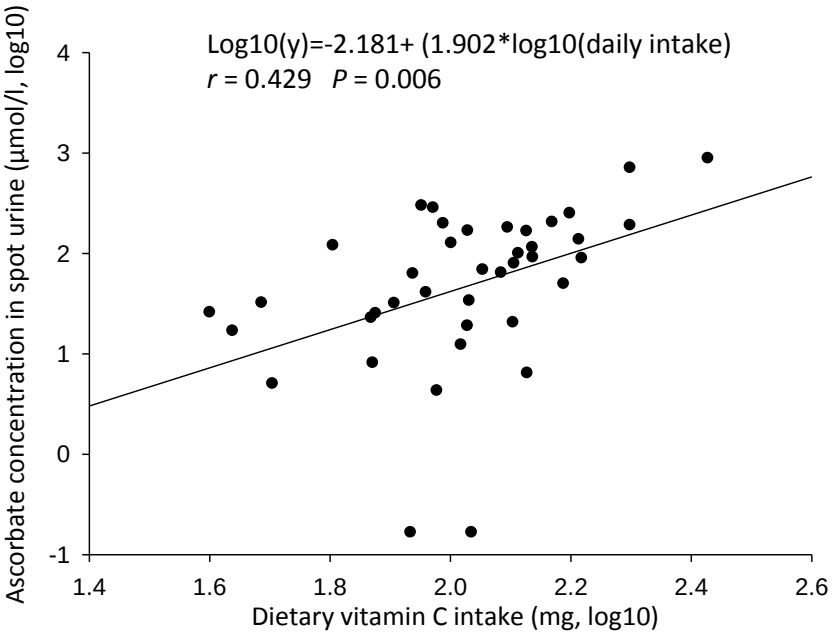

Figure 11: *The concentrations of ascorbate in spot urine samples were plotted against the dietary vitamin C intake. There was a significant dependence of ascorbate concentrations in spot urine on GFR. Please note that log10 transformed data were displayed.*

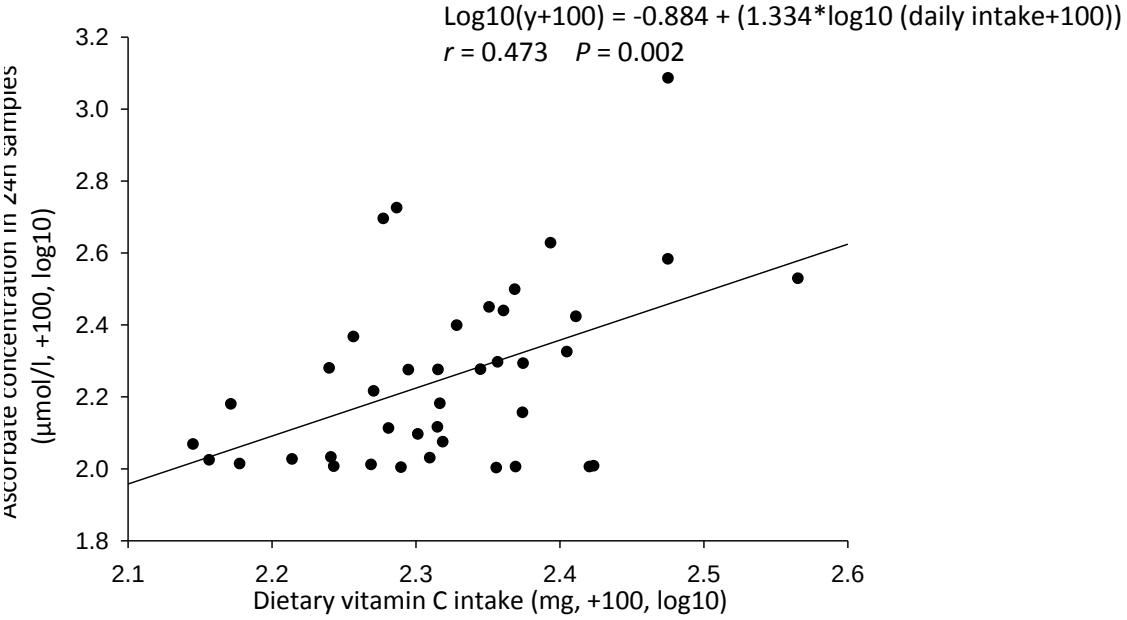

Figure 12: *The ascorbate concentrations in 24-h urine samples were plotted against the dietary vitamin C intake. There was a significant dependence of ascorbate concentrations in 24-h urine on dietary vitamin C intake. Please note that log10 and +100 transformed data were displayed.*

Plasma versus spot urine and 24-h urine

It was tested whether there was a correlation between the ascorbate concentrations in plasma and the ascorbate concentrations in spot urine and 24-h urine. There were significant relations between ascorbate concentrations in plasma and spot urine (r = 0.456, P = 0.003) as well as between ascorbate concentrations in plasma and 24-h urine (r= 0.635, P < 0.001), indicating that the higher the ascorbate concentrations in plasma, the higher the concentrations of ascorbate in spot urine (Fig. 13) and 24-h urine (Fig. 14).

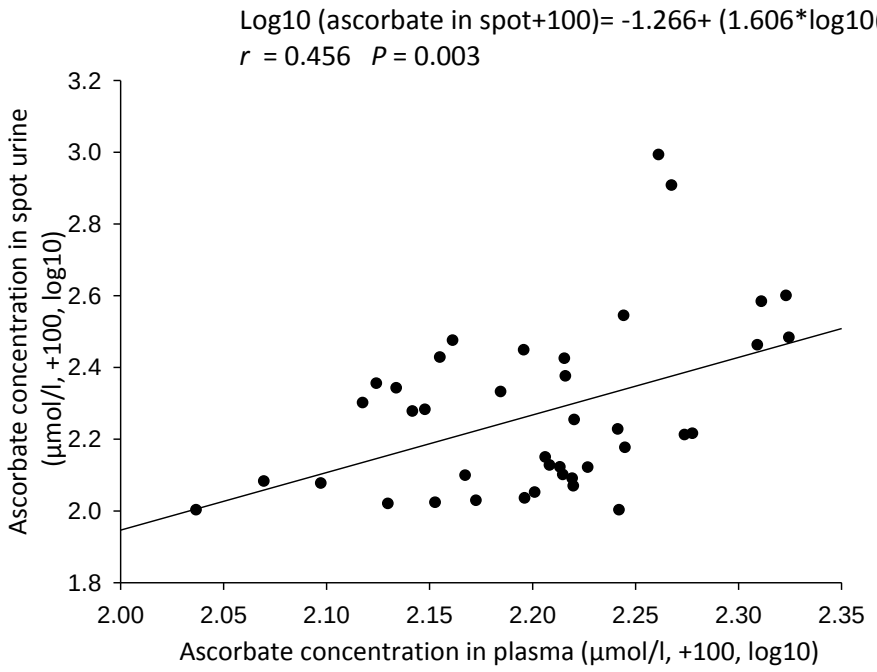

Figure 13: *The ascorbate concentrations in spot urine samples were plotted against the ascorbate concentrations in plasma. There was a significant dependence of ascorbate concentrations in spot urine on ascorbate concentrations in plasma. Please note that log10 and +100 transformed data were displayed.*

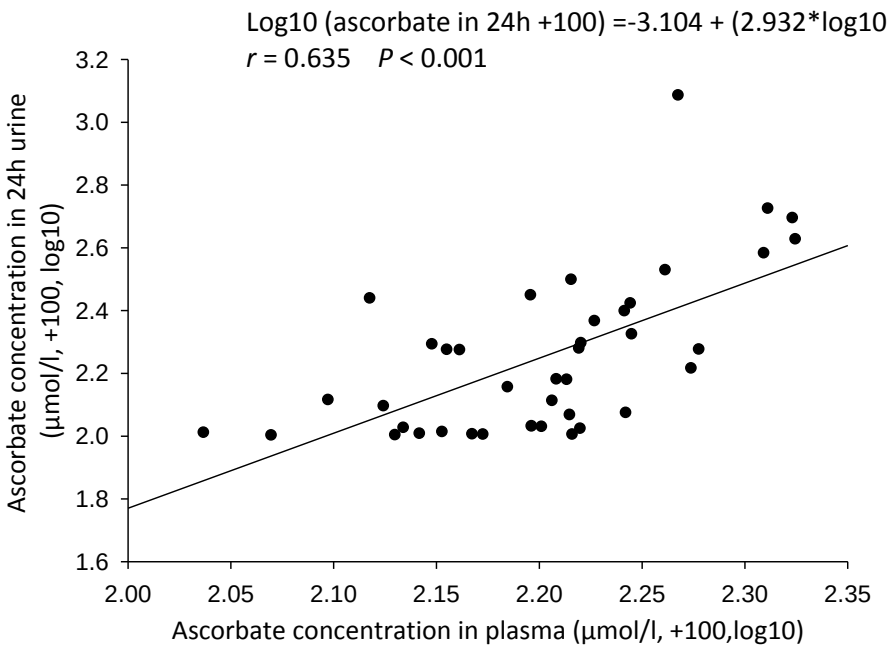

Figure 14: *The ascorbate concentrations in 24-h urine samples were plotted against the ascorbate concentrations in plasma. There was a significant dependence of 24-h urine concentrations on plasma concentrations. Please note that log10 and +100 transformed data were displayed.*

Ascorbate clearance versus GFR, plasma ascorbate concentrations and dietary vitamin C intake

Ascorbate clearance was calculated based on the formula [Urine ascorbate (mg/dl)/ plasma ascorbate (mg/dl)] * urine volume (ml/min) * 1.73 m^2, (modified from Hirsch, et al., 1998). Potential determinants of the ascorbate clearance, including GFR, plasma ascorbate concentrations and dietary vitamin C intake during the 5 days prior to the investigations, were tested using regression analysis. As shown in Fig. 15, GFR was not a significant determinant of ascorbate clearance, in contrast to plasma ascorbate concentrations (r = 0,44, P = 0.007) and dietary vitamin C intake (r = 0.411, P = 0.013), both of which showed significant effects on ascorbate clearance.

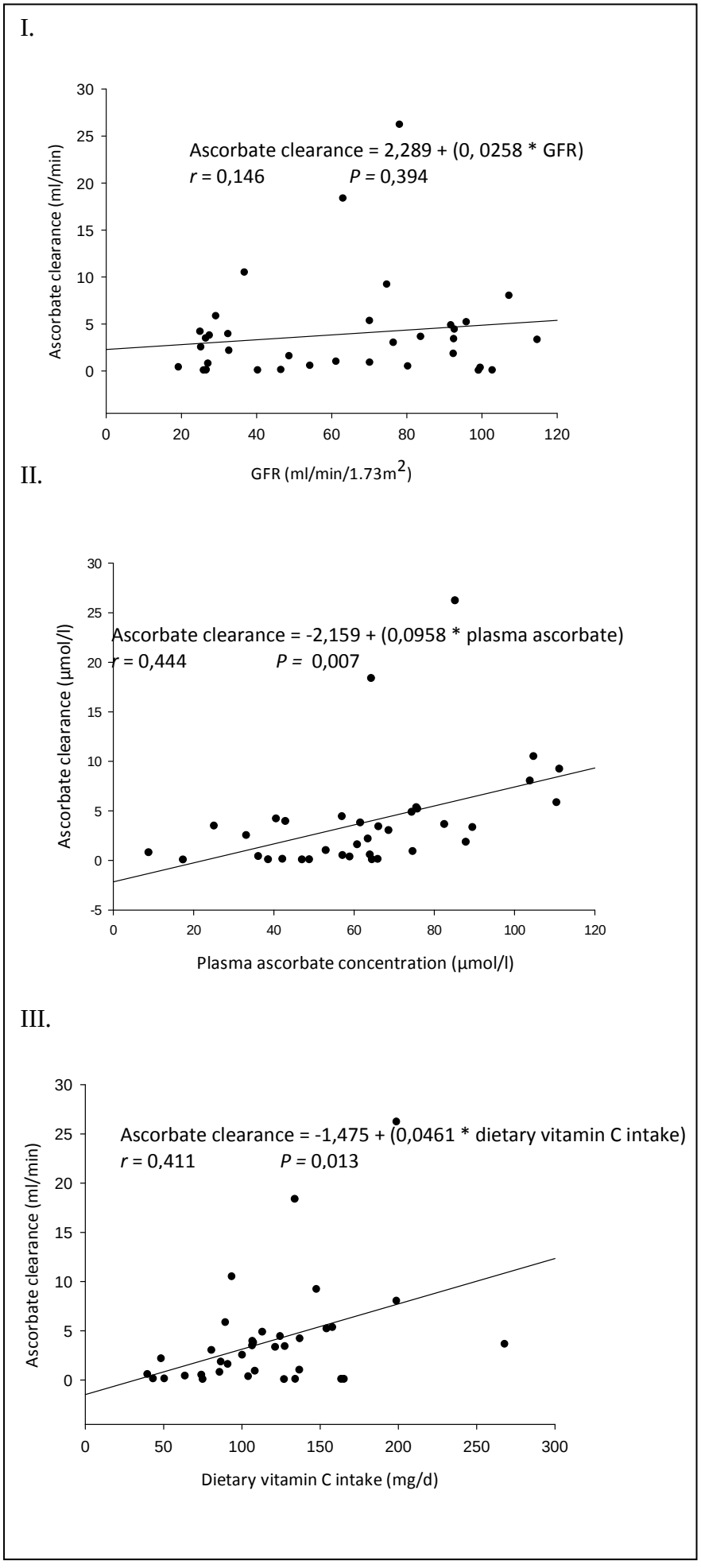

Figure 15: *The ascorbate clearance was plotted against GFR (I), plasma ascorbate concentrations (II) and dietary vitamin C intake (III). There was a significant dependence of ascorbate clearance on plasma concentrations and dietary intake, but not on GFR.*

Ascorbate clearance versus ascorbate concentrations in plasma and GFR and ascorbate clearance versus dietary vitamin C intake and GFR

Multiple regression analysis was used to test whether the ascorbate clearance can be predicted from a linear combination of the dietary ascorbate intake and GFR (Fig. 16) or from the linear combination of the plasma ascorbate concentrations and GFR (Fig. 17). The results showed significant effects on ascorbate clearance only for dietary vitamin C intake (*P* = 0.021) and plasma ascorbate concentrations (*P = 0.011),* but not for GFR, confirming the results of (simple) linear regression analysis.

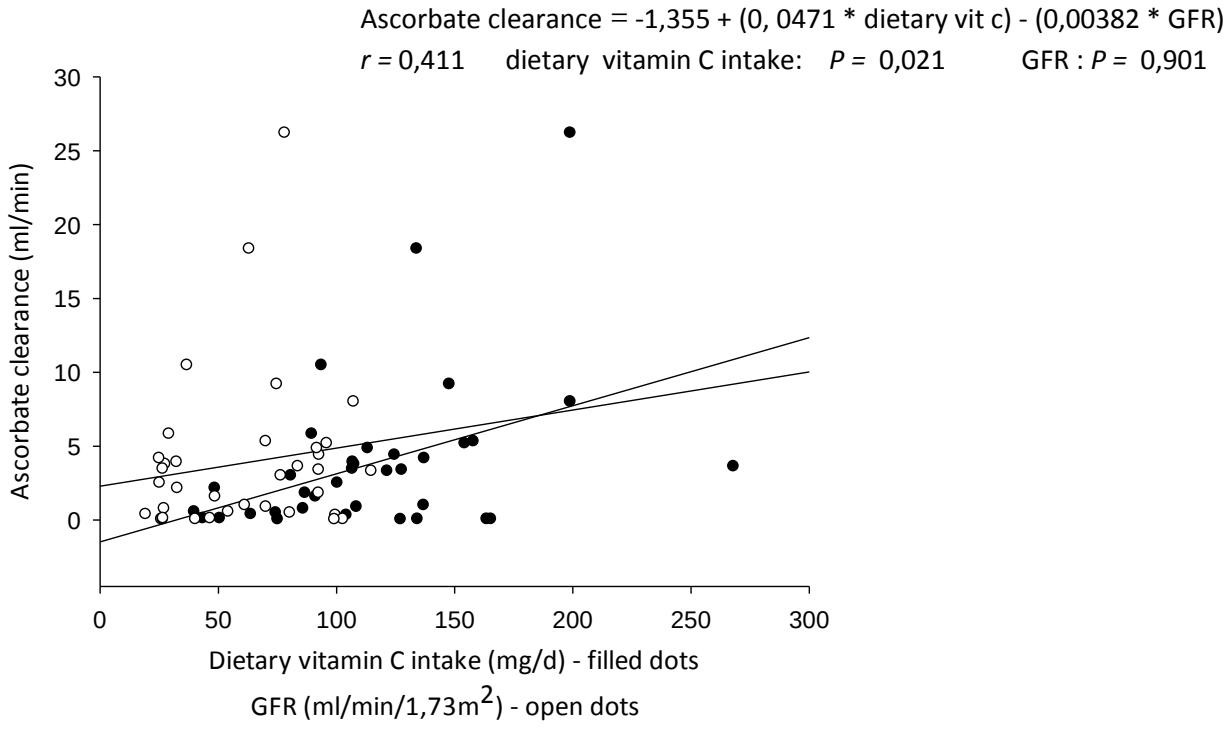

Figure 16: *The ascorbate clearance was plotted against two variables: the dietary vitamin C intake and GFR. Only the relation with dietary vitamin C intake was statistically significant.*

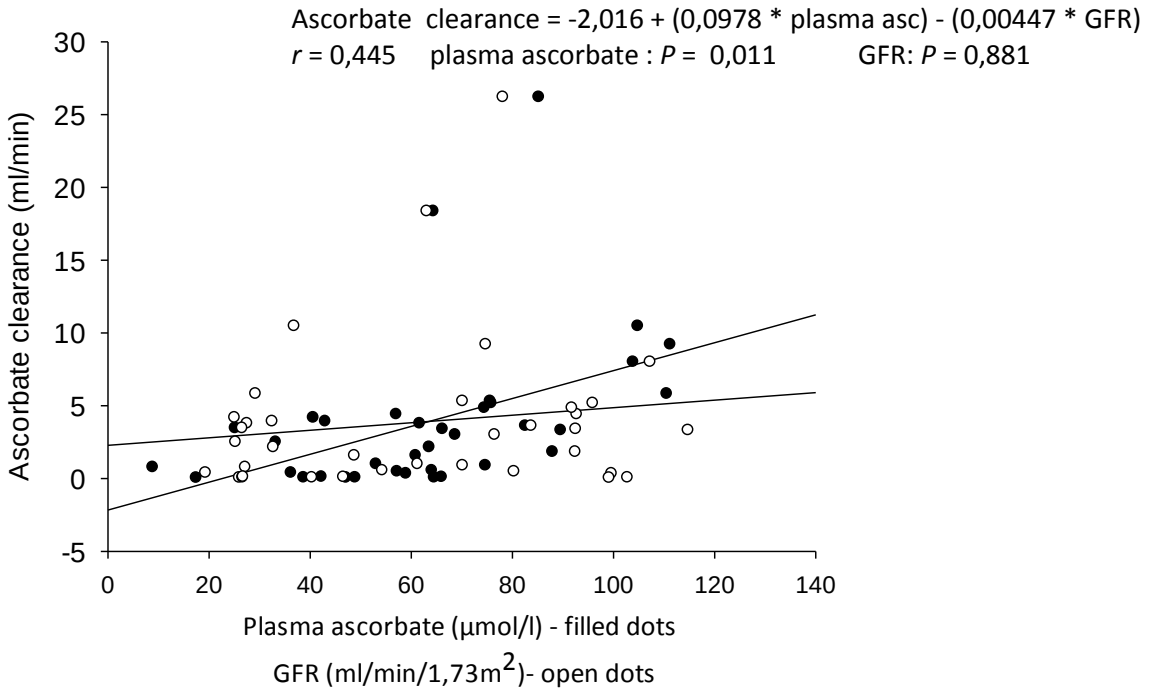

Figure 17: *The ascorbate clearance was plotted against two variables: the plasma ascorbate concentrations and GFR. Only the relation with plasma ascorbate concentrations was statistically significant.*

Gender differences

Given that 50 % of the study subjects were males and 50 % were females, it was possible to investigate whether gender exerts a significant effect on the concentrations of ascorbate in plasma, spot urine and 24-h urine. The differences in the median values between males and females for all three variables (ascorbate concentrations in plasma, spot urine, and 24-h urine concentrations) were not great enough to exclude the possibility that the differences were due to random sampling variability, indicating that there was not a statistically significant difference ($P > 0.05$) between genders.

Part II:

The aim of the second part was to determine the differences in the ascorbate concentrations between spot urine samples and urine collected over 24 hours. From each of the eight subjects six spot urine samples were collected during a 24-hour period. Five of the samples were left standing in the laboratory up to the end of the 24-hour experimental period, the first sample was standing for 24 hours and the following for shorter periods with the sixth, which was the last sample obtained, collected at the end of the period. At the time of each void, an aliquot was collected and processed immediately.

Stripe tests

The stripe tests performed directly when the spot urine samples were collected delivered no significant results when the estimated leukocytes concentrations and the pH values of each spot urine sample were compared with the ascorbate concentrations.

Ascorbate losses in urine samples kept at room temperature

An aliquot from each sample was collected at the end of the 24-hour experimental period. This procedure was followed in order to be able to determine the ascorbate losses after specific time intervals by measuring the concentrations in the fresh aliquots and the concentrations in the aliquots collected at the end of the experimental period (Table 5 and 6). Each sample of each subject was left standing for approximately the same length of time. At the end of the experimental period, after the second aliquot was collected from each individual sample, all the remaining urine samples of each subject were combined. This was performed in order to determine the total volume of the urine samples collected and to measure the ascorbate concentrations in an aliquot of the 24-hour urine collection. The actual amount of ascorbate in the 24-h aliquot was measured in order to compare it with the calculated amount and to determine the ascorbate losses (Table 7).

Table 5: *Ascorbate data retrieved from HPLC measurements and calculations for subjects A to D.*

	Fresh urine sample Initial ascorbate concentrations (µmol/l)	Urine sample volumes (ml)	Ascorbate amounts (mg)	Times elapsed[1] (hours)	Old urine samples[2] Final ascorbate concentrations (µmol/l)	Ascorbate Losses[3] (µmol/l)	Ascorbate Losses[4] (%)
A1 [5,6]	25.31	61	0.27	23	1.09	24.22	95.70
A2	136.89	272	6.56	21	104.85	32.04	23.40
A3	216.38	339	12.91	18	133.43	82.94	38.33
A4	194.14	246	8.41	13	105.10	89.04	45.86
A5	160.45	379	21.41	8	132.86	27.59	17.20
A6	947.06	111	18.42	0	NA[7]	NA[7]	NA[7]
B1	1261.79	83	18.43	23	1145.22	116.57	9.24
B2	1454.00	109	27.90	21	1273.68	180.32	12.40
B3	863.40	227	34.50	18	544.37	319.03	36.95
B4	1328.42	203	47.48	13	896.39	432.03	32.52
B5	1644.80	212	61.40	8	1624.36	20.44	1.24
B6	1777.55	228	71.34	0	NA	NA	NA
C1	1070.34	89	16.77	23	902.54	167.81	15.68
C2	1123.85	145	28.70	21	932.64	191.22	17.01
C3	1877.58	52	17.19	18	1656.59	221.00	11.77
C4	1009.15	116	20.61	13	892.91	116.24	11.52
C5	736.41	85	11.02	8	682.36	54.05	7.34
C6	698.01	98	12.04	0	NA	NA	NA
D1	37.29	19	0.12	23	9.28	28.02	75.13
D2	17.26	248	0.75	19	1.73	15.53	89.98
D3	28.95	458	1.46	16	7.87	21.08	72.82
D4	37.25	322	4.99	13	19.37	17.88	48.00
D5	87.98	343	1.75	4	61.53	26.45	30.06
D6	18.00	376	2.47	0	NA	NA	NA

[1]Time elapsed indicates the time interval between the collection of the first aliquot and the collection of the second aliquot at the end of the experimental period from each urine sample.

[2]Final concentration presents the concentrations of the second aliquot, collected at the end of the experimental period.

[3,4]Loss is the amount of ascorbate that was lost during the time elapsed between the collection of the first and second aliquot (presented in µmol/l and in percentage).

[5] A-D indicate the different subjects.

[6] A1-6, B1-6,C1-6, D1-6 indicate the different samples at time points 1 to 6.

Time interval is the amount of hours elapsed between the collection of a specific sample until the end of the experimental period and the collection of the second aliquot.

[7]The final concentration and loss for all samples at time point 6 are not applicable because this sample was the last one collected and thus was not left standing.

Table 6: *Ascorbate data retrieved from HPLC measurements and calculations for subjects E to H.*

	Fresh urine sample Initial ascorbate concentration (µmol/l)	Urine sample volume (ml)	Ascorbate amount (mg)	Time elapsed[1] (hours)	Old sample Final ascorbate concentration[2] (µmol/l)	Ascorbate loss[3] (µmol/l)	Ascorbate loss[4] (%)
E1[5,6]	1511,18	48	12.77	23	1251.85	245.15	17.16
E2	1266,03	150	33.44	19	909.04	356.99	28.20
E3	144.38	413	10.50	16	95.33	49.04	33.97
E4	305,7	621	33.41	13	138.63	167.07	54.65
E5	476,74	362	30.37	4	383.03	84.71	19.66
E6	136.09	244	5.85	0	NA[7]	NA[7]	NA[7]
F1	36.33	54	0.35	23	4.09	32.24	88.74
F2	31.20	576	3.16	19	18.14	13.06	41.85
F3	100.27	792	13.98	16	99.85	0.42	0.41
F4	53.06	534	4.99	13	26.94	26.12	49.24
F5	129.40	561	12.78	4	87.96	41.44	32.03
F6	46.85	437	3.61	0	NA	NA	NA
G1	67.22	490	5.80	25	32.88	34.35	51.09
G2	56.20	375	3.71	19	26.53	29.67	52.80
G3	179.43	440	13.90	15.5	138.27	41.16	22.94
G4	422.47	295	21.95	13	381.33	41.14	9.74
G5	383.95	240	16.22	8	322.68	61.28	15.96
G6	870.26	440	67.41	0	NA	NA	NA
H1	39.16	225	1.55	25	3.88	35.29	90.09
H2	274.59	175	8.46	23	188.04	86.55	31.52
H3	272.55	160	7.68	19	149.00	123.54	45.33
H4	141.48	350	8.72	18	121.36	20.13	14.22
H5	330.60	295	17.17	12	201.45	129.15	39.06
H6	682.10	385	46.23	0	NA	NA	NA

[1]Time elapsed indicates the time interval between the collection of the first aliquot and the collection of the second aliquot at the end of the experimental period from each urine sample.

[2]Final concentration presents the concentrations of the second aliquot, collected at the end of the experimental period.

[3,4]Loss is the amount of ascorbate that was lost during the time elapsed between the collection of the first and second aliquot (presented in µmol/l and in percentage).

[5] A-D indicate the different subjects.

[6] A1-6, B1-6,C1-6, D1-6 indicate the different samples at time points 1 to 6.

Time interval is the amount of hours elapsed between the collection of a specific sample until the end of the experimental period and the collection of the second aliquot.

[7]The final concentration and loss for all samples at time point 6 are not applicable because this sample was the last one collected and thus was not left standing.

Table 7: *The differences between the calculated amounts of ascorbate and the amounts measured in urine collected over 24 hours (experimental period).*

	Ascorbate concentrations measured (µmol/l)	Sample volumes measured (ml)	Ascorbate amounts measured (mg)	Ascorbate amounts calculated[1] (mg)	Ascorbate Losses[2] (%)
A24h	228.27	1380	55.43	67.98	18.46
B24h	997.73	995	174.82	261.06	33.03
C24h	91.17	540	8.69	106.33	91.82
D24h	18.13	1680	5.36	11.54	53.57
E24h	201.77	1750	62.16	126.33	50.80
F24h	55.26	2900	28.22	38.77	27.40
G24h	278.46	2230	109.31	128.99	15.25
H24h	186.25	1515	49.68	89.81	44.69

[1] *The sums calculated from individual urine samples (shown in Tables 1, 2 under the column: Amount)*
[2] *Differences between calculated amounts and measured amounts expressed as % of initial amounts*

In order to compare the ascorbate losses between the various standing times, the mean percent ascorbate losses were calculated at each of the five time points of all eight subjects and were compared with the length of time the sample were standing on the bench (Fig. 18). There was a significant relation (r = 0.920, P = 0.027) between the average percent losses of ascorbate and the elapsed times since voids, indicating that the ascorbate losses in the stored samples were dependent on the length of time the samples were left standing on the bench.

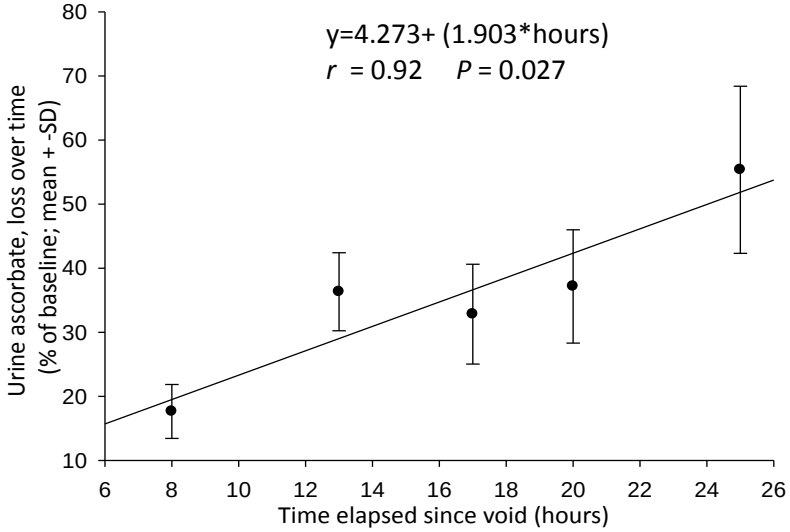

Figure 18: *The losses over time in urine ascorbate concentrations were plotted against the time elapsed since the voids. There was a significant relation between ascorbate losses and times elapsed since void.*

Furthermore, we hypothesized that the volume of each sample separately, as well as the total urine volume of each person, could potentially play an important role regarding the initial concentrations and the ascorbate losses. The ascorbate losses were both calculated as percentage of initial concentrations and amounts (mg). The total urine volumes of each subject were also documented after combining all the individual samples.

Ascorbate concentrations in urine samples versus volume of urine samples

The relation between the initial concentrations, namely the concentrations of the freshly collected aliquots, and the volumes of the samples collected, was studied. There was a significant relation between the two variables (r = 0.403, P = 0.04), indicating that the larger the volume of the urine samples, the lower the ascorbate concentrations (Fig. 19).

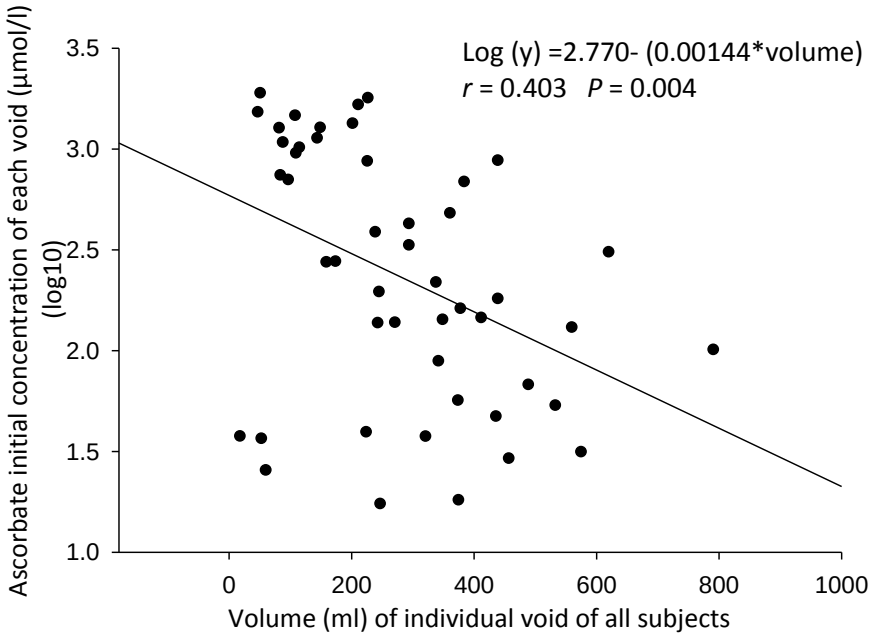

Figure 19: *The initial concentrations of ascorbate in each urine sample were plotted against the volumes of the corresponding samples. There was a significant relation between the ascorbate concentrations and the sample volumes. Please note that log10 transformed data were displayed.*

Absolute ascorbate losses versus volumes of urine samples

In addition to the losses expressed as % of initial concentrations mentioned above, the absolute losses (mg) were also calculated and used to examine whether there was a relation between the absolute ascorbate losses and the volumes of each individual sample. There was a significant relation between absolute losses and volumes (r = 0.394, P < 0.05), indicating that the absolute losses in the individual void are dependent on the volume (Fig. 20).

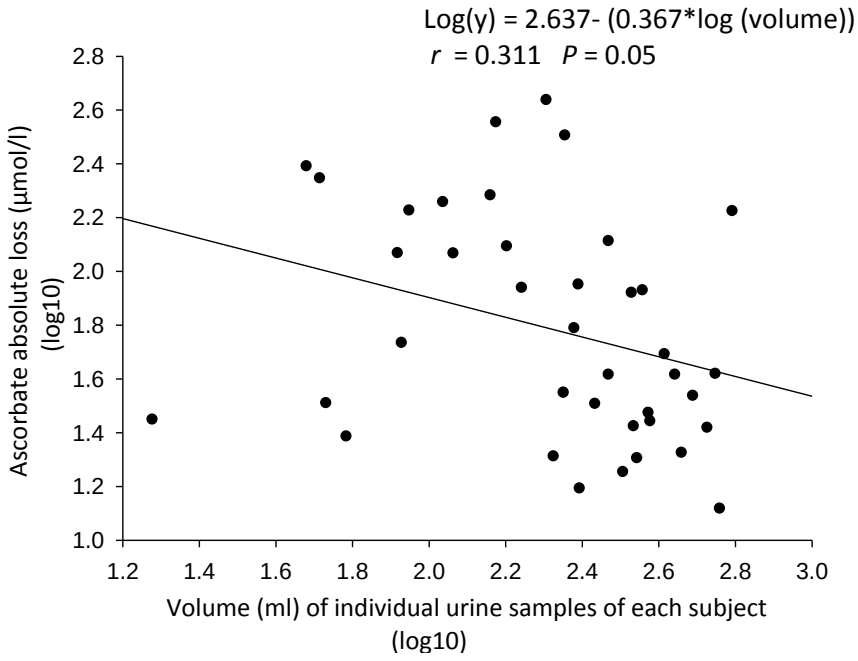

Figure 20: *The absolute losses of ascorbate in urine were plotted against the volumes of the corresponding urine samples of each subject. There was a significant relation between the absolute ascorbate losses in urine and the volumes of the samples. Please note that log10 transformed data were displayed.*

Absolute ascorbate losses in urine samples versus initial ascorbate concentrations

A significant relation (r = 0.672, P < 0.001) between the absolute losses of ascorbate and the initial ascorbate concentrations was observed, indicating that the higher the initial concentrations of ascorbate, the higher the losses during the experimental period (Fig. 21).

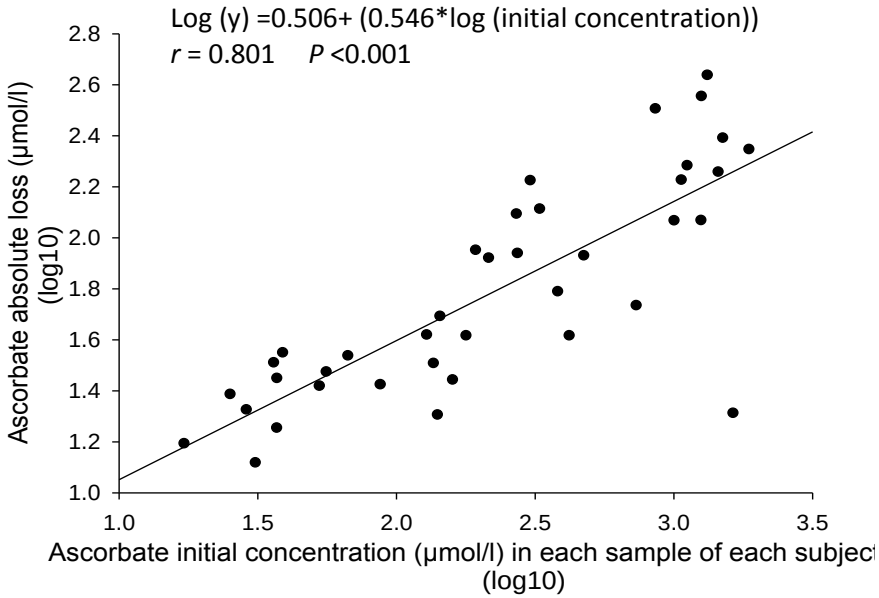

Figure 21: *The absolute losses of ascorbate in urine were plotted against the initial ascorbate concentrations in each sample of each subject. There was a significant relation between the absolute losses and the initial concentrations of ascorbate Please note that the log10 of the concentrations has been displayed.*

Ascorbate concentrations versus volumes of urine samples

The mean ascorbate concentrations of all the individual urine samples of each subject were calculated and used for examining their relation with the mean values of the volumes of each subject's samples. The relation was significant (r = 0.719, P = 0.044), indicating that the larger the mean volume, the smaller the mean ascorbate concentrations of the study subjects (Fig.22).

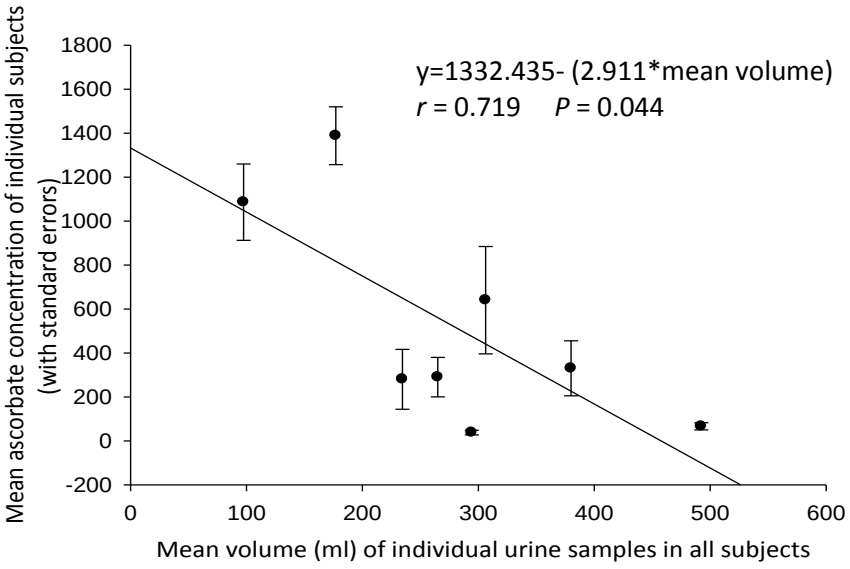

Figure 22: *The means of urine ascorbate concentrations of individual subjects were plotted against the volumes of urine samples of individual subjects were presented as means ± standard errors. There was a significant relation between the mean ascorbate concentrations and the mean volumes.*

Ascorbate losses versus initial concentrations and times elapsed

Additionally, it was tested whether the percent losses or the absolute losses can be predicted from a linear combination of the length of time the samples were left standing and the initial ascorbate concentrations of the samples. The results for the percent losses were significant for both the length of time (r = 0.689, P < 0.05) and the initial concentrations (r = 0.689, P < 0.001), while for the absolute losses the relation was significant only for the initial concentrations (r = 0.708, P < 0.001), but not for the length of time (r = 0.708, P > 0.05). This indicates that both the initial concentrations and the length of time each sample was left standing contributed to predicting the percent ascorbate losses (Fig. 23), while only the initial ascorbate concentrations of the samples appeared to account for the ability to predict the absolute ascorbate losses (Fig. 24).

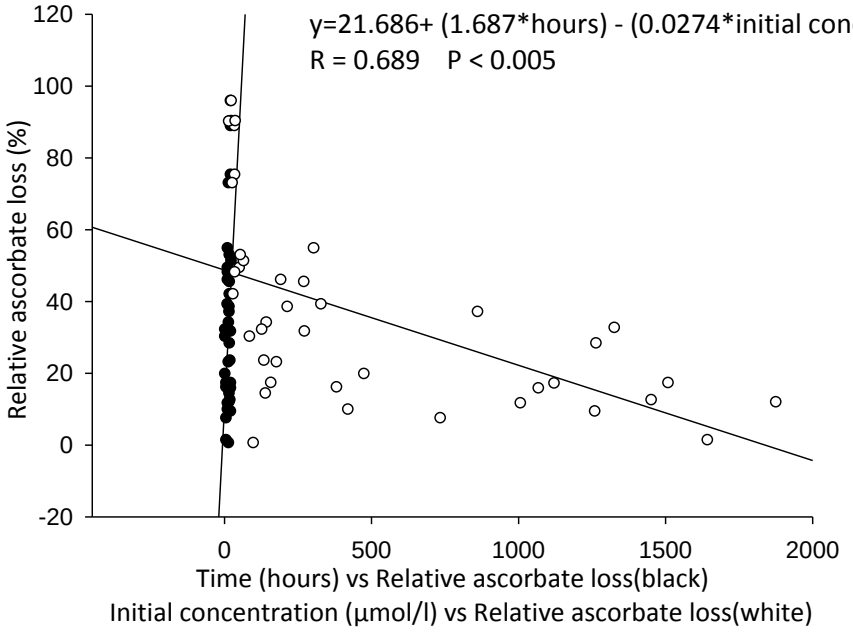

Figure 23: *The relative ascorbate losses in urine were plotted against two variables: the time elapsed since voidand the initial ascorbate concentrations in urine. Both relations were significant (P < 0.05) and both independent variables appeared to contribute to predicting the relative losses.*

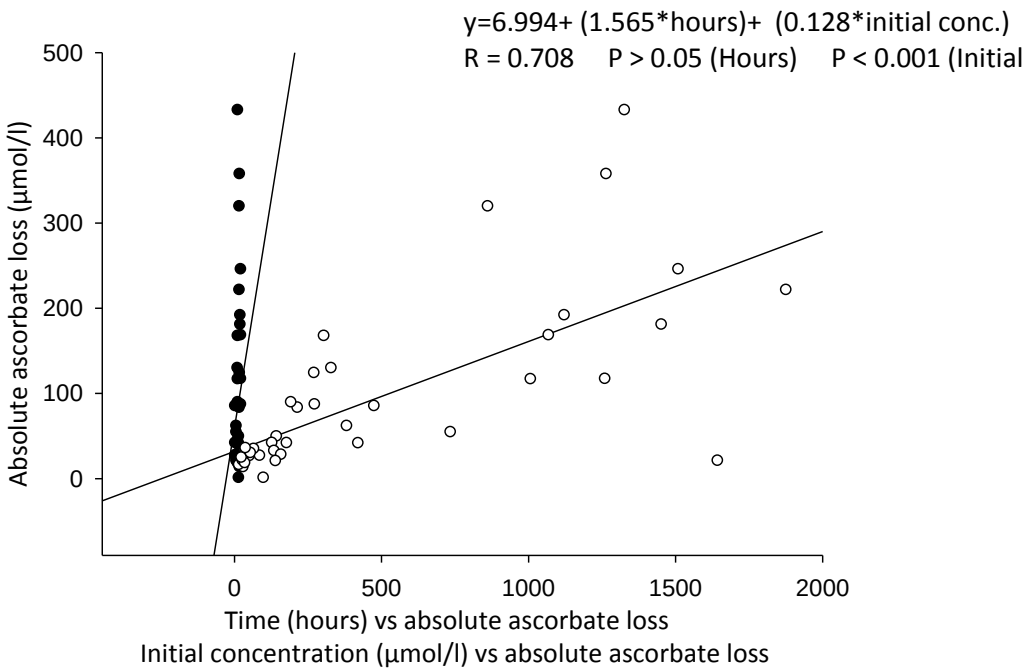

Figure 24: *The absolute ascorbate losses in urine were plotted against two variables: the time elapsed since void (hours) and the initial ascorbate concentrations in urine. Only the initial concentrations appeared to account for the ability to predict the absolute ascorbate losses (P < 0.001). Time did not appear necessary for prediction (P > 0.05).*

DISCUSSION

Vitamin C is a water-soluble vitamin which is readily excreted in urine. Determination of vitamin C status is based on ascorbate concentrations in plasma and urine. Frequently, 24-h urine samples are collected to study the excretion of substances in urine and so are 24-h vitamin C excretion and renal ascorbate clearance used. However, ascorbate is known to be unstable due to redox reactions.

The findings of **Part I** that the ascorbate concentrations in plasma were dependent on GFR as an indicator of renal function is consistent with data in the literature, showing that patients with impaired renal function had lower ascorbate concentrations in plasma and increased renal clearance of ascorbate (Hirsch, et al., 1998). In this study, there was no evidence of increased renal losses due to increased renal clearance, since there was no significant relation between GFR and the ascorbate concentrations, neither in spot urine nor in 24-h urine samples and there was no relation between GFR and ascorbate clearance. The 24-h urine collections provided also the basis for the calculations of the ascorbate clearances.

Another objective of this study was to determine whether there was a dependency of the ascorbate concentrations in plasma, spot urine and 24-h urine samples on the amounts of 5-day dietary vitamin C intake prior to the investigation. Dietary vitamin C intake of the study subjects was between 40 and 268 mg/d, indicating substantial between-subject variability. The hypothesis that the more vitamin C is consumed, the more is excreted in urine was proven by significant relations between the amounts of dietary vitamin C intakes and the concentrations of ascorbate in both spot urine and 24-h urine samples. These findings demonstrate that vitamin C is readily excreted in urine when greater amounts of vitamin C are ingested.

In contrast, plasma concentrations of ascorbate did not show a dependency on dietary vitamin C intakes. This finding was surprising because in a study on healthy nonsmokers conducted previously in the same region a significant association had been identified (Sundl, et al., 2003). However, the current finding is consistent with the fact that plasma concentrations of vitamin C rarely exceed 100 µmol/l, when food is the exclusive source of vitamin C intake (Levine, 2011). In the present study, only 4 out of 40 study subjects showed

plasma concentrations greater than 100 µmol/l (103, 104, 110 and 111 µmol/l), and none of the study subjects was taking vitamin C supplements.

The excretion of ascorbate in urine increased with increasing ascorbate concentrations in plasma. The fact that the plasma concentrations of ascorbate reached saturation was further explained by the observation that ascorbate concentrations in spot urine and 24-h urine samples increased in response to increases in plasma concentrations of ascorbate, indicating that ascorbate is readily excreted in urine.

The main aim of **Part II** of this study was to investigate the losses of ascorbate in urine samples that were kept at room temperature for up to 24 hours. The hypothesis that the longer a sample is kept in the laboratory at room temperature the more ascorbate is degraded was confirmed by the finding that losses increased with time.

Additional variables such as the volumes of the samples, initial ascorbate concentrations and absolute losses were investigated in order to clarify which factors might have a significant effect on the ascorbate concentrations in urine samples collected over a 24-h period.

In addition, a dependency between the absolute losses and the initial ascorbate concentrations in the fresh urine samples was revealed, indicating that the higher the initial concentrations of ascorbate, the higher the absolute losses.

Multiple regression analysis revealed that percent losses increased significantly both with time elapsed since void and initial concentrations, while absolute losses increased significantly only with increasing initial ascorbate concentrations. It was concluded from these findings that high absolute losses may only occur when initial concentrations are high, while percent losses take into account the proportion that is being lost and small absolute amounts may still be relevant relative losses. It is therefore suggested that percent losses should preferably be used in future studies.

In this study it has been demonstrated that the larger the volumes of the urine samples, the smaller the ascorbate concentrations of the urine samples and the smaller the absolute losses of ascorbate. Here, it could be hypothesised that in urine there exist factors which contribute to the chemical stabilisation of ascorbic acid, leading to a slower degradation and

therefore a smaller absolute loss. However, future studies are needed to prove this hypothesis.

The volumes of the urine samples seem to play an important role in the concentrations of ascorbate. The mean of the ascorbate concentrations of each subject's urine samples was compared with both the mean volume and the total volume of each subject's urine samples. It was observed that the concentrations decreased when the mean of the individual spot urine samples or the total volume increased.

CONCLUSIONS

In the first part of this study, GFR has been shown to influence the ascorbate plasma concentrations, leading to the conclusion that the proper function of the kidneys, as indicated by GFR, plays an important role in the maintenance of vitamin C status in the human body.
Moreover, it was shown that the amounts of ascorbate excreted through urine were dependent on the dietary vitamin C intakes, indicating that the human body can only maintain a certain amount of ascorbate and the excess amounts are readily excreted.

From the second part of the study it was concluded that the longer a urine sample stays unprocessed at room temperature, the higher the ascorbate losses that will occur.

A further important conclusion is that both the ascorbate concentrations and the ascorbate absolute losses are influenced by the volume of the urine samples. This is a finding which could trigger future studies, in order to find out the mechanisms by which the urine volume affects ascorbate concentrations and absolute losses. It was also interesting to find out that the initial ascorbate concentrations of a urine sample plays an important role in the amount of ascorbate that will be lost over time.

Because the initial ascorbate concentrations in fresh urine samples are the most important determinant of the absolute losses, ascorbate losses cannot be predicted from the time elapsed between void and preanalytical sample processing.

Taken together, the results of this study indicate that 24-h urine collections are not a reliable tool for assessing urinary ascorbate excretion, because an unpredictable amount of ascorbate is lost in the urine samples prior to analysis. The same seems to hold true for the ascorbate clearance, because it is also based on 24-h collections.

The vitamin C maintenance in subjects with impaired renal function, the urinary losses of vitamin C and how the concentrations measured in the laboratory are altered over time are clinically highly relevant topics, which should be further investigated in larger scale studies, taking into consideration additional variables.

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ABBREVIATIONS

24-h U: Urine collected over 24 hours

BIL: Bilirubin

BMI: Body mass index

CRP: C-reactive protein

DHA: Dehydroascorbic acid

DMT1: Divalent metal transporter

ERY: Erythrocytes

GFR: Glomerular filtration rate

GLO: Gulonolactone oxidase

GLU: Glucose

Hb: Hemoglobin

KET: Ketones

LDL: Low density lipoprotein

LEU: Leukocytes

MDCK-MDR1: Madin-Darby canine kidney cells transfected with the human MDR1 gene

MDRD: Modification of Diet in Renal Disease Study Group

mg: Milligram

ml: milliliter

µmol: micromol

NIT: Nitrates

PRO: Proteins

%: Percent

RDA: Recommended daily allowance

SG: Specific gravity

Spot U: Spontaneous urine sample

SVCT1/2: Sodium-dependent vitamin C transporter

UBG: Urobilinogen

WHO: World Health Organization

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I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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