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Detection and improvement of fat soluble vitamin status in patients with short bowel syndrome using two different supplement formulations

Diploma thesis
submitted by
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Contents

Abbreviations

AP	alkaline phosphatase
ATTP	α-tocopherol transfer protein
BGP	bone Gla protein
BHT	butylhydroxytoluene
BMC	buccal mucosa cells
BMI	body mass index
BSA	bovine serum albumin
CRP	C-reactive protein
d	day
DNA	desoxyribonucleic acid
DNP	dinitrophenylhydrazine
EDTA	ethylendiaminetetraacetic acid
EFA(D)	essential fatty acid (deficiency)
EIA	enzyme imuno assay
ELISA	enzyme linked immuno sorbent assay
HPLC	high pressure liquid chromatography
HPN	home parenteral nutrition
IgG	immunoglobulin G
i.m.	intra muscular
i.v.	intra venous
INR	interational normalized ratio
IU	international units
KDS	Kurzdarmsyndrom
LDL	low density lipoprotein
MCT	medium chain triglycerides
MGP	matrix Gla protein
MRDR	modified relative response test
OPDA	o-phenylendiamine
p.a.	pro analysi
PBS	phosphate buffered saline
PIVKA	proteins induced by vitamin K absence
PN	parenteral nutrition
PTH	parate hormone
RAR/ RXR	retinoic acid receptors
RBP	retinol binding protein
RDR	relative dose response test
SBS	short bowel syndrome
SR-A	scavenger receptor A
TBARS	thiobarbituric acid reactive substances
TEMPO	4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy free radical
TG	triglycerides

TPGS	$d-\alpha$-tocopheryl polyethylene glycol 1000 succinate
TPN	total parenteral nutrition
TTR	transthyretin
UKT	Universitäts Klinikum Tübingen
VLDL	very low density lipoproteins
WHO	World Health Organisation

Chapter 1

Introduction

1.1 Short bowel syndrome (SBS)

1.1.1 Definition

Short bowel syndrome (SBS) is a rare disease resulting from either massive small bowel resections or loss of intestinal function. It occurs when less than 200 cm of functioning small bowel are available and it is clinically defined by malabsorption, diarrhea, electrolyte imbalance, fluid disturbance and malnutrition [1]. In a shortened bowel the resorptive area is reduced, different specialized bowel segments and structures such as the terminal ileum or ileocecal valve are missing and the transit time of intestinal content is severely diminished.

1.1.2 Causes for SBS

SBS in children is commonly congenital: many authors name volvulus and intestinal atresia as main causes for short bowel in infants [2, 3, 4]. In adult patients, SBS is mostly acquired: small bowel resections are mainly performed because of chronic inflammatory bowel disease such as Crohn‘s diseases (50 to 60 % of all cases) [1]. Tumor resections as well as mesenterial infarction and trauma also result in SBS. A loss of intestinal function can be seen in irradiation enteritis after radiotherapy of the lower abdomen or villus atrophy in celiac disease. Women are more prone to develop SBS than men due to a shorter premorbid bowel-length [9].

1.1.3 Prevalence and incidence

1.1.3.1 Prevalence

Little is known about the prevalence of SBS. Most authors calculate the number of short bowel patients from epidemiological data on home parenteral nutrition (HPN), but this is not accurate since many short bowel

patients can be weaned off total parenteral nutrition after adapting to complete oral nutrition (50 to 70 % of all SBS patients [3]). Furthermore some SBS patients never require total parenteral nutrition and thus never appear in such statistics.

Estimations from the U.S. state that the prevalence of HPN is 120 per million inhabitants and that about 25 % of HPN-users are SBS patients. About 290.000.000 people live in the U.S. therefore the prevalence for SBS can be calculated to 30 SBS-patients per 1 million inhabitants [5]. Kurlberg et al. [6] name 400 to 500 SBS patients in Sweden, within a population of estimated 9 million people indicating 44 to 55 cases per million inhabitants.

1.1.3.2 Incidence

Estimations about the incidence range from 2 to 5 new SBS-cases per 1 million inhabitants in Germany [7]. Hebuterne et al. state 163 new HPN patients per 1 million inhabitants in Europe [8], 5% of these having gastrointestinal disorders including SBS ($\approx$ 8 patients per 1 million inhabitants).

1.1.4 Pathophysiology

The complex clinical picture of SBS presents itself depending on the site of resection, the length of remaining small bowel, accompanying colon resections, intestinal adaptation and the patients' age and nutritional status. Resections of more than 50 % of the small bowel in adults result in impaired nutrient absorption, but if more than 75 % of the small bowel are lost, a severe undernourishment is likely to occur [2]. In the following sections the different types of intestinal resections and their influence on the nutritional status of the SBS patient are described (refer to **figure 1.1**).

1.1.4.1 Colonic resection

Most authors distinguish two anatomical types of SBS [9, 3]:

1. Patients with a (partially) resected jejunum and ileum with a colon in continuity, with or without preserved ileocecal valve.

2. Patients without a colon in continuity having a jejunostomy.

The colon is an important organ for the fluid and electrolyte balance. It absorbs sodium, water and energy (short chain fatty acids) and becomes therefore an important digestive organ for SBS-patients. If it is missing severe diarrheas occur, and the patient must be supplemented with oral glucose-saline solution [9].

1.1.4.2 Ileal resection

Resections of the jejunum are mostly better tolerated than ileal resections. The ileum is capable of adapting to jejunal functions whereas the ileal functions can not be adapted by the jejunum. The ileum is the site of resorption for cobalamin (vitamin B_{12}) and bile acids which are recycled in the enterohepatic circulation. A loss of more than 100 cm of ileum results in severe fat malabsorption since the mediators of fat digestion, the bile acids, are lost with feces. This bile acid loss generates chologenic diarrhea and - since fat absorption is disturbed - also steathorrhea. Unabsorbed long chain fatty acids bind free magnesium and calcium leading to increased losses of these minerals and to an increased absorption of oxalate. This happens because calcium and magnesium bind oxalate by forming insoluble salts, that can not be absorbed. An increased oxalate absorption puts the patient at great risk for calcium oxalate renal stones. 25 % of patients with a colon in continuity have calcium oxalate renal stones [9].

As described, a loss of bile acids results in fat maldigestion and therefore fat malabsorption. This leads to a reduced uptake of essential fatty acids [10] and fat soluble vitamins. A deficiency of these nutrients is likely to occur in ileum-resected patients [11].

1.1.4.3 Resection of the ileocecal valve

A special problem arises, when the ileocecal valve is resected. This valve prevents reflux of colon content as well as bacterial colonization of the small intestine starting from the colon. A bacterial overgrowth deteriorates the bile acid loss, since a bacterial bile acid deconjugation occurs. Furthermore, the intestinal transit time is sped up, worsening diarrheas and fluid loss. Patients with a preserved ileocecal valve experience better clinical outcome than patients without [12].

1.1.4.4 Jejunal resection

Resections of the jejunum alone are very rare and as described above usually well tolerated [13]. A deficiency of water soluble vitamins and minerals is not very likely, since the ileum can adapt to absorption of these nutrients.

1.1.5 Nutritional needs of the SBS patient

The nutritional needs of the SBS patient have been described in detail in many reviews [13, 4, 11]. There are usually three stages of SBS characterized in the literature: the postoperative acute phase, the phase of intestinal adaptation and the stabilization-stage [7, 5]. They are presented in **table 1.1**. It is obvious that the nutritional needs of a SBS patient differ a lot between the three stages.

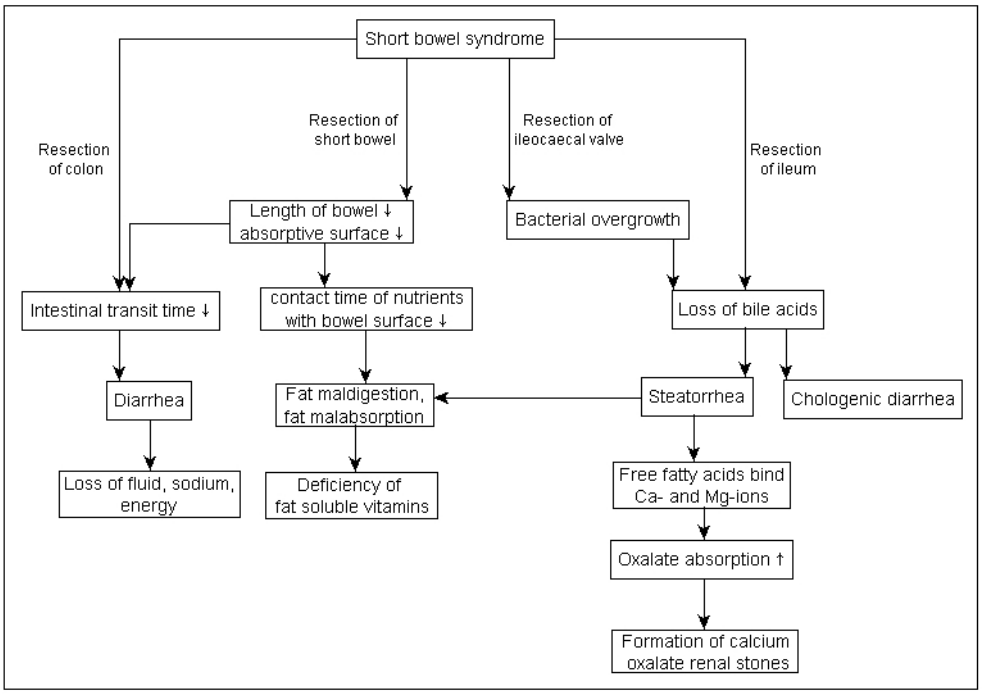

Figure 1.1: Pathophysiology of SBS

Table 1.1: Stages of SBS

Stage	**Duration**	**Characteristics**	**Nutritional requirements**
Postoperative acute phase	Up to 1 month	Loss of fluid (> 2,5 L/d), electrolytes, large stool volumes	Total parenteral nutrition
Intestinal adaptation	3 weeks to 1 year	Structural and functional adaptation, fluid loss (< 2,5 l/d)	Weaning off PN, introduction of oral diet enhancing the intestinal adaptation
Stabilization	After 3 months up to 2 years	No further adaptation, decline of diarrhea and steathorrhea	Oral feeding, adequate supplementation of micronutrients

1.1.5.1 Postoperative acute phase

Immediately after massive bowel resections the patient relies completely on total parenteral nutrition (TPN). Today, standardized therapy protocols for the treatment of this SBS-stage are available [7].

1.1.5.2 Intestinal adaptation

The second stage of SBS, the intestinal adaptation, is currently subject of many studies. Intestinal adaptation is the structural and functional modification of the remaining bowel segments. Enterocytes are proliferating at an increased rate resulting in heightened villi and deeper crypts thus increasing the absorptive surface of the bowel [7]. Decelerated intestinal transit time of bowel content is a sign of functional adaptation [5]. A third aspect of adaptation is hyperphagia, often recognizable in SBS-patients [14]. Intestinal adaptation is mainly stimulated by intraluminal nutrients but non-nutritional components are currently investigated for their gut-stimulating ability as well.

In general, complex nutrients that require a whole set of digestive enzymes have a higher potential to increase adaptation than diets with high content of monomers [5, 15]. Complete intestinal adaptation is usually achieved within 2 years after resection [14]. For this reason I included only patients into my study whose last bowel resection was at least 2 years ago.

1.1.5.3 Stabilization

In this stage of SBS the adaptation process is completed. The patient has to maintain weight and appropriate status of macro- and micronutrients. Depending on the site of resection and remaining bowel length either complete oral nutrition or partly oral nutrition can be achieved. As described

in section 1.1.4, fat soluble vitamin deficiency due to fat malabsorption is common in SBS. A recommendation for supplementation of these vitamins and other micronutrients is given in the literature [15, 1, 13]. Furthermore, dietary fat is partially substituted by medium chain triglycerides (MCT) to decrease steatorrhea.

In this diploma thesis I focus on the third stage of SBS.

1.2 Vitamins

As explained in the previous sections, SBS patients are at great risk of developing fat soluble vitamin deficiency. In the following sections, the fat soluble vitamins are characterized and examples for deficiencies in SBS patients are presented. In addition, vitamin C will be described in short since it has been investigated in this study along with the fat soluble vitamins.

1.2.1 Vitamin E

1.2.1.1 Chemistry and biological function

The term vitamin E comprises a group of eight fat soluble molecules, the tocopherols and tocotrienols. Vitamin E is a derivative of the 6-chromanol with a saturated phytol sidechain (tocopherols) or a isoprenoid side-chain (tocotrienols) respectively. The isoforms differ in number and position of their methyl-groups on the chromanol ring system (see **figure 1.2**). Natural vitamin E, i.e. non synthetical vitamin E, is a mixture of high amounts of RRR-α-tocopherol and smaller quantities of RRR-β-tocopherol, RRR-γ-tocopherol and RRR-δ-tocopherol [16].

Synthetic vitamin E is a racemic mixture of the 8 isomers of α-tocopherol in equal amounts, called all-*rac* α-tocopherol.

Vitamin E is mainly stored as α-tocopherol in adipose tissue [17]. All eight isoforms have potent antioxidative capacity, but α-tocopherol has the greatest biological importance. In plasma, α-tocopherol states the largest fraction of all tocopherols, since it is homeostatic regulated by action of the α-tocopherol-transfer protein (ATTP) in the liver [18]. This protein transports exclusively α-tocopherol into nascent VLDLs, which carry vitamin E to muscle and adipose tissues.

The main function of α-tocopherol lies in its antioxidative property. Vitamin E protects lipids in the cell membrane, lipoproteins and storage fats from oxidative damage by action of free radicals [16]. Oxidative changes of cellular structures facilitate a large number of pathological downstream effects. Among these are atherosclerosis (through formation of oxidized LDL), cancer (through oxidative damage of the DNA) [19] and aging processes. Vitamin E also shows non-antioxidant functions: it may have important gene

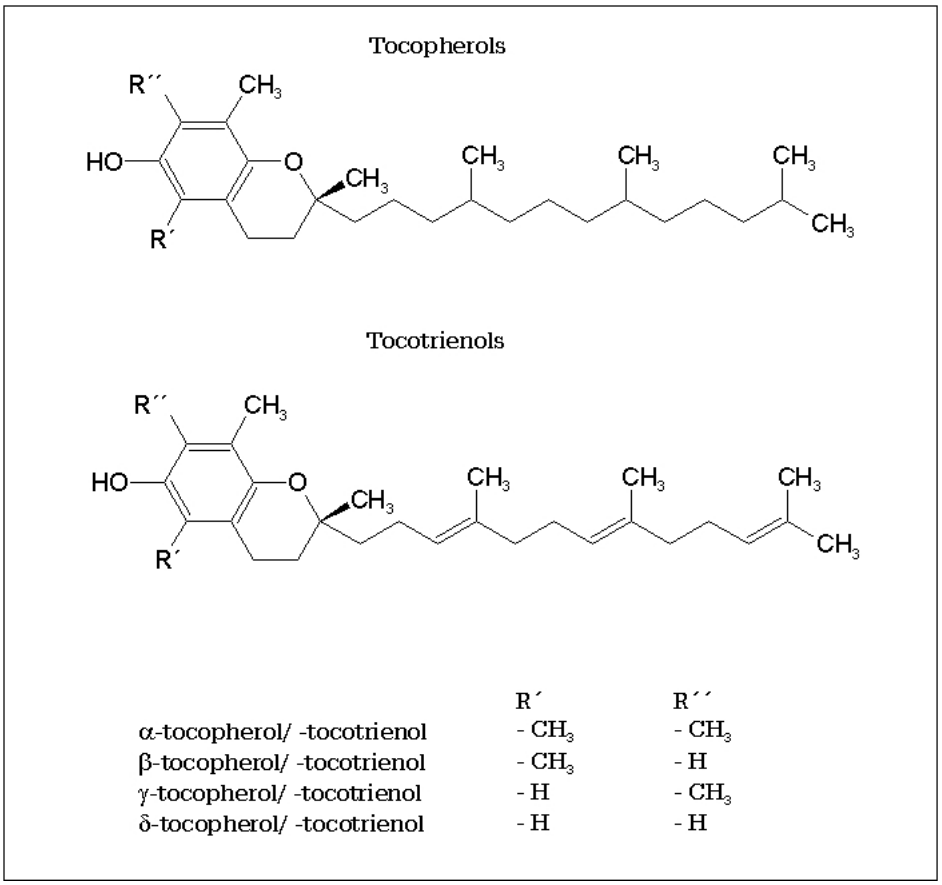

Figure 1.2: Molecular structure of tocopherol and tocotrienol isoforms

regulation properties (e.g. down regulation of SR-A and CD36 scavenger receptors [19]), it has been shown to regulate immune-response by modulating the generation of prostaglandins and lipid peroxidation products [21] and vitamin E can inhibit protein kinase C, thus affecting a number of signaling pathways [16].

A deficiency of vitamin E results in neuromuscular dysfunctions such as neuropathies and myopathies [16]. Patients with low α-tocopherol plasma concentrations ($<$ 11,6 μmol/L) are more susceptible to peroxide induced hemolysis [20, 21].

1.2.1.2 Vitamin E and SBS

There are single case reports described in the literature concerning vitamin E deficiencies in SBS. Howard and his colleagues [22] presented a patient with SBS suffering from gait ataxia and generalized motor weakness in proximal

muscle groups. He showed diminished proprioception in all extremities and visual disturbances, especially scotopic vision. The patient had a 25-year history of Crohn's disease, which required several bowel resections, leaving him with 30 cm of jejunum anastomosed to the transversal colon. The authors assessed the patient's nutritional status and a severe vitamin E deficiency was apparent. The patient was aggressively substituted with large doses of vitamin E intramuscularly (3200 mg *dl*-α-tocopherol acetate in sesame oil), which showed no great effect on serum vitamin E levels and clinical parameters such as peroxide hemolysis. After that, they administered water miscible *dl*-α-tocopherol acetate (200 mg/d) orally and within two years the patient showed normalized vitamin E concentrations as well as clinical improvement. The authors strongly suggest a metabolic connection between the vitamin E deficiency and the neurological disturbances.

Traber et al. [23] treated a SBS patient aged 71 with longstanding fat malabsorption and consecutive vitamin E deficiency with a water soluble form of vitamin E, tocopheryl succinate polyethylene glycol 1000 (TPGS). Before intervention the patient showed impaired neurological function such as proximal muscle weakness, absent deep tendon reflexes and unsteady gait. Orally administered TPGS in high doses (4000 IU/d) proved to be more efficient to treat severe vitamin E deficiency in this case of fat malabsorption than parenteral application. The authors concluded that much of intramuscularly injected vitamin E remains in the injection site and cannot be absorbed completely thus being inappropriate to correct a deficiency.

A third case was recently presented by Walker et al. in 2004 [24]. The patient described was a 51 year old man who suffered from SBS due to small bowel resections because of Crohn's disease. He showed extremely low vitamin E levels and the neurological symptoms described for vitamin E deficiency, i.e. ataxia, tremor in upper extremities, decreased proprioception and vibratory sensation in distal extremities as well as absent deep tendon reflexes. The symptoms appeared not until roughly 30 years after small bowel resection. The patient received an aggressive vitamin E supplementation with TPGS (final dose 1000 IU twice daily). The plasma α-tocopherol concentration could be corrected, however, the neurological dysfunctions did not disappear completely.

These case reports indicate, that vitamin E deficiency occurs years up to decades after the resection. This might be explained by the fact that α-tocopherol is stored in adipose tissue and slowly released from these sites [25]. In humans, it takes several years to develop clinical symptoms of vitamin E deficiency [23]. In adults, they are mostly irreversible, therefore it is imperative to observe and correct the α-tocopherol concentrations in plasma as early as possible in SBS patients.

1.2.2 Vitamin A

1.2.2.1 Chemistry and biological function

Vitamin A is the term used for retinol and the retinylesters. [26]. All other derivatives like retinoic acid and retinal are summarized as retinoids. The structures of the mentioned molecules are presented in **figure 1.3**.

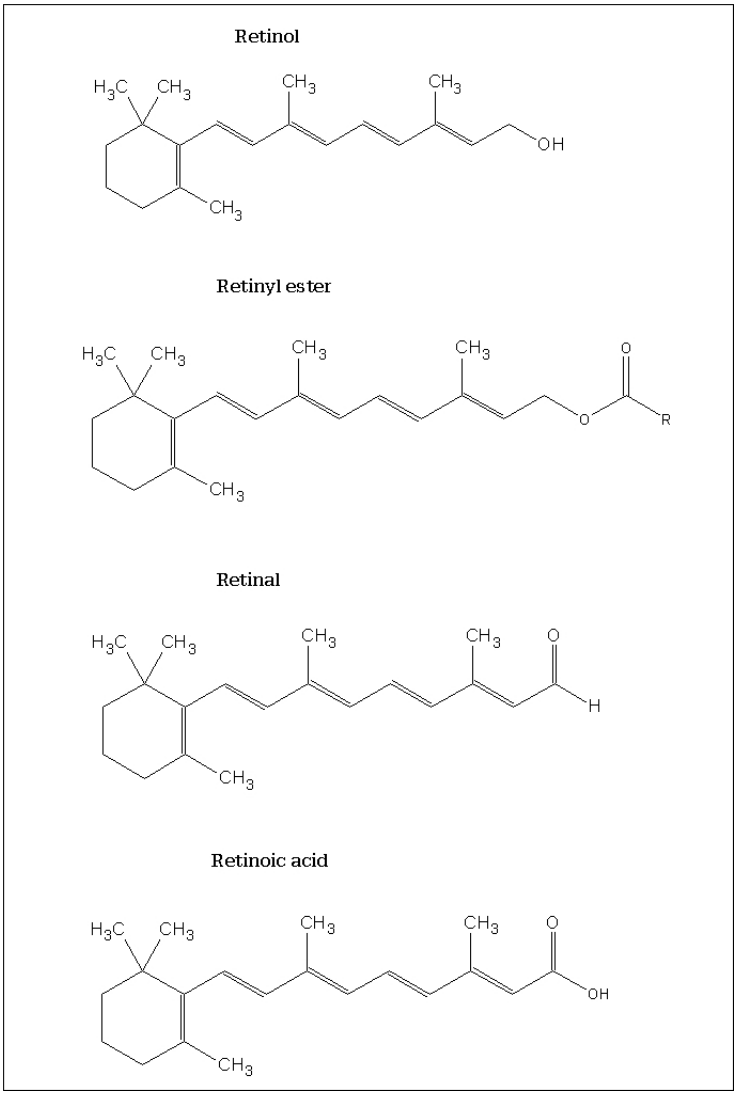

Figure 1.3: Molecular structure of retinol and its derivatives

Retinol and its different derivatives have specific functions in the human organism. Retinol itself is the main metabolite of vitamin A found in plasma. Bound to retinol binding protein (RBP) and transthyretin (TTR) it serves

as transport form. Vitamin A is stored as retinyl ester (e.g. retinyl palmitate or -stearate) mainly in the Ito cells of the liver and in small quantities in all other tissues [21]. Retinal serves as ligand of opsin forming rhodopsin, which is substantial for vision. Retinoic acid in its *cis*- and *trans*-form binds to specific receptors (RXR and RAR) and is an important transcription factor. It has a distinct effect on proliferation and differentiation of epithelia and different tumor cells.

A prolonged undersupply with vitamin A results in the clinical picture of xerophthalmia. This pathological state includes nyctalopia, dry conjunctiva, Bitot's spots, epithelial lesions and finally keratomalacia. If a vitamin A deficiency remains untreated the condition is irreversible and the patient will go blind. Other deficiency symptoms are impaired immune response [21] and respiratory tract infections [16].

A marginal supply with vitamin A is difficult to detect due to homeostatic regulation of retinol in plasma. Decreased plasma concentrations can be detected only after the vitamin A stores in the liver are emptied to less than 100 μg/g liver tissue [16]. When the organism is depleted of vitamin A the typical clinical symptoms as described above occur. A marginal vitamin A status can be assessed by the relative dose response test (RDR) or the modified relative dose response test (MRDR) [21]. Furthermore there are biochemical methods available, for instance measurement of the retinol binding protein (RBP) in plasma. Retinol plasma concentrations are usually decreased during inflammation [27, 28]. Therefore a parallel assessment of C reactive protein (CRP) is useful to evaluate the vitamin A status. In the present study, a CRP-value greater than 5 mg/mL was considered as positive for inflammation and the retinol concentration of that patient was not considered in statistical analyzes.

1.2.2.2 Vitamin A and SBS

Mokete et al. [29] described a case of xerophthalmia caused by vitamin A deficiency secondary to SBS in a child. The young patient was small bowel resected shortly after birth and developed a deficiency within 8 years of life. He showed all of the above described ocular symptoms for vitamin A deficiency. He was treated according to WHO-standards with 100.000 IU of vitamin A intramuscularly and several oral multivitamin supplements. Since he suffered from bacterial overgrowth he was also treated with systemic antibiotic therapy. The authors suggest a general review and screening for micronutrient deficiencies in SBS patients.

An almost identical case was described by Cella et al. [30]. A 9-year-old girl with SBS developed xerophthalmia after nutritional guidance was discontinued two years earlier. She had undergone small bowel resection soon after birth because of congenital intestinal atresia. The girl was successfully treated with a single dose of vitamin A (100.000 IU) i.m. and within 1

month the signs of xerosis had disappeared. From this example one can derive, that nutritional follow up and adequate maintenance of micronutrient status after resections of the bowel is crucial for good clinical outcome.

1.2.3 Vitamin D

1.2.3.1 Chemistry and biological function

Vitamin D is the name for a group of lipophilic molecules which possess antirachitic activity [21]. The human organism is able to synthesize vitamin D, therefore it is not a vitamin per definition, but it is still classified as fat soluble vitamin for historical reasons. The endogenous synthesis is as follows: Through UV-irradiation of the skin, the vitamin D precursor 7-dehydrocholesterol is converted to cholecalciferol (= vitamin D_3). Vitamin D is then hydroxylated twice in the human organism: firstly, on its carbon number 25 in the liver forming $25(OH)D_3$ and secondly, controlled by parathormone (PTH), in the kidneys on carbon number 1 providing $1{,}25(OH)_2D_3$ which is the active metabolite. The above mentioned molecules are presented in **figure 1.4**. In case of insufficient dermal sun exposure, vitamin D becomes essential and exogenous vitamin D sources from plant and animal foods are required.

The major role of vitamin D is its calcium providing function. This is accomplished by increased calcium absorption from the intestine or increased bone resorption in case of nutritional calcium shortage. Both pathways are regulated by $1{,}25(OH)_2D_3$, which binds to its nuclear receptor in its various target cells [21, 26].

A vitamin D deficiency therefore affects the calcium metabolism of the organism. Bone will be increasingly resorbed to provide the adequate calcium plasma concentration. This results in rickets (children) and osteomalacia (adults). Prior to bone symptoms, myopathy occurs. This includes muscle weakness and increased body sways [16, 31]. Hypovitaminosis D is characterized by increased PTH and alkaline phosphatase (AP) plasma concentrations. A reduction of circulating $1{,}25(OH)_2D_3$ has been reported as well [21]. However, most authors state, that a reduction of $1{,}25(OH)_2D_3$ occurs not until $25(OH)D_3$ concentrations are very low [32, 33, 34].

1.2.3.2 Vitamin D and SBS

Vitamin D deficiency is very common in patients with short bowel syndrome. Haderslev et al. [32] reported in a study with 42 SBS patients in Denmark that 38.1 % of these patients were vitamin D deficient, whereas 23.8 % had low normal serum vitamin D concentrations and 38.1 % where within normal range. In this study vitamin D deficiency was defined as ≤ 8 ng/ mL $25(OH)D_3$. In addition to serum parameters bone resorption markers and PTH where analyzed as well. These turned out to be increased in the state

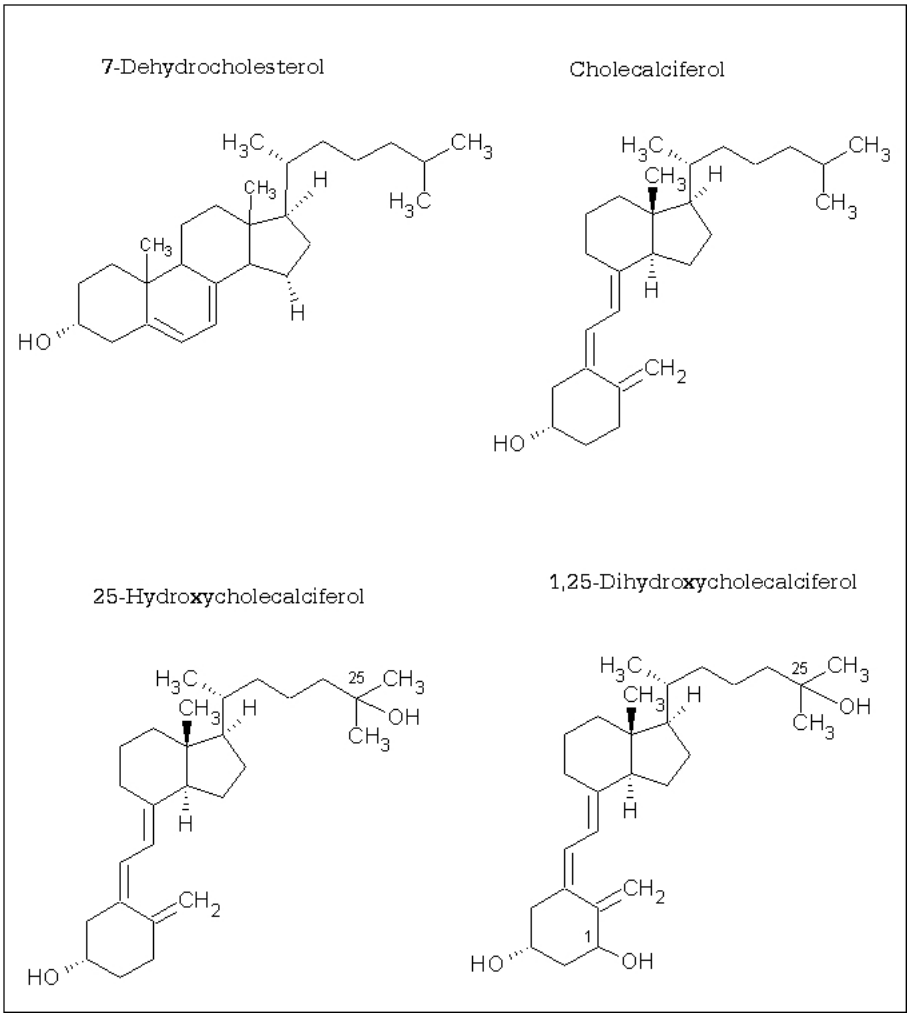

Figure 1.4: Molecular structure of 7-Dehydrocholesterin, Cholecalciferol, 25-Hydroxycholecalciferol and 1,25-Hydroxycholecalciferol

of vitamin D deficiency. The authors name fat malabsorption and reduced sunlight exposure as main causes for the insufficient supply with vitamin D. They recommend supplementation as most rational approach to reduce the deficiency.

There are other single case reports concerning vitamin D deficiency in SBS patients. Metabolic disturbances (especially related to divalent cations) due to vitamin D deficiency and subsequent acute renal failure are described by Banerjee and Warwicker [35] in five patients. Koutika et al. [31] presented a single case report of SBS subsequent to Crohn's disease with very low $25(OH)D_3$ concentrations and osteoporosis of the hip. The patient could not be treated with oral vitamin D supplementation since she had a very short remaining small bowel and was dependent on TPN. Therefore she was successfully treated with UV B irradiation via a tanning bed.

1.2.4 Vitamin K

1.2.4.1 Chemistry and biological function

Vitamin K is the term used for 2-methyl-1,4-naphtoquinone and all its derivatives that exhibit antihemorrhagic activity in animals fed a vitamin K deficient diet [21]. There are two important vitamin K forms: vitamin K_1 (phylloquinone) is synthesized by plants and larger quantities are found in green vegetables; vitamin K_2 (menaquinone) is produced by some gram-positive bacteria (refer to **figure 1.5**). It is hypothesized that some intestinal bacteria produce vitamin K which can then be utilized by the human organism [16]. However, it is not clear how much this enteral production is contributing to the vitamin K supply of the organism [36]. The mainpart of bacteria are found in the colon but the site for vitamin K absorption is in more proximal regions like the jejunum and ileum [37]. Therefore a enteral supply is rather unlikely.

Vitamin K functions as cofactor in the γ-carboxylation of glutamyl-residues of so called vitamin K dependent proteins. The γ-carboxylation, which is facilitated by the vitamin K dependent carboxylase, is crucial for correct protein function. The most important vitamin K dependent proteins are clotting factors: prothrombin, factors II, V, VII, IX, X, protein C and protein S. These factors are proteases that activate each other in a cascade system. The carboxylation enables the proteins to bind Ca^{2+} ions that are needed for activation of protease activity. There are other vitamin K dependent proteins: in skeletal tissues osteocalcin (also named bone Gla protein, BGP) and matrix Gla protein (MGP). They can bind Ca^{2+} ions and seem to be involved in the regulation of bone mineralisation [21]. A higher level of undercarboxylated osteocalcin has been associated with an decreased bone density and increased fracture rate. Osteocalcin is a vitamin D responsive protein and supplementation with vitamin D and vitamin K in combination

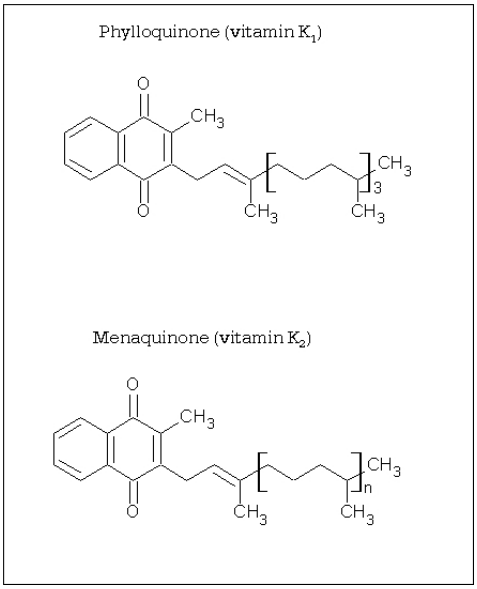

Figure 1.5: Molecular structure of phylloquinone and menaquinone

proved to be of additive nature [16].

Deficiencies of vitamin K are rare and mostly described in infants [26]. In adults vitamin K responsive bleedings occur along with intestinal diseases, such as chronic diarrhea, steatorrhea and pancreatic insufficiency [21]. A marginal supply with vitamin K results in increased levels of under-γ-carboxylated proteins, they are referred to as PIVKAs (proteins induced by vitamin K absence). The assessment of these PIVKAs can be used to detect a subclinical vitamin K deficiency, but this is a very costly method. If the deficiency prolongs and the organism stores are depleted, the hemostatic system is affected resulting in prolonged coagulation time and bleeding into tissues and organs. At that stage vitamin K deficiency can be detected by assessment of the prothrombin time via the Quick value. The Quick value is laboratory specific and can be converted into the international normalized ratio (INR). I used both parameters in this pilot study to screen for vitamin K deficiency.

1.2.4.2 Vitamin K and SBS

Vitamin K deficiency in SBS patients is not often described, but widely assumed by many authors due to fat malabsorption, steatorrhea and use of antibiotics [38, 13]. A case report from India was presented by Chandra et al. in 2001 [39]. He described a child (aged 10 years), that had undergone intestinal resection from the duodenal-jejunal flexure to 30 cm proximal to ileocecal junction two years earlier. The patient exhibited subcutaneous

bleeding and a prolonged prothrombin time. He was on antibiotic therapy for recurring diarrheas. The patient was treated with 5 mg vitamin K per day for three days and his diet was modified to improve the nutritional status. After vitamin K therapy the patient's prothrombin time improved and subcutaneous bleeding stopped. The authors conclude that the vitamin K deficiency had developed due to SBS and frequent use of antibiotics and that a prevention of vitamin K deficiency in SBS patients should be kept in mind by the treating physician.

1.2.5 β-carotene

1.2.5.1 Chemistry and biological function

β-carotene is the most prominent substance of the carotenoid group. It consists of 2 β-ionon rings, that are connected by a system of conjugated doublebonds. These doublebonds contribute to the orange color of β-carotene and to its lipophilic properties (**figure 1.6**).

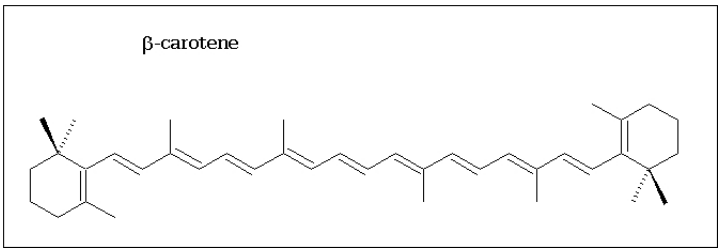

Figure 1.6: Molecular structure of β-carotene

β-carotene functions as provitamin A. It can be cleaved in the enterocytes by 15,15-dioxygenase yielding 2 molecules retinal. Apart from that, β-carotene has its own antioxidant effects. It quenches singulett oxygen ($^1O_2$), which is generated especially in the skin by influence of UV-radiation. By that β-carotene is turned into a radical itself, but it can be converted into β-carotene again by deduction of the surplus energy as thermal energy. Furthermore β-carotene is capable of reacting directly with lipidperoxides, which are generated by oxidative stress. The lipids are regenerated but β-carotene is consumed in this process and needs to be replaced. Thus β-carotene can be classified as essential for humans.

1.2.5.2 β-carotene and SBS

There is not much information available on the general β-carotene status in SBS-patients. Edes et al. [40] describe a patient with short bowel syndrome who was examined for essential fatty acid deficiency (EFAD). Apart from his EFA-status the authors investigated his ability to absorb β-carotene from

two different preparations (both fat soluble). It turned out that none of the two β-carotene supplements could increase the serum β-carotene concentrations of the patient. Since β-carotene plasma concentrations are usually responsive to dietary intake and since the patient also exhibited a vitamin K deficiency, the authors concluded, that this patient suffered from fat malabsorption evident by the unsuccessful β-carotene supplementation.

1.2.6 Vitamin C

1.2.6.1 Chemistry and biological function

Vitamin C is a water soluble vitamin that can be synthesized from glucose in all animals except guinea pigs and primates. Thus vitamin C is essential for humans. Chemically vitamin C is L-(+)-ascorbic acid and its derivatives, that show some vitamin C activity. Its structure is presented in **figure 1.7**.

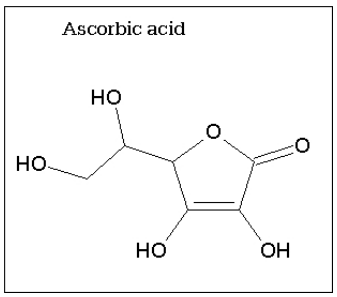

Figure 1.7: Molecular structure of ascorbic acid

The most prominent function of vitamin C is its reducing capacity. It is therefore acting as antioxidant in biological systems. Apart from these functions, ascorbic acid is cofactor in many enzyme dependent reactions, for instance the collagen biosynthesis. A deficiency of ascorbic acids leads to the well known disease scurvy.

1.2.6.2 Vitamin C and SBS

As mentioned in section 1.1.4.4 a deficiency of water soluble vitamins and therefore of vitamin C is not likely in SBS-patients. Nevertheless I investigated ascorbic acid in this study in order to show to show that the supply with water-soluble vitamins is usually not a problem in SBS-patients.

1.3 Pilot study

The pilot study consisted of two parts: a basic assessment and a double blind, controlled, randomized intervention trial with fat soluble vitamins. The study was conducted at the University hospital Tübingen (UKT) as follows: patients were asked to participate by a study assistant via telephone 4 weeks prior to the study start. After agreement, the patients received an invitation and a description of the study. Seven days before the start of the study the patients had to stop taking vitamin supplements containing any of the study vitamins (vitamin A, E, C and β-carotene) and they had to start brushing teeth exclusively with a vitamin A free toothpaste (Elmex® Sensitive). On day 1, the study participants were examined and informed in the UKT and the samples for the basic assessment were taken. On the same day, the patients started taking the study supplement and continued to do so for the next 27 days. On day 28, again blood and BMC samples were taken.

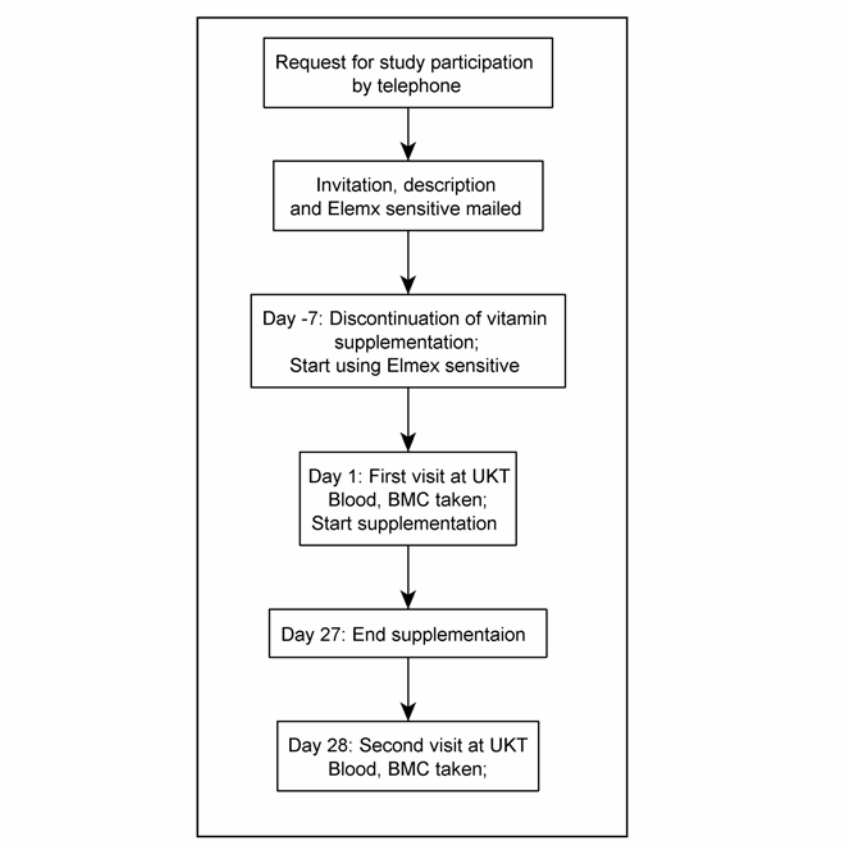

Figure 1.8: Study course

1.3.1 Subjects

18 patients with small bowel resections were enrolled in this pilot study. Inclusion criteria were:

- age $\geq$ 18 years
- a former prescription of ADEK-Falk® [1] **or** a proven deficiency of fat soluble vitamins **or** a current prescription of medium chain triglycerides (as an indicator of existing steathorrhea)
- last bowel resection $\geq$ 2 years ago
- discontinued use of all supplements containing one of the study vitamins during the course of the study

Patients were excluded from the study for fulfilling one of the following exclusion criteria:

- known incompatibility to one or more of the gummi bears ingredients
- pregnant or lactating woman
- acute inflammation in patients with Crohn‘s disease
- pancreatic insufficiency
- total parenteral nutrition

1.3.2 Aims

In the pilot study several aims were pursued:

1. A basic assessment of the fat soluble vitamin status in a cohort of SBS-patients had to be conducted. As described in section 1.2, there are only single case reports available (except for vitamin D) on extreme deficiencies of fat soluble vitamins in SBS patients. To my knowledge there has been no comparable study that investigated a general deficiency of all fat soluble vitamins in SBS so far.
2. The effect of fat soluble vitamins with improved galenics on plasma vitamin levels and markers for oxidative stress was investigated in a 4-week intervention trial in the same group of SBS patients. A vitamin supplement that is currently being tested in cystic fibrosis patients (see section 1.3.3) was used.

[1] A supplement containing the vitamins A, D, E and K that was administered by intramuscular injection to patients with fat malabsorption. ADEK-Falk® is not available in Germany any more since July 1st, 2003.

3. A 'proof of principle' for the superior bioavailability of micellised fat soluble vitamins compared to normal fat soluble vitamins in SBS patients.

1.3.3 Study supplement

The study supplement has been described earlier by Back et al. [45]. The supplement contained vitamin A and E as well as β-carotene as water-soluble, pH-stable micelles (AQUANOVA® solubilisates, Aquanova German solubilisate technologies GmbH, Darmstadt, Germany) (= verum) or as common fat soluble vitamin formulations (= positive control). Additionally, vitamin C was added in order to complement the antioxidant function of vitamin E and β-carotene. The composition of the study supplement is shown in **table 1.2**. Originally these supplements were designed for use in cystic fibrosis patients because these patients tend to have higher levels of oxidative stress and lower levels of antioxidant vitamins than healthy subjects. Thus, a special composition of antioxidant vitamins was chosen to improve the antioxidant-prooxidant imbalance that these patients experience.

Table 1.2: Composition of the study supplement

Vitamin	**Applied chemical form**	**Quantity in 6 gummi bears (daily ration)**
A	Retinyl palmitate	1000 mg RE
E	α-tocopheryl acetate	100 mg TE
β-carotene	β-carotene	1,6 mg
C	Ascorbic acid	400 mg

1.3.4 Properties of solubilized fat soluble vitamins

As described in the previous section, the verum supplement that I used in the pilot study contained solubilized, i. e. micellised fat soluble vitamins. Micellised means that the fat soluble vitamins are already packed into a small micelle of less than 50 nm of diameter thus yielding a water soluble form of these vitamins. This is accomplished by use of polysorbate as emulgator. The micelles are able to pass the unstirred water layer to the intestinal surface and they are small enough to fit between the microvilli, where they can be - according to the manufacturer - directly absorbed by the enterocytes.

Common fat soluble vitamins, as used in the positive control supplement, from natural or synthetic sources require the normal course of fat digestion,

i.e. formation of micelles by bile acids, that allow the various digestive enzymes to act on the lipids (triglycerides, cholesterolesters, phospholipids), cleaving ester bondes. In that process the micelle is decreased in size and turned into a mixed micelle containing both lipids and cleavage products. The mixed micelle diffuses through the unstirred water layer and is absorbed by the enterocytes.

In case of fat maldigestion and reduced intestinal surface, the advantage of micellised fat soluble vitamins over common fat soluble vitamins becomes obvious. Since the micellised vitamins can be absorbed independently from bile acids and digestive enzymes, their absorption can start already in proximal bowel sections and pass off much faster, thus increasing their bioavailability.

Chapter 2

Materials and methods

2.1 Sample collection

2.1.1 Venous blood

2.1.1.1 Plasma

Venous blood was drawn into a 2,7 mL EDTA-Plasma-Monovette and immediately centrifuged in a table centrifuge at 3000 x g for 10 minutes. The centrifuge was not capable of cooling therefore the rotor was cooled with dry ice packages before each run. After separating the plasma from blood cells, plasma was aliquoted and frozen in dry ice. Until analyzes the samples were stored at - 80°C.

2.1.1.2 Serum

Venous blood was drawn into a 2,7 mL Serum-Monovette and kept in the dark at room temperature for 20 minutes for coagulation. Then the blood was centrifuged for 10 minutes at 3000 x g and the serum was aliquoted and frozen on dry ice. Like plasma samples, the serum aliquots were stored at -80°C.

2.1.2 Buccal Mucosa Cells (BMC)

All study participants performed BMC-sample collection on their own after instruction. Cells were collected in a pain-free manner using a soft surgical toothbrush to scrub the inside of every cheek. After rinsing the mouth with tap water, the patients brushed the inside of one cheek twenty times. The participants then rinsed the mouth with 20 mL of NaCl-solution (0,9 %) and the toothbrush was washed in the same solution. After that the whole procedure was repeated with the other cheek. The NaCl-solutions that were used for rinsing and washing were pooled and filled into a 50 mL plastic conical tube that was coated with 200 μL of a BHT-solution (2% in

ethanol). The cells were then subjected to repeated cycles of centrifugation (1000 x g, 5 minutes, 4 °C) and washing with PBS (phosphate buffered saline). After three cycles the cells were sealed by flooding the tube with gaseous nitrogen, frozen in dry ice and stored at -80°C.

2.2 HPLC-Analysis of fat soluble vitamins

Retinol, α-tocopherol and carotenoids were analyzed by use of a HPLC-method described by Jürgen Erhardt et al. [41] with slight modifications.

2.2.1 Sample preparation

Plasma aliquotes were thawed at room temperature in the dark. All following steps were performed on ice. To each sample 100 μL of extracting agent (50% ethanol, 50% butanol + 5 mg BHT/mL extracting agent + 0,008 μmol/L tocol + 0,1 μL β-apocarotenacid-ethylester) was added and the mixture was vortexed for 20 seconds. The vial was centrifuged at 24000 x g for 5 minutes at 4°C and the supernatant was transferred into a brown plastic autosampler vial and sealed with a cap.

BMC-samples were thawed in the dark at room temperature. To break the cells open they were alternately put into fluid nitrogen and into a hand-warm waterbath each step for two minutes. These two steps were repeated for a total of 3 times. After that 200 μL of extracting agent (ethanol + 5 mg/mL BHT + 0,004 μmol/L tocol + 0,05 μmol/L β-apocarotenacid-ethylester) were added and the samples were vortexed. The fat soluble vitamins were extracted by adding 1000 μL hexane. The samples were vortexed for 30 seconds and then placed onto a shaker incubator for 15 minutes in the dark at 4 °C. After centrifugation at 3000 g for 5 minutes at 4 °C the hexane-phase was transferred into a reactant tube and dried under nitrogen. In the next extraction step once again 1000 μL hexane were added and the sample was placed onto the shaker incubator for 5 minutes. The mixture was centrifuged at 13000 x g for 5 minutes and the hexane phase was again dried under nitrogen. The residue was resolved into 100 μL of a ethanol/butanol-solution (50/50, v/v) and transferred into a brown plastic autosampler vial. The remaining cell pellet was dried in an Automatic Environmental Speed Vac System AES 1010 over night and the cell pellet was stored at -80°C for further DNA-analysis.

2.2.2 HPLC-system

The HPLC-system consisted of a ProStar pump Model 210, a Waters 474 fluorescence detector and a Waters 2487 Dual λ absorbance detector. A cooled autosampler (Dynamax Automatic sample injector AI-200) was used. Samples were analyzed with a Spherisorb ODS-2 3 μm column (250 x 4,6 mm).

The column was kept at a constant temperature of 40 °C by a column oven. The eluent consisted of 82% acetonitrile, 15 % dioxan and 3 % methanol that contained 100 mM ammonium acetate and 0,1 % triethylamine. The mobile phase was delivered at a rate of 1,6 mL/ min. α-tocopherol, γ-tocopherol and the internal standard tocol were measured with the fluorescence detector. Retinol was measured at 392 nm, the carotenoides at 450 nm. HPLC-runs were quantified by use of the STAR chromatography workstation Version 5.31 HPLC software.

2.3 Analysis of Ascorbic acid

Ascorbic acid was determined with a colorimetric method described by Ihara et al. [42]. The method was adapted to use in a fully selective analyzer Cobas Mira S available at the institute.

2.3.1 Sample preparation

100 μL serum-aliquots were transferred into a 1,5 mL reaction-tube containing 10 μL of metaphosphoric acid (40%). The sample was vortexed, frozen in dry ice and stored at -80°C. For analyzes aliquoted serum samples were thawed at room temperature in the dark and centrifuged at 4 °C and 3000 x g for 5 minutes. 50 μL of the supernatant were transferred to a Cobas Mira reactant tube and placed into the analyzer.

2.3.2 Analysis

A 15 μL sample was mixed with 10 μL of potassium phosphate buffer. The ascorbic acid was oxidized with 200 μL TEMPO (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy free radical) in potassium phosphate buffer (1 mg TEMPO/5 mL buffer) to dehydroascorbic acid. 85 μL OPDA (o-phenylendiamine) in potassium buffer (1 mg OPDA/2 mL buffer) were added forming a colored product. This products absorption was measured at 340 nm wavelength. The magnitude of absorption was proportional to the concentration of ascorbic acid. To determine the concentration of a sample, its absorption was compared to a standard of 56,60 μmol/L.

2.4 Analysis of vitamin D

Vitamin D-metabolite 1,25$(OH)_2D_3$ was analyzed from 500 μL serum using an EIA-kit (1,25 Dihydroxy Vitamin D EIA) according to the manufacturers instructions.

2.5 Analysis of DNA-content of BMC

The DNA-content of buccal mucosa cells was assessed using the method of Natarajan et al. [43]. Dried cell pellets were incubated overnight with 200 μL of 0,016% acetaldehyde in PBS diluted 1:5 in 85% perchloric acid and 320 μL of a 4% solution of diphenylamine in acetic acid. 100 μL of the mixture were transferred to a 96 well plate and measured at 590 nm, correction 750 nm. The DNA-content was quantified with a 10 point standard curve made from fish sperm DNA (2 μg - 40 μg DNA).

2.6 Analysis of protein carbonyls

The content of protein carbonyls in plasma samples was assessed using the method described of Buss et al. [44]

2.6.1 Assessment of protein content

Plasma-aliquots of 20 μL were thawed in the dark an then kept on ice. First, the total protein content was assessed in order to dilute the samples to 4 mg protein per mL. Protein assessment was performed using the Bio-Rad Protein Assay Dye Reagent Concentrate that was diluted 1:5. 4 μL of an 1:100 dilution of the samples were transferred to a 96 well plate. Each sample was mixed with 200 μL of the diluted protein reagent and the absorption was measured at 590 nm. The protein content of samples was quantified using a 8-point standard curve made from BSA in PBS (range: 0 mg/mL - 1,4 mg/mL).

2.6.2 Assessment of protein carbonyl content

Samples were diluted to the desired 4 mg Protein/mL PBS and incubated for 45 minutes in the dark with 45 μL DNP-solution, that consisted of 2 mg DNP (dinitrophenyhydrozine) per 1 mL DNP-buffer (6 M guanidiniumhydrochlorid and 0,5 M KH_2PO_4, pH 2,5). 15 μL of the incubate were mixed with 1000 μL of coating buffer (10 mM sodiumphosphate buffer + 140 mM NaCl, pH 7,0) and 200 μL of this mixture were transferred to a 96-well-plate (Nunc Immuno Plate Maxisorb). The plate was incubated overnight at 4°C.

After incubation the coating-buffer was decanted and 250 μL per well 0,1 % reduced BSA (diluted in PBS) as blocking agent was added. The plate was incubated in the dark at room temperature. After 90 minutes incubation the blocking agent was decanted and 200 μL of an anti-DNP-antibody (1:1000 dilution in 0,1 % BSA + 0,1 % Tween 20) was pipetted into each well. The plate was incubated at 37 °C for 60 minutes. Before the next step the plate was washed 3 times with PBS. After that 200 μL of an anti-IgG-POD-marked antibody (1:10000 dilution in 0,1 % BSA + 0,1

% Tween 20) was added to each well and the plate was incubated for 60 minutes in the dark. Again the plate was washed 3 times with PBS and finally 200 μL per well developing agent was added. The developing agent consisted of 50 mM Na_2HPO_4 + 24 mM citric acid with 0,6 mg/ mL o-phenylendiamine and 0,04 % H_2O_2. The plate was developed at 37 °C for 15 minutes. The development was stopped by adding 100 μL 2,5 M H_2SO_4. The absorbance of the plate was measured at 490 nm, corrected 750 nm. For quantification a 8-point standard curve made from reduced and oxidized BSA was used ranging from 0,637 to 2,0 nmol proteincarbonyl/mg protein.

2.7 Analysis of total cholesterol, LDL and triglycerides

Total cholesterol, LDL (low density lipoproteins) and triglycerides were analyzed on Cobas Mira S using the following kits: ABX Pentra Cholesterol CP for cholesterol, ABX Pentra LDL Direct CP for LDL and ABX Pentra Triglycerides CP for determination of triglycerides. All determinations were performed according to the manufacturers instructions. For calibration ABX Pentra Multical (cholesterol and triglycerides) as well as ABX Pentra LDL Cal (for LDL) was used.

2.8 Analysis of CRP

C reactive protein (CRP) was analyzed on Cobas Mira S with ABX Pentra CRP CP according to the manufacturers instructions. ABX Pentra CRP Cal was used to calibrate the analyzer. CRP-concentrations above 5 mg/L point to an acute infection.

2.9 Statistical analysis

All statistical tests were performed using SPSS 11.0 for Windows. In order to achieve sufficient number of samples for statistical analysis, plasma-concentrations of fat soluble vitamins below the detections limit of the HPLC systems were assigned half the value of the detection limit. This was only necessary for plasma-β-carotene samples of day 1. However, this method was not applicable for BMC-vitamin concentrations because the detection limit refers to the vitamin concentration in the extracts made from each BMC aliquot. Assigning the same value to those extracts would introduce a large error as the BMC aliquots did not all have the same number of cells [45].

All variables were tested for normal distribution by use of the Shapiro-Wilk test. Since non of the variables were normally distributed only non-

parametric tests were applied. To describe the differences between the different study groups at basic assessment and after intervention the Mann-Whitney test was used. The Wilcoxon signed rank test was used to evaluate the difference of the variables in each group after intervention. A p-value < 0.05 was considered significant. Results are expressed as median with minimum and maximum in brackets unless stated otherwise.

2.10 Ethical approval

The study protocol was approved by the ethical committee of the Medical faculty of the University of Tübingen and written informed consent was obtained from each subject.

Chapter 3

Results

3.1 Basic assessment

3.1.1 Characteristics of study subjects

Table 3.1 shows the general and clinical characteristics of the study participants. Results are expressed as median with minimum and maximum in parentheses unless declared otherwise. Altogether, 18 patients were included into the study and were therefore eligible for basic assessment.

The remaining bowel length was known in only 10 patients. For the other 8 patients, data were available only on the length of the resected bowel segment (e.g. 30 cm of terminal ileum) or the type of resection (e.g. ileocecal resection, ileotransversostomy) which provided limited information on the remaining bowel length. Since the size of the human adult small bowel is approximately 4 to 5 m, a resection of about 30 cm results not in a bowel length less than 200 cm. These patients were therefore placed into the group of 200 cm - max remaining bowel length. As described in section 1.1.1, it has been agreed to define SBS by the remaining small bowel length being less than 200 cm. This case is given in 5 patients only. These patients will be investigated separately in section 3.2.5, since they fulfill the SBS-definition.

Table 3.1: General and clinical characteristics of study subjects at baseline

Total n	18
Female/male	9/9
Age (years)	54.5 (38 - 69)
BMI (kg/m^2)	23.66 (19.47 - 36.85)
Resection due to Morbus Crohn	17/18 (94.4 %)[1]
Resection due to cancer	1/18 (5.6 %)[1]
Small bowel < 200 cm	5/18 (27.7 %)[1]
Small bowel > 200 cm	13/18 (72.2 %)[1]
Remaining bowel length (cm)	190 (100 - 400) (n=10)
Resection of ileum	5/18 (27.7 %)[1]
Resection of ileocecal valve	16/18 (88.9 %)[1]
Resection of cecum	15/18 (83.3 %)[1]
Resection of colon	2/18 (11.1 %)[1]
Prior intake of ADEK-Falk®	15/18 (27.7 %)[1]
Cholesterol (mmol/L)	3.92 (2.53 - 5.12)
LDL (mmol/L)	1.93 (1.05 - 3.04)
TG (mmol/L)	1.7 (0.46 - 3.48)
CRP positive	2/18 (11.1 %)[1]
Neutrophils (%)	65.5 (57.3 -73.8)
Leukocytes (1/μmol)	6355 (2880 - 5440)

[1] n of total (%)

3.1.2 Baseline status of fat soluble vitamins and ascorbic acid

Table 3.2 and **table 3.3** show an overview of concentrations of vitamins in plasma, serum and BMC as well as values for coagulation parameters (Quick, INR), that were used to assess the vitamin K status. The patients were grouped depending on their remaining short bowel length (group 1 = remaining small bowel length < 200 cm; group 2 = remaining small bowel length 200 cm - maximum).

Table 3.2: Vitamins status at basic assessment

	Remaining short bowel length		
	Group 1 (0-199 cm)	**Group 2 (200 cm - max)**	**p**[1]
Plasma			
α-tocopherol (μmol/L)	17.28 (11.95 - 27.21)	32.28 (18.11 - 66.91)	0.016
Retinol[2] (μmol/L)	1.65 (1.08 - 2.06) n = 5	2.35 (1.4 - 4.04) n = 11	0.02
β-carotene (μmol/L)	0.113 (0.03 - 0.18)	0.13 (0.04 - 0.59)	NS
Serum			
Ascorbic acid (μmol/L)	45.73 (21.75 - 80.50)	64.06 (41.16 - 111.75)	NS
1,25$(OH)_2D_3$ (pmol/L)	82.83 (49.65 - 105.49)	72.23 (34.60 - 112.64)	NS
BMC			
α-tocopherol (pmol/μg DNA)	33.83 (27.48 - 102.95)	51.21 (27.26 - 125.56)	NS
Retinol (pmol/μg DNA)	0.15 (0.11 - 0.85) n = 4	0.15 (0.07 - 0.19) n = 7	NS
β-carotene (pmol/μg DNA)	0.13 (0.11 - 0.17) n = 3	0.17 (0.11 - 1.3) n = 9	NS

[1] p-value (Mann-Whitney-test); NS = not significant
[2] only CRP-negative subjects

Table 3.3: Coagulation parameters at basic assessment

	Remaining short bowel length		
	Group 1 (0 - 199 cm)	**Group 2 (200 cm - max)**	**p**[1]
Quick (%)	98 (87 - 120)	118 (87 - 120)	NS
INR	1.0 (0.9 - 1.0)	0.9 (0.8 - 1.0)	NS

[1] p-value (Mann-Whitney-test); NS = not significant

None of the 18 enrolled patients exhibited abnormal coagulation parameters. Quick and INR were in each patient within normal range (refer to section B.6 in the appendix) and therefore no sign for a vitamin K deficiency was apparent.

3.1.2.1 Vitamin E

Plasma **Figure 3.1** shows the α-tocopherol concentrations in plasma.

Patients of group 1 (small bowel length < 200 cm) had significantly lower vitamin E concentrations in plasma compared to patients of group 2 (p=0.016). Two patients of group 1 had marginal plasma vitamin E concentrations, the other three in that group were within normal range (refer to **table B.1** in the appendix). In group 2 all patients had either normal or even preventive plasma concentrations.

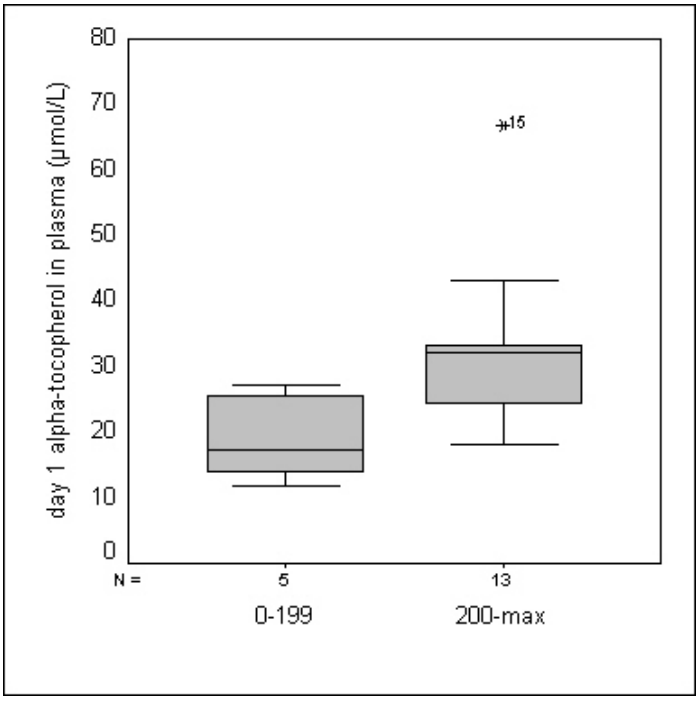

Figure 3.1: Basic assessment of α-tocopherol in plasma. The figure shows data as boxplots of group 1 and 2 with median, interquartile range, minimum and maximum concentrations of α-tocopherol. *15 is an outlier.

BMC **Figure 3.2** displays the tissue Vitamin E concentrations, which were generally low in both groups when compared to a healthy population (refer to **table B.2** in the appendix). However, in group 1 the concentration was even lower than in group 2, even when this difference reached not significance level due to wide scattering of values in group 2.

3.1.2.2 Vitamin A

Plasma **Figure 3.3** shows the retinol plasma concentrations of CRP-negative patients. As pointed out in **table 3.2** there was a significant difference between concentrations of group 1 and 2 (p=0.02). However, none

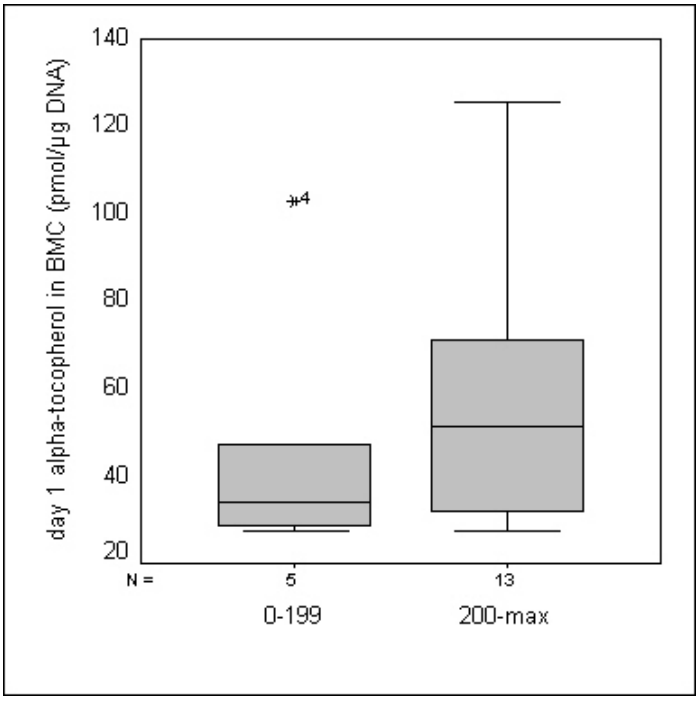

Figure 3.2: Basic assessment of α-tocopherol in BMC. The figure shows data as boxplots of group 1 and 2 with median, interquartile range, minimum and maximum concentrations of α-tocopherol. *4 is an outlier.

of the patients exhibited a biochemical retinol deficiency.

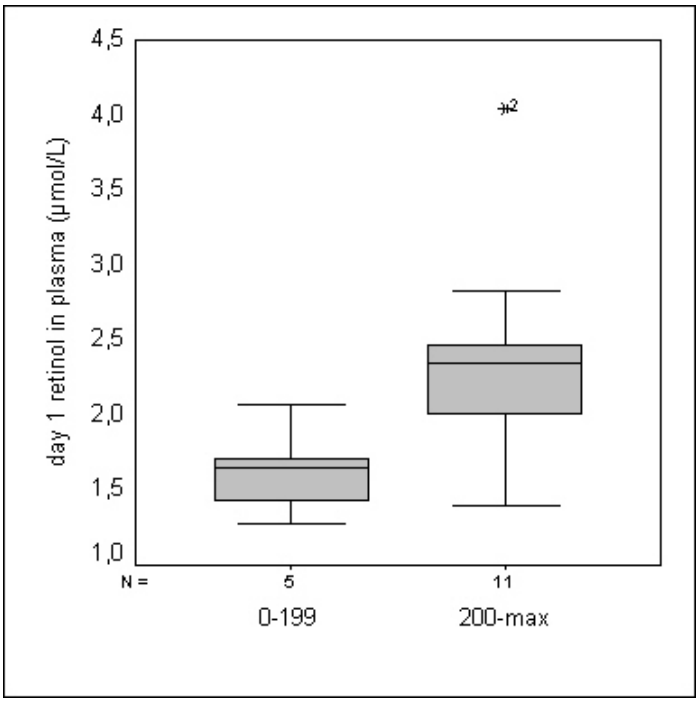

Figure 3.3: Basic assessment of retinol in plasma. The figure shows data as boxplots of group 1 and 2 with median, interquartile range, minimum and maximum concentrations of retinol. *2 is an outlier.

BMC Retinol concentrations in tissue samples could not be assessed in 7 patients, since the concentrations were below the detection limit of 0.08 μmol/L. The data for the other 11 patients are displayed in **figure 3.4**.

3.1.2.3 β-carotene

Plasma **Figure 3.5** shows the β-carotene concentrations in plasma. The median value is lower in group 1 (less than 200 cm remaining small bowel), but the difference to group 2 is not significant. Overall, 14 of 18 patients showed plasma-β-carotene concentrations below 0,3 μmol/L which is insufficient (refer to **table B.4** in the appendix) and indicative of fat malabsorption.

BMC **Figure 3.6** displays the tissue β-carotene concentrations of 12 patients. 6 patients had β-carotene concentrations below the detection limit and where therefore omitted from analysis. The median concentrations of both groups differed slightly, but not significantly.

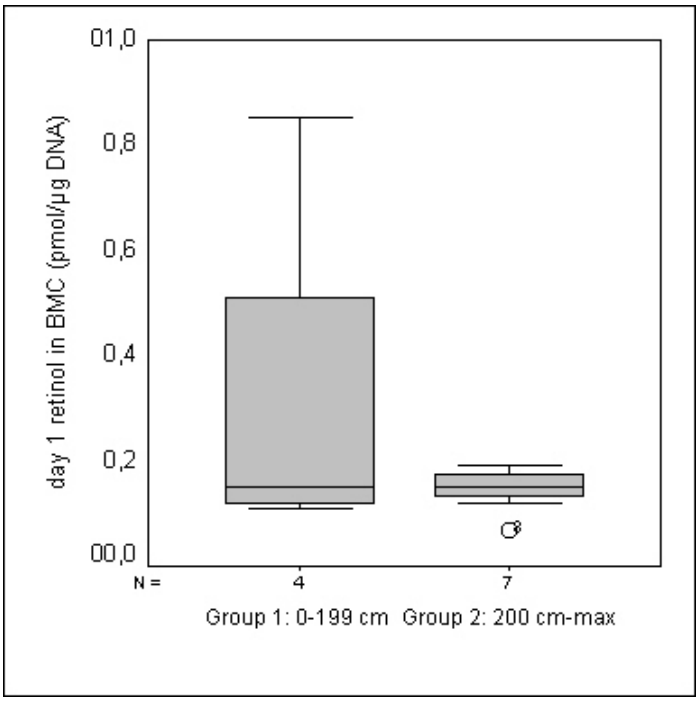

Figure 3.4: Basic assessment of retinol in BMC. The figure shows data as boxplots of group 1 and 2 with median, interquartile range, minimum and maximum concentrations of retinol. ∘8 is an outlier.

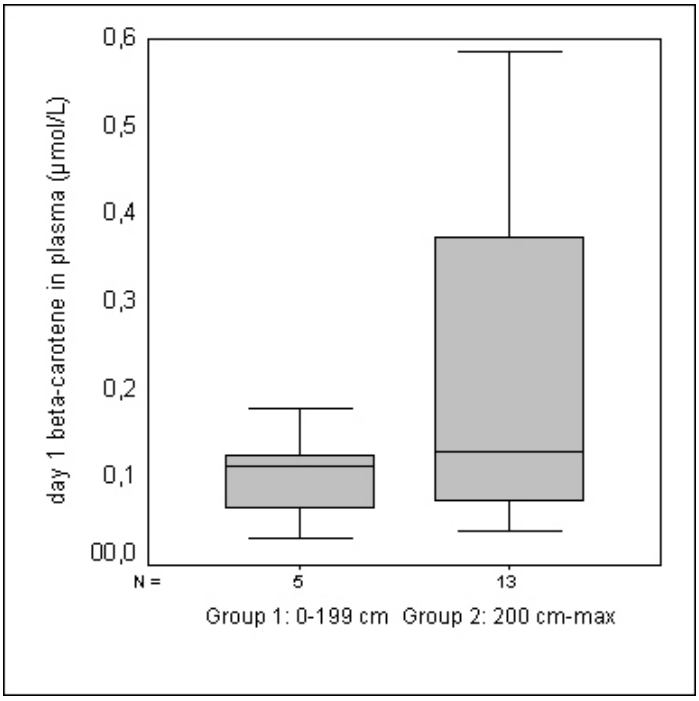

Figure 3.5: Basic assessment of β-carotene in plasma. The figure shows data as boxplots of group 1 and 2 with median, interquartile range, minimum and maximum concentrations of β-carotene.

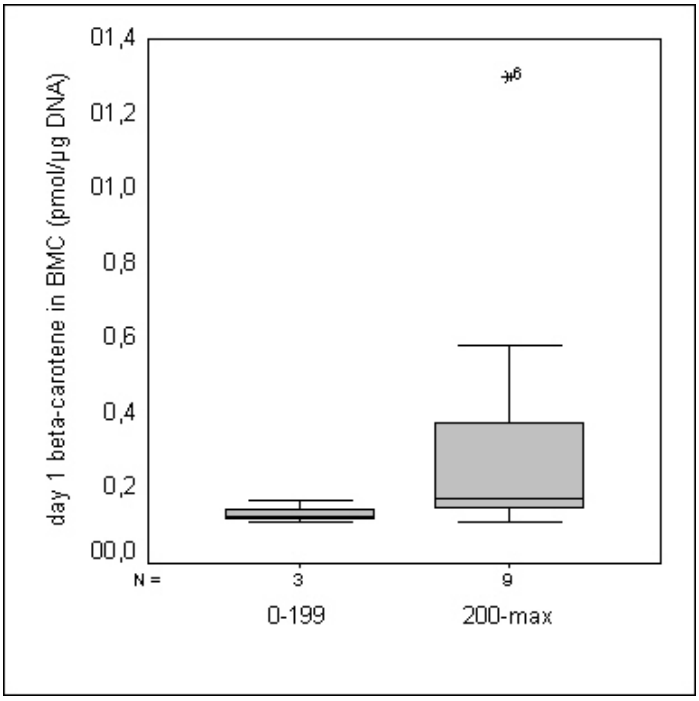

Figure 3.6: Basic assessment of β-carotene in BMC. The figure shows data as boxplots of group 1 and 2 with median, interquartile range, minimum and maximum concentrations of β-carotene. *6 is an outlier.

3.1.2.4 Ascorbic acid

All patients except one showed sufficient serum-ascorbic acid concentrations at baseline according to data from Biesalski et al [16] (refer to **table B.6** in the appendix). One patient in group 1 had a marginal serum concentration of 21.75 μmol/L. There was a difference between the medians of the two bowel-length groups, but it was not significant.

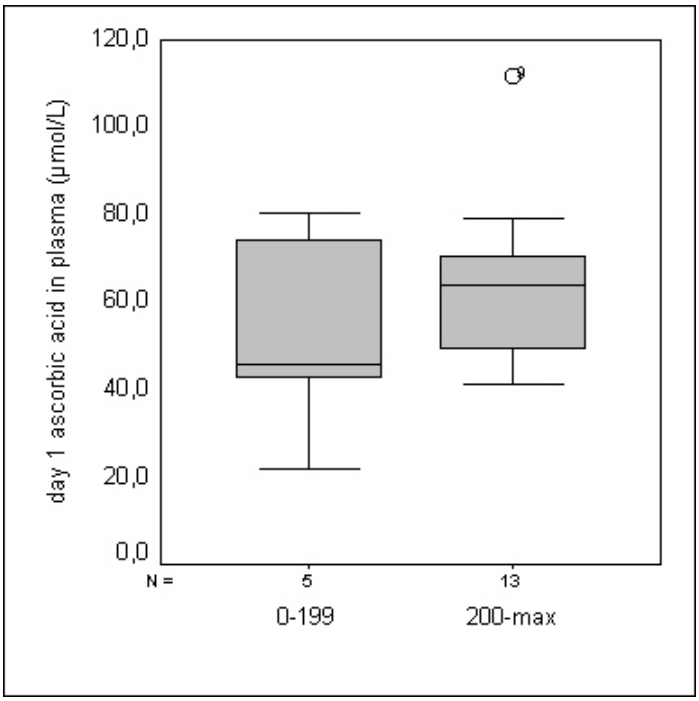

Figure 3.7: Basic assessment of ascorbic acid in serum. The Figure shows data as boxplots of group 1 and 2 with median, interquartile range, minimum and maximum concentrations of ascorbic acid. ∘9 is an outlier.

3.1.2.5 1,25(OH)$_2$D$_3$

Figure 3.8 displays the results of 1,25(OH)$_2$D$_3$ analysis in serum at basic assessment. There was no significant difference between the two bowel length groups. The normal range for this vitamin D metabolite is 80 - 180 pmol/L [46]. Therefore 11 patients were below the lower limit and 7 patients were within normal range but close to the lower limit.

3.1.3 Baseline status of oxidative stress

The concentrations of protein carbonyls as marker for oxidative stress are shown in **table 3.4**.

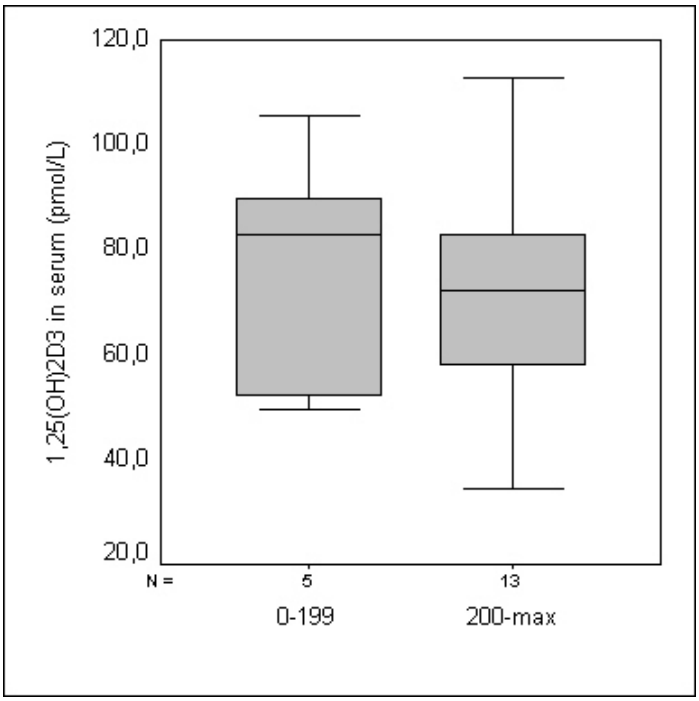

Figure 3.8: Basic assessment of $1{,}25(OH)_2D_3$ in serum. The figure shows data as boxplots of group 1 and 2 with median, interquartile range, minimum and maximum concentrations of $1{,}25(OH)_2D_3$.

Table 3.4: Oxidative stress at basic assessment

	Remaining short bowel length		
	Group 1 (0 - 199 cm)	**Group 2 (200 cm - max)**	**p**[1]
Protein carbonyls (nmol/mg protein)	0.457 (0.422 - 0.464)	0.445 (0.431 - 0.466)	NS

[1] p-value (Mann-Whitney-test); NS = not significant

The concentration of protein carbonyls was not elevated in any of the groups when compared to a healthy adult population (0.536 (0.513 - 0.552 nmol/mg protein [45]). There was a difference between the medians of the two groups but it was not significant.

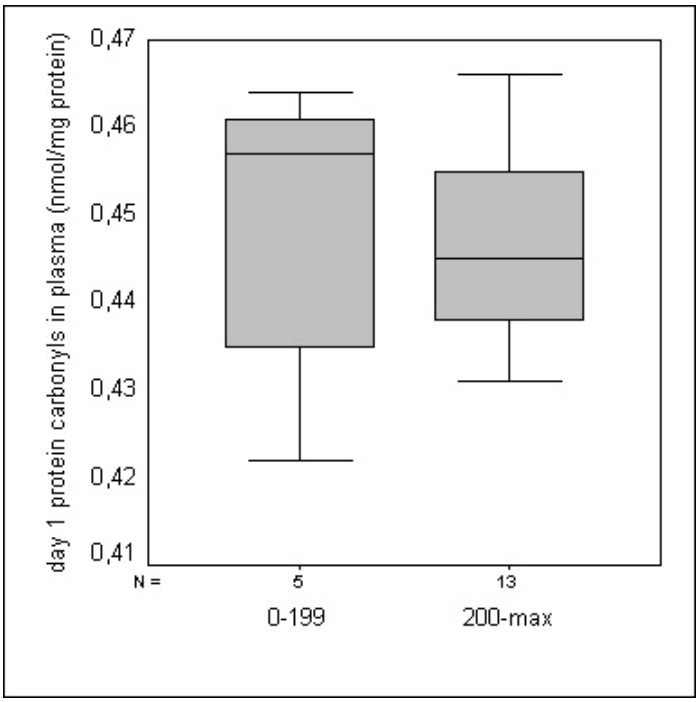

Figure 3.9: Basic assessment of Protein carbonyl content in plasma. The figure shows data as boxplots of group 1 and 2 with median, interquartile range, minimum and maximum concentrations of protein carbonyls.

3.2 Intervention trial

3.2.1 Subjects

From 18 included study participants 16 remained in the study for the intervention trial. The drop-out patients belonged one each to the verum and positive control group as well as to group 1 (remaining small bowel length less than 200 cm) and group 2 (remaining small bowel length more than 200 cm) respectively (refer to **table 3.5**).

Table 3.5: Distribution of subjects in the intervention trial

	Remaining bowel length		
	Group 1: < 200 cm	Group 2: 200 cm - max.	Total
Verum	2	6	8
Positive control	2	6	8

3.2.2 Changes in plasma, serum and tissue vitamin concentrations after intervention

The intervention with the study vitamins A, E, C and β-carotene was effective in each of the two intervention groups, except for retinol in the positive control group. There were statistically significant increases in plasma and tissue concentrations of α-tocopherol and β-carotene. Retinol concentrations in plasma increased significantly in the verum group but decreased in the positive control group. In tissue the retinol concentration rose slightly but insignificantly in the verum group and decreased in the positive control group. There was also a significant increase of ascorbic acid in the verum group. The increment for each vitamin was calculated by subtracting the concentration of that vitamin measured at day 1 from the concentration measured at day 28. All data on increase are presented as median with minimum and maximum in parentheses in **table 3.6** and **table 3.7**.

Table 3.6: Increase of study vitamins in plasma.

Vitamin	**d_1**	**d_{28}**	**Increase in plasma (d_{28}-d_1)**	**p[1] (d_1 vs. d_{28})**
Verum				
α-tocopherol (μmol/L)	29.98 (17.28-66.91)	59.69 (37.96-92.28)	27.19 (20.67-36.24)	0.012
retinol[2] (μmol/L)	2.02 (1.28-2.52)[3]	2.43 (1.37-3.24)[3]	0.25 (0.1-0.86)	0.018
β-carotene (μmol/L)	0.15 (0.04-0.59)	0.39 (0.11-1.35)	0.22 (0.03-0.76)	0.012
ascorbic acid (μmol/L)	63.83 (46.85-80.50)	77.95 (63.07-107.25)	16.63 (1.66-41.10)	0.012
Positive control				
α-tocopherol (μmol/L)	27.13 (11.95-43.15)	46.54 (22.64-58.94)	17.51 (10.69-23.98)	0.012
retinol[2] (μmol/L)	2.26 (1.7-4.04)[3]	2.16 (1.69-4.75)[3]	-0.14 (-0.19-0.83)	NS
β-carotene (μmol/L)	0.10 (0.04-0.55)	0.23 (0.1-0.68)	0.13 (0.03-0.23)	0.012
ascorbic acid (μmol/L)	63.26 (41.16-111.75)	68.94 (57.07-104.40)	16.65 (-12.38-25.71)	0.05

[1] p-value (Wilcoxon signed rank test); NS = not significant
[2] only CRP-negative subjects
[3] n=7

Table 3.7: Increase of study vitamins in tissue.

Vitamin	**d_1**	**d_{28}**	**Increase in tissue (d_{28}-d_1)**	**p[1] (d_1 vs. d_{28})**
Verum				
α-tocopherol (μmol/L)	37.79 (27.48-125.56)	81.44 (30.97-190.82)	41.27 (0.03-148.5)	0.012
retinol (μmol/L)	0.11 (0.07-0.13)[2]	0.103 (0.1-0.11)[2]	0.005 (-0.03-0.03)	NS
β-carotene (μmol/L)	0.17 (0.11-1.3)[5]	1.18 (0.23-5.85)[5]	0.94 (0.06-4.55)	0.028
Positive control				
α-tocopherol (μmol/L)	40.66 (27.26-71.04)	78.84 (35.70-93.16)	27.45 (1.46-53.16)	0.012
retinol (μmol/L)	0.17 (0.15-0.18)[3]	0.103 (0.08-0.13)[3]	-0.07 (-0.09- -0.02)	NS
β-carotene (μmol/L)	0.13 (0.11-0.37)[4]	0.72 (0.48-1.77)[4]	0.57 (0.37-1.39)	0.043

[1] p-value (Wilcoxon signed rank test); NS = not significant
[2] n=3
[3] n=4
[4] n=5
[5] n=6

3.2.3 Comparison of the supplementation effect in the two intervention groups

3.2.3.1 α-tocopherol

Plasma In **figure 3.10** the results for the intervention effects in verum and positive control group are displayed. There was a remarkable difference of increase between the two intervention groups: the increase in the verum group was about 27.19 μmol/L vs. the increase in the positive control group was about 17.51 μmol/L (refer to **table 3.6**). This difference of increase in the two groups reached significance with a p-value of 0.02. In **figure 3.11** the individual plasma increases for each patient are presented. All patients of the respective group had similar concentration increases.

Tissue In **figure 3.12** the α-tocopherol concentrations for the two intervention groups are displayed. As in plasma α-tocopherol concentrations, the BMC-α-tocopherol-concentration increased in the verum group more than in the positive control-group (41.27 μmol/L vs 27.45 μmol/L). However, due to a high degree of scattering in the verum group after intervention this difference in increase was not significant (refer to **figure 3.13**).

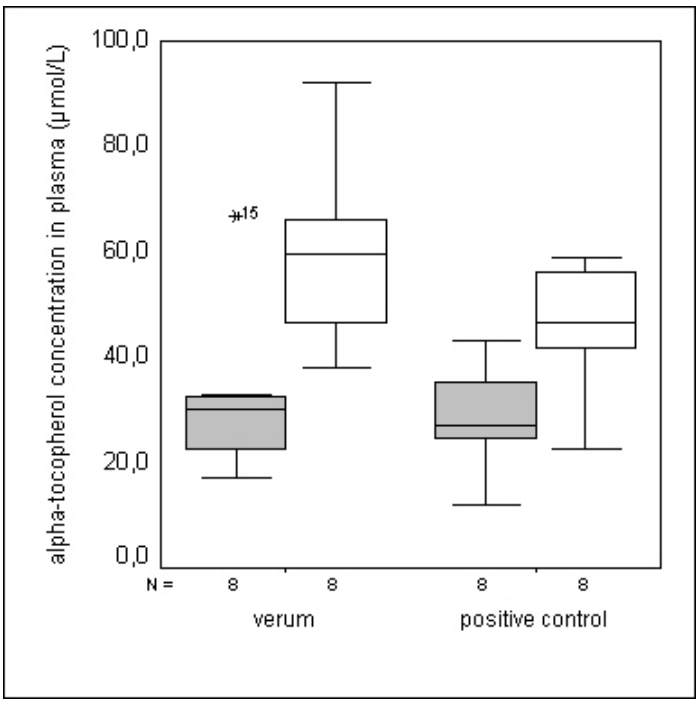

Figure 3.10: Effect of supplementation with vitamin E on α-tocopherol concentrations in plasma. The figure shows data as boxplots of verum and positive control groups with median, interquartile range, minimum and maximum concentrations of α-tocopherol. Grey boxes represent data of basic assessment (day 1), white boxes show data assessed on day 28. *15 is an outlier.

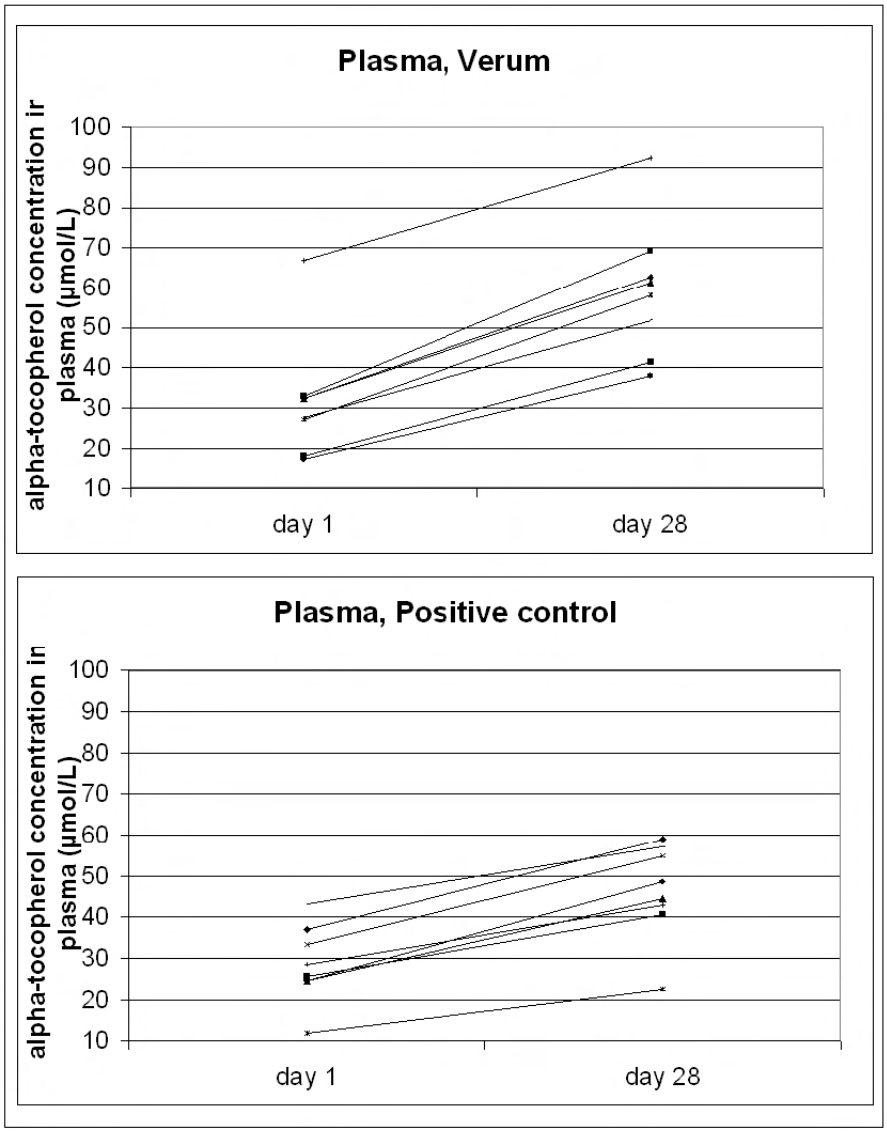

Figure 3.11: Individual increases of α-tocopherol in plasma for patients of verum and positive control group. Each data set (day 1 and day 28) represents one patient.

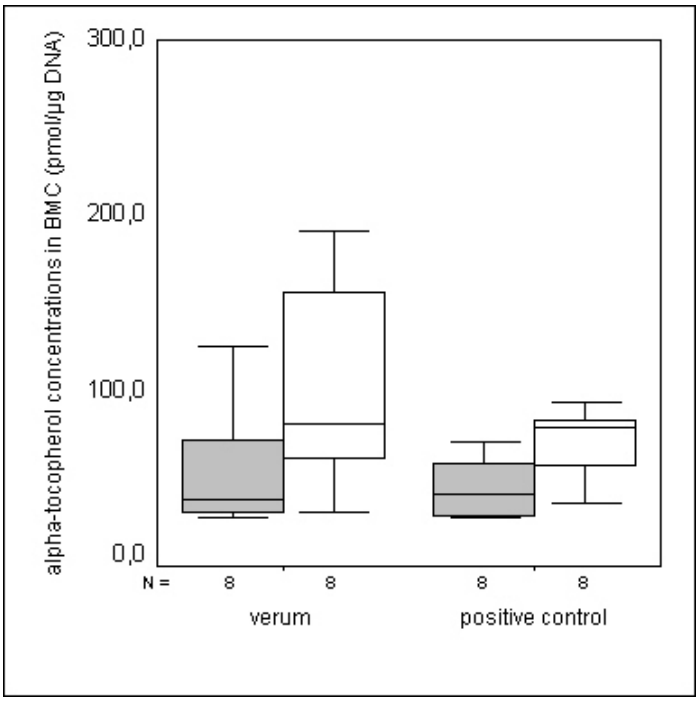

Figure 3.12: Effect of supplementation with vitamin E on α-tocopherol concentrations in tissue. The figure shows data as boxplots of verum and positive control groups with median, interquartile range, minimum and maximum concentrations of α-tocopherol. Grey boxes represent data of basic assessment (day 1), white boxes show data assessed on day 28.

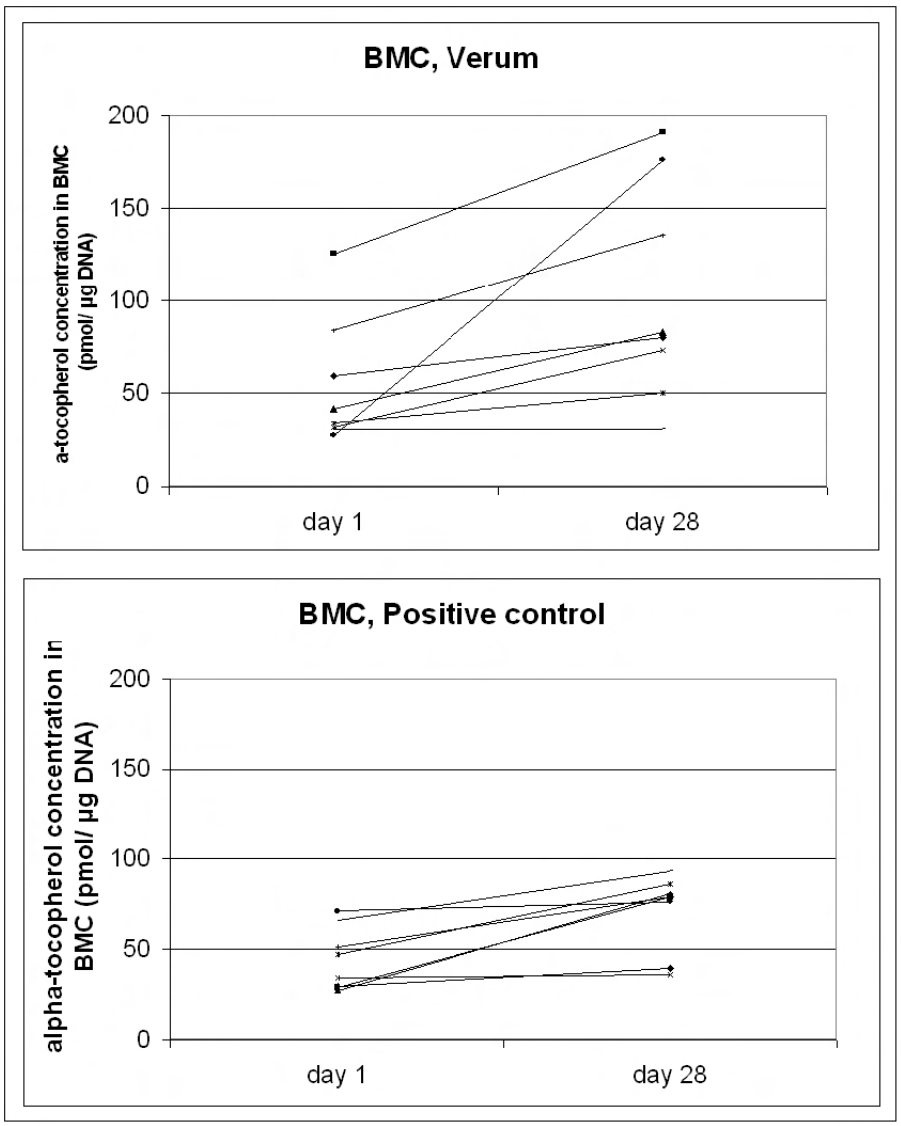

Figure 3.13: Individual increases of α-tocopherol in BMC for patients of verum and positive control group. Each data set (day 1 and day 28) represents one patient.

3.2.3.2 Retinol

Plasma The results of supplementation with vitamin A on plasma concentrations is shown in **figure 3.14**. Since inflammatory processes lead to increased retinol plasma concentrations, CRP-positive plasma samples were omitted from statistical analysis and display. **Figure 3.14** shows an increase of retinol in the verum group and a slight decrease in the positive control group. Despite this contrary effect of vitamin A supplementation in the two groups, the difference between the intervention groups is not significant (refer to **table A.2** in the appendix). The individual plasma concentration changes are displayed in **figure 3.15**.

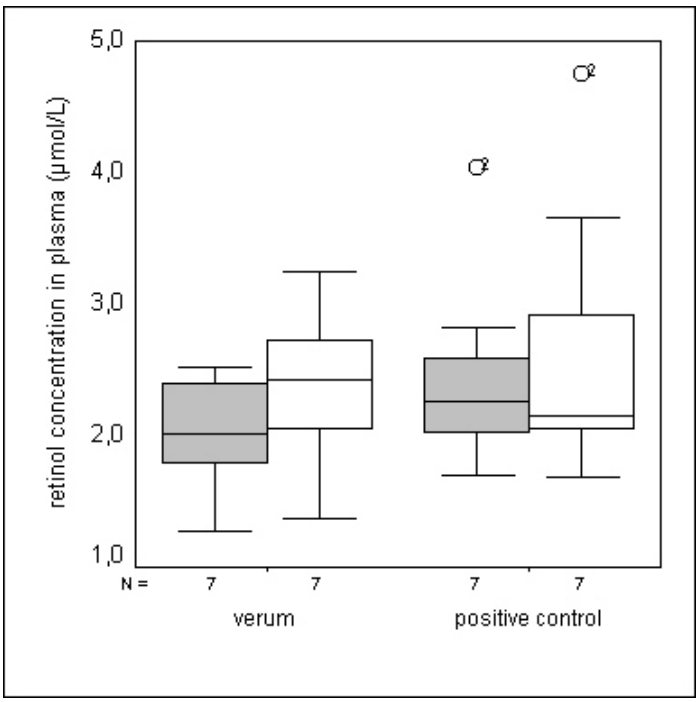

Figure 3.14: Effect of supplementation with vitamin A on retinol concentrations in plasma. The figure shows data as boxplots of verum and positive control groups with median, interquartile range, minimum and maximum concentrations of retinol. Grey boxes represent data of basic assessment (day 1), white boxes show data assessed on day 28. ∘2 is an outlier.

Tissue The supplementation effect of vitamin A in tissue is quite surprising (refer to **figure 3.16**). From three analyzable samples of the verum group, an increase of retinol was detectable in only 1 patient. In the other 2 patients of that group a decrease was observed, as well as in all 4 analyzable

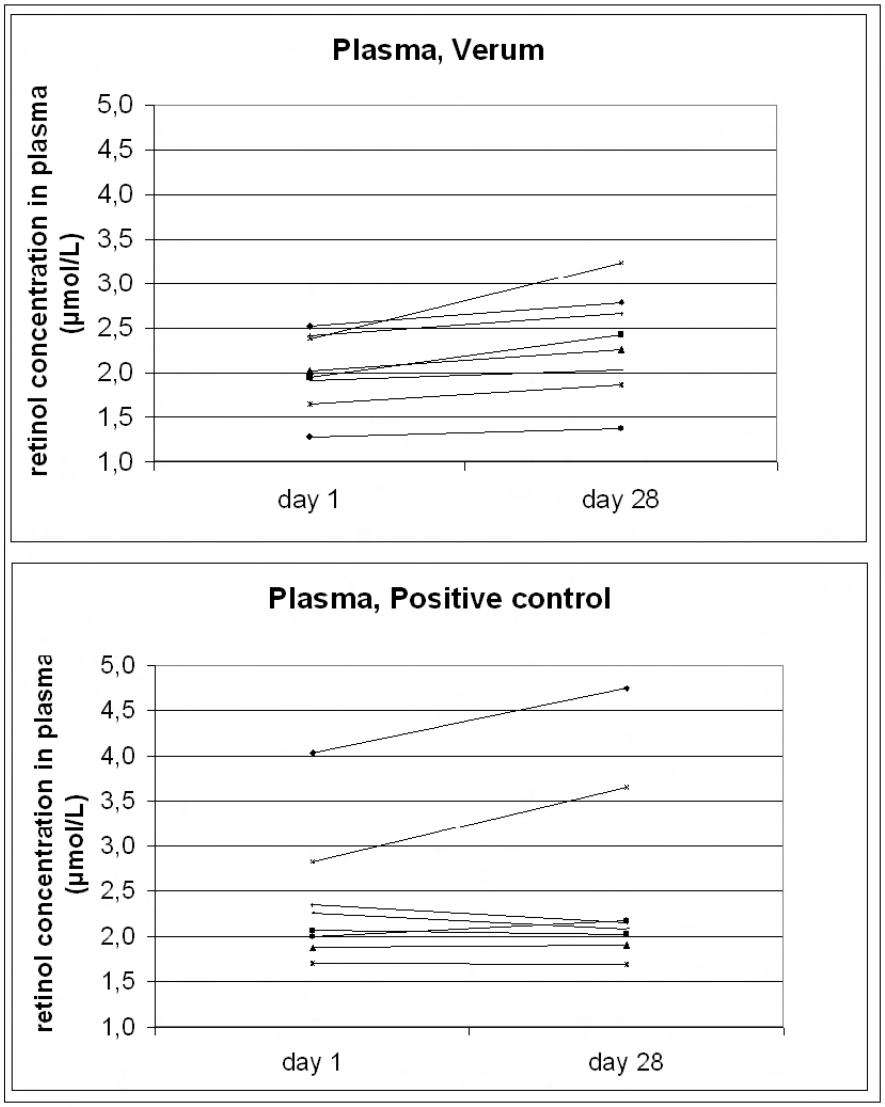

Figure 3.15: Individual increases of retinol in plasma for patients of verum and positive control group. Each data pair (day 1 and day 28) represents one patient.

samples from the positive control group. However, the picture is not complete, since all the samples that lay below the detection limit on either day 1 or day 28 were excluded from statistical analysis. From 16 patients that completed the study, 8 patients (4 verum, 4 positive control) showed BMC retinol concentrations below the detection limit (0.07 pmol/μg DNA) at basic assessment and only 5 patients (3 verum, 2 positive control) after the intervention trial. On the other hand 7 patients (3 verum, 4 positive control) had decreased BMC retinol concentrations after intervention. This shows, that the supplementation led to improved retinol status in BMC in some of the patients but in others decreasing BMC concentrations were observed. To provide a complete picture on individual increases and decreases, BMC retinol concentrations below the detection limit were assigned to 0 pmol/μg DNA. These data are displayed in **figure 3.17**.

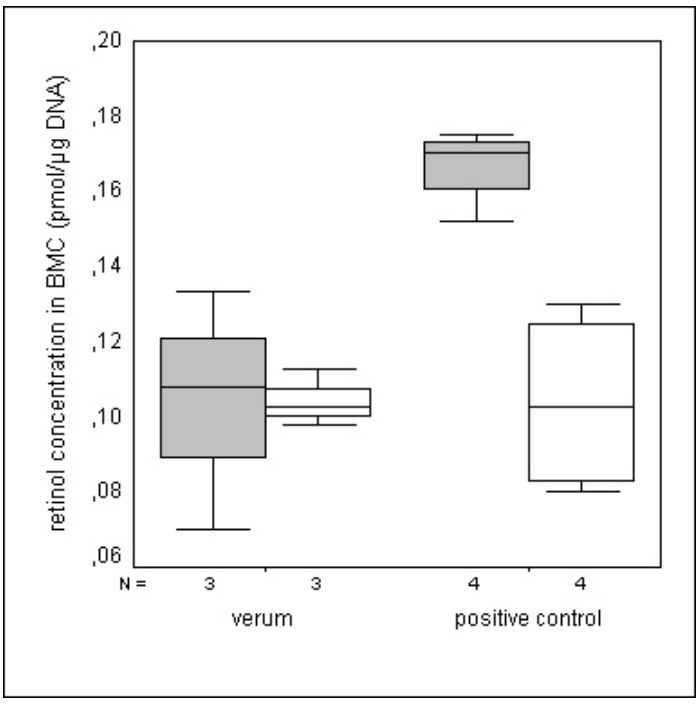

Figure 3.16: Effect of supplementation with vitamin A on retinol concentrations in BMC. The figure shows data as boxplots of verum and positive control groups with median, interquartile range, minimum and maximum concentrations of retinol. Grey boxes represent data of basic assessment (day 1), white boxes show data assessed on day 28.

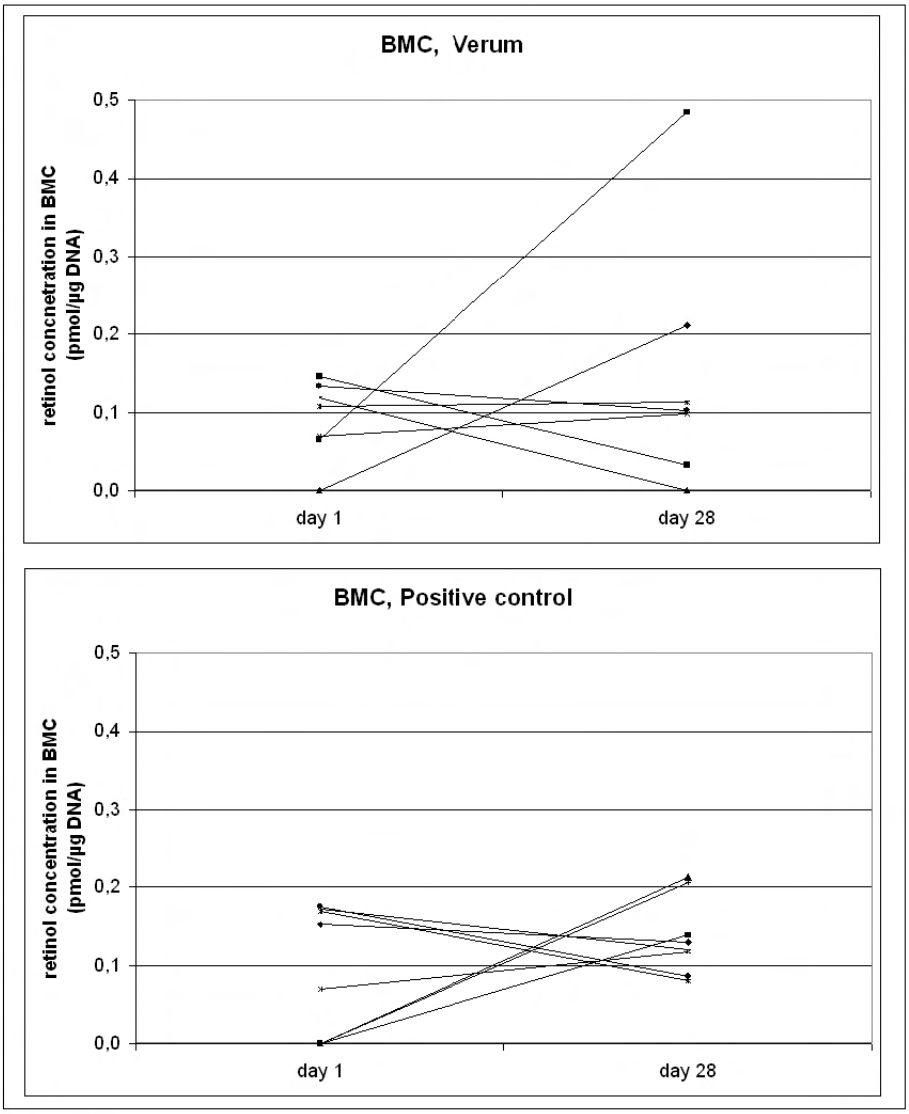

Figure 3.17: Individual increases and decreases of retinol in BMC for patients of verum and positive control group. Each data set (day 1 and day 28) represents one patient. Values below the detection limit of the HPLC-system were assigned to 0 pmol/μg DNA.

3.2.3.3 β-carotene

Plasma **Figure 3.18** displays the increase of β-carotene in plasma, which is higher in the verum than in the positive control group (0.22 μmol/L vs. 0.13 μmol/L). However, this difference in increase is not significant due to a high degree of scattering in the verum group (refer to **figure 3.19**).

It is very important to mention, that 14 out of 18 patients (77.8 %; 7 verum, 7 positive control) exhibited insufficient β-carotene plasma concentrations (< 0.3 μmol/L) at baseline. This changed during the intervention trial to only 9 out of 16 patients (56.25 %; 3 verum, 6 positive control) being insufficiently supplied.

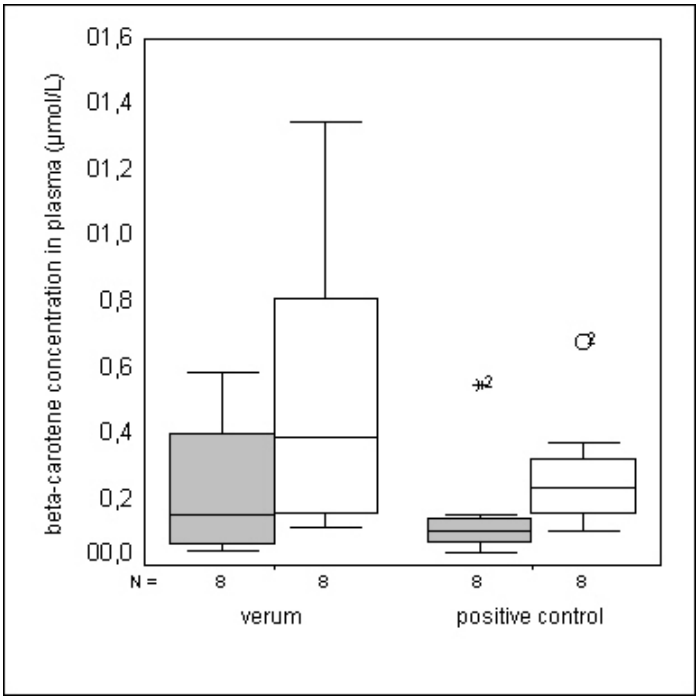

Figure 3.18: Effect of supplementation with β-carotene on β-carotene concentrations in plasma. The figure shows data as boxplots of verum and positive control groups with median, interquartile range, minimum and maximum concentrations of β-carotene. Grey boxes represent data of basic assessment (day 1), white boxes show data assessed on day 28. *2 and ∘2 are outliers.

Tissue In tissue (**figure 3.20**) the intervention effect with β-carotene was different between the groups (0.94 μmol/L in verum group vs. 0.57 μmol/L in positive control group), but did not reach statistical significance. The individual BMC retinol concentration changes are presented in **figure 3.21**.

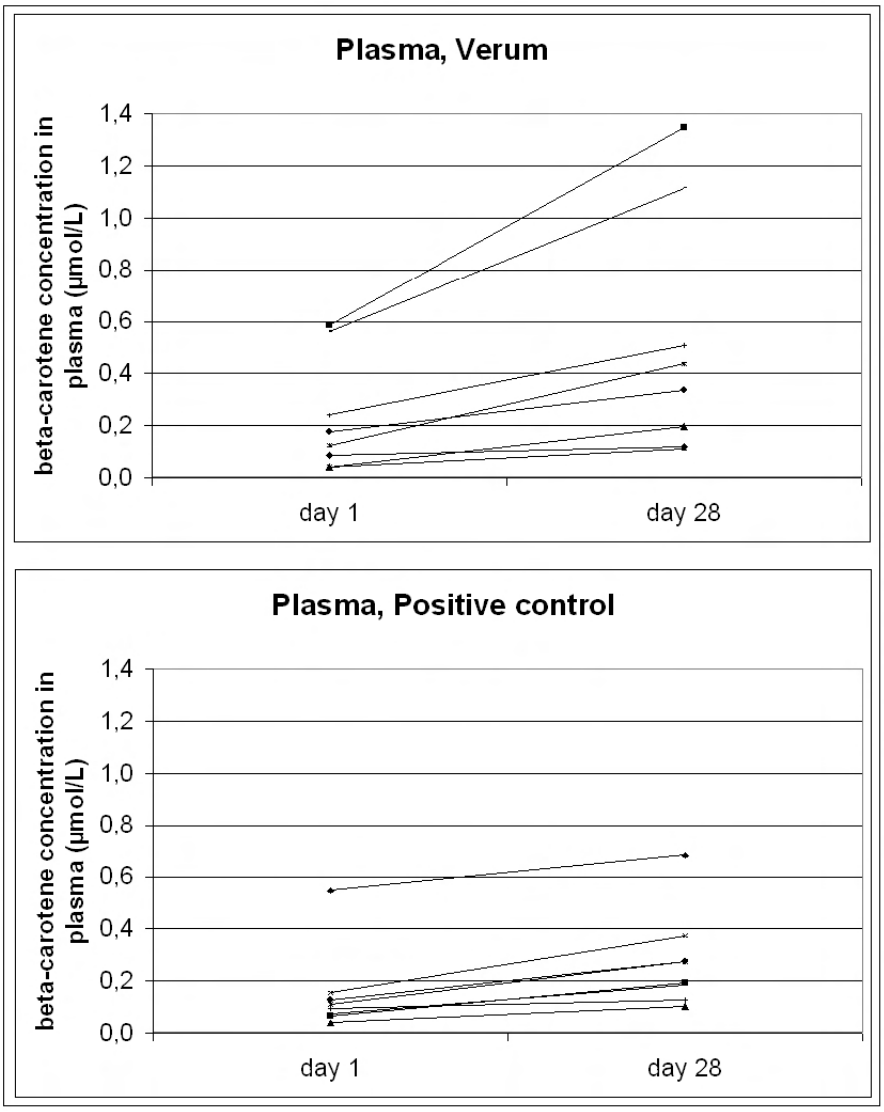

Figure 3.19: Individual increases of β-carotene in plasma for patients of verum and positive control group. Each data set (day 1 and day 28) represents one patient.

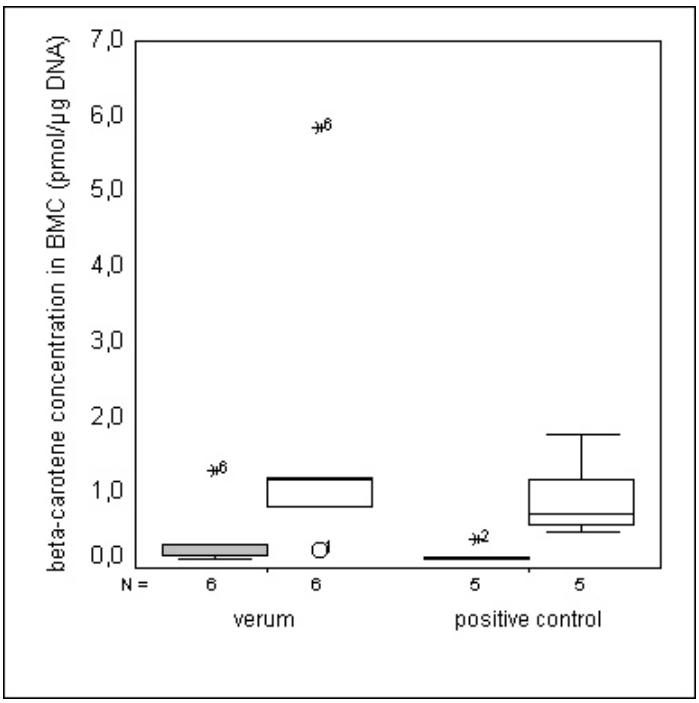

Figure 3.20: Effect of supplementation with β-carotene on β-carotene concentrations in BMC. The figure shows data as boxplots of verum and positive control groups with median, interquartile range, minimum and maximum concentrations of β-carotene. Grey boxes represent data of basic assessment (day 1), white boxes show data assessed on day 28. *6, *2 and ∘1 are outliers.

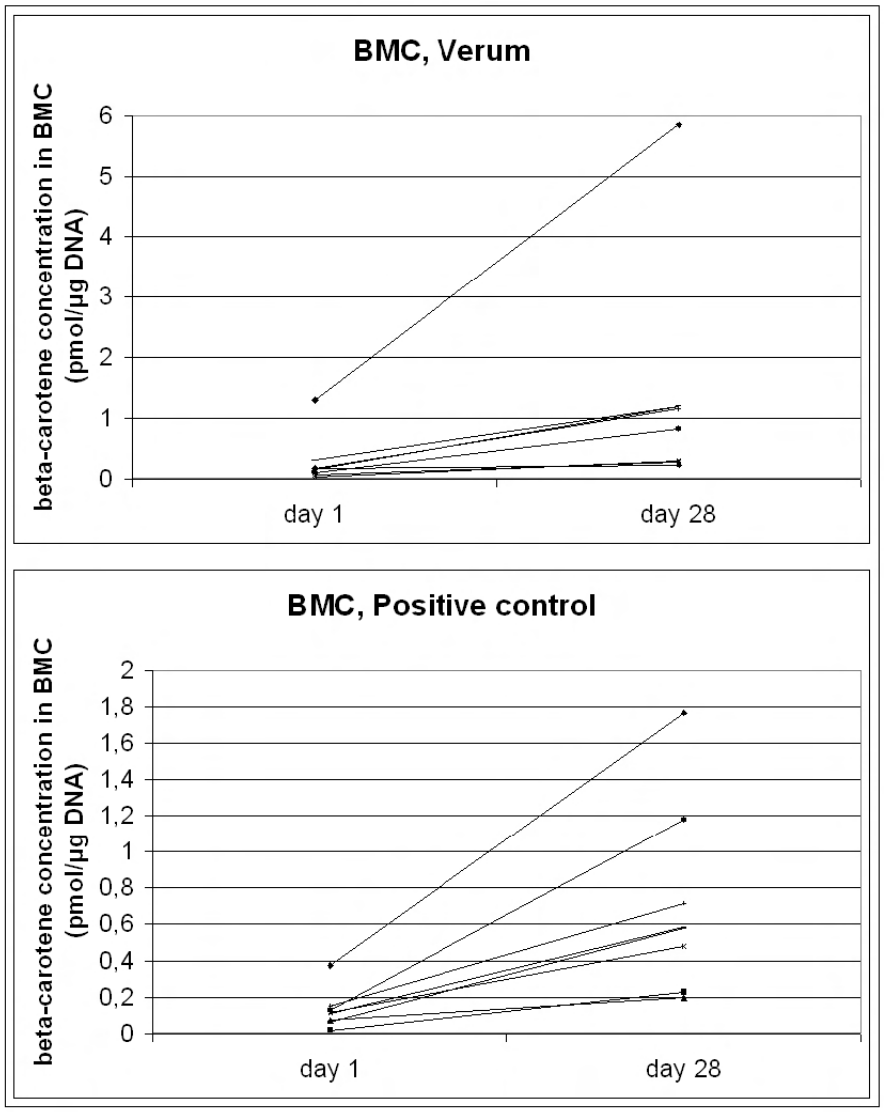

Figure 3.21: Individual increases of β-carotene in plasma for patients of verum and positive control group. Each data set (day 1 and day 28) represents one patient.

3.2.3.4 Ascorbic acid

As expected, the median increase of the ascorbic acid serum concentrations was in both intervention groups almost identical (16.63 μmol/L in verum group vs. 16.65 μmol/L in the positive control group).

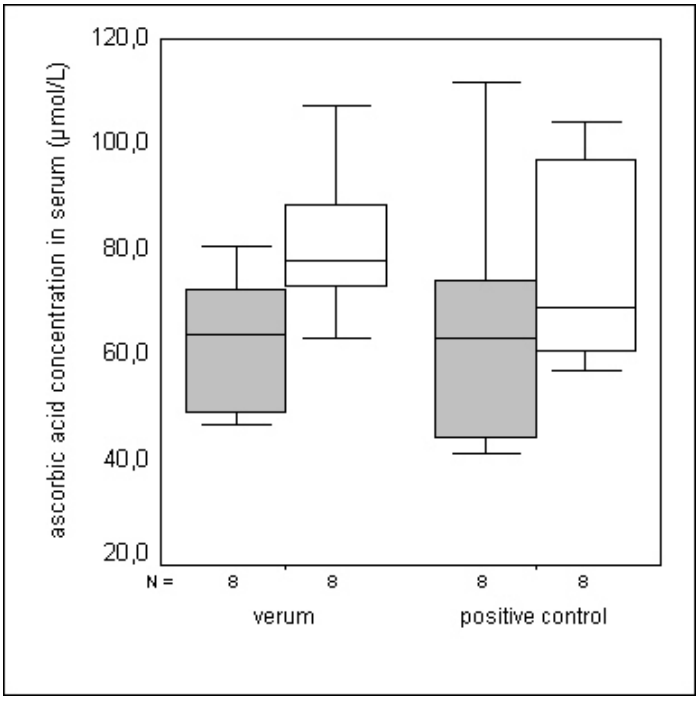

Figure 3.22: Effect of supplementation with vitamin C on ascorbic acid concentrations in serum. The figure shows data as boxplots of verum and positive control groups with median, interquartile range, minimum and maximum concentrations of ascorbic acid. Grey boxes represent data of basic assessment (day 1), white boxes show data assessed on day 28.

3.2.4 Changes in plasma protein carbonyl concentrations after intervention

Table 3.8 presents the data on the plasma protein carbonyl concentration changes in the two intervention groups during the 4 week intervention period. A minor median increase was observed in both groups, but it did not reach significance level in neither group.

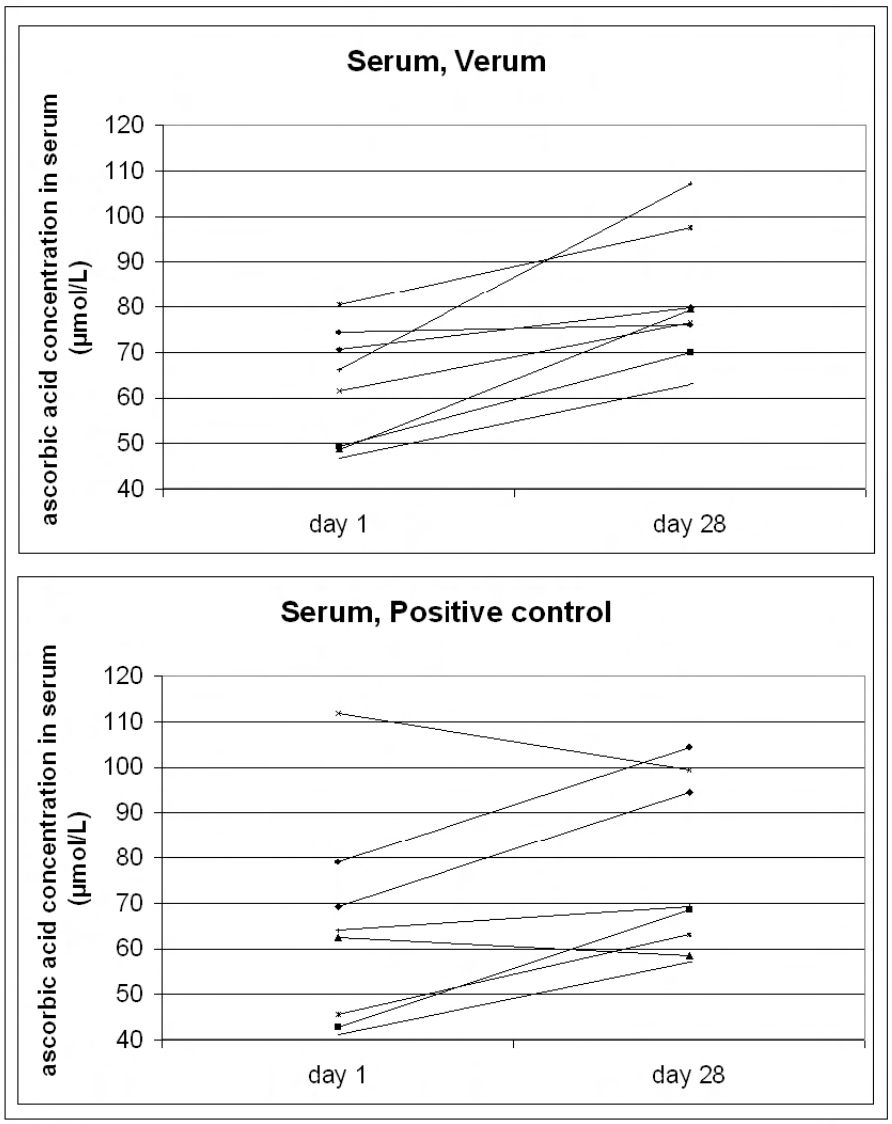

Figure 3.23: Individual increases and decreases of ascorbic acid in serum for patients of verum and positive control group. Each data set (day 1 and day 28) represents one patient.

Table 3.8: Change of protein carbonyl concentration in plasma during intervention.

Intervention group	**d_1**	**d_{28}**	**Change in plasma (d_{28}-d_1)**	**p[1] (d_1 vs. d_{28})**
Verum	0.443 (0.435-0.466)	0.471 (0.447-0.5)	0.03 (0-0.05)	NS
Positive control	0.444 (0.422-0.464)	0.478 (0.457-0.482)	0.03 (0-0.05)	NS

[1] p-value (Wilcoxon signed rank test); NS = not significant

Figure 3.24 displays the overall effect of intervention with antioxidant vitamins on the protein carbonyl concentrations in the patients. Surprisingly there was an slight increase of about 0.03 nmol carbonyls/mg protein in both groups rather than an expected decrease (refer to **table 3.2.4** for data). This can be explained by an intra-assay variation of 5 % detected by use of known controls for each assay. The controls differed about 0.027 nmol carbonyls/mg protein in the same way as the two corresponding data pairs of day 1 and day 28. Taken this intra-assay variation into account, there was neither increase nor decrease of protein carbonyl content after intervention.

3.2.5 Analysis of supplementation effect in patients with a remaining small bowel length of less than 200 cm (group 1)

It is of outstanding interest to examine the patients of group 1 with a remaining small bowel length of less than 200 cm as they are at the highest risk of all patients in the study to develop malnutrition, fat malabsorption and deficiency of fat soluble vitamins. However, there are only 2 patients for each intervention group available (refer to **table 3.5**) and these are too few for statistical analysis. Therefore the data on these 4 patients are presented as individual data sets (day 1 and day 28) for each of the investigated study vitamin in plasma and BMC.

The trend described in previous sections (section 3.2.2) of concentration increase in blood and tissue could be confirmed in the patients with a small bowel length of less than 200 cm.

The plasma α-tocopherol increase in the verum group was in both patients higher than in the positive control group (30.89 and 20.67 μmol/L vs. 10.69 and 15.02 μmol/L) In BMC, however, only one patient in the verum group showed a distinct α-tocopherol concentration increase (148.5 μmol/L), the others had modest concentration increases (38.82 and 50.12 μmol/L).

Retinol plasma concentrations could be increased only in the verum group, patients that received the positive control supplement exhibited de-

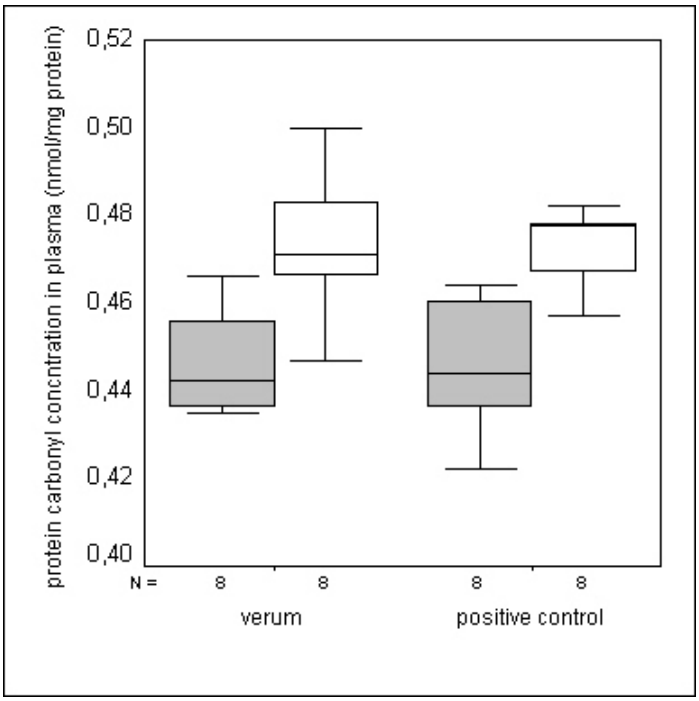

Figure 3.24: Change of protein carbonyl content in plasma over the trial period. The figure shows data as boxplots of verum and positive control groups with median, interquartile range, minimum and maximum concentrations of ascorbic acid. Grey boxes represent data of basic assessment (day 1), white boxes show data assessed on day 28.

creasing plasma concentrations. This fits very well into the trend that was described for the patients altogether in section 3.2.3.2.

The supplementation with β-carotene produced in th verum group slightly higher increases in plasma compared to the positive control group. Higher β-carotene concentration increases were achieved in tissue of patients in the verum group as well, but also one patient of the positive control group had a distinct increases.

For ascorbic acid, which was the same crystalline form in both study supplements all patients achieved serum concentration increases of about the same amount (approximately 20 μmol/L)) only one had a weak increase of 1.7 μmol/L.

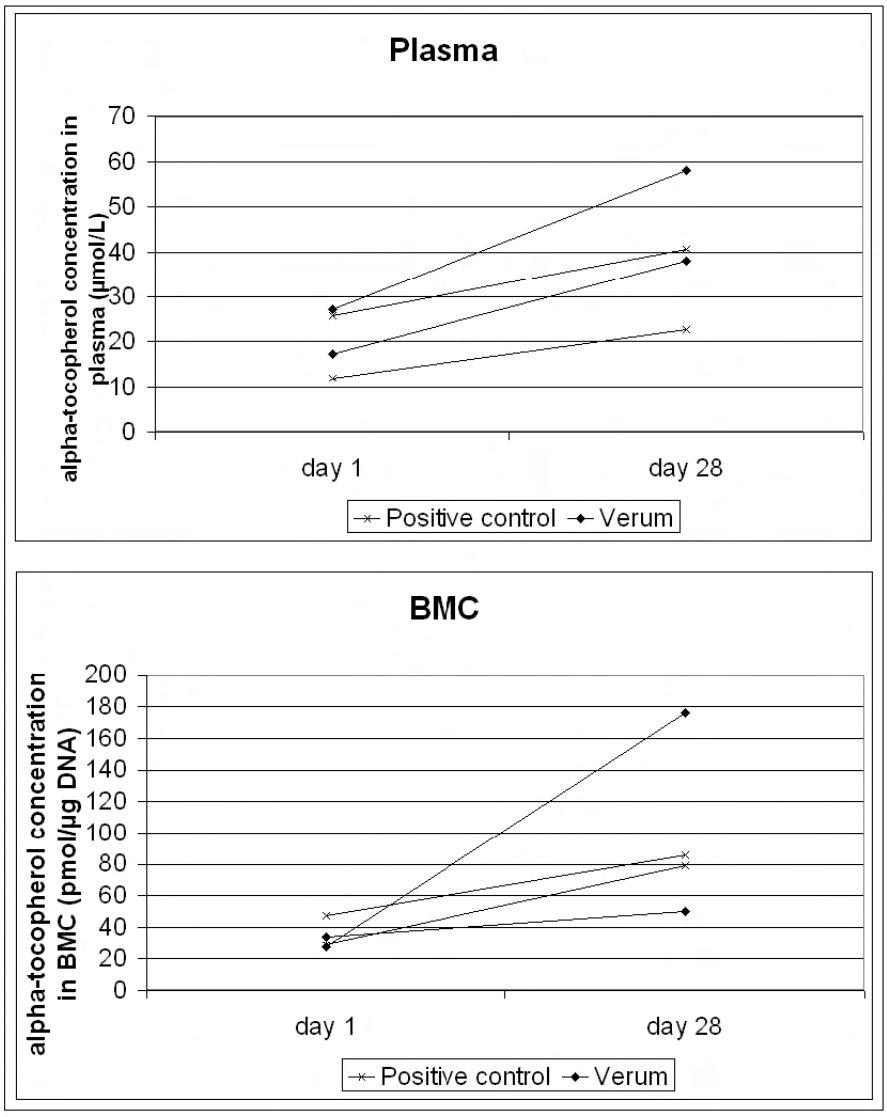

Figure 3.25: Individual increases of α-tocopherol in plasma and BMC for patients with less than 200 cm of remaining small bowel. Each data set (day 1 and day 28) represents one patient.

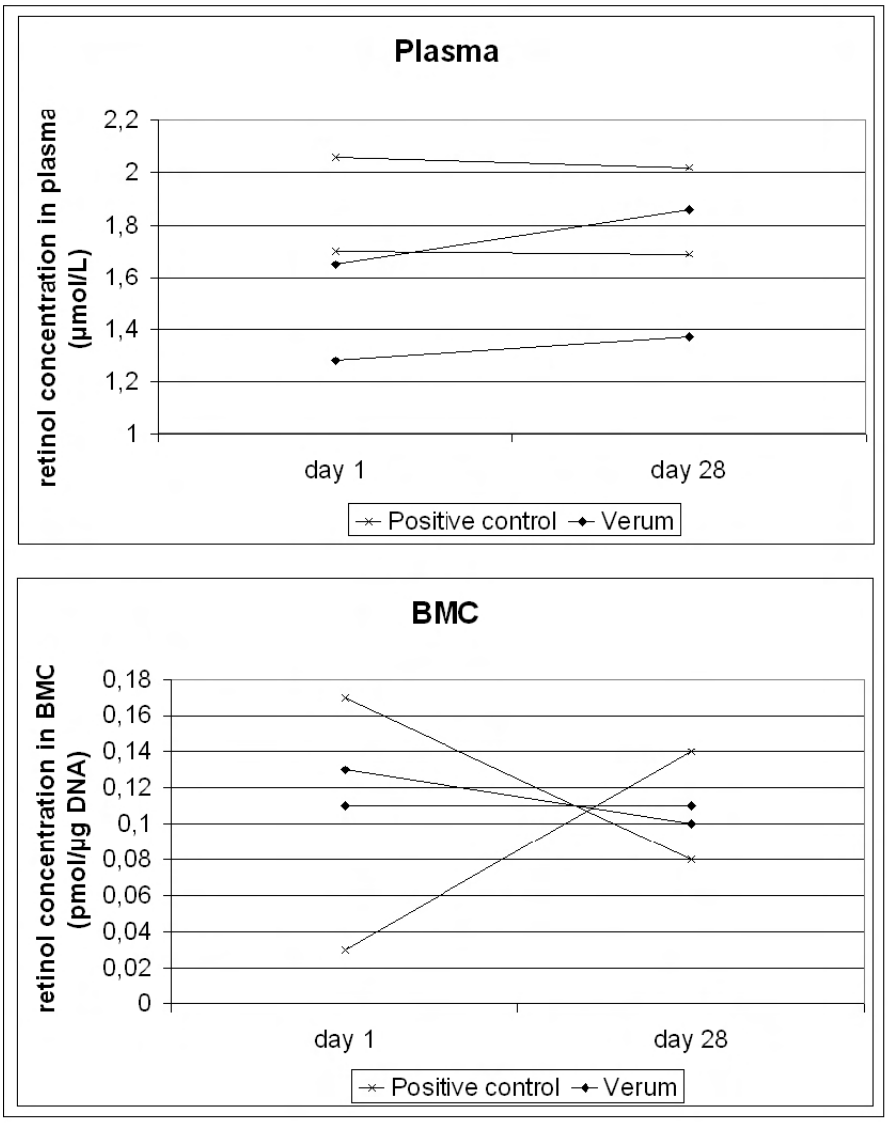

Figure 3.26: Individual increases of retinol in plasma and BMC for patients with less than 200 cm of remaining small bowel. Each data set (day 1 and day 28) represents one patient.

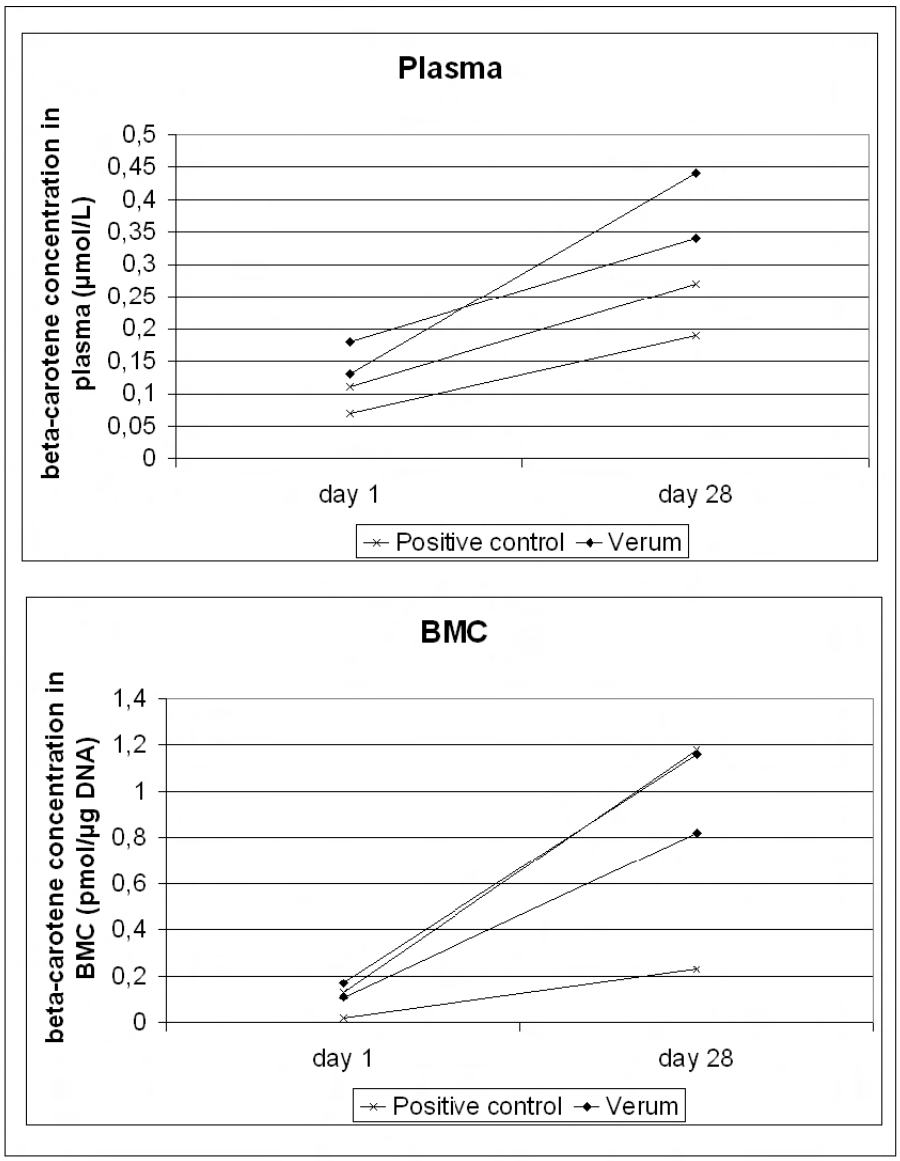

Figure 3.27: Individual increases of β-carotene in plasma and BMC for patients with less than 200 cm of remaining small bowel. Each data set (day 1 and day 28) represents one patient.

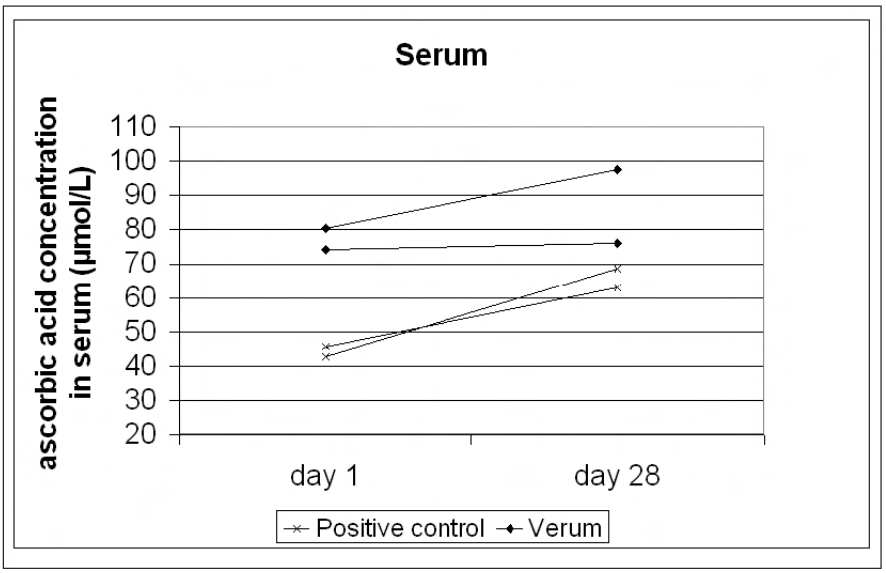

Figure 3.28: Individual increases of ascorbic acid in serum for patients with less than 200 cm of remaining small bowel. Each data set (day 1 and day 28) represents one patient.

Chapter 4

Discussion

4.1 Basic assessment

4.1.1 Vitamin E status

The results of the pilot study met my expectations to some extend: first of all, there were differences regarding the plasma concentration of α-tocopherol between the two bowel length groups and secondly, the lowest plasma concentrations were observed in patients with the shortest small bowel. The difference in concentration between groups was significant.

However, I had expected a more pronounced deficiency situation in the patients of group 1 (less than 200 cm of remaining small bowel). Regarding the standard plasma concentrations (**table B.1**), only two patients of that group were marginally supplied with vitamin E. The other three had low normal plasma concentrations. All other patients in group 2 showed normal to preventive plasma vitamin E concentrations. How can this be explained?

It seems, that a moderately shortened small bowel, as in patients of group 2 ($>$ 200 cm remaining small bowel), yields a intestinal surface still sufficient for vitamin E absorption. For this reason, these patients had normal and preventive plasma-concentrations. One patient of that group had a very high baseline plasma concentration (66.91 μmol/L), but he had been supplementing vitamin E (400 IU/d) regularly during the months prior to study start. The patients of group 1 ($<$ 200 cm remaining small bowel) however, had decreased, but not yet deficient plasma concentrations, pointing towards an insufficient vitamin E absorption from the bowel. As outlined in section 1.2.1 vitamin E is stored in adipose tissue and slowly released from these sites [25]. It takes years to decades before the storage sites are depleted [24]. It's therefore likely, that the patients of group 1 have a subclinical vitamin E deficiency, but their storage sites are still sufficient to provide normal plasma concentrations.

This would be consistent with observations of Walker et al. [24] and Traber et al. [23] who reported on SBS patients with a long history of SBS

but a rather late appearance of clinical vitamin E deficiency symptoms.

In addition, vitamin E intake and fasting plasma concentrations are not well correlated. Booth at al. [47] who investigated the diet-plasma relationships for fat soluble vitamins found no significant relationship for α-tocopherol. In her study, 36 healthy subjects provided fasting plasma samples and three sets of weighed diet records. The study participants did not take any vitamin supplements and did not suffer from any kind of maldigestion or malabsorption. The results of this study indicate that the quantity of vitamin E that is actually absorbed from the intestine is not immediately apparent in the plasma concentrations.

In contrast to plasma, the α-tocopherol content of the buccal mucosa cells is well correlated with the current vitamin E supply according to Peng et al. [48]. He investigated among other parameters the concentration of α-tocopherol in BMC after supplementation with 800 (first 4 days) and 400 IU *dl*-α-tocopherol (day 5-11) respectively. Samples were taken on day 0, 4, 7 and 11 and a steady increase of a-tocopherol was observed. Peng's data proved that there is a response towards vitamin E supplementation apparent in the immediately rising BMC-content.

Reversing this conclusion, a low tissue-concentration of vitamin E would reflect a low vitamin E intake with the diet. In my pilot-study, almost all study participants had low α-tocopherol tissue concentrations. The patients with the shortest small bowel had, however, the lowest concentrations which were comparable with tissue-vitamin E concentrations of adult CF-patients (**table B.2**) , who suffer from continuous fat malabsorption. But also patients of group 2 had lower tissue-concentrations than healthy adult subjects. Taken the results of Peng [48] into consideration the SBS-patients seem to be vitamin E deficient apparent by the very low BMC-concentrations.

4.1.2 Vitamin A status

Retinol is homeostatically regulated in plasma and decreases only in case of prolonged severe vitamin A deficiency leading to depletion of liver stores ($<$ 100 μg/g liver tissue [16]). None of the patients enrolled in the pilot study showed plasma concentrations indicating a deficiency according to **table B.3**, but nevertheless, patients of group 1 had significantly lower plasma concentrations than patients of group 2.

Tissue concentrations of retinol are quite difficult to assess, first of all because the BMC-content is rather low and often below the detection limit and secondly, there is an unknown substance co-eluting with retinol at the same time when using the above (section 2.2) described HPLC-method. Sometimes, this additional peak makes it impossible to analyze retinol. For these reasons, only samples of 11 patients could be analyzed in the pilot study. No significant difference was detectable between group 1 and 2. The median of both groups was identical (0,15 pmol/μg DNA) but in group 1

a great interindividual variability was observed. It is unknown if this variability reflects a different state of retinol supply, since data on correlation of retinol status with BMC retinol concentration in humans are not available. I could identify one comparable study of Sobeck et al. [50]. She assessed BMC retinol concentrations in healthy adult subjects who did not take any vitamin A supplements. These subjects had a mean value of 0.002 pmol/μg DNA, which is below the value assessed for the SBS patients.

Since the assessment of plasma retinol concentrations is not a very reliable tool to evaluate vitamin A deficiency either a clinical examination such as an electroretinography (ERG) or a different testmode like the relative dose response test (RDR) should be applied. Impaired scotopic vision is reportedly the first clinical sign of vitamin A deficiency [51]. In the pilot study one patient of group 2 reported a subjective impaired night vision, but this observation could not be validated since an ERG was not conducted. However, this single case shows that it is important to use more reliable assessment parameters for retinol in the future.

4.1.3 β-carotene status

β-carotene is a useful parameter to assess fat malabsorption. The concentration of β-carotene reflects the recent dietary uptake [40] and a plasma-concentration of > 0.3 μmol/L has been defined as adequate [25].

In the pilot study 78 % (14/18) of the patients had plasma β-carotene concentrations below the limit of 0.3 μmol/L. Of these 14 patients six even had undetectable plasma concentrations. The results therefore met my expectations. The lack of sufficient β-carotene concentrations points to fat malabsorption, which is common in SBS patients as outlined in the introduction part. The 5 patients in group 1 (small bowel length < 200 cm) had concentrations ranging from 0.03 to 0.18 μmol/L, whereas the values of group 2 scattered over a wide range from deficiency to preventive plasma concentrations (0.04 to 0.59 μmol/L).

These findings are in accordance with data from Wendland et al. [59]. She investigated the status of antioxidants in plasma of patients with Crohn's disease and conducted a subgroup analysis for patients who had a small bowel resection. The patients with Crohn's disease and with SBS had significantly lower β-carotene concentrations than the patients with no small bowel resections (0.23 μmol/L $\pm$ 0.06 vs. 0.45 μmol/L $\pm$ 0.08, $p < 0.05$).

These data can be confirmed by a study of Geerling et al. [52] who investigated patients with Crohn's disease in remission for their complete nutritional status and compared the data with a healthy control population. The patients showed significant lower plasma β-carotene concentrations than the control subjects. SBS patients were included in the study as well, but unfortunately, no subgroup analysis was conducted.

The two studies of Wendland and Geerling show that Crohn's disease

contributes to malabsorption since these patients had lower concentrations than the healthy control population. In the case of Wendland's study, patients with Crohn's disease plus SBS had even lower β-carotene concentrations. I assume that Crohn's disease (even when in remission) is per se a risk factor for the development of fat malabsorption. A shortened small bowel, however, acts as a strengthener causing an even worse plasma-β-carotene status. This information can be transferred to my pilot study since 17 out of 18 patients in the pilot study were suffering from Crohn's disease.

For the β-carotene status in tissue the same picture as for the plasma-concentration was apparent: patients of group 1 and 2 had very low BMC-β-carotene concentrations when compared to healthy adult subjects (refer to **table B.5**) and patients of group1 had even lower tissue concentrations than group 2.

According to Peng and colleagues [53], the BMC concentrations of β-carotene correlate very well with the plasma β-carotene concentrations (r=0.909). Therefore, the data from the pilot study fulfill the expectations and complete the picture of β-carotene deficiency in SBS patients, especially in patients of group 1.

4.1.4 Vitamin D status

The vitamin D status had been assessed using an ELISA for determination of $1,25(OH)_2D_3$. It turned out that 12 of 18 patients(= 64 %) showed concentrations below the lower limit of normal serum concentrations (=80 pmol/L [46]). The other 36 % had normal serum concentrations.

As described in section 1.2.3, $1,25(OH)_2D_3$ concentration in serum is only affected when the vitamin D status of the organism is very low. Thus only a late stage of vitamin D deficiency can be detected by assessment of this vitamin D metabolite. In the pilot study, most of the patients showed low concentrations of $1,25(OH)_2D_3$ indicating a deficiency. To clarify this deficiency further it would be very useful to assess more parameters: first, $25(OH)D_3$ should be measured, since this metabolite is more dependent on dietary vitamin D supply than $1,25(OH)_2D_3$ [34]. Second, to achieve a complete clinical picture, an assessment of parat hormone (PTH) should be addressed [32].

There was no significant difference between the two bowel length groups regarding the serum concentrations of $1,25(OH)_2D_3$, but this is explainable by the employed parameter $1,25(OH)_2D_3$. There will be probably more distinct differences between groups when measuring $25(OH)D_3$. A deficiency in the group with a very short small bowel is likely when taking other studies into consideration. As explained in section 1.2.3.2, Haderslev et al. [32] found 23.8 % of SBS patients (bowel length $<$ 200 cm or more than 150 cm of small bowel resected) having low normal plasma concentrations and 38.1 % being vitamin D deficient.

4.1.5 Vitamin K status

In this pilot study, coagulation time was used to screen for vitamin K deficiency in SBS patients. With this method, a vitamin K deficiency was not detectable in any of the study participants.

Coagulation time was used since it is a very easy and inexpensive screening tool. The disadvantage of this method is its limited informative value regarding the vitamin K status. Prothrombin time prolongs when there is less than 50 % of regular prothrombin in plasma present [54, 55] and this happens only in case of a prolonged and distinct deficiency of vitamin K [56]. Thus the prothrombin time is a late indicator for vitamin K deficiency.

Vitamin K status in plasma is dependent on dietary supply. Insufficient uptake of vitamin K results within a short period of time in a subclinical vitamin K deficiency, apparent by decreased plasma vitamin K_1 concentrations. A study with 32 healthy adult subjects conducted by Ferland et al. [36] revealed the direct dependency of plasma vitamin K concentrations on dietary supply. In this study a subclinical deficiency was induced within 13 days by a diet that contained very little vitamin K. After the depletion period the study participants were repleted with vitamin K_1 supplements. During the study several vitamin K dependent parameters were assessed, among these the prothrombin time, which did not change during the course of study.

Ferland's study dealt with healthy persons in which a subclinical deficiency was easily inducible. In the light of these results, it seems very likely that a subclinical vitamin K deficiency is existent in patients with fat maldigestion and fat malabsorption - like SBS patients. There are other conditions, that lead to fat malabsorption, in which a vitamin K deficiency is known. Such conditions are for instance cystic fibrosis (CF). Rashid et al. [56] demonstrated in a study with 98 CF patients that 72 % of them showed abnormal PIVKA-II concentrations indicating an insufficient supply with vitamin K. PIVKA-II (protein induced by vitamin K absence), which can be assessed by an ELISA method, is a very sensitive parameter to detect an early vitamin K deficiency [56]. To elucidate vitamin K deficiency further a direct assessment of vitamin K in plasma should be approached. However, due to the limited budget of this pilot study I could analyze neither PIVKAs nor vitamin K directly.

4.1.6 Vitamin C status

The median ascorbic acid concentration in serum was different in both groups, but the difference was not statistically significant. All patients except one in group 1 had sufficient to preventive serum concentrations.

There are several studies reporting generally lower vitamin C concentrations in Crohn's disease patients (in remission) compared with healthy

subjects (Hoffenberg [57], Imes [58] and Wendland [59]). These reduced vitamin C concentrations were explained by reduced vitamin C intake [58] and increased utilization of ascorbic acid as an antioxidant [57]. However, subgroup analyzes of patients with Crohn's disease with short bowel resection revealed no further ascorbic acid deficiencies in SBS patients [59]. Taking the explanations of section 1.2.6 into consideration a worsened vitamin C status is yet not likely in SBS patients compared to patients with Crohn's disease because the absorption of the water soluble ascorbic acid from the intestines is usually not impaired. Patients of the pilot study showed no general vitamin C deficiency. Nevertheless only 50% of all patients had preventive serum vitamin C concentrations (> 50 μmol/L).

4.1.7 Oxidative stress

Plasma concentration of protein carbonyls was measured to assess the extent of oxidative stress in SBS patients.

At basic assessment all patients exhibited equally low plasma protein carbonyl concentrations. "Low" is here defined by comparison with healthy and chronically ill adult subjects respectively (Back [45]) and furthermore with data from Buss et al. [44]. Buss states, that healthy individuals usually show plasma protein carbonyl concentrations between 0 to 0.5 nmol/mg protein. These low concentrations indicate no sign of excessive oxidative stress.

I could not identify any literature regarding the status of plasma protein carbonyls in SBS patients. Yet there are studies that dealt with the problem of oxidative stress in patients with Crohn's disease and SBS patients as subgroups. A study by Sampietro et al. [60] revealed, that patients with complicated Crohn's disease show a disbalance of pro- and antioxidative mechanisms apparent by assessment of peroxidative plasma levels (thiobarbituric acid reactive substances (TBARS), stimulated thiobarbituric acid reactive substances, plasma peroxidation susceptibility) on the one hand and vitamins A and E on the other hand. The patients of this study were treated with conservative surgery and showed 2 months and 1 year after treatment respectively significantly reduced peroxidative plasma concentrations. However, basal levels of peroxidation and antioxidant scavengers remained disregulated when compared to healthy subjects. This shows that Crohn's disease patients are highly oxidativly stressed when they suffer from acute bowel inflammation and this state can be improved, yet not normalized, by relief of obstructive complications through surgery.

Other parameters for the assessment of oxidative stress were used in a study with 37 Crohn's disease patients and 37 healthy subjects of Wendland and colleagues [59]. These were F_2-isoprostane in plasma and breath output of ethane and pentane (all lipid peroxidation products). Furthermore, antioxidant micronutrients (carotenoids, vitamin C, E) and vitamin A as well

as retinoids were assessed. The results showed that patients with Crohn's disease had significantly higher levels of lipid peroxidation and significantly lower micronutrient status than healthy control subjects. A subgroup analysis was conducted for small bowel resected patients. They showed no significant differences when compared to the other patients with Crohn's disease.

These two studies demonstrate that oxidative stress is existent in patients suffering from active Crohn's disease. However, the study of Sampietro showed a decrease of oxidative stress after removal of afflicted bowel segments, meaning that the patients achieved remission. This demonstrates, that oxidative stress is mainly caused by inflammation of the gut, but oxidative stress is still elevated (compared to healthy subjects), even when patients are in remission, as the results of Wendland's study show.

In my pilot study, the low level of oxidative stress can be explained by the chosen exclusion criteria. Patients were not included into the study when they suffered from acute inflammation of the bowel. Furthermore as explained in the previous sections, the status of antioxidants (vitamin C and E) was in most patients within normal range. Therefore, an extreme imbalance of pro- and antioxidants could not be demonstrated.

To obtain yet more information on oxidative stress in SBS patients and to compare the data with other findings, other parameters should be assessed like the mentioned TBARS and F_2-isoprostane.

4.2 Intervention trial

4.2.1 Vitamin E

In the pilot study, the application of both test supplements - verum and positive control - was successful, i. e. in patients of both groups a significant increase of plasma and tissue α-tocopherol concentrations was achieved. However, the increase in the verum group was higher when compared with the positive control group in each plasma and tissue and this difference was significant for plasma (p=0.02), but not for tissue concentrations (p=0.382).

The use of water soluble vitamin E and water miscible vitamin E respectively in single SBS patients has been described earlier by Howard et al. [22], Traber et al. [23] and Walker et al. [24] (refer to section 1.2.1.2). They administered these forms of vitamin E because common fat soluble vitamin E preparations either applied orally or i.m. did not improve their patients vitamin E status.

Howard treated his patient with water miscible *dl*-α-tocopherol acetate. Water miscible means that vitamin E is dispersible in water by use of an emulsifier, but it is not water soluble.

Traber and Walker used TPGS (*d*-α-tocopheryl polyethylene glycol 1000 succinate) a substance not approved for use in dietary supplements in Germany. TPGS is an amphiphilic derivative of tocopherol which has the ability

to form its own micelles independently from bile salts [61], it is therefore a water soluble vitamin E. TPGS has been successfully applied in the treatment of vitamin E deficiency in diseases with fat malabsorption (early childhood cholestasis [62], short bowel syndrome [23]).

These examples prove that water soluble and water miscible vitamin E formulations were superior to normal fat soluble vitamin E in correcting plasma α-tocopherol concentrations in case of fat malabsorption.

The supplement used in the pilot study is not completely comparable to water miscible vitamin E and TPGS respectively. The fat soluble vitamins in the verum supplement are already packed into micelles of physiological size (approximately 30 nm diameter, according to the manufacturer). In addition, the micelles are stable in a pH-range of 1 to 7 and therefore, they reach unaltered the intestine where they are immediately absorbed by the enterocytes. Because the normal course of fat digestion (i.e. formation of micelles by action of bile acids) is bypassed, micellised vitamin E has a higher bioavailability than common fat soluble vitamin E. This has been proven in adult healthy subjects by Back et al. [63].

In this pilot study, I could demonstrate that supplementation with micellised vitamin E increased the patients' plasma concentrations significantly higher than the normal fat soluble vitamin of the positive control group.

In contrast to the homogeneous increase in plasma in both intervention groups (refer to **figure 3.11**) a high degree of interindividual variability towards the verum supplement was observed in the BMC. This variability is visible in the higher degree of scattering after supplementation (refer to **figure 3.13**) in the verum group when compared to the positive control.

The same effect has been observed in plasma and BMC concentrations in children with cystic fibrosis, who are currently enrolled in a study that uses the same supplement as the SBS-pilot study (personal communication with Dr. E. Back). The cause for this phenomenon remains unclear.

In addition, the supplementation with vitamin E was successful in raising the tissue α- tocopherol concentration of all patients although the majority of the patients still had tissue-concentrations below that of healthy adult subjects. A longstanding supplementation should improve the patients tissue-concentrations further.

However, longstanding high dose vitamin E supplementation is not risk free and currently subject of critical discussion. A meta-analysis conducted by Miller et al. [49] revealed that high dose vitamin E supplementation ($>$ 400 IU/d, over 1 year) was associated with a significantly ($p=0.035$) increased overall mortality compared with control supplements. On the other hand, low dose vitamin supplementation ($<$ 400 IU/d) was associated with slightly but not significantly decreased mortality risk ($p>0.2$). The studies that were investigated in this meta-analysis dealt mainly with chronically ill subjects (cancer, cardiovascular disease), therefore the results are limited to this population. The reasons for the increased mortality remain unclear, but

the authors suggest prooxidative properties of high vitamin E dosages or its anticoagulant properties leading to hemorrhagic stroke. In the light of these results I would recommend cautious supplementation with vitamin E not exceeding 400 IU per day and not in patients, whose plasma concentrations are already at preventive concentrations (>30 μmol/L). However, the daily dose of vitamin E applied to SBS patients in the pilot study was rather low (100 mg tocopherol equivalents =150 IU) and the supplementation period lasted only 4 weeks.

4.2.2 Vitamin A

In the pilot study, a median increase in retinol plasma concentrations was only observed in the verum group whereas in the positive control group the median concentration decreased. But here, like for α-tocopherol, it is useful to examine the individual plasma retinol changes (**figure 3.15**). In the verum group all patients had slightly to moderately increased plasma retinol concentrations after the 4 week supplementation period, but in the positive control group only 2 patients showed appreciable increases, the others remained at about the same plasma value as on day 1. The latter observation is not surprising, considering that plasma retinol is homeostatically regulated.

Unlike in other supplementation studies the greatest increase in plasma retinol concentration was observed in patients with the highest baseline retinol concentrations. Willett et al. [64] made the opposite observation in women who where supplemented with 10.000 IU vitamin A daily for 4 weeks. In his study the subjects with the lowest concentrations had the largest increases in plasma concentration after the treatment. He assumed that they had a low vitamin A intake prior to the study and concluded, vitamin A supplementation was most useful in subjects with low vitamin A plasma concentrations and low vitamin A intake.

Therefore, the rather drastic increases of the two patients in the positive control group seem surprising since they already started from high baseline retinol concentrations. These patients did not take any vitamin A supplements prior to study start. I suggest that the high baseline concentrations point to a generally higher plasma retinol status in these individuals, that was even elevated through supplementation. Furthermore it is conceivable that these two patients consumed foods high in vitamin A, for instance liver, on a regular basis thus creating higher baseline retinol concentrations.

In contrast to the positive control group an increase in **every** patient could be achieved in the verum group, which indicates a superior ability of the vitamin A solubilisates to enhance the plasma retinol concentrations.

For BMC retinol concentrations, no consistent results were achieved through supplementation. The patients of both groups had increased as well as decreased tissue retinol concentrations. As apparent in **figure 3.17** it is

clear that patients responded very differently towards the supplementation with either verum or positive control. To my knowledge, there are no data available on the effect of oral vitamin A supplementation on BMC retinol concentration. Therefore, these results are difficult to explain. However, a study of Sobeck et al. [50] proved hat BMC responds to topical application of retinyl palmitate with increased retinol concentration, which indicates that buccal mucosa cells are capable of accumulating retinol. Whether these cells also increasingly take up retinol from the bloodstream upon supplementation remains to be elucidated.

4.2.3 β-carotene

The median β-carotene concentrations in plasma increased significantly in each intervention group. The median increase in the verum group was higher than in the positive control group, but did not reach the significance level (p=0.105).

The bioavailability of β-carotene supplements is varying in a high degree and is differing from the bioavailability of β-carotene from foods [65]. In general, β-carotene supplements yield a higher bioavailability than foods, especially when they are consumed along with a fat containing meal. β-carotene in natural sources is incorporated into subcellular structures from which it is extracted only to a small percentage during digestion [66].

I expected the β-carotene plasma concentrations to change in the same manner as the α-tocopherol plasma concentrations did, i. e. to observe a significantly higher increase in the verum group compared to the positive control group. However, this effect was spoiled because of the high degree of scattering in the verum group after intervention, causing a non significant difference between the groups.

This scattering can be explained by individual variations in β-carotene absorption and resulting increase in plasma concentration. This has been observed in a number of studies that investigated the effect of β-carotene supplementation, e.g. by Stich et al [67]. These variations were explained by lifestyle habits (smoking, alcohol consumption), different supplementation status, fat consumption along with the supplement, gender and age [68, 48]. These explanations are applicable to the patients of the pilot study, but since the highest degree of scattering occurred in the verum group, I suggest, that the type of supplement is contributing strongly to the high variability.

Unlike in plasma, BMC-β-carotene increases in both groups were comparable (refer to **figure 3.21**) except for one patient in the verum group whose BMC β-carotene concentrations started from 1.3 pmol/μg DNA and rose to 5.8 pmol/μg DNA. As observed for retinol, the greatest β-carotene plasma and BMC concentration increases occurred in the patients with the highest baseline values.

Some patients did respond only weakly towards β-carotene supplemen-

tation, whereas in others a clear improvement was observed. But still all patients (except for the one mentioned above) achieved BMC concentrations below that of healthy adult subjects (**table B.5**). I suppose that the supplementation period was too short to elevate BMC-concentrations even higher. The different response to β-carotene supplementation can be explained again by individual variations. This has been observed earlier by Gilbert and colleagues [68] as well as Stich et al. [67] and Peng et al.[48]. Gilbert investigated the BMC-concentration changes after a 3-d supplementation period with 30 mg β-carotene and observed in 17 % of patients a high change (>9.01 ng/10^6 cells) and in 31 % a weak increase (< 1.8 ng/10^6 cells). The rest of the study participants had increases in between. A comparable study of Stich [67] revealed similarly varying results under supplementation with even higher β-carotene dosages (90 mg/d). Gilbert explained the different responses with lifestyle and accompanying dietary habits: e.g. an increased bioavailability of β-carotene when the supplement was taken together with a high fat diet. This explanation is not completely transferable to the results of the pilot study, because of fat malabsorption. I would emphasize the explanation that BMC tissues of individuals have a highly different ability to accumulate and store β-carotene in the cells.

4.2.4 Fat soluble vitamins

Looking at the above presented results the advantage of the micellised fat soluble vitamins when compared to common fat soluble vitamins is apparent. The supplementation with water soluble forms of these vitamins for SBS patients with ileal resections of more than 100 cm has been already recommended earlier by Bernhard and Shaw [11].

At the present time, there is no adequate substitute for ADEK-Falk® available in Germany. Patients who need supplementation of fat soluble vitamins have to take several preparations in order to obtain a sufficient supply with all required vitamins.

Moreover, ADEK-Falk® was administered i.m. which is inconvenient, for some patients painful and requiring a physician for application. An oral supplementation is therefore much more preferable to patients.

To my knowledge, there is no other vitamin supplement available in Germany that uses water soluble vitamin A, D, E or K, except for β-carotene which is available for beverage production as water dispersible formulation (so called "powder") [65].

It is important to supplement SBS-patients, especially those with less than 200 cm of small bowel remaining, in order to maintain optimal micronutrient status and health. The pilot study proved, that the patients have some absorptive bowel surface left, because they could absorb sufficient amounts of the supplemented vitamins. Micellised fat soluble vitamins provided higher increases in plasma and in part also in tissue thus, improv-

ing plasma and BMC concentrations faster than the common fat soluble vitamins.

4.2.5 Vitamin C

The vitamin C supplementation increased the patients' serum ascorbic acid concentrations. This increase was as expected, because the absorption of water soluble vitamin C is usually not affected in SBS patients [4, 13].

However, vitamin C supplementation seems to be not completely risk-free in SBS patients. A small percentage (1.5 % [69]) of absorbed ascorbic acid is converted into oxalate and ascorbic acid increases in predisposed subjects (so called "stoneformers") the oxalate absorption from the diet. Oxalate is subsequently excreted via the kidney and high amounts of urinary oxalate increase the risk for the formation of calcium oxalate renal stones. As outlined in section 1.1.4.2, SBS patients tend to absorb more oxalate from the diet than healthy subjects due to steatorrhea. A study from Chai et al. [70] investigated the impact of high dose supplementation (2000 mg) with ascorbic acid in a population with a history of calcium oxalate renal stones. The results of this study point to increased oxalate absorption and increased oxalate formation after ascorbic acid supplementation and the authors suggest that high dose supplementation is a risk factor for lithogenesis in predisposed subjects.

The influence of i.v. administered ascorbic acid on urinary oxalate excretion has been investigated recently by Pena da la Vega [71] and colleagues. They observed an increase in urinary oxalate excretion in TPN-dependent patients when the daily vitamin C dose was increased from 100 mg to 200 mg.

I could not identify any study that investigated the oral supplementation with vitamin C in SBS patients under the aspect of stone formation, but in the light of the results of Chai and Pena de le Vega, a careful observation of urinary oxalate concentrations and a limited vitamin C supplementation together with limited intake of fat should be considered in the treatment of SBS patients.

4.3 Importance of the pilot study findings for SBS patients

SBS patients should be aware of certain vitamin deficiency risks because of their disease. To meet such risks one would advise the SBS patients to increase their consumption of vegetables and fruit and other foods rich in specific vitamins. However, its comprehensible that SBS patients cannot adopt such proposals for a healthy diet, since they frequently experience various digestive problems. These problems can cause modified dietary habits, for instance avoiding foods with high fat content to prevent steatorrhea, which

is of course inconvenient and unpleasant. Even if a healthy and balanced diet is consumed, malabsorption leads to insufficient micronutrient supply.

Therefore, SBS patients should be advised to supplement vitamins additionally. As the results of the pilot study indicate, a regular supplementation with β-carotene is recommended. The same accounts for vitamin E and A, because subclinical deficiencies are difficult to detect and a preventive supplementation is preferable, because some clinical vitamin deficits, like neuropathies in case of prolonged vitamin E deficiency are scarcely reversible in adults [24]. The supplementation with vitamin D is necessary too, in order to maintain bone health.

4.4 Summary and conclusion

In summary, the basic assessment of vitamin status in SBS patients revealed deficits of certain vitamins in plasma, serum and tissue. Prolonged vitamin deficiencies create a number of clinical symptoms, some of these beeing irreversible. A careful supervision of fat soluble vitamin status in SBS patients and an early correction of deficits must be accomplished to maintain health and wellbeing apart from the underlying disease.

The results of the intervention study showed that the reduced intestinal surface of SBS patients was sufficient to absorb vitamins from verum and positive control supplement respectively. Increases of plasma and tissue concentrations were generally higher in the verum than in the positive control group, which makes the micellised fat soluble vitamins preferable to common fat soluble vitamins.

4.5 Future prospects

The pilot study showed that SBS patients need additional supplementation of certain vitamins. With the withdrawal of ADEK-Falk® from the german market an adequate supplement, that meets the requirements of SBS patients is not available. Therefore a new dietary supplement with a specific composition of micellised vitamins is the next step that should be approached. Such a vitamin preparation has to be tested in a study over a longer period of time (several months) with a larger cohort of SBS patients. In the following paragraphs I will outline the properties of such a study.

4.5.1 Supplement

A new supplement should contain β-carotene, since there were deficits in plasma and tissue in the majority of the SBS patients in the pilot study. The daily dose of β-carotene was rather low (1.6 mg) when compared to other studies who applied up to 30 mg β-carotene per day (for instance

Peng et al. [48]). However, I would not increase this dose further, because the supplement is indented for longterm use.

Despite the mostly sufficient plasma concentrations of retinol and α-tocopherol, I would still supplement low doses of vitamin A and E, because the majority had low tissue-concentrations compared to healthy subjects. Furthermore, this improved supplement is for "true" SBS patients, that have only 200 cm or less of small bowel left and these patients had significantly lower retinol and α-tocopherol plasma concentrations than the subjects with more than 200 cm remaining small bowel. A supplementation of these vitamins in this group of patients is therefore justified. The supplement should contain vitamin D as well, since $1{,}25(OH)_2D_3$ was low in the majority of the patients.

The pilot study showed that SBS patients are sufficiently supplied with vitamin C. For that reason I would refrain from adding ascorbic acid to a new supplement. Moreover, as outlined in section 4.2.5 the supplementation with ascorbic acid can increase the risk for formation of calcium oxalate renal stones in predisposed subjects thus, supplementing vitamin C might not be a safe approach.

4.5.2 Selection of subjects for further studies

To investigate the effect of supplementation in SBS patients it is necessary to chose the study subjects more carefully. Only patients that fulfill the SBS definition, i.e. patients with less than 200 cm of small bowel, should be included into the study. A larger study population should be investigated as well in order to achieve statistically relevant results.

4.5.3 New parameters to investigate

In a future SBS study I would use far more parameters to assess the effect of vitamin supplementation in the patients than in the pilot study.

As described in section 4.1.2 it is necessary to use a reliable method to assess the vitamin A status accurately. An electroretinography (ERG) would be the preferred choice. Moreover, it is advisable to establish a reference range for retinol and also retinylpalmitate concentrations in BMC, because there are no data available yet and it would be another promising way to supervise the vitamin A status in patients.

The means to assess oxidative stress should be reconsidered. The measurement of protein carbonyls did not yield much useful information on the status of oxidative stress in the SBS patients. I would recommend the use of other markers for oxidative stress, i. e. thibarbituric acid reactive substances (TBARS) and F_2-isoprostane.

Vitamin D supplementation should be assessed by measuring $25(OH)D_3$ instead of $1{,}25(OH)_2D_3$. The positive effect of vitamin D on bone health

should be assessed by analysis of bone density, a method easily realizable in medical facilities.

To complete the assessment of the patients' vitamin status, a dietary protocol should be conducted to obtain information on the total of ingested vitamins. It might be that the vitamin deficiencies and suboptimal vitamin concentrations are partly caused by insufficient micronutrient uptake. For some patients, at least for those that show signs of malnutrition like underweight, this seems to be a likely explanation

Chapter 5

Summary

5.1 Summary

Short bowel syndrome is a rare disease either resulting from massive small bowel resection or loss of function. Patients commonly develop fat maldigestion and malabsorption due to reduced bowel length, loss of bile acids caused by ileum resections and bacterial overgrowth. Patients with less than 200 cm remaining small bowel are at high risk developing a deficiency of fat soluble vitamins. Common enteral diets are inadequate to substitute these vitamins in SBS patients, thus supplementation is frequently needed. At the present time an appropriate supplement that meets the patients needs for all fat soluble vitamins is not available in Germany. In this study a vitamin supplement in form of gummi bears was used that contained the fat soluble vitamins E, A and β-carotene as pH-stable micelles, so-called solubilisates (AQUANOVA®) and vitamin C. The solubilisates do not require the normal course of fat digestion, thus they can be absorbed immediately and independent from bile acids.

In this monocentric controlled randomized double blind pilot study I investigated: 1. the status of fat soluble vitamins and vitamin C in SBS patients in relation to their remaining small bowel length and 2. whether solubilised fat soluble vitamins or common fat soluble vitamins can improve the plasma vitamin concentrations for retinol, α-tocopherol and β-carotene in short bowel patients in a period of 4 weeks. All patients received a daily dose of 1000 mg retinol equivalents, 100 mg tocopherol equivalents, 1,6 mg β-carotene and 400 mg ascorbic acid.

18 short bowel patients with a median small bowel length of 190 cm (100 - 400 cm) were recruited by the University hospital of Tuebingen (F/M = 9/9), 16 completed the trial. Plasma and tissue (BMC) concentrations of retinol, α-tocopherol and β-carotene were analyzed as well as serum concentration of ascorbic acid. Additionally $1,25(OH)_2D_3$ and coagulation time (quick and INR) were measured at basic assessment.

Patients with less than 200 cm remaining small bowel exhibited lower vitamin E, A and β-carotene concentrations in plasma and tissue compared to patients with longer small bowel and in case of plasma concentrations of vitamin E and A the difference was significant. No differences in ascorbic acid-, $1,25(OH)_2D_3$-status and coagulation time was found. After 4 week of supplementation increased concentrations of the applied vitamins in both intervention groups were observed. The median increase of α-tocopherol in plasma was significantly higher in the the verum group compared to the positive control group ($p=0.02$). The median increases of β-carotene and retinol in plasma were also higher in the verum than in the positive control group, but not significantly. In BMC similar trends were observed except for retinol concentrations, which were slightly decreasing in both intervention groups.

The effect of supplementation with micellised fat soluble vitamins in SBS patients was superior to conventional fat soluble vitamins, although both forms were capable of increasing the plasma and tissue concentrations. This proves that the remaining resorptive surface of SBS patients has the ability to absorb fat soluble vitamins but this ability depends on the remaining bowel length. The application of micellised vitamins in nutritional therapy of SBS patients seems to be very promising because of the better bioavailability and the greater plasma- and tissue-concentration increases.

5.2 Zusammenfassung

Das Kurzdarmsyndrom (KDS) ist eine seltene Erkrankung, die entweder durch ausgedehnte Resektionen oder durch Funktionsverlust des Dünndarms verursacht wird. KDS-Patienten entwickeln häufig Fettmaldigestion und Fettmalabsorption aufgrund der verringerten Dünndarmlänge, bakterieller Überbesiedelung und Gallensäureverlust bei Ileum-Resektionen. Patienten, die mit weniger als 200 cm Dünndarm leben, haben ein hohes Risiko einen Mangel an fettlöslichen Vitaminen zu entwickeln. Herkömmliche Diäten reichen nicht aus um diese Vitamine bei KDS-Patienten zu substituieren, daher ist ein zusätzliche Supplementierung häufig erforderlich. Momentan gibt es in Deutschland kein Vitaminpräparat, welches den Bedarf an allen fettlöslichen Vitaminen deckt. In der vorliegenden Studie wurde ein Vitamin-Supplement in Form von Gummibärchen verwendet, welches die fettlöslichen Vitamine A, E und β-Carotin als pH-stabile Mizellen, sogenannte Solubilisate (AQUANOVA®), sowie Vitamin C enthält. Die Solubilisate bedürfen nicht der normalen Fettverdauung, sie können vielmehr unmittelbar und unabhängig von Gallensäuren absorbiert werden.

In dieser monozentrischen, kontrollierten, randomisierten, doppelt blinden Pilotstudie wurde folgendes untersucht: 1. Versorgung von KDS-Patienten mit fettlöslichen Vitaminen und Vitamin C in Bezug auf die verbliebene Dünndarmlänge und 2. ob solubilisierte oder herkömmliche fettlösliche Vitamine die Plasma- und Gewebe-Konzentrationen von Retinol, α-Tocopherol und β-Carotin innerhalb von 4 Wochen erhöhen können. Alle Patienten erhielten als tägliche Dosis 1000 mg Retinoläquivalente, 100 mg Tocopheroläquivalente, 1,6 mg β-Carotin und 400 mg Ascorbinsäure.

18 Kurzdarmpatienten (W/M = 9/9) mit einer mittleren Dünndarmlänge von 190 cm (100 - 400 cm) wurden von dem Univeristätklinikum Tübingen rekrutiert, 16 Patienten beendeten die Studie. Im Plasma und Gewebe wurden die Konzentrationen von Retinol, α-Tocopherol and β-Carotin sowie die Serum-Konzentration von Ascorbinsäure bestimmt. Zur Basiserhebung wurde zusätzlich $1{,}25(OH)_2D_3$ und die Gerinnungszeit (Quick und INR) gemessen.

Patienten, die weniger als 200 cm Restdünndarmlänge haben, zeigten niedrigere Konzentrationen an Vitamin E, A und β-Carotin in Plasma und Gewebe verglichen mit den Konzentrationen von Patienten mit mehr als 200 cm Restdünndarmlänge. Die Plasma-Konzentrationen für Vitamin E und A waren dabei signifikant unterschiedlich. Dagegen konnten keine Unterschiede bei der Versorgung mit Ascorbinsäure und $1{,}25(OH)_2D_3$ sowie bei der Gerinnungzeit festgestellt werden. Nach der 4-wöchigen Supplementation wurden in beiden Interventionsgruppen erhöhte Konzentrationen der verabreichten Vitamine gemessen. Der mittlere Anstieg der α-Tocopherol-Konzentrationen in Plasma war in der Verum-Gruppe signifikant höher als in der Positiv-Kontroll-Gruppe (p=0.02). Die mittleren Anstiege von β-

Carotin und Retinol im Plasma waren ebenfalls in der Verum-Gruppe höher als in der Positiv-Kontroll-Gruppe, jedoch waren diese Unterschiede nicht signifikant. Im Gewebe wurden ähnliche Trends beobachtet mit Ausnahme der Retinol-Konzentration, die in beiden Interventionsgruppen leicht absank.

Der Effekt der Supplementierung mit solubilisierten fettlöslichen Vitaminen war dem der herkömmlichen Vitamine überlegen, allerdings konnten beide Formen der Vitamine die Plasma- und Gewebe-Konzentrationen erhöhen. Dies beweist, dass die verbliebene Resorptionsfläche der KDS-Patienten die Fähigkeit besitzt fettlösliche Vitamine zu absorbieren, jedoch hängt diese Absorptionsfähigkeit von der verbliebenen Dünndarmlänge ab. Der Einsatz mizellisierter Vitamine bei der Ernährungstherapie von KDS-Patienten scheint aufgrund der besseren Bioverfügbarkeit und der größeren Konzentrationsanstiege in Plasma und Gewebe sehr aussichtsreich.

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Appendix A

Additional data

The following tables present an overview on the plasma, serum and tissue concentrations of the study vitamins and of protein carbonyls before and after the intervention trial. All data are given as median with minimum and maximum in parentheses. The significance level for the difference between the groups is presented in each table.

Table A.1: Changes of α-tocopherol concentrations in plasma and tissue in the two intervention groups and their statistical significance.

	Intervention group	$\mathbf{d_1}$	$\mathbf{d_{28}}$	**Difference**	**p**[1]
Plasma (μmol/L)	Verum	29.98 (17.28-66.91)	59.69 (37.96-92.28)	27.19 (20.67-36.24)	0.02
	Positive control	27.13 (11.95-43.15)	46.54 (22.64-58.94)	17.51 (10.69-23.98)	
BMC (pmol/μg DNA)	Verum	37.79 (27.48-125.56)	81.44 (30.97-190.82)	41.27 (0.03-148.5)	NS [2]
	Positive control	40.66 (27.26-71.04)	78.84 (35.70-93.16)	27.45 (1.46-53.16)	

[1] Verum vs. positive control; Mann-Whitney-Test
[2] NS = not significant

Table A.2: Changes of retinol concentrations in plasma and tissue in the two intervention groups and their statistical significance.

	Intervention group	$\mathbf{d}_1$	$\mathbf{d}_{28}$	**Difference**	**p** [1]
plasma (μmol/L)	Verum	2.02 (1.28-2.52)	2.43 (1.37-3.24)	0.25 (0.1-0.86)	NS [2]
	Positive control	2.26 (1.7-4.04)	2.16 (1.69-4.75)	-0.14 (-0.19-0.83)	
BMC (pmol/μg DNA)	Verum n=3	0.11 (0.07-0.13)	0.103 (0.1-0.11)	0.005 (-0.03-0.03)	NS [2]
	Positive control n=4	0.17 (0.15-0.18)	0.103 (0.08-0.13)	-0.07 (-0.09- -0.02)	

[1] Verum vs. positive control; Mann-Whitney-Test
[2] NS = not significant

Table A.3: Changes of β-carotene concentrations in plasma and tissue in the two intervention groups and their statistical significance.

	Intervention group	$\mathbf{d}_1$	$\mathbf{d}_{28}$	**Difference**	**p** [1]
plasma (μmol/L)	Verum	0.15 (0.04-0.59)	0.39 (0.11-1.35)	0.22 (0.03-0.76)	NS [2]
	Positive control	0.10 (0.04-0.55)	0.23 (0.1-0.68)	0.13 (0.03-0.23)	
BMC (pmol/μg DNA)	Verum n=6	0.17 (0.11-1.3)	1.18 (0.23-5.85)	0.94 (0.06-4.55)	NS [2]
	Positive control n=5	0.13 (0.11-0.37)	0.72 (0.48-1.77)	0.57 (0.37-1.39)	

[1] Verum vs. positive control; Mann-Whitney-Test
[2] NS = not significant

Table A.4: Changes of ascorbic acid concentrations in serum in the two intervention groups and their statistical significance.

	Intervention group	**d_1**	**d_{28}**	**Difference**	**p** [1]
Plasma (μmol/L)	Verum	63.83 (46.85-80.50)	77.95 (63.07-107.25)	16.63 (1.66-41.10)	NS [2]
	Positive control	63.26 (41.16-111.75)	68.94 (57.07-104.40)	16.65 (-12.38-25.71)	

[1] Verum vs. positive control; Mann-Whitney-Test
[2] NS = not significant

Table A.5: Changes of protein carbonyl concentrations in plasma in the two intervention groups and their statistical significance.

	Intervention group	**d_1**	**d_{28}**	**Difference**	**p** [1]
Plasma (nmol/ mg protein)	Verum	0.443 (0.435-0.466)	0.471 (0.447-0.5)	0.03 (0-0.05)	NS [2]
	Positive control	0.444 (0.422-0.464)	0.478 (0.457-0.482)	0.03 (0-0.05)	

[1] Verum vs. positive control; Mann-Whitney-Test
[2] NS = not significant

Appendix B

Reference data

B.1 α-tocopherol

B.1.1 Plasma

Table B.1: Reference plasma α-tocopherol concentrations [16]

Deficiency	$<$ 11.6 μmol/L
Marginal	11.6 - 16.2 μmol/L
Normal	$>$ 16.2 μmol/L
Preventive	$>$ 30 μmol/L

B.1.2 Tissue

Table B.2: BMC α-tocopherol concentrations in healthy adult subjects and adult CF-patients

Healthy adult subjects[1] (n = 12)	101.5 (80.3 - 136.4) pmol/μg DNA[2]
Adult CF patients[1] (n = 5)	57.1 (32.7 - 84.8) pmol/μg DNA [2]

[1] Data from Back 'Antioxidant systems and vitamin A in cystic fibrosis' [45]

[2] median; interquartile range in parentheses

B.2 Retinol

Table B.3: Reference plasma retinol concentrations [16]

High risk for vitamin A deficiency	< 0.35 μmol/L
Medium risk for vitamin A deficiency	0.35-0.7 μmol/L
Small risk for vitamin A deficiency	0.7-1.05 μmol/L
Preventive	> 1.05 μmol/L

B.3 β-carotene

B.3.1 Plasma

Table B.4: Reference plasma β-carotene concentration [25]

Deficiency	$<$ 0,3 μmol/L
Normal	0,3 - 0,4 μmol/L
Preventive	$>$ 0,4 μmol/L

B.3.2 Tissue

Table B.5: BMC β-carotene concentrations in healthy adult subjects

Healthy adult subjects[1] (n = 9)	3.15 (2.16 - 5.75) pmol/μg DNA[2]

[1] Data from Back 'Antioxidant systems and vitamin A in cystic fibrosis' [45]

[2] median; interquartile range in parentheses

B.4 Ascorbic acid

Table B.6: Reference serum ascorbic acid concentration [16]

Deficiency	$<$ 11 μmol/L
Marginal	11 - 23 μmol/L
Normal	23 - 85 μmol/L
Preventive	$>$ 50 μmol/L

B.5 1,25(OH)$_2$D$_3$

Reference range in serum: 80 - 180 pmol/L [46]

B.6 Quick and INR

Quick: reference range: 70 - 125 % [46]
INR: reference range: around 1 [46]

B.7 Protein carbonyls

Table B.7: Reference protein carbonyl concentrations

Healthy adult subjects[1] (n = 14)	0.536 (0.513 - 0.536) nmol/mg protein[2]
Adult CF patients[1] (n = 4)	0.623 (0.536 - 0.686) pmol/μg DNA [2]
Healthy adult subjects[3]	0 - 0.5 nmol/mg protein

[1] Data from Back 'Antioxidant systems and vitamin A in cystic fibrosis' [45]
[2] median; interquartile range in parentheses
[3] Reference data from Buss et al. [44]

B.8 Blood lipids

Table B.8: Reference blood lipid concentrations

Total cholesterol[1]	4.14-5.18 mmol/L
LDL-cholesterol[1]	2.59-3.89 mmol/L
Triglycerides[1]	< 2.26 mmol/L

[1] Data from http://www.labor-keeser-arndt.de/

Appendix C

Purchase information

Table C.1: Chemicals

Product	Supplier
1,4-Dioxane	VWR International GmbH, Darmstadt, Germany
1-Butanol	VWR International GmbH, Darmstadt, Germany
Butylhydroxytolene (BHT)	Sigma-Aldrich, Steinheim, Germany
4-hydroxy-2,2,6,6-tetramethyl-piperidinyloxy free radical (TEMPO)	Sigma-Aldrich, Steinheim, Germany
Acetaldehyde, $\geq$ 99.5 % (GC)	Sigma-Aldrich, Steinheim, Germany
Acetic acid	VWR International GmbH, Darmstadt, Germany
Acetonitrile LiChrosolv®, gradient grade for liquid chromatography	VWR International GmbH, Darmstadt, Germany
Ammonium acetate	VWR International GmbH, Darmstadt, Germany
Anti-DNP-antibody from rabbit	Sigma, Taufkirchen, Germany
AQUANOVA® β-carotene solubilisate	AQUANOVA German solubilistae technologies GmbH, Darmstadt, Germany
AQUANOVA® vitamin A solubilisate	AQUANOVA German solubilistae technologies GmbH, Darmstadt, Germany
AQUANOVA® vitamin E solubilisate	AQUANOVA German solubilistae technologies GmbH, Darmstadt,

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Product	Supplier
	Germany
Bovine Serum Albumine	VWR International GmbH, Darmstadt, Germany
Citric acid	VWR International GmbH, Darmstadt, Germany
Dinitrophenylhydrazine	VWR International GmbH, Darmstadt, Germany
Diphenylamine	Sigma-Aldrich, Steinheim, Germany
Elmex® sensitive	GABA GmbH, Lörrach, Germany
Ethanol Rotipuran® $\geq$ 99.8% p.a.	Carl Roth GmbH & Co, Karlsruhe, Germany
Fish sperm DNA	Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany
Guanidine hydrochloride	VWR International GmbH, Darmstadt, Germany
Hydrogen peroxide, 30 % Isotonic NaCl (0.9%)	Fluka, Neu-Ulm, Germany Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany
KCl	VWR International GmbH, Darmstadt, Germany
KH_2PO_4	VWR International GmbH, Darmstadt, Germany
Methanol LiCHrosolv® for liquid chromatography	VWR International GmbH, Darmstadt, Germany
Mouse monoclonal anti-rabbit IgG conjugated to horseraddish peroxidase	Sigma, Taufkirchen, Germany
m-Phosphoric acid	VWR International GmbH, Darmstadt, Germany
Na_2HPO_4	VWR International GmbH, Darmstadt, Germany
Na_2HPO_4 x 2 H_2O	VWR International GmbH, Darmstadt, Germany
$NaBH_4$	VWR International GmbH, Darmstadt, Germany
NaCl	VWR International GmbH, Darmstadt, Germany
NaH_2PO_4	VWR International GmbH, Darmstadt, Germany

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Product	Supplier
n-Hexane LiCHrosolv®	VWR International GmbH, Darmstadt, Germany
o-Phenylendiamine	Sigma, Taufkirchen, Germany
o-Phosphoric acid	VWR International GmbH, Darmstadt, Germany
Perchloric acid (20%) Sulfuric acid (2.5 M)	Sigma-Aldrich, Steinheim, Germany VWR International GmbH, Darmstadt, Germany
Tocol	Matreya, Inc. State College, PA, USA
Triethylamine, min. 99%	Sigma-Aldrich, Steinheim, Germany
Tween 20	VWR International GmbH, Darmstadt, Germany
"Vitamin C, crystalline & pure ascorbic acid"	Krüger, Bergisch-Gladbach, Germany

Table C.2: Kits

Product	Supplier
1,25 Dihydroxy Vitamin D EIA	Immunodiagnostic Systems (IDS) GmbH
ABX Pentra Cholesterol CP	Axon Lab AG, Stuttgart, Germany
ABX Pentra LDL Direct CP	Axon Lab AG, Stuttgart, Germany
ABX Pentra Triglycerides CP	Axon Lab AG, Stuttgart, Germany
ABX Pentra Multical	Axon Lab AG, Stuttgart, Germany
ABX Pentra LDL Cal	Axon Lab AG, Stuttgart, Germany
ABX Pentra CRP CP	Axon Lab AG, Stuttgart, Germany
ABX Pentra CRP Cal	Axon Lab AG, Stuttgart, Germany
Bio-Rad® protein assay	BioRad Labarotories, Hercules, California, USA

Table C.3: Disposables

Product	**Supplier**
15 mL plastic conical tubes	Greiner bio-one, Frickenhausen, Germany
50 mL plastic conical tubes	Greiner bio-one, Frickenhausen, Germany
autosampler vial caps	VWR International GmbH, Bruchsal, Germany
EDTA-coated S-Monovettes®	Sarstedt AG & Co. Nuembrecht, Germany
Serum-Monovettes®	Sarstedt AG & Co. Nuembrecht, Germany
Micro tube 1 mL 9NC	Sarstedt AG & Co. Nuembrecht, Germany
Micro tube 1 mL EDTA coated	Sarstedt AG & Co. Nuembrecht, Germany
Multi-Fly set	Sarstedt AG & Co. Nuembrecht, Germany
Pierce SnakeSkin T Dialysis Tubing, 10 K MWCO	PERBIO SCIENCE , Deutschland GmbH Bonn, Germany
Nunc Immuno Plate Maxisorb 96-well plate	Nunc GmbH & Co. KG, Wiesbaden, Germany
Polypropylene autosampler vial	VWR International GmbH, Bruchsal, Germany
PS Microplate 96 flat bottom well	Greiner bio-one, Frickenhausen, Germany
Surgical Toothbrush, select TE 227 special care	Medico-Service Beyer GmbH, Herdorf, Germany

Table C.4: Equipment and software

Product	**Supplier**
Automatic Environmental1010 SpeedVac® System AES mit VaporNet®	Savant Instruments, Inc., Holbrook, NY, USA
Bio Kinetics Microplate Reader EL 340	BioTekR Instruments, Vermont, USA
Cobas Mira S	Roche, Grenzach-Wyhlen, Germany
Labofuge 200	Kendro Laboratory, International Products Sales, Hanau, Germany
ProStar Pump 210	Varian Deutschland GmbH, Darmstadt, Germany
Scanning Fluorescence Detector Waters	Waters, Arcade, New York, USA
Spherisorb ODS-2 column, 3 μm, 250 x 4,6 mm	Grom, Rottenburg-Hailfingen, Germany
SPSS 11.0 for Windows	SPSS Inc., Chicago, IL, USA
STAR chromatography workstation Version 5.31 HPLC software	Varian Deutschland GmbH, Darmstadt, Germany
UV-VIS Dual λ Absorbance Detector Waters 2487	Waters, Arcade, New York, USA

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Declaration

I hereby certify that the present diploma thesis with the title "Detection and improvement of fat soluble vitamin status in patients with short bowel syndrome using two different supplement formulations" was written independently and that all references and aids which were required are indicated.

This thesis was not submitted in the same or a similar form to any other board of examiners.

Erklärung

Hiermit bestätige ich, dass ich die vorliegende Diplomarbeit mit dem Titel "Detection and improvement of fat soluble vitamin status in patients with short bowel syndrome using two different supplement formulations" selbstständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt habe.

Diese Diplomarbeit wurde in gleicher oder ähnlicher Form in keinem anderen Studiengang als Prüfungsleistung vorgelegt.

Ort, Datum

Unterschrift