IMMUNOLOGICAL PERSPECTIVES ON **NUTRITIONAL SUPPORT** DURING CANCER

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COVER
Different types of white blood cells (immune cells)

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IMMUNOLOGICAL PERSPECTIVES ON NUTRITIONAL SUPPORT DURING CANCER

Een immunologisch perspectief op voedingsondersteuning tijdens kanker

(met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op

woensdag 11 januari 2012 des middags te 2.30 uur.

Door
Joyce Faber

Geboren op 17 december 1979 te Hengelo

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PREFACE

Cancer! An awful disease which is diagnosed in more than 5 million people per year worldwide. Everyone will know somebody with cancer in its immediate environment. Some people will feel perfectly healthy, others will feel seriously ill. Nevertheless, the diagnosis of cancer will have a big impact on the patients' life, family and friends. With the research that I performed in the past 6 years at Nutricia Advanced Medical Nutrition on the immunological aspects of nutritional support during cancer, I hope to further contribute to the development of new products for cancer patients. My aim is to support these people during their disease, during their treatment and during the many associated complications they experience. Some people can be cured and some people will die within weeks months or even years, but it is important to acknowledge that the major goal is to provide optimal nutritional support and to give them the best possible quality of life.

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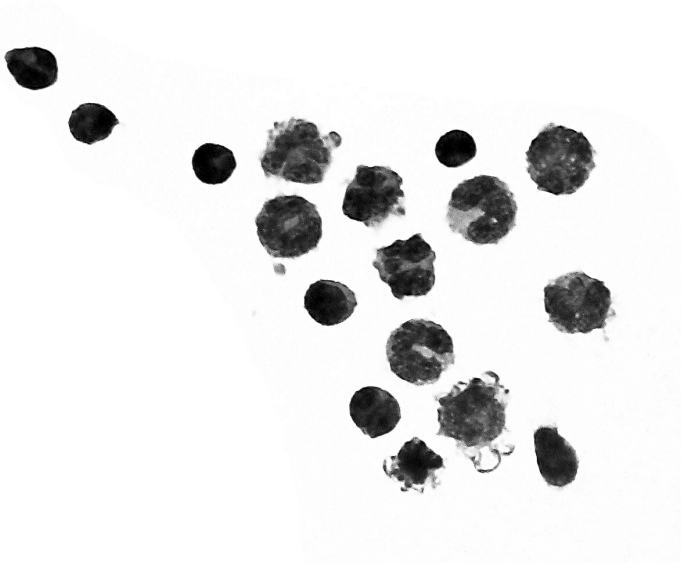
CHAPTER ONE



Introduction

CANCER

DIAGNOSIS CANCER

Cancer is a collective name for more than one hundred types of distinct diseases. Nevertheless, a common feature of the different types of cancer is the uncontrolled growth of "abnormal" cells. In the human body, regulatory mechanisms exist to ensure the controlled growth and development of new cells and for death (apoptosis) of damaged or outdated cells. Once alterations arise in the cellular genome that affect the expression or function of genes controlling cell growth and differentiation, a normal cell can become a cancer cell (initiation phase, Figure 1) and a tumor might develop (promotion phase). The nature of a tumor can be benign or malignant. Malignant tumors continue to grow and will, by definition, invade surrounding tissue and organs (progression phase). In addition, certain tumor cells can spread throughout the body by metastasizing to other organs via the bloodstream or lymph vessels, growing out to new tumors (metastasis phase). Not all cancers will lead to the development of a solid tumor in tissue or organs, non-solid tumors can appear in the blood, the lymphatic system or in the bone-marrow fluid and often spread throughout the whole body (1-4).

The immune system can specifically identify and eliminate tumor cells on the basis of the expression of tumor-specific antigens, and destroy them before they can cause harm (immune surveillance) (5). Consequently, to develop cancer, six essential alterations have to occur at the tumor site, that are shared by almost all cancer types (6). Tumor cells have to be self-sufficient in producing growth signals, insensitive to growth inhibitory signals, able to evade programmed cell death (apoptosis), unrestricted in the potential to replicate,

able to sustain angiogenesis and able to invade other tissues by invasive growth or metastasis. Fortunately, due to the multitude and diversity of alterations that are necessary to sustain malignant growth and the existence of redundant control and repair mechanisms, the occurrence of a cancer lesion is a relatively rare event in a human lifetime (6).

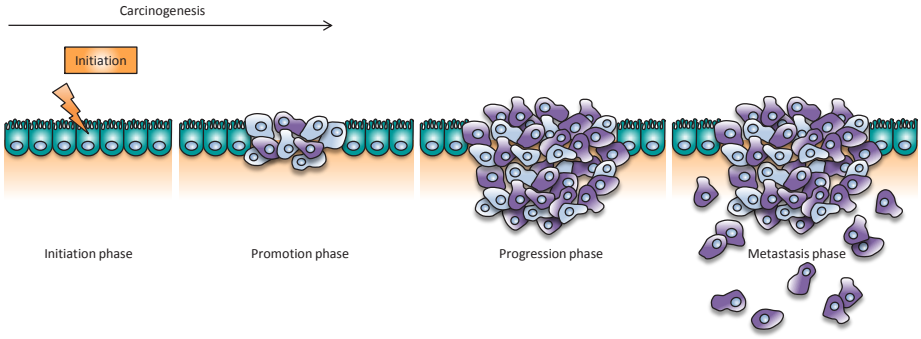


Figure 1 Carcinogenesis.

Incidence

In the year 2008, worldwide 6.6 million men and 6.0 million women developed a malignant tumor and altogether 7.5 million people died from cancer (7). Lung cancer is the most common cancer worldwide, accounting for 1.6 million new cases annually; followed by cancer of the breast, 1.4 million; colorectal cancer, 1.2 million; stomach cancer, 1.0 million and prostate cancer, accounting for 0.9 million new cases per year. The three leading cancer killers are however different from the three most common types, with lung cancer responsible for 17.8% of all cancer deaths; stomach, 10.4% and liver, 8.8%. Significant differences can be observed in both cancer incidence and mortality between male and female (Figure 2). But, because cancer is a collective name for several distinct diseases that occur at different ages, these numbers will change with age (7).

The global incidence of cancer is predicted to increase from 12.6 million people in 2008 to 15.5 million in 2030 accompanied by an increase of 50% in cancer deaths from 7.5 million deaths in 2008 to 11.5 million deaths in 2030. This increase is mainly due to a steadily ageing populations in both developed and developing countries and also to current trends in smoking prevalence and the growing adoption of unhealthy lifestyles (8).

Classification and disease staging

The different types of cancer can be classified in two ways; by the type of tissue in which the cancer originates (histological type) and by primary site (location in the body where the cancer first developed). On basis of histological type, the different cancer types can be divided into six categories: carcinoma (internal or external lining of the body, e.g. epithelium), sarcoma (connective tissues, e.g. bones, muscle, fat), myeloma (bone marrow), leukemia (blood cancers), lymphoma (glands or lymph nodes, e.g. spleen, tonsils) and mixed types (cancer types from different categories, e.g. adenosquamous carcinoma, carcinosarcoma) (9, 10).

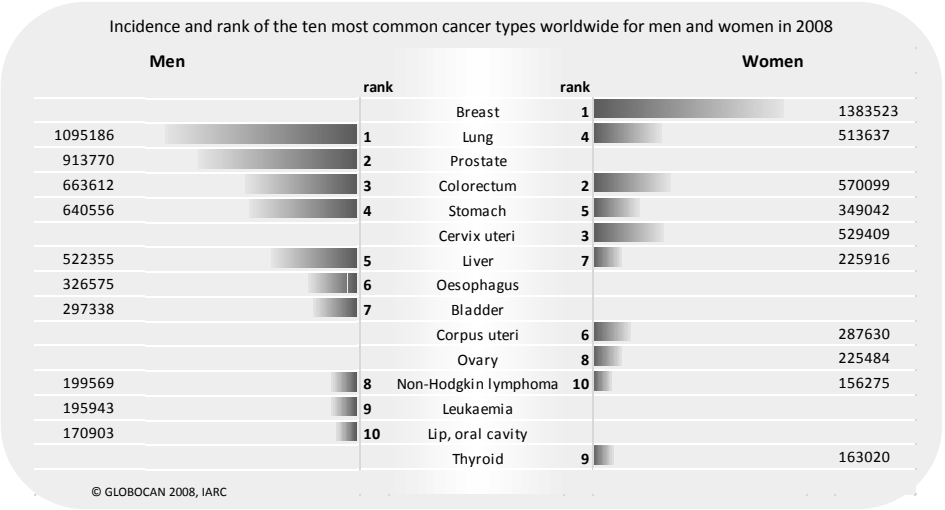


Figure 2 Incidence and rank of the ten most common cancer types worldwide for men and women in 2008 (GLOBOCAN 2008, IARC, WHO).

For most cancer types, the stage of the disease is expressed using the TNM classification, an anatomically based system that primarily records the primary and regional nodal extent of the tumor and the absence or presence of metastases (11). Each individual aspect of TNM is termed a category: the T category describes the extent of the primary tumor, the N category describes the regional lymph node involvement and the M category describes the presence or otherwise of distant metastatic spread. Once the values for T, N and M have been determined, they are combined to an overall stage, in general a Roman number from I to IV. For example, a T1, N0, M0 breast cancer indicates that the primary breast tumor is less than 2 cm across (T1), does not have lymph node involvement (N0), and has not spread to distant parts of the body (M0). This would make it a stage I cancer (12). However, criteria for stages differ for different types of cancer; bladder cancer T3 N0 M0 is stage III, whereas colon cancer T3 N0 M0 is stage II.

CAUSES OF CANCER

The development of cancer is a multifactorial process, there is no single cause for any one type of cancer. Genetic factors are involved in this process, as well as lifestyle factors including tobacco use, diet and physical activity, certain types of infections and environmental exposure to carcinogenic substances and radiation (2, 3, 8, 13). Ageing is another fundamental factor in the development of cancer. The incidence of cancer increases with age, most likely due to an accumulation of risk factors in combination with less effective repair mechanisms in older people (8). Moreover, also immune suppression (caused by either primary or secondary immunodeficiencies) and therapy to prevent transplant rejection are often associated with a heightened risk of cancer. Immune suppression to prevent transplant rejection is clearly associated with a heightened risk (3- to 100-fold increase) of developing certain types of cancer (5).

Genetic factors

In some families, persons have a genetic predisposition for a specific type of cancer and are already born with a genetic mutation. This will increase the risk to develop cancer during their life significantly. For example, women who carry one of the breast cancer genes BRCA1 and BRCA2 have a higher chance to develop breast cancer than women who do not (1, 14). But on the other hand, a minority of 5-10% of all breast cancers can be ascribed to these genes, so most breast cancers are not caused by a specified genetic predisposition (14).

Lifestyle factors

Nowadays, lifestyle factors are recognized as major risk factors for cancer. Smoking is responsible for almost 9 out of 10 lung cancer deaths. Lung cancer is the leading cause of cancer deaths in both men and women, and is one of the hardest cancers to treat. However, smoking is also related to other types of cancer such as head- and neck cancers, liver cancer and pancreatic cancer and is estimated to cause 21% of the cancer deaths worldwide (15, 16). Alcohol abuse is another risk factor for cancer, it might lead to a higher risk of liver cancer, head and neck cancers and breast cancer (15) and causes 5% of the cancer deaths worldwide. The Western lifestyle is characterized by a highly caloric diet, rich in fat, refined carbohydrates and animal proteins, combined with low physical activity, resulting in an overall energy imbalance and eventually in obesity. Malignancies associated with these diet related factors are colorectal cancers, breast cancer, cancer of the uterus, gallbladder and kidney but also prostate cancer and esophageal cancer are frequently observed. Moreover, low fruit and vegetable intake may increase the risk on colorectal cancer, stomach cancer, lung cancer and esophageal cancer and is estimated to cause 5% of the cancer deaths worldwide (15, 17, 18).

Infections

Certain types of infections can play a role in the development of specific cancer types. Bacterial infections as for example *Helicobacter pylori,* are one of the major causes of gastritis and have proven to be related with the occurrence of stomach cancer. In addition, infections with the Human Papilloma Virus (HPV) or the Hepatitis B and C viruses increase the risk of cervical and liver cancer, respectively (19, 20).

Environmental factors

The environmental exposure to carcinogenic chemicals and radiation is described as a major risk factor as well, occurring mostly in an occupational setting. Examples of carcinogenic chemicals include asbestos, benzene and arsenic (21), in which asbestos is highly related to cancer of the lung and pleural mesothelioma (22) that appear most of the times in mine workers, plumbers, pipe fitters and in the insulation and asbestos cement industry. Benzene can induce leukemia and is frequently used as a solvent or chemical in the pharmaceutical industry as well as in the printing industry (23). Examples of radiation are exposure to X-rays, Y-rays, neutrons and radon which can cause bone cancer, leukemia, as well as lung, liver and thyroid cancer, which are frequently observed in radiologists, nuclear workers and aircraft crew (24). The most commonly known example of environmental

cancer triggers is ultraviolet (UV) radiation. The primary source of UV radiation is sunlight, but tanning lamps and booths are also sources of UV radiation. Exposure of light from these sources is linked to a higher risk for both melanoma as well as non-melanoma skin cancers (25).

Prevention and early detection

In total, 35% of all cancers can be prevented by avoiding above mentioned risk factors (e.g. smoking, alcohol) and by making healthy lifestyle choices (e.g. high consumption of fruit and vegetables)(15). Moreover, it is important to control occupational hazards (asbestos, radiation), as occupational cancers are largely preventable (26), but it is also true for skin cancers which can be inhibited by less exposure to sunlight. Furthermore, the clinical course of the disease may be improved by early detection, diagnosis and treatment. Therefore, it is important to recognize early signs of cancer and to have screening programs in place to identify early cancer stages before symptoms become overt, including cytology tests for cervical cancer and mammography for breast cancer (27, 28). For most of these cancers early detection has been shown to reduce the number of deaths (29).

CANCER TREATMENT

The purpose of cancer treatments is to cure patients, prolong life or improve quality of life for the patients. The most common treatments used alone or in combination are surgery, chemotherapy, radiotherapy, hormone therapy, bone marrow- and stem cell transplants and immunotherapy (or biological therapy). Once patients are diagnosed with cancer, the different treatment options are discussed. The treatment schedule is designed based on the type and stage of cancer but other important factors include the overall health of the patient, its age, performance status, side effects of the treatment and the probability of curing the disease, extending life or relieving symptoms (1, 30-32).

Surgery

Many cancer patients will undergo some type of surgery as it offers optimal curative potential in many types of cancer. Initially, preventive surgery can be performed to remove body tissue that is at risk for developing a tumor, due to the presence of a genetic predisposition, even though there are no signs of cancer at the time of surgery (33). Surgery is used also to diagnose cancer or to determine the stage of cancer (34). In relation to cancer treatment, surgery is mostly used to remove the tumor and an area of healthy tissue surrounding it, also known as a clear margin or clear excision, in order to prevent the cancer from recurrence (35). In the case that curative resection is not possible, surgery is used to treat problems caused by advanced cancer. Finally, reconstructive surgery is used to improve the look or to restore the function of an organ or body part after an earlier tumor removal, for example a breast reconstruction after removal of the breast due to breast cancer (36).

Radiotherapy

Radiotherapy is commonly applied for the treatment of cancer and makes use of high energy X-rays or other types of radiation with the aim to destroy the cancer cells in a specific treatment area. Since the repair mechanisms of cancer cells are less effective and

they divide faster than normal cells, cancer cells are destroyed selectively.

Radiotherapy is applied to the patient frequently; about 40% of the patients receive radiotherapy as part of their treatment (37). Radiotherapy can be applied in two ways, either externally or internally (38). External radiotherapy is administered using a machine outside the body that directs its radiation bundle on a specific location of the body affected by the tumor. The radiotherapy destroys the cancer cells by damaging the DNA in the cells so they are unable to multiply (39). By contrast, internal radiotherapy makes use of a radioactive substance sealed in needles, seeds, metal wires or tubes which are placed directly into or near the tumor (brachytherapy). The radioactive material is left in the body for a period of time, e.g. minutes or days, depending on the type of cancer and radiotherapy. The radioactivity from most implants only beams a few millimetres through the body, while for other internal radiotherapies patients have to be isolated and avoid contact with other people during the presence of the radioactive material in the body. In addition to the implants, internal radiotherapy can be given by a drink as well. Examples include radioactive phosphorus in blood disorders or radioactive strontium in some secondary bone cancers.
Besides these two types of radiotherapy, it is also possible to irradiate the total body which is sometimes performed in patients suffering from leukemia or lymphoma having a bone marrow or stem cell transplantation. In this case, all bone marrow cells will be destroyed before transplantation with either autologous bone marrow cells or bone marrow cells from a donor (37, 38).

Chemotherapy

Unlike surgery and radiotherapy, which are in most cases considered local treatments, chemotherapy is a systemic treatment and affects the entire body. Currently, there are more than 100 different chemotherapeutic drugs available which can be used separately or in combination and new types are continuously being developed (40, 41). Chemotherapy is most commonly given by tablets or intravenously and targets rapidly dividing cancer cells (32, 42).

Chemotherapeutics can be divided into several groups based on their working mechanism and their chemical structure, but with a common goal to prevent cancer cells from multiplying, invading, metastasizing, and ultimately killing the host. The major categories of chemotherapeutic agents are alkylating agents, antimetabolites, anthracyclines, plant alkaloids, antitumor antibiotics, taxanes, and monoclonal antibodies (43). Most of these agents exert their effect primarily on cell multiplication and tumor growth. They interfere with the synthesis of either DNA, RNA, or proteins, or with the appropriate functioning of the preformed molecule. Many cells die because of the direct effect of the chemotherapeutic agent, while in other cases, the chemotherapy may trigger differentiation, senescence or apoptosis, affecting the mechanism of programmed cell death (1, 43-45). However, because cell division is a characteristic of many normal cells as well, most chemotherapeutic agents have a toxic effect on normal cells as well. In particular cells with a rapid turnover rate, such as cells in hair follicles, bone marrow cells and cells in the lining of the digestive system, which are constantly renewing themselves, carry a risk of chemotherapy-induced side effects as hair loss and diarrhea (32, 44-46). Therefore, each time chemotherapy is prescribed, a balance has to be found between destroying the cancer cells (in order to cure or control the disease) and sparing the normal cells.

Combination therapy

To maximize the chance to destroy all cancer cells and to decrease the risk of cancer recurrence, different types of cancer treatment can be combined. Chemotherapy or radiotherapy can be applied before surgery to shrink the tumor and make it easier to remove, or to reduce the risk of spreading during surgery. This type of treatment is called neoadjuvant treatment and is frequently used in different types of cancer. Chemotherapy and radiotherapy can be applied after surgery as well; then it is called adjuvant treatment and it is applied with the aim to destroy any remaining cancer cells that may have been left after the operation (47).

Palliative care

In an early stage of cancer, the above described treatments are applied with a curative intention. However, in more advanced cancer stages, the same treatment options are used, but with another goal. Palliative care is aimed to relieve disease symptoms and to improve length and quality of life. Important goals are the prevention and relief of suffering by means of early identification and optimal treatment of pain and other problems (e.g. side effects of cancer treatments) (48). Palliative care can consist of diverse types of treatment, including application of analgesics, anti-nausea drugs, chemotherapy, radiotherapy and even surgery. The ultimate goal of such an approach is to shrink the tumor, relieve the symptoms and to give the patient a longer but acceptable life (37, 49).

Complications and side effects

Cancer treatment can induce a large number of complications and side effects, especially due to the use of increasingly aggressive methods during the last 20 years (50). This can affect quality of life of the patient and may influence the length of hospital stay (51). However, the most commonly observed side effects differ per type and duration of treatment. The most common chemotherapy-related side effects include fatigue, alopecia, anorexia, alterations in taste and smell, nausea, vomiting, mucositis, constipation, diarrhea, early satiety and weight loss, resulting in a decreased performance status impairing the ability to work or to perform household duties (52-54). Moreover, chemotherapy can suppress the function of the patient's immune system, leading to a higher risk of infectious complications and fever. The side effects of radiotherapy are determined by the type, quantity, duration and site of exposure to the body, since areas of highly proliferative cells, as the gastrointestinal (GI) mucosa and hair follicles, are most vulnerable to side effects (1, 39, 55). Radiation of the head and neck area is associated with anorexia, stomatitis, nausea, xerostomia, vomiting, dysphagia and alterations in taste and smell, whereas radiation of the abdomen may cause diarrhea, mucositis and colitis. When a larger area is radiated, a general malaise with fatigue, nausea, vomiting and immune suppression may occur. Side effects of surgery can be diverse, but patients generally suffer from fatigue up to 8 weeks after the operation. Moreover, surgery may have several nutritional implications, leading to weight loss and malabsorption of essential nutrients and vitamins. Surgery-related complications can occur as well; bleedings, wound infections and pneumonia are most often observed (56).

The survival of cancer patients strongly depends on the type of cancer as well as on the stage in which the disease is diagnosed. Approximately 50% of all cancer patients can be

cured from the disease, but many others will die after some months, years or even decades (7). Statistics demonstrate that the survival of cancer patients has improved significantly in the past decades. Nowadays, cancer is more often diagnosed in an early stage, and prognostic factors as malnutrition and CRP are defined leading to a more effective treatment approach (57-59). In addition to a higher survival, quality of life during treatment has improved as well compared to previous decades. Surgical techniques have improved, chemotherapeutics with fewer side effects are available and new treatment procedures are continuously being developed.

CANCER-RELATED MALNUTRITION

The majority of patients with advanced cancer experience involuntary weight loss at some points during the course of their disease, which can be caused by anorexia, reduced appetite, treatment-related malnutrition, or cancer cachexia (wasting disease) (60, 61). Cachexia is a term derived from the Greek words kakos, meaning bad, and hexis, meaning condition. Cancer cachexia is a complex metabolic, multi-factorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment. The pathophysiology is characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism. However, details of its definition have been the topic of a long-lasting and ongoing debate (60, 62-66). Tumor-derived factors, therapeutic strategies, but also nutritional status, age and even stress and depression are involved in this process, resulting in a chronic inflammatory state and impaired immune responsiveness (62, 67, 68). Weight loss is observed in up to 80% of cancer patients and is associated with a poor prognosis, leading to reduced survival rates in these patients (62, 63, 69, 70).

PREVALENCE OF CANCER CACHEXIA

The process of cancer cachexia starts early. In the literature it has been described that 85% of patients with pancreatic or stomach cancer and 60% of patients with lung cancer have experienced weight loss by the time of diagnosis and in 30% of the patients this body weight loss is severe (71, 72). In addition, metabolic alterations leading to cancer cachexia are already present before diagnosis (73) and recent findings show that impaired immune responsiveness and muscle protein degradation may even occur already before the onset of weight loss (74, 75).

The global incidence of malnutrition during the course of cancer ranges from 30% to 90% and depends on type, location, stage and spread of the tumor as well as on anti-cancer treatment, age, gender and individual characteristics (76-78). Weight loss is most apparent in patients suffering from stomach (83%) and pancreatic cancer (83%), but also in patients with esophageal cancer (79%), head and neck (72%) and lung cancer (50-61%) the incidence is high (77). Patients with sarcomas (39-66%), testicle cancer (25%) and breast cancer (9-36%) have the lowest frequency of weight loss. Moreover, anti-cancer treatment can negatively affect body weight (63, 79), as described by Langius et al. in early stage laryngeal cancer patients receiving radiotherapy (80) and by chemotherapy-induced nausea and vomiting as described by Navari et al (81).

CAUSES OF CANCER CACHEXIA

Weight loss in cancer patients is due to depletion of both adipose tissue as well as skeletal muscle mass, while the non-muscle protein compartment is relatively preserved, thus distinguishing cachexia from simple starvation (82). In case of starvation (weight loss due to caloric deficiency), metabolism shifts from glucose to fatty acids and ketone bodies as a fuel source, thereby protecting lean body mass. By contrast, in cancer cachexia, nutrients are used inefficiently and lean body mass decreases significantly (83).

However, an inadequate intake of energy and nutrients by itself does not account for the changes in nutritional status observed in cachectic cancer patients. Cachexia is considered a multi-factorial syndrome, caused by an inadequate food intake and metabolic alterations as summarized in Figure 3.

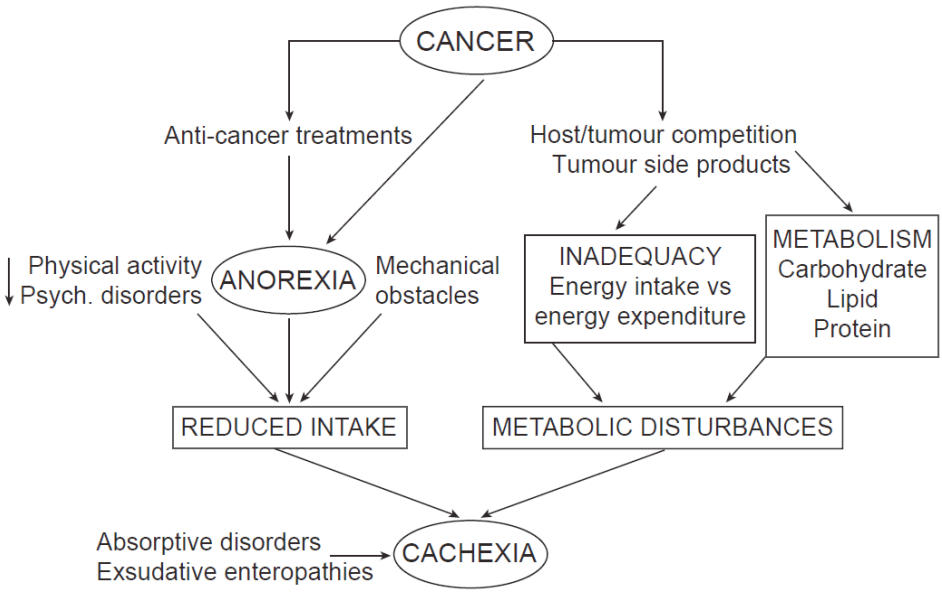


Figure 3 Multi-factorial causes of cancer cachexia, adapted from Nitenberg et al (76).

Food intake

Food intake is frequently reduced in cancer patients due to disease- and treatment-related factors affecting both energy and protein intake, including systemic effects of the disease (e.g. anorexia, alterations in taste and smell, nausea, vomiting, pain), local effects of the tumor (e.g. odynophagia, dysphagia, gastrointestinal obstruction, early satiety, malabsorption), psychological factors (e.g. fear, depression, anxiety) and side effects of anti-cancer treatment (e.g. radiation-induced tissue damage, cytotoxic drugs). Moreover, surgery of the oral cavity, esophagus and the gastrointestinal tract can significantly reduce nutritional intake and status, resulting in impaired mastication, dysphagia, taste changes, early satiety, regurgitation, malabsorption, and diarrhea in these patients (84-87).

Metabolic alterations

The metabolic alterations that are associated with cancer cachexia involve numerous organs, affecting energy expenditure and the metabolism of carbohydrates, protein and fat,

although the underlying mechanisms are still not fully understood (86, 88). In the literature, resting energy expenditure (REE) of cachectic patients with advanced pancreatic cancer was increased compared to the predicted values for healthy individuals, whereas total energy expenditure remain unchanged due to a reduction in physical activity (89). Moreover, an elevated REE was described in cachectic lung cancer patients as well, whereas in patients suffering from gastric or colorectal cancer no changes on REE were observed (90). Although the underlying mechanism still needs to be resolved, there is a general consensus that cachectic patients are often (mildly) hypermetabolic, indicated by a higher REE (140-290 kcal/day) (91-93). If this increase in expenditure is not compensated by an increase in caloric intake, this can result in loss of up to 1 kg body fat and/or loss of more than 2 kg muscle mass per month (93).

Changes in carbohydrate metabolism appear similar to type II diabetic patients. Cancer patients have an increased turnover and production of glucose by the liver (gluconeogenesis) formed by the increased and highly efficient conversion of lactate, which is produced in the relative low-oxygen environment of the tumor and contributes to a high REE of the patients (94). Moreover, the insulin-stimulated glucose uptake by muscles is decreased due to peripheral insulin resistance and redirects glucose to the liver (95). Alterations in protein metabolism of cancer patients include nitrogen depletion and skeletal muscle degradation through activation of the ATP-ubiquitine-proteasome pathway. In addition, the unavailability of glucose to the muscles leads to oxidation of amino acids and consequently to the loss of lean body mass (86, 93, 96). This results in specific alterations in the plasma amino acid profile with decreased levels of arginine, valine and leucine and increased levels of tryptophan (93, 97). The altered lipid metabolism of cancer patients is characterized by an increased lipolysis (the breakdown of body fat) and lipid oxidation, a decreased lipogenesis and an overall depletion of fat stores (98). Moreover, an increased activity of lipoprotein lipase, an enzyme required for triglyceride clearance, has frequently been observed in cancer patients, leading to breakdown of adipose tissue (99). Another important factor in the metabolic alterations associated with cancer cachexia is the hepatic acute-phase response (APR). An APR is seen n half of the cancer patients at presentation, and is most often determined clinically by raised plasma C-reactive protein (CRP) concentrations, although the synthesis of other hepatic proteins (e.g. serum amyloid A, fibrinogen, α1-acid glycoprotein, α1- antichymotrypsin and haptoglobulin) is increased as well. The APR consists of a complex series of reactions, initiated in response to infection, physical trauma or malignancy. These reactions prevent ongoing tissue damage, isolate and destroy infective microorganisms and activate the repair process in order to restore normal function (100). The APR is triggered by the pro-inflammatory cytokines Interleukin (IL)-6, IL-1β, Tumor Necrosis Factor (TNF)-α and Interferon (IFN)-γ and is linked to adverse outcome and shortened survival in advanced cancer patients (101).

Potential mediators

Several mediators that are either tumor- or host-derived have been implicated in the pathogenesis of cancer cachexia. Tumor-derived factors such as proteolysis-inducing factor (PIF), have been described to cause breakdown of skeletal muscle (proteolysis) and weight loss (90). PIF was shown to induce muscle protein degradation through an increased

activity and expression of the ubiquitin-proteasome pathway, consequently leading to the breakdown of skeletal muscle proteins (63, 90). Lipid-mobilizing factor (LMF), another tumor-derived factor, has been detected in the urine of patients with cancer and has been shown to stimulate lipolysis, increase metabolic rate and energy expenditure and cause loss of adipose tissue in mice (102, 103). Moreover, serum and urinary levels of LMF has been correlated with weight loss in cancer patients (104).

As already mentioned, several pro-inflammatory cytokines are involved in the metabolic disturbances associated with cancer cachexia (63, 105-107). Pro-inflammatory cytokines are protein mediators that are secreted by tumor cells, immune cells and other cells. These cytokines stimulate the APR, but are also able to exert a variety of nutritional, behavioral and physiologic effects in addition to their immunological functions. Although no consensus exists for the exclusive roles of any of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ (also called pro-cachectic cytokines), it has become clear that overlapping biological activities and synergistic interactions between them lead to a progressive cachectic state (Figure 4) (69, 105, 108).

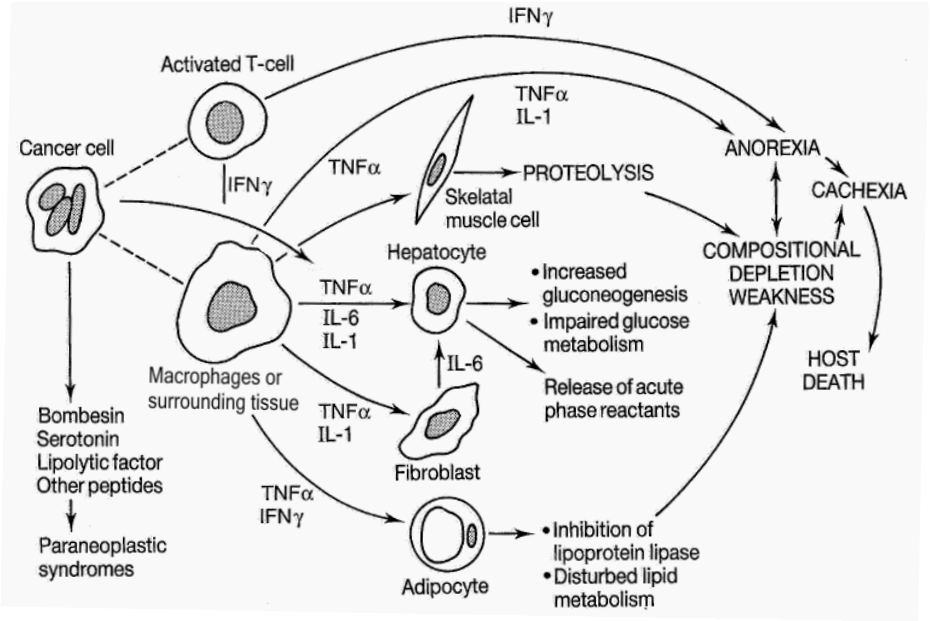


Figure 4 The central role of cytokines in the (patho)physiological processes leading to the development of cancer cachexia, adapted from Bozzetti et al (93).

Indirect evidence for the role of the pro-inflammatory cytokines is based on the observation that administration of these cytokines can produce similar host responses as observed in cancer cachexia (109). For example, in preclinical studies in healthy animals, injection of exogenous IL-1β and TNF-α caused increased energy expenditure, protein turnover and weight loss. In addition, improved food intake, whole lipid content, lean tissue retention and decreased tumor volume have been observed in tumor-bearing animals after an injection of neutralizing antibodies against TNF-α, IL-1β, IL-6 and IFN-γ (63). Moreover, several alterations in gastrointestinal function, indirectly affecting the nutritional status, have been

ascribed to the release of pro-inflammatory cytokines, including changes in gastric emptying and changes in small bowel motility (83).

Clinical data suggest that pro-inflammatory cytokines play a key role in the promotion of cancer cachexia since serum IL-6 levels are increased in malnourished patients with colorectal cancer compared with well-nourished patients and are associated with hypermetabolism and weight loss in patients with lung cancer. In addition, elevated serum levels of TNF-α are associated with malnutrition and reduced quality of life in gastric cancer patients (63, 110). Moreover, cytokines as IL-1β and TNF-α have been suggested to be involved in cancer-related anorexia, affecting food intake directly or through other mediators as corticotrophin-releasing hormone, serotonin or leptin (83, 109). However, the predominant effect of the pro-inflammatory cytokines is local and may become more important than the actual circulating levels of the cytokines, at least in humans (83, 109). Nevertheless, the production of the pro-inflammatory cytokines can lead to a severe inflammatory state, which may play a central role in cancer progression and the reduced immune responsiveness observed in cancer patients as described in detail later on.

CONSEQUENCES OF CANCER CACHEXIA

Cancer cachexia occurs in the majority of cancer patients and is a major contributor to morbidity and mortality during advanced disease (62, 63). It has been estimated to account for 10–30% of cancer deaths, but might also contribute to other death causes such as opportunistic infections (111-113). Tumor and host-derived factors, therapeutic strategies, but also nutritional status, age and even stress and depression are involved in this process, resulting in weight loss, a chronic inflammatory state and impaired immune responsiveness (62, 67, 68). Immune suppression is a major problem in these cancer patients leading to disease progression, increased complications and a delayed or suboptimal treatment protocol (e.g., surgery, chemotherapy, radiotherapy) resulting in a reduced quality of life and a poor prognosis (58, 67, 68, 114, 115). Moreover, cachectic cancer patients were described to have a reduced functional capacity and performance status caused by reduced muscle function (58, 72, 116). This might lead to a prolonged hospital stay, higher prescription and consultation rates, eventually leading to higher costs (117-120).

CANCER AND THE IMMUNE SYSTEM

The immune system has three primary roles in the action against the development of cancer. First of all, the immune system can specifically identify and eliminate tumor cells on the basis of their expression of tumor-specific antigens and destroy them before they can cause harm (immune surveillance). Secondly, the immune system can protect the host from virus-induced tumors by eliminating or suppressing viral infections. Thirdly, the elimination of pathogens and the prompt resolution of inflammation can prevent the establishment of an inflammatory environment, which is favorable in tumor development (5).

CANCER AND INFLAMMATION

In 1863 Rudolf Virchow was the first describing a link between inflammation and cancer and he suggested, based on the finding of leucocytes in neoplastic tissue, that the

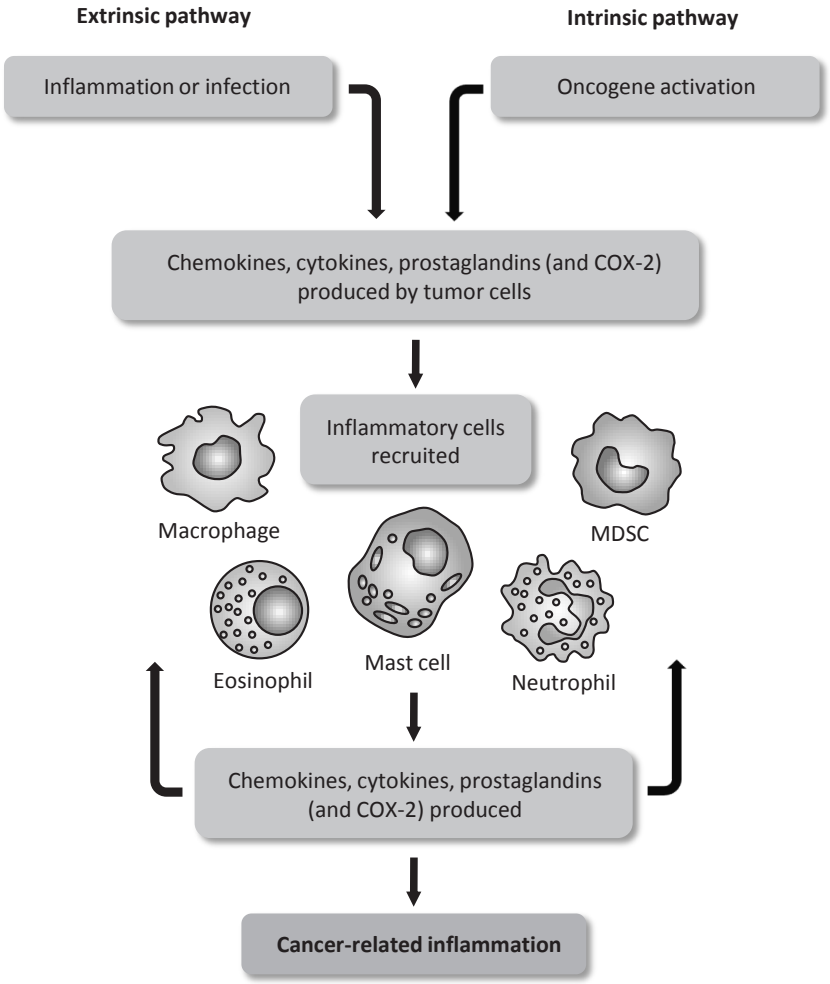


Figure 5 Pathways that connect inflammation and cancer, partly adapted from Mantovani et al (125).

tumorigenesis is generally accepted and it has become evident that an inflammatory microenvironment is an essential setting for all tumors, including some in which a direct relationship with infections has not been proven yet (122). As already mentioned before, a minor part of all cancers is primarily caused by hereditary factors, whereas the majority are linked to environmental and lifestyle factors (e.g. chronic infections, tobacco smoking and asbestos) which are associated in diverse ways with the process of chronic inflammation (123). Chronic inflammation may be one of the driving forces for the initiation and development of cancer. Moreover, once inflammatory conditions are present at the tumor site, they may further support the progression of the tumor into more advanced stages and also promote metastasis (124).

Acceleration of tumor development by chronic inflammation can be observed in many types of cancer (extrinsic pathway). For example, persistent Helicobacter pylori infection is associated with gastric cancer and infections with hepatitis B or C viruses increase the risk of hepatocellular carcinoma, respectively (123, 124). The cancer-associated chronic

inflammation is the result of normal protective mechanisms in order to eliminate pathogens. Inflammatory cells are recruited by both host- and tumor-derived factors, of which macrophages are predominantly present (Figure 5). Once activated, macrophages produce a large variety of bioactive products, providing an important defense mechanism against tumor cells. Subsequently, progression to an overall inflammatory state is accompanied by the production of several pro-inflammatory mediators, including cytokines, chemokines, prostaglandins and reactive oxygen/nitrogen species. In general, these processes are self-limiting, however, dysregulation of any of the converging factors can lead to a continuous stimulation and eventually to pathogenesis and progression towards advanced disease (122, 124).

However, also in cancer without an underlying primary inflammatory condition, in which neoplasia is caused by genetic events as a mutation, chromosomal rearrangement or amplification and the inactivation of tumor-suppressor genes, inflammation plays a central role (intrinsic pathway). Transformed cells produce several pro-inflammatory mediators as well, thereby generating an inflammatory microenvironment comparable to the extrinsic pathway. The inflammatory mediators have been characterized as key players in tumor promotion, since they are able to induce a variety of factors that support angiogenesis, tumor cell growth and tissue remodeling. In addition, the immune suppressive activities of the inflammatory mediators give rise to the inhibition of immune reactions by T-lymphocytes, Natural Killer (NK) cells, macrophages and neutrophils against the tumor, skewing the immune response to a suppressed phenotype (124, 126).

INFLAMMATORY CELLS IN THE TUMOR MICROENVIRONMENT

As a result of cancer-associated chronic inflammation, the tumor microenvironment contains innate immune cells, including macrophages, myeloid-derived suppressor cells (MDSC), neutrophils, dendritic cells (DC) and Natural Killer (NK) cells, and adaptive immune cells, including T- and B-lymphocytes, in addition to the cancer cells and surrounding stroma (consisting of fibroblasts, endothelial cells, pericytes and mesenchymal cells) (123, 127). The different cell types communicate via cell-cell contact or via the production of cytokines and chemokines in order to control tumor development. Consequently, the balance between the production of these immune modulators and the activation state of the different cell types in the tumor microenvironment determines the induction of tumor-promoting inflammation and immune suppression or even anti-tumor immunity (123, 128).

Macrophages, TAM and MDSC

Monocytes and macrophages are derived from myeloid progenitor cells. These precursor cells are located in the bone marrow and after maturation, monocytes are released into the bloodstream. Circulating blood monocytes migrate to different tissues and differentiate into resident macrophages (129). Macrophages play an important role in all aspects of tumor immunity. They are involved in tumor destruction and can facilitate tumor growth and metastasis, depending on their phenotype. Classically activated M1 macrophages are induced by IFN-γ and by bacterial lipopolysaccharides (LPS). These are able to destroy tumor cells by the production of nitric oxide (NO) and T-helper (Th) 1 cytokines (e.g. TNF-α, IL-1, IL-6, IL-12 and IL-23) and chemokines. Moreover, these macrophages can act as antigen

presenting cells (APC) to activate cytotoxic $CD8^+$ T-cells. For that reason, M1 macrophages are generally considered as potent defense factors against the attack of pathogens and tumor cells and accordingly, these cells predominate during the early stages of carcinogenesis (Figure 6) (121, 123, 130, 131). By contrast, in established and advanced cancer, when tumor cells have escaped the attack from the immune system, M2-polarized macrophages then predominate. M2 macrophages are induced and activated by IL-4, IL-10 and IL-13 in a process called alternative activation. M2 macrophages have a poor antigen presenting capacity, they suppress the immune responses and Th1 immunity and they promote angiogenesis and tissue remodeling (Figure 6) (123, 130, 131).

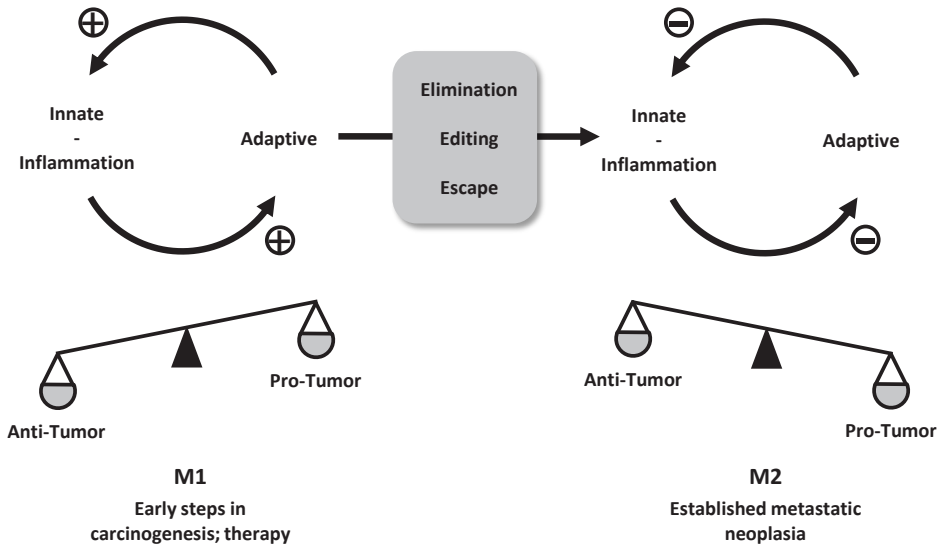


Figure 6 M1 and M2 macrophages in tumor progression, adapted from Allavena et al (130).

A specific type of macrophages, found in many tumors and skewed to the M2 phenotype, are tumor-associated macrophages (TAM). These cells are derived from circulating monocytes that infiltrate the tumor by attraction through chemotactic factors as CCL2 (monocyte chemotactic protein (MCP)-1), vascular endothelial growth factor (VEGF) and macrophage colony stimulating factor (M-CSF), and differentiate to macrophages (121, 130). In most types of cancer TAM promote tumor growth and support angiogenesis, invasion and metastasis. Moreover, high levels of infiltrating TAM have been shown to be associated with a poor prognosis (123, 130). However, in some cases TAM are able to express some characteristics of M1 macrophages, expressing anti-tumor immunity as well (124, 130, 131).
Another important cell type in the chronic inflammation of the tumor microenvironment, with a common myeloid precursor as the TAM, are the myeloid derived suppressor cells (MDSC) (Figure 7). Human stem cells (HSC) give rise to common myeloid precursors (CMP), which subsequently originate at least three subsets of cells circulating in tumor-bearing hosts that can be identified by specific markers: monocytes, granulocytes, and MDSC. Circulating monocytes are recruited by tumors and differentiate into TAM, acquiring tumor-promoting functions. During tumor progression, MDSC that accumulate in blood and in

lymphoid organs such as the spleen may be recruited to the tumor microenvironment as well, however this depends on the type of cancer. It has been hypothesized that immature forms of granulocytes might differentiate into MDSCs or condition their function and/or further differentiation.

MDSC have, like TAM, a phenotype similar to that of the alternatively activated M2 macrophages and are elevated in most patients and experimental mice suffering from cancer (131). In mice MDSC are characterized by the expression of the cell surface markers GR1 (granulocytic macrophage/neutrophil marker) and CD11b (myeloid marker), but other surface markers were described as well, depending on the subtype of MDSC (129). In cancer patients, MDSC are defined as $CD11b^{+}CD33^{+}CD14^{-}HLA^{-}DR^{-}$ and vary in their expression of CD15 and other markers, dependent on the intermediate stages of myeloid cell differentiation (129, 133). Moreover, MDSC are induced by tumor-secreted and host-secreted mediators and consequently contribute to the severe inflammatory state and down-regulation of immune surveillance and anti-tumor immunity (133). MDSC suppress immune responses by affecting both innate and adaptive immunity. MDSC suppress T-cells by the production of arginase, consequently inhibiting levels of arginine, an essential amino acid for T-cell activation. Moreover, MDSC produce reactive oxygen species (ROS) and peroxynitrite which are able to inhibit $CD8^{+}$ T-cells, but they also induce regulatory T-cells (Treg) through an IL-10 and IFN-γ-dependent process. Furthermore, MDSC skew the immune system to a Th2 phenotype by secreting high levels of IL-10, decrease macrophage production of the Th1-polarizing cytokine IL-12, suppress NK cell cytoxicity and inhibit antigen presentation by the limited expansion of MDSC at the expense of DC (129, 131, 133).

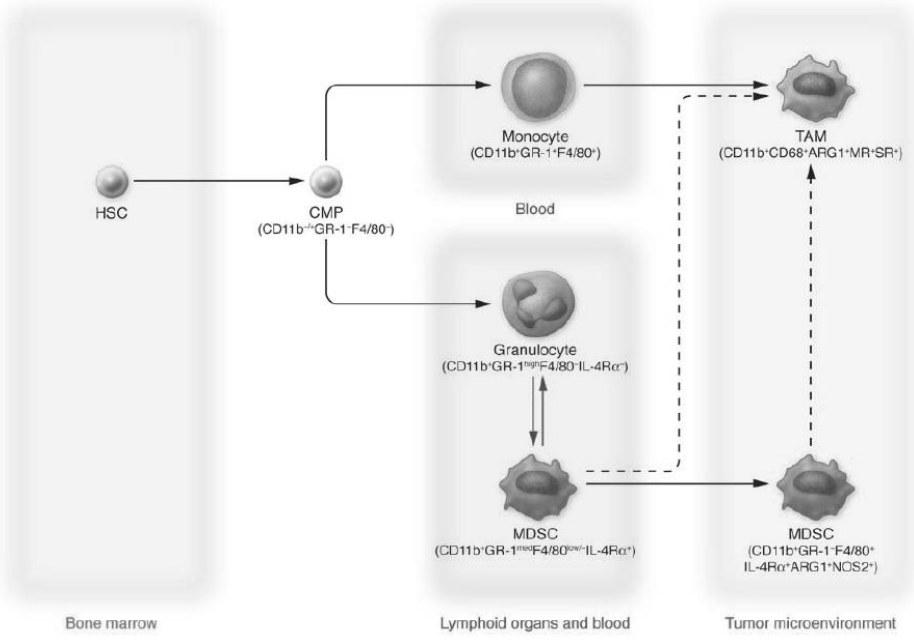


Figure 7 Current view of TAM and MDSC differentiation, adapted from Sica et al (132).

Neutrophils

Neutrophils are the most abundant type of circulating leukocytes in the blood and play an important role in the first-line defense against infections and they are important effectors of inflammation as well (129, 134). In a cancer setting, neutrophils are described to be active mediators of immune surveillance against several tumors. This might be related to the production of cytokines and chemokines by the tumor and the degree of recruitment and activation of neutrophils. Consequently, activated neutrophils are involved in tumor destruction by the release of several factors (e.g. cytokines, chemokines, oxidants) inducing direct tumor killing, extracellular lysis, inhibition of angiogenesis and activation of other immune cells such as NK^- cells and T-cells, and antibody-dependent cytoxicity (134). However, there is increasing evidence that, like TAM or M2 macrophages, neutrophils in the tumor microenvironment can acquire a "N2" tumor-promoting phenotype, through a process that is largely driven by Transforming Growth Factor (TGF)-β. It appears that, like macrophages, neutrophils are capable of being pro- or anti-tumorigenic, depending on the tumor microenvironment (135, 136).

Dendritic cells

DC are specialized antigen presenting cells (APC) that take up, process and present tumor associated antigens to activate tumor-specific T-cells (129). Initially, monocytes can differentiate into immature DC in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. Subsequently, DC migrate to the affected and inflamed tissue where they capture the antigens. After maturation, the DC migrate to the lymph nodes to stimulate T-cell activation (122). During cancer, infiltration of DC is associated with an immune response against the tumor and moreover high levels were correlated with an improved prognosis (67). However, in neoplastic infiltrates, the differentiation and activation of DC is inhibited by immune suppressive signals as IL-10 and accordingly, mainly immature and defective T-cells are found, leading to less effective responses to tumor antigens (67, 122, 125).

Natural Killer-cells

NK-cells are part of the innate immune system. They are involved in the initial phase of defense against infections and play an important role in immune surveillance against tumors and virus infected cells. NK-cells comprise approximately 15% of all circulating blood lymphocytes, but are also found in lymph nodes, spleen, bone marrow, lung, liver, intestine, omentum and placenta (137). In general, resting NK-cells circulate in the blood and after activation by cytokines, they infiltrate into tissues that contain pathogen-infected or malignant cells. The activation of NK-cells is regulated by a balance of signals, which are produced by inhibitory receptors, binding class I major histocompatibility complex (MHC) molecules and by activating receptors, binding ligands on tumors and infected cells (131, 138, 139). In addition, NK-cells respond to many cytokines, including IL-2, IL-12, IL-15, IFN-α and IFN-β, inducing their cytolytic, secretory, proliferative and anti-tumor functions (138). The ability of NK-cells to kill target cells (tumor cells) is mediated by a variety of effector mechanisms, including the perforin/granzyme-containing granule-mediated pathway, releasing cytoplasmic granules to lyse target cells, the death receptor pathway, expressing

TNF-α or NO, and the IFN-γ mediated pathway, producing TNF-α to restrict tumor angiogenesis and stimulate adaptive immunity (138). Consequently, NK-cells are involved in rejection of the tumor and can act against tumor initiation, growth and metastasis and until now, NK-cells are the only immunological cells that have never been described to take on a tumor-promoting role under the influence of tumor-associated factors (67, 123, 138).

Lymphocytes

Acute activation of the innate immune system by the recruitment of the above described leukocytes from the circulation into the damaged tissue, additionally induce the activation of the adaptive immune system (127, 140). APC acquire foreign (tumor) antigens and subsequently migrate to lymphoid organs, where they present the antigens to adaptive immune cells. Upon recognition of the specific unique antigen presented by the APC, clonal expansion of the adaptive immune cells is required to obtain sufficient antigen-specific immune cells as $CD4^+$ T-helper lymphocytes, $CD8^+$ cytotoxic T- lymphocytes (CTL) and B-lymphocytes to counteract infection (127, 140). During cancer, T-lymphocytes can infiltrate the tumor micro environment in order to eradicate and destroy the tumor, depending on the type of tumor. However, they can also exert tumor-promoting effects (123). $CD8^+$ CTL are able to kill the tumor cells directly by binding to a specific receptor. $CD4^+$ T-helper cells are activated in response to soluble factors and can be classified generally into two categories as either T-helper 1 (Th1) or T-helper 2 (Th2) cells. Initially, an acute anti-tumor response is demonstrated and after activation, Th1 cells secrete IFN-γ, TNF-α and IL-2. Through the production of these cytokines, Th1 cells regulate immune surveillance programs by up-regulating antigen processing by APC and activation of tumor inhibitory responses in recruited macrophages and CTL in order to destroy tumor cells. Moreover, Th1 cells can directly kill tumor cells by releasing high levels of IFN-γ, TNF-α and cytolytic granules (131, 140, 141). By contrast, chronic activation of the immune response (chronic inflammation), results in the activation of Th2 cells, regulatory T-cells (Treg) and activated B-cells secreting IL-4, IL-5, IL-6, IL-10 and IL-13 and immunoglobulins that enhance tumor promoting responses in innate immune cells and inactivate CTL cytotoxicity (140). In addition to the Th1 and Th2 cells, another T-helper cell subset might be involved in the tumor responses. Th17 is differentiated by a combination of IL-6, IL-23 and TGF-β and mediates its effects through the production of IL-17, IL-21 and IL-22. Th17 cells may play a role in the protection against pathogens and have been implicated with the development of inflammation-associated colonic tumors in response to pathogenic bacteria (131, 141). Moreover, infiltration of Th17 cells has been observed in patients with colon, ovarian and prostate cancer in which high numbers of IL-17 producing cells are correlated with a poor prognosis (141).

INFLAMMATORY MEDIATORS IN THE TUMOR MICROENVIRONMENT

Cytokines

As previosly described, cytokines play an important role in the tumor micro environment and can promote and/or inhibit tumor development. They can be produced by different types of immune cells and tumor cells, and their profile persisting at the inflammatory site determines the activation of either an anti-tumor or a tumor progressive response (122, 128, 142). TNF-α is one of the major mediators of inflammation. It can be produced during the

acute inflammatory reaction against the tumor, but is also able to induce other pro-inflammatory cytokines and chemokines (121, 143). TNF-α has been associated with all steps of carcinogenesis, including initiation, promotion, survival, proliferation, invasion, angiogenesis and metastasis. Moreover, TNF-α is produced during chronic inflammation and is related to the impairment of immune surveillance by the suppression of T-cell response and the cytotoxic activity of M1 macrophages as described earlier (127, 128, 143).
TNF-α can be detected in ovarian, breast, prostate, bladder and colorectal cancer, lymphomas and leukemias, often in association with the production of IL-1β and IL-6 (121).

High levels of IL-1β are also associated with the secretion of angiogenic factors by tumor and stromal cells that promote growth lung carcinoma *in vivo* (144). IL-6 is an inflammatory cytokine that is considered a key growth-promoting and anti-apoptotic factor. Its target genes are all involved in cell cycle progression and suppression of apoptosis. Moreover, IL-6 is described to play a major role in multiple myeloma and high levels of IL-6 may be associated with an increased risk of developing Hodgkin lymphoma (128, 143). The pro-inflammatory cytokine IL-8 may be involved in the promotion of tumor growth and metastasis of several tumor types as well. In addition, high levels of IL-8 were detected in cancers of the central nervous system (143).

IL-12 and IL-23 are both pro-inflammatory heterodimeric cytokines that are composed of IL-12p40/IL-12p35 and IL-23p40/IL-23p19 subunits, respectively. These cytokines are mainly produced by activated APC and DC and bind to their receptors on T-cells, NK-cells and NKT cells. IL-12 plays an important role in anti-tumor immunity based on its ability to promote Th1 adaptive immunity and CTL responses. Moreover, IL-12 induces high levels of IFN-γ, which has a direct toxic effect on tumor cells and anti-angiogenic activity (128, 145). IL-23 can even enhance the production of IL-12 and IFN-γ and is able to increase the proliferation of memory T-cells. By contrast, M2 macrophages down-regulate the production of the pro-inflammatory cytokines TNF-α, IL-6, IL-8, IL-1β, IL-12 and IL-23, whereas they induce the production of IL-10 and TGF-β (141). IL-10 is a Th2-derived cytokine, but can also be produced by Treg-cells. It plays an important role in the inhibition of the inflammatory responses by the suppression of activated immune cells. Accordingly, IL-10 is involved in the reduction of antigen presentation by APC, it can inhibit Th1-mediated immune responses and can suppress the function of NK cells and macrophages (124). Similarly, TGF-β is involved in the inhibition of inflammatory responses, but plays a major role in the regulation of Treg proliferation and function as well. In accordance to that, TGF-β can inhibit the growth of many cell types, including immune cells and tumor cells. Paradoxically, TGF-β can contribute to the immune suppressive effects of the tumor as well, eventually leading to the induction of inflammation (124).

Chemokines

Inflammatory cytokines are the major inducers of chemo-attractant molecules called chemokines. Chemokines have initially been described as potent attractants for leukocytes such as neutrophils and monocytes, and therefore are generally regarded as mediators of acute and chronic inflammation. Chemokines can be classified into "homeostatic" or "inflammatory" chemokines (121, 143). Homeostatic chemokines are constitutively expressed and regulate leukocyte attraction during immune surveillance, whereas the inflammatory

chemokines (vast majority) are inducible and control the recruitment of cells to sites of inflammation (146, 147). Moreover, despite the similar structure, the different chemokines can elicit other distinct cellular responses, regulated via different pathways. Accordingly, cancer cells must both provide and derive signals in order to recruit cells, modulate angiogenesis, and stimulate proliferation and survival of tumor cells, eventually leading to cancer progression and metastasis (147).

In this regard, chemokines are responsible for the recruitment of a number of different cell types to the tumor microenvironment, including TAM, MDSC, neutrophils, lymphocytes, fibroblasts and endothelial cells. In addition, these infiltrating cells produce a secondary source of chemokines that are able to affect tumor growth and survival (146). For example, macrophages are recruited to the tumor site by CCL2 (MCP-1), which is observed in many cancer types, including breast cancer, pancreas cancer, lung cancer, cervix cancer, ovary cancer, melanomas and sarcomas, but CCL2 also play an important role in angiogenesis (147, 148). In addition, CCL5 (RANTES) produced by MDSC upon contact with breast cancer cells, is able to promote metastasis to the lung and both CCL2 and CCL5 expression levels might be related to advanced disease, early relapse and poor prognosis (124, 147, 149, 150). In the tumor microenvironment, TGF-β can induce a so-called N2 state in local neutrophils, leading to an increased expression of arginase and CCL2 and CCL5 that contributes to the suppressive state of the immune system (146).

Prostaglandin E_2

Prostaglandin E_2 (PGE_2) is a lipid-derived mediator that contributes to the inflammatory state and immune suppression during the course of cancer (114, 151). It is involved in several human malignancies including colon, lung, breast and head and neck cancer and is produced during inflammation in response to growth factors, hormones and inflammatory cytokines (126, 142, 151). PGE_2 is produced by the enzyme cyclooxygenase (COX)-2, using the polyunsaturated fatty acid (PUFA) arachidonic acid (AA) as substrate. It is produced by various types of cancer cells and their surrounding cells, leading to a range of oncogenic effects including stimulation of cell proliferation, protection against apoptosis, and induction of migration and invasion (124, 152). In addition, it can induce epithelial cells to secrete growth factors, pro-inflammatory mediators and angiogenic factors, switching a normal microenvironment to a tumor-supporting environment (126, 133, 151). Pro-inflammatory PGE_2 produced by tumor epithelial cells and/or their surrounding stromal cells induces immunosuppression through several mechanisms. First of all, PGE_2 can down regulate anti-tumor Th1 cytokines and up regulate immunosuppressive Th2 cytokines. Moreover, PGE_2 is able to inhibit $CD8^+$ T-cell proliferation and activity, suppress the anti-tumor activity of NK-cells and stimulate the expansion of Treg-cells and MDSCs. Furthermore, PGE_2 can inhibit $CD8^+$ T-cell anti-tumor functions by impairing the ability of tumor cells to directly present tumor antigen, inhibit DC differentiation and switch the function of DC from induction of immunity to T cell tolerance (Figure 8).

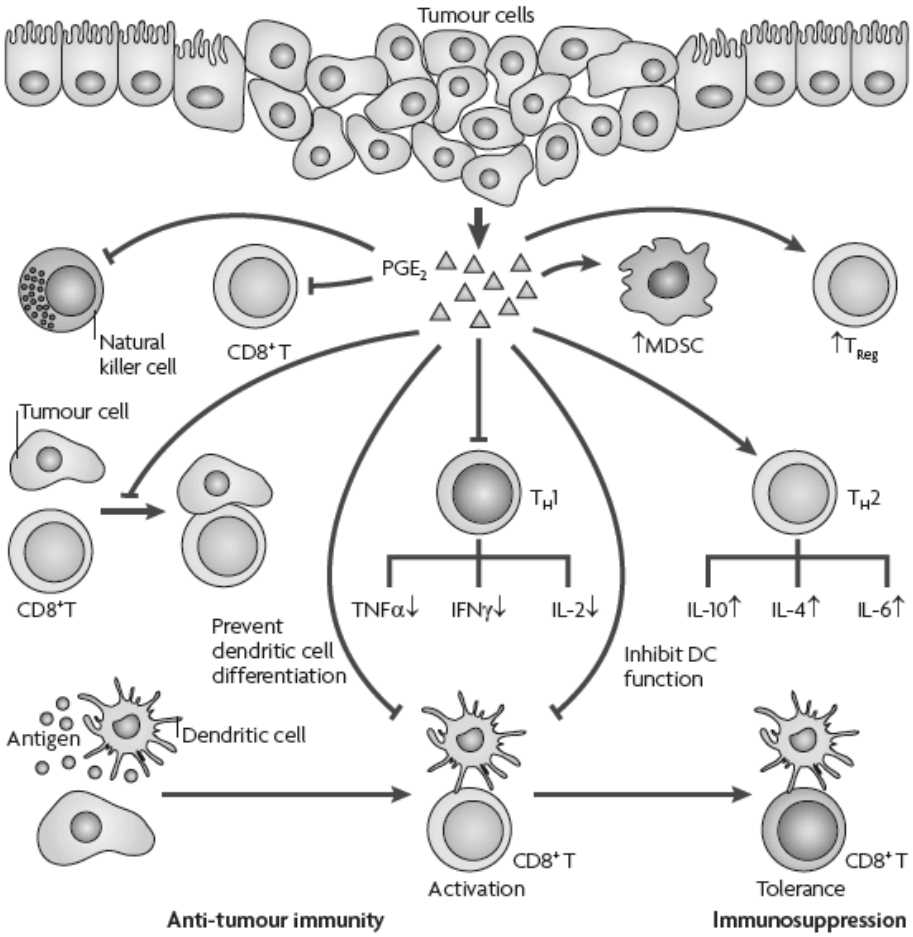


Figure 8 The role of PGE_2 in the regulation of immune suppression, adapted from Wang et al (126).

PGE_2 contributes to the shift of the tumor microenvironment from an anti-tumor Th1 response to an immunosuppressive Th2 response by down regulating Th1 cytokines (IFN-γ, TNF-α and IL-2) and up regulating Th2 cytokines (IL-4, IL-6 and IL-10) and has a clear role in the regulation of immune suppression (114, 126). Another mechanism by which PGE_2 is involved in the inflammation promoting tumor progression is through the induction of MDSC (126, 142). PGE_2 can act as a chemotactic factor for MDSCs and therefore regulate the recruitment of these cells to the tumor (133, 153). In turn, MDSCs can produce PGE_2 by themselves, thereby maintaining the inflammatory vicious circle (126, 151).

CHRONIC INFLAMMATION AND SEVERE IMMUNE SUPPRESSION: A PARADOX

In summary of the immune responses during the course of cancer, as previously mentioned; an acute inflammatory reaction occurred initially with the recruitment of several immune cells and the production of a large variety of inflammatory mediators to initiate an effective anti-tumor response (Figure 9). However, during progressive tumor development, these inflammatory reactions become chronic and the interactions between innate and adaptive immune responses become disturbed. Still, immune cells are recruited and

inflammatory mediators are produced, however, leading to a switch from an anti-tumor to a tumor-promoting setting, accompanied paradoxically by a state of immune suppression (127, 140, 141, 154).

As a consequence of the chronic inflammatory state and reduced immune responsiveness in advanced disease, angiogenesis is stimulated and tumor cells can metastasize to other parts of the body (121, 123). Moreover, a significant correlation between circulating MDSC and clinical cancer stage was described in newly diagnosed solid tumor patients (stages I-IV). Among stage IV patients, those with extensive metastatic tumor burden had the highest percent and absolute number of MDSC (155). In addition, elevated serum CRP levels were associated with a reduced survival time and increase with the progression of the disease (156).

Impaired function of the immune system can lead to severe (infectious) complications and even to delayed or suboptimal anti-cancer treatment, ultimately resulting in a decreased quality of life and even reduced survival rates in the patients (68, 77, 83, 114, 115).

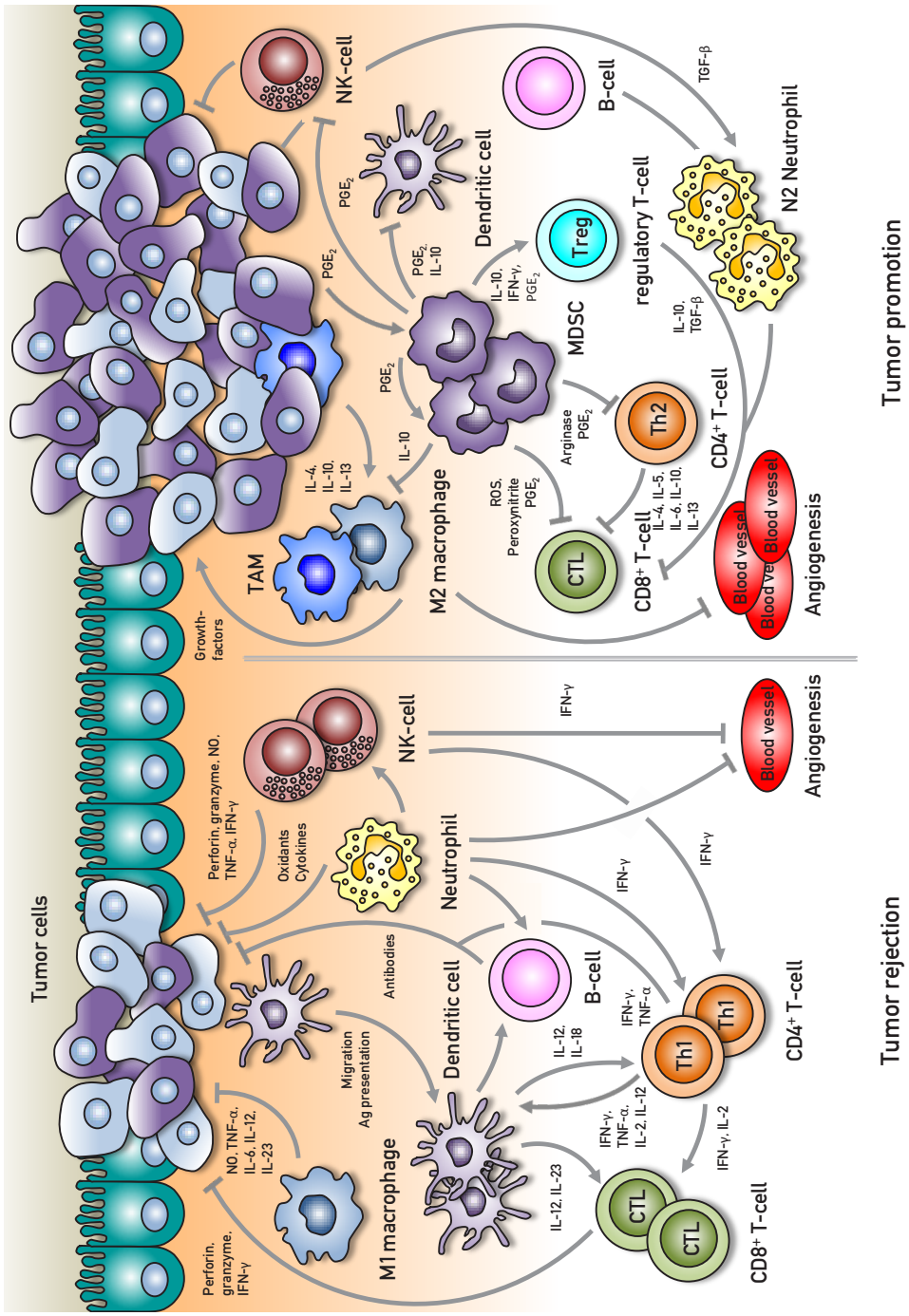


Figure 9 The immune system during carcinogenesis, presenting an anti-tumor response in the early stages towards a pro-tumor response and chronic inflammation during the later stages.

NUTRITIONAL SUPPORT

As previously discussed, many cancer patients suffer from cachexia during the course of their disease. Immune suppression is closely related to the presence of cachexia in these patients leading to disease progression, increased (infectious) complications and a delayed or suboptimal treatment regimen resulting in a reduced quality of life and a poor prognosis (58, 67, 68, 114, 115). Moreover, cachectic cancer patients have been described to develop a reduced functional capacity and performance status caused by reduced muscle function (58, 72, 116). To provide optimal treatment support for these patients, nutritional support plays an important role in disease management. Sufficient amounts of energy and nutrients can be provided in order to preserve or restore an adequate nutritional status (74, 76). This is an important step in the recovery of the patients and to tolerate aggressive therapeutic regimens, since the presence of cachexia is described to affect both treatment toxicity and survival (74, 157).

These cancer-related weight loss and other cachexia features can however, not be reverted by simple nutrient provision, which is in contrast by the weight loss induced after simple starvation. In cancer patients, a multidisciplinary approach should be applied in which nutritional intervention is recommended as an integral part of anti-cancer therapy (74). However, recent findings suggest that impaired immune responsiveness and muscle protein degradation may already occur before the onset of weight loss (74, 75). Early nutritional and metabolic intervention is therefore important to effectively manage both nutritional status and physical conditions in cancer patients. Therefore, a multi-disciplinary approach is recommended, which is initiated at the moment of diagnosis and continues in parallel to the pathway of cancer therapies (74).

A first key step in nutritional support is to identify patients who might benefit by routine screening using a thorough nutritional assessment by a dietician. This will include the determination of the severity and causes of malnutrition, risk of treatment-related complications and (expected) efficacy of nutritional support. Nutritional assessment must be combined with a careful evaluation of performance status and quality of life, so that nutritional management is correctly adapted to the patient's real needs and entails a minimum of constraints (74, 76). Afterwards, an individual nutritional support plan has to be developed, since no single nutritional therapy is applicable for all cancer patients. Accordingly, a nutritional support plan should be tailored to the patient's individual needs, nutritional status, dietary restrictions, tolerance and feasibility, gastrointestinal function, medical condition and the current and expected side effects of treatment (158).

In practice, patients can be supported by different types of nutrition, including dietary counseling, oral nutritional supplements (ONS), enteral nutrition or parenteral nutrition.

DIETARY COUNSELING

If nutritional intake is inadequate, dietary counseling is an important first step in nutritional support. Dieticians and nurses play a major role in dietary counseling offering the cancer patients information and advice in order to improve their food intake via normal foods and beverages or via oral nutritional supplements (159). It appears that, dietary counseling is important enabling an optimal anti-cancer treatment, since in patients with colorectal cancer receiving radiotherapy, clinical outcomes such as complications and

quality of life were improved, probably as a result of an increased overall dietary intake, including protein (158, 160). Accordingly, regular contact between the healthcare professional and the patient is important, since regular nutritional assessment and dietary advice may beneficially affect their compliance. Moreover, the use of a diary by patients or their families, to record food intake and factors that influence their appetite, can have a positive influence as well. Accordingly, a shift in eating patterns can be recognized in an early stage and is a useful tool for nurses to involve a dietician when additional support is needed (84, 158, 161).

ORAL NUTRITIONAL SUPPLEMENTS

Oral nutritional supplements (ONS) can be provided when oral protein-calorie intake is insufficient and patients are unable to meet their nutritional requirements, despite dietary counseling (76, 158). ONS is a relatively simple and less invasive method of increasing nutritional intake of the patients and the success of ONS depends on sufficient quantities being consumed over an extended period of time. In general, many ONS are available, varying in the type and amount of protein, energy density, osmolarity, lactose and gluten content and in vitamin and mineral content. In addition, specific nutritional ingredients can be added to the product, each with their own features, focusing on specific physiological targets (e.g. n-3 PUFA, leucine, beta-hydroxy-betamethylbutyrate, arginine, glutamine and fibers). Beneficiall effects of ONS include an increased protein and energy intake, reduced weight loss and improved weight gain, improved appetite, decreased GI toxicity and an improved performance status. Moreover, increased immune responses and an improved quality of life were reported in patients using ONS (93, 162-170). In addition, ONS are available in different commercial formulations, from liquid and powder to soup and even ice-creams, but the flavors of the different products play a major role as well, since taste fatigue is the major drawback of ONS. However, the use of ONS is also determined by their costs and reimbursement. In many countries ONS are reimbursed, but in others oral supplementation is still considered as a comfort measure and not as a medication (76).

TUBE FEEDING AND TOTAL PARENTERAL NUTRITION

Enteral tube feeding (TF) is appropriate for patients who are unable to meet their nutritional requirements by oral intake through the diet or ONS, but who have retained, at least partly, GI function. TF is indicated in patients with an incapacity or limited ability to eat, as a result of dysphagia, upper GI obstruction, partial lower GI obstruction or central nervous system pathology (158). Moreover, TF is also provided when patients suffer from increased nutritional losses due to impaired digestion and absorption. Enteral TF can be administered into multiple sites of the GI tract, depending on the functional status and the safety of accessing the gastrointestinal tract, and the risk of aspiration (171). In total parenteral nutrition (TPN), nutrition is provided through a catheter in the bloodstream. Parenteral nutrition does not use the digestive system and may be given to people who are unable to absorb nutrients through the intestinal tract because of severe vomiting, severe diarrhea, or intestinal disease. It may also be given to patients undergoing high-dose chemotherapy or radiation and /or bone marrow transplantation. It is possible to give all of the proteins, calories, vitamins and minerals a person needs using TPN (158, 172-175).

TIMING OF NUTRITIONAL SUPPORT

As previously described, cachexia is an important risk factor in the disease process of cancer patients. However, it is also recognized as a significant source of postoperative morbidity and to aggravate toxicity of chemotherapy and radiotherapy, resulting in increased length of hospital stay, increased treatment costs, decreased performance status and quality of life (176). Consequently, early provision of nutritional support is essential in order to prevent rather than reverse the severe catabolic state in these patients (159). Moreover, beneficial effects have been described using nutritional support during chemotherapy, showing that n-3 fatty acids are able to enhance the efficacy of chemotherapy (177-179). Furthermore, nutritional support before, during and/or after surgery and radiotherapy, can contribute to the overall condition of the patient by improving the nutritional status, reducing complications and improving quality of life (176, 180). Additionally, when patients gain in weight and have a better immunological and overall performance status, higher doses of chemotherapy and radiotherapy can be provided, increasing the chance of survival. Consequently, supportive care, upon indication, should be provided at the earliest time-point possible, to maximize the chance of reducing disease progression, reducing the frequency and severity of complications and improving treatment adherence (74, 75, 159, 176).

GUIDELINES

The evidence that shows the efficacy of nutritional support, has strongly increased within the last decades. For that reason, the European Society for Clinical Nutrition and Metabolism (ESPEN) has decided to publish guidelines with recommendations for nutritional support in cancer patients (181). These guidelines form a consensus document designed by a group of experts providing evidence-based information regarding specific problems like timing, dosing, composition and route of application. The general indication and efficacy of ONS in patients who cannot fulfill their nutritional needs adequately is therefore well established and the whole consensus group strongly agreed on this, however results may vary according to diagnosis, prior nutritional status, age, technical adequacy of treatment and patient selection (individual characteristics). Below a summary is presented of the guidelines for both non-surgical patients and surgical cancer patients (182, 183).

Table 1 Summary of guidelines for non-surgical cancer patients (182).

Subject	Recommendations
General	Nutritional assessment of cancer patients should be performed frequently, and nutritional intervention initiated early when deficits are detected. There are no reliable data that show any effect of enteral nutrition on tumor growth. Such theoretical considerations should, therefore, have no influence on the decision to feed a cancer patient.
Indication	
General	Start nutritional therapy if under nutrition already exists or if it is anticipated that the patient will be unable to eat for >7 days. Start enteral nutrition if an inadequate food intake (<60% of estimated energy expenditure for >10 days) is anticipated. It should substitute the difference between actual intake and calculated requirements. In weight losing patients due to insufficient nutritional intake enteral nutrition should be provided to improve or maintain nutritional status.
Peri-operative	Patients with severe nutritional risk benefit from nutritional support 10-14 d prior to major surgery even if surgery has to be delayed.
During radio- or radiochemotherapy	Use intensive dietary advice and oral nutritional supplements to increase dietary intake and to prevent therapy-associated weight loss and interruption of radiation therapy. Routine enteral nutrition is not indicated during radiation therapy.
During chemotherapy	Routine enteral nutrition during chemotherapy has no effect on tumor response to chemotherapy or on chemotherapy-associated unwanted effects and, therefore, is not considered useful.
Incurable patients	Provide enteral nutrition in order to minimize weight loss as long as the patient consents and the dying phase has not started. When the end of life is very close most patients only require minimal amounts of food and little water to reduce thirst and hunger. Small amounts of fluid may also help to avoid states of confusion induced by dehydratation. Subcutaneously infused fluids in hospital or at home may be helpful and also provide a vehicle for the administration of drugs.
Application	Prefer the enteral route whenever feasible. Administer preoperative enteral nutrition preferably before admission to the hospital.
Route	Use tube feeding if an obstructing head or neck or esophageal cancer interferes with swallowing or if severe local mucositis is expected.
During radio- or radiochemotherapy	Tube feeding can either be delivered via transnasal or percutaneous routes. Because of the radiation induced oral and esophageal mucositis a percutaneous gastrostomy (PEG) may be preferred.
Type of formula	
General	Use standard formulae. Regarding n-3 fatty acids, randomized clinical trial evidence is contradictory/ controversial and at present it is not possible to reach any firm conclusion with regard to improved nutritional status/physical function. It is unlikely that n-3 fatty acids prolong survival in advanced cancer.
Peri-operative	Use preoperative enteral nutrition preferably with immune modulating substrates (arginine, n-3 fatty acids,nucleotides) for 5-7 d in all patients undergoing major abdominal surgery independent of their nutritional status.
Drug treatment	In the presence of systemic inflammation pharmacological efforts are recommended in addition to nutritional interventions to modulate the inflammatory response. In cachectic patients steroids or progestins are recommended in order to enhance appetite, modulate metabolic derangements, and prevent impairment of quality of life. Administer steroids for short-term periods only weighing their benefits against their adverse side-effects. Consider the risk of thrombosis during progestin therapy.

In summary, the type of nutritional intervention of cancer patients depends on many factors, including: nutritional requirements, nutritional status, medical condition, GI function, current and expected side effects of treatment that may affect nutritional status, oral intake, dietary restrictions and tolerance.

Table 2 Summary of guidelines for surgical patients including cancer patients (183).

Subject	Recommendations
General	Preoperative fasting from midnight is unnecessary in most patients. Interruption of nutritional intake is unnecessary after surgery in most patients.
Indications	
Peri-operative	Use nutritional support in patients with severe nutritional risk for 10-14 days prior to major surgery even if surgery has to be delayed. Severe nutritional risk refers to at least one: - Weight loss >10-15% within 6 months - BMI <18.5 kg/m2 - Subjective Global Assessment Grade C - Serum albumin <30 g/l (with no evidence of hepatic or renal dysfunction) Initiate nutritional support (by the enteral route if possible) without delay: - even in patients without obvious undernutrition, if it is anticipated that the patient will be unable to eat for more than 7 days peri-operatively - in patients who cannot maintain oral intake above 60% of recommended intake for more than 10 days. Consider combination with parenteral nutrition in patients in whom there is an indication for nutritional support and in whom energy needs cannot be met (<60% of caloric requirement) via the enteral route C.
Contraindications	Prefer the enteral route except for the following contraindications: Intestinal obstructions or ileus, severe shock, intestinal ischemia.
Application	
Pre-operative	Encourage patients who do not meet their energy needs from normal food to take oral nutritional supplements during the preoperative period. Administer preoperative enteral nutrition (EN) preferably before admission to the hospital. Patients undergoing surgery, who are considered to have no specific risk for aspiration, may drink clear fluids until 2 h before anesthesia. Solids are allowed until 6 h before anesthesia.
Post-operative	Use preoperative carbohydrate loading (the night before and 2 h before surgery) in most patients undergoing major surgery. Initiate normal food intake or enteral feeding early after gastrointestinal surgery. Oral intake, including clear liquids, can be initiated within hours after surgery to most patients undergoing colon resections. Oral intake should, however, be adapted to individual tolerance and to the type of surgery carried out. Apply tube feeding in patients in whom early oral nutrition cannot be initiated, with special regard to those: - undergoing major head and neck or gastrointestinal surgery for cancer - with severe trauma - with obvious undernutrition at the time of surgery - in whom oral intake will be inadequate (<60%) for more than 10 days Initiate tube feeding for patients in need within 24 h after surgery. Start tube feeding with a low flow rate (e.g. 10-max.20 ml/h) due to limited intestinal tolerance. It may take 5-7 days to reach the target intake and this is not considered harmful. Reassess nutritional status regularly during the stay in hospital and, if necessary, continue nutritional support after discharge, in patients who have received nutritional support peri-operatively.
Type of tube feeding	Placement of a needle catheter jejunostomy or naso-jejunal tube is recommended for all candidates for TF undergoing major abdominal surgery. When anastomoses of the proximal gastrointestinal tract have been performed, deliver EN via a tube placed distally to the anastomosis. Consider placement of a percutaneous endoscopic tube (e.g. PEG) if long term tube feeding (>4 weeks) is necessary, e.g. in severe head injury.

DISEASE-SPECIFIC NUTRITIONAL SUPPORT

As prescribed by the guidelines and can be concluded from the literature, a standard protein formula can lead to reduced weight loss in cancer patients. In a study in oropharyngeal cancer patients receiving chemoradiation or radiotherapy, individualized dietary

counseling including encouraged ONS use resulted in a reduced body weight loss (184). Moreover, in a post-operative setting in mostly cancer patients, a hospital diet ad lib and ONS use reduced weight loss significantly. This effect was only observed when the patients were still in the hospital, whereas in the out-patient setting, no effect was observed of ONS (185). By contrast, in cancer patients receiving radiotherapy, dietary counseling and ONS supplementation stabilized weight in outpatients during a 12 week intervention (186).

However, to obtain more pronounced effects or to achieve weight gain, and to provide nutritional support in a disease specific setting, provision of protein and energy is not sufficient. For that reason specific nutrients have to be added in order to affect the broad range of problems including several metabolic alterations, inflammation and reduced immune responsiveness. In the literature some studies were described demonstrating beneficial effects on the immune system after nutritional support containing n-3 PUFA, vitamin E or arginine.

In a study with sixty patients with generalized solid tumors, immune function was suppressed in malnourished patients compared to healthy controls as measured by the ratio of $CD4^+$/ $CD8^+$ T-cells and a reduced TNF-α production in LPS-stimulated mononuclear cells. Nutritional intervention with either 18 g fish oil (providing 3.1 g eicosapentaenoic acid (EPA) and 2.1 docosahexaenoic acid (DHA) daily) and 200 mg vitamin E daily, resulted in immune modulatory effects by increasing the $CD4^+/CD8^+$ ratio in malnourished patients and an enhanced TNF-α production in LPS-stimulated mononuclear cells. Consequently, n-3 PUFA reversed immunodeficiency and seemed to prolong the survival of malnourished cancer patients (187). Moreover, in a 4-week study in six consecutive patients with unresectable carcinoma of the pancreas, receiving high purity EPA in a dose of 1 g/day in week 1, 2 g/day in week 2, 4 g /day in week 3 and 6 g/day in week 4, serum CRP levels were significantly reduced compared to baseline ($p < 0.05$). Moreover, IL-6 production in LPS-stimulated peripheral blood mononuclear cells (PBMC) was significantly decreased after 4 weeks of intervention (188). In a study in esophageal patients after surgery, subjects received 1.8 g EPA/day orally or enterally in addition to TPN with a soybean oil emulsion. Postoperative levels of serum IL-6 were significantly reduced compared with patients receiving only the TPN with soybean oil emulsion. Moreover, intervention with EPA and soybean oil emulsion significantly improved lymphocyte proliferation and NK-cell activity on postoperative day 21 (189). When combining n-3 fatty acids with arginine in an immune-enhancing diet, provided to sixty patients with gastric cancer compared to a standard formula, this even resulted in attenuation of the inflammatory response and a lower incidence and duration of postoperative infectious complications ($p < 0.05$) (190).

SPECIFIC NUTRITIONAL COMBINATION

To provide the optimal treatment support for cancer patients, thereby reducing the inflammatory state, supporting immune function, and preserving muscle mass and function, recently a specific nutritional combination has been developed. This specific nutritional combination is high in protein and leucine and is enriched with emulsified fish oil (containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) and a specific oligosaccharide mixture. These ingredients were identified by literature search and selected from extensive previous *in vitro* and *in vivo* studies.

High protein and leucine

High protein and the branched chain amino acid (BCAA) leucine have been selected because of their potential to influence in protein metabolism in cancer patients and they may in combination with fish oil have a positive effect on skeletal muscle function, lean body mass and daily activity. Leucine has been selected to provide an anabolic trigger for muscle protein synthesis and high levels of protein were selected in order to provide sufficient amounts of protein building blocks (191, 192). In addition, in cancer patients BCAA supplementation after surgery demonstrated a reduction in length of hospital stay, a better performance status at 3 months and an increased body weight at 1 year after major surgery (193, 194). In patients receiving chemotherapy, BCAA supplementation for up to 1 year after the chemotherapy treatment, resulted in decreased overall morbidity, and improved nutritional status and quality of life (195).

The acute effect of the specific nutritional combination as application in a medical food on muscle protein synthesis has recently been shown in a clinical study in catabolic cancer patients with involuntary weight loss (196) and the effects on muscle mass and function have been demonstrated in previous pre-clinical studies using an animal model of tumor induced cachexia (197).

Fish oil (EPA and DHA)

Fish oil is a generally used ingredient in immune-modulating nutritional interventions, containing the conditionally essential long-chain n-3 PUFA EPA and DHA. These long chain n-3 PUFA play a major role in the regulation of immune responses and inflammation during cancer (198, 199). Increased consumption of n-3 PUFA results in their incorporation into inflammatory cell phospholipids, which occur in a dose-response fashion and will be more efficient by the use of pre-emulsified fish oil (200, 201). This incorporation is partly at the expense of the n-6 PUFA arachidonic acid (AA), although levels of other n-6 PUFA are decreased as well (Figure 10). Subsequently, a decreased amount of AA will lead to a decreased synthesis of AA-derived eicosanoids by the enzyme COX-2, including the reduced production of PGE_2, which might be beneficial for cancer patients. Moreover, the n-3 PUFA reduce the production of pro-inflammatory cytokines as TNF-α, IL-1β, IL-6 and IL-8 by several mechanisms and may positively influence the cancer cachectic state (198). Clinical evidence suggest that nutritional intervention with n-3 PUFA beneficially affects nutritional status during multimodality treatment in patients with non-small cell lung cancer (170) and reduces the loss of weight and lean tissue in pancreatic cancer patients (202) and in patients following esophageal cancer surgery (203). Moreover, in studies with pancreatic cancer patients, the use of EPA-enriched interventions was described to reduce serum levels of CRP and IL-6 production by PBMC (167, 188). EPA also appears to have immune enhancing effects, including a stimulation of lymphocyte proliferation and NK-cell activity in patients after surgery for esophageal cancer (189, 204). Consequently, these anti-cachectic, anti-inflammatory and immune supporting effects have been shown to translate into improved clinical outcomes (158), since nutritional intervention with n-3 PUFA was associated with a reduction in (infectious) complications, an improved performance status, improved quality of life and prolonged survival (158, 187, 202, 205). However, results are not consistent for

all cancer populations, therefore, further studies are required to confirm these findings in different cancer groups, but the combination with other nutritional ingredients might also lead to more obvious effects on clinical outcome (158, 206, 207).

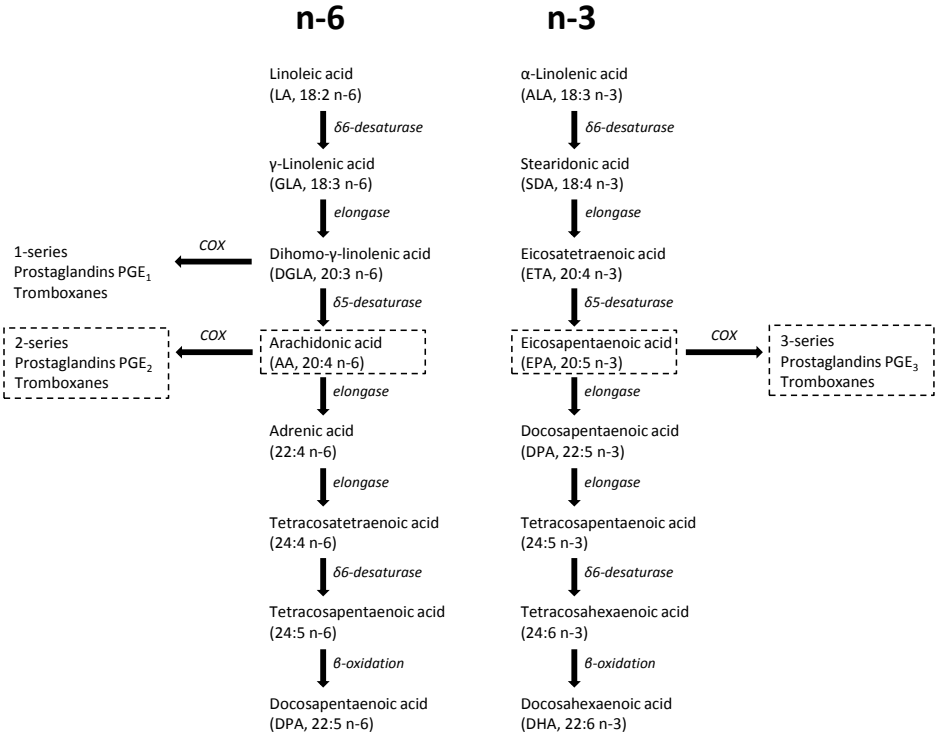


Figure 10 Metabolism of n-6 and n-3 PUFA and the subsequent production of prostaglandins

Specific oligosaccharides (GOS/FOS)

The use of non-digestible carbohydrates, especially of prebiotic oligosaccharides, is based on the immuno-modulating activities observed in several animal experiments and clinical trials as reviewed by Vos et al (208). Moreover, these prebiotic oligosaccharides also have been associated with other health benefits, including an improved gut barrier function (209). This might be the result of intestinal lactate and SCFA production, including butyrate, acetate, and propionate, formed by the fermentation of the oligosaccharides by the colonic microbiota, which can contribute to a restoration of the intestinal barrier function (208, 210, 211). Accordingly, a reduced pH in the intestinal lumen can lead to the inhibition of pathogen growth and adhesion. Furthermore, the fermentation of the specific oligosaccharides can stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacilli, which in turn can inhibit pathogens by the production of antimicrobial substances (208, 212). Thus, the specific oligosaccharides can modulate the immune system via a microbiota-dependent (prebiotic) mechanism, but they may also directly affect immune function by blocking or activating specific receptors on immune cells, leading to improved immune responses and eventually to enhanced resistance to systemic infections.

In an influenza vaccination model in healthy C57BL/6 mice, a specific mixture of oligosaccharides stimulated the vaccine-specific delayed-type hypersensitivity response as a marker for T-helper 1 (Th1) immunity (213). Enhancing systemic Th1-dependent adaptive immune responses would lead in theory to better immune responses against infections that have been confirmed in clinical trials in infants (214, 215).

In cancer patients, only a few studies examined the effect of prebiotics and most of them look at their preventive capacities. Accordingly, mechanisms for beneficial effects of prebiotics might include changing the activity of exogenous carcinogens through modulating metabolic activation and/or detoxification, or stimulating the production of SCFA such as butyrate (216, 217). In addition, modern analytical techniques suggest that an important consequence of a modified bacterial community could be a change in the expression not only of a range of different bacterial genes in bowel contents, but also in the bowel mucosa of the host. Analogous with observations with probiotics, the stimulation of cytokines and modification of immune responses could be important in producing beneficial effects (217). However, the beneficial effects of the specific oligosaccharides mixture in cancer patients might decrease the incidence and severity of secondary infections as well. Moreover, they can contribute to the reduction of the inflammatory state and improve the gut barrier function in cancer patients dealing with chemotherapy-induced damage of the GI tract.

As described, each of the ingredients in the specific nutritional combination play a specific role, but overlapping biological activities and synergistic interactions between them eventually lead to a multi-target approach. Together these features might reduce inflammation, support immune function, improve the nutritional status, and preserve muscle mass and function, leading to reduced (infectious) complications, enhanced performance status and improved quality of life, with the ultimate goal to provide optimal treatment support to cancer patients.

COMPLIANCE AND PALATABILITY

The success of nutritional support depends, besides its composition, on sufficient quantities being consumed over an extended period of time (158, 218). Bauer et al demonstrated that patients who are compliant with the prescribed amount of nutritional supplementation showed improved nutrition related outcomes (219). This was in agreement with a study, which demonstrated an increase in lean body mass only in these patients who were compliant with the daily recommended dose (202). Accordingly, the palatability of a nutritional supplement is important to reach a high level of compliance and is thus a key factor in effectiveness. Taste alterations are common in patients with cancer (76, 220). The changes in taste and smell can be the result of the disease itself and/or its treatments and can vary from food tasting like cardboard or sandpaper to food tasting too salty, sweet, sour or bitter (161, 221, 222). In a study of Wickham et al, 193 out of 284 patients (68%) undergoing chemotherapy reported taste changes. The most reported changes were loss of taste acuity, changes in threshold and metallic aftertaste (161). Acceptability and palatability of a nutritional supplementation is extremely important in its effectiveness and the flavor of the nutritional supplement should therefore be adjusted to the preference of cancer patients.

AIM AND OUTLINE OF THE THESIS

Cancer is an important cause of morbidity and mortality worldwide. As was described in Chapter 1, it is a complex set of conditions and factors that can affect the clinical course, including the type and stage of the tumor, individual characteristics, anti-cancer treatment and the presence of cancer cachexia. Cachexia is a major problem in many cancer patients resulting in a chronic inflammatory state and impaired immune responsiveness. Moreover, cancer patients are described to have a reduced functional capacity and performance status due to reduced muscle function. Together, these factors may lead to disease progression, increased complications and a delayed or suboptimal treatment regimen resulting in a reduced quality of life and a poor prognosis. Accordingly, nutritional support is recommended as an integral part of anti-cancer therapy (74). For that reason, a specific nutritional combination has been developed for application in cancer patients. This concept is high in protein and leucine and is enriched with emulsified fish oil (containing EPA and DHA) and a specific oligosaccharide mixture and is designed to reduce complications and to provide optimal treatment support by reducing the inflammatory state, supporting immune function, and preserving muscle mass and function.

In order to get insight into the efficacy of nutritional ingredients, a recently modified and validated animal model for cancer cachexia was used, based on the colon-26 tumor model, in which parameters of immune function were measured as well. In Chapter 2, immune suppression during the course of the cachexia process was studied to identify potential early risk factors. The results indicated that immune function was already suppressed before weight loss was apparent. Providing nutritional support as early as possible, preferably starting at diagnosis and running parallel to the pathway of anti-cancer therapies might therefore be of high importance to support immune function and to reduce inflammation and other cachexia features. To evaluate the potential benefits of specific nutritional support for immune competence, the effects of the individual ingredients as well as of the complete mixture of ingredients of the specific nutritional combination (SNC) were investigated on inflammatory status, immune function and on different parameters of cachexia in the colon-26 tumor model (Chapter 3). Only the combination of ingredients appeared to reduce the inflammatory state and improve Th1-mediated immune function in this early phase compared to an iso-caloric control diet. To translate these beneficial immune modulatory effects to a setting investigating the resistance to infections with a relevant living pathogen, the effects of the SNC on *Pseudomonas aeruginosa* infections was studied in a model for chemotherapy-induced neutropenia, dealing with severe immune suppression (Chapter 4). This resembles the situation in humans suffering from chemotherapy-induced infectious complications, which have been associated with a higher morbidity and mortality in advanced disease. Infections were measured by the incidence and severity of bacterial translocation to liver and lungs and confirmed by plasma levels of pro-inflammatory cytokines.

The next step to a clinical application in cancer patients is to study the efficacy as well as the safety of the specific nutritional combination in a medical food in healthy volunteers, in which immune function was measured by the *ex vivo* immune responsiveness to LPS in whole blood cultures (Chapter 5). An additional objective of this study was to investigate

the incorporation kinetics of EPA and DHA (from fish-oil) into white blood cell phospholipids within one week of intervention, since a rapid and effective incorporation of these n-3 PUFA might be very important for cancer patients starting a treatment regimen as soon as possible after diagnosis. Consequently, it may be beneficial to supply a fast-acting product to patients to reduce complications and provide optimal treatment support.

In Chapter 6, the effect of a 4-week nutritional intervention with the specific medical food on immune function was investigated in an early phase in a group of newly diagnosed esophageal cancer patients before the start of anti-cancer therapy. In this exploratory study, the group receiving the specific medical food was compared with a control group that received routine nutritional support. Secondary objectives were to assess the effects of the medical food on parameters of inflammation, nutritional status, product palatability and safety. Data on immune function, inflammation and nutritional status of healthy volunteers were obtained to compare baseline values and allow an adequate interpretation of the data.

However, the risk of immune suppression is even higher after anti-cancer treatment leading to a reduced treatment efficacy and a higher frequency and severity of infectious and other complications. Therefore, the aim of the study presented in Chapter 7 was to investigate the rapid-acting effects of the medical food in cancer patients receiving radiotherapy. EPA and DHA incorporation into white blood cell phospholipids and the subsequent changes on parameters of immune function were analyzed and compared with the effects of an iso-caloric and iso-nitrogenous control product. Secondary objectives of the study were to assess the effects of the medical food on inflammatory status, nutritional status, safety and compliance.

Finally, this thesis is summarized in Chapter 8 and conclusions are drawn on the outcome and the translation from pre-clinical to clinical data. Subsequently, recommendations for additional research have been provided in order to investigate the possibility for an application in cancer patients as an integral part of disease management to provide optimal treatment support.

*A medical food is in USA defined in 21 U.S.C. § 360ee(b)(3) as ‘‘a food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognizable scientific principles, are established by medical evaluation’’ (223). A comparable definition exists in the harmonized legislation of the European Union (cf. Article 1, 2(b) of Commission Directive 1999/21/EC of 25 March 1999 on dietary foods for special medical purposes).

REFERENCES

1 Kumar P, Clark M. Clinical Medicine: Elsevier Saunders; 2005.

2 Pitot HC. The molecular biology of carcinogenesis. Cancer. 1993;72:962-70.

3 Pitot HC, Dragan YP. Facts and theories concerning the mechanisms of carcinogenesis. FASEB J. 1991;5:2280-6.

4 Vincent TL, Gatenby RA. An evolutionary model for initiation, promotion, and progression in carcinogenesis. Int J Oncol. 2008;32:729-37.

5 Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest. 2007;117:1137-46.

6 Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57-70.

7 Ferlay J, Shin H, Bray F, Forman D, Mathers C, D.M. P, GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC CancerBase No.10, Lyon, France: International Agency for Research on Cancer, 2010, Available from: http://globocan.iarc.fr

8 World Health Organization, Cancer fact sheet no. 297, WHO Media Centre, 2011, Available from: http://www.who.int

9 Ko AH, Dollinger M, Rosenbaum EH. Everyone's guide to cancer therapy: AndrewsMcMeel; 2008.

10 National Cancer Institute, Cancer Classification, U.S. National Cancer Institute's Surveillance Epidemiology and End Results Training module, U.S. National Institutes of Health, 2011, Available from: http://www.cancer.gov

11 Editors: Sobin L, Gospodarowicz M, Wittekind C. UICC International Union Against Cancer, TNM Classification of Malignant Tumors: Wiley-Blackwell; 2009.

12 American Cancer Society, Understanding your diagnose: staging, 2011, Available from: http://www.cancer.org

13 American Cancer Society, Stay healthy, 2011, Available from: http://www.cancer.org

14 Palacios J, Robles-Frias MJ, Castilla MA, Lopez-Garcia MA, Benitez J. The molecular pathology of hereditary breast cancer. Pathobiology. 2008;75:85-94.

15 Danaei G, Vander Hoorn S, Lopez AD, Murray CJ, Ezzati M. Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. Lancet. 2005;366:1784-93.

16 Gandini S, Botteri E, Iodice S, Boniol M, Lowenfels AB, Maisonneuve P, Boyle P. Tobacco smoking and cancer: a meta-analysis. Int J Cancer. 2008;122:155-64.

17 van Duijnhoven FJ, Bueno-De-Mesquita HB, Ferrari P, Jenab M, Boshuizen HC, Ros MM, Casagrande C, Tjonneland A, Olsen A, et al. Fruit, vegetables, and colorectal cancer risk: the European Prospective Investigation into Cancer and Nutrition. Am J Clin Nutr. 2009;89:1441-52.

18 Anand P, Kunnumakkara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, Sung B, Aggarwal BB. Cancer is a preventable disease that requires major lifestyle changes. Pharm Res. 2008;25:2097-116.

19 Saha A, Chaudhury AN, Bhowmik P, Chatterjee R. Awareness of cervical cancer among female students of premier colleges in Kolkata, India. Asian Pac J Cancer Prev. 2010;11:1085-90.

20 Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol. 2006;45:529-38.

21 Fucic A, Gamulin M, Ferencic Z, Rokotov DS, Katic J, Bartonova A, Lovasic IB, Merlo DF. Lung cancer and environmental chemical exposure: a review of our current state of knowledge with reference to the role of hormones and hormone receptors as an increased risk factor for developing lung cancer in man. Toxicol Pathol. 2010;38:849-55.

22 Harding AH, Darnton AJ. Asbestosis and mesothelioma among British asbestos workers (1971-2005). Am J Ind Med. 2010;53:1070-80.

23 Khalade A, Jaakkola MS, Pukkala E, Jaakkola JJ. Exposure to benzene at work and the risk of leukemia: a systematic review and meta-analysis. Environ Health. 2010;9:31.

24 Weiderpass E, Boffetta P, Vainio H, Editor-in-chief: Alison MR. Occupational causes of cancer, The Cancer handbook 2nd edition: John Wiley & Sons, Ltd; 2007.

25 de Gruijl FR. Skin cancer and solar UV radiation. Eur J Cancer. 1999;35:2003-9.

26 Rushton L, Hutchings S, Brown T. The burden of cancer at work: estimation as the first step to prevention. Occup Environ Med. 2008;65:789-800.

27 World Health Organization, Cancer prevention, screening and early detection of cancer, 2011, Available from: http://www.who.int

28 Adams EK, Breen N, Joski PJ. Impact of the National Breast and Cervical Cancer Early Detection Program on mammography and Pap test utilization among white, Hispanic, and African American women: 1996-2000. Cancer. 2007;109:348-58.

29 Levin B, Lieberman DA, McFarland B, Andrews KS, Brooks D, Bond J, Dash C, Giardiello FM, Glick S, et al. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. Gastroenterology. 2008;134:1570-95.

30 Newcomb PA, Carbone PP. Cancer treatment and age: patient perspectives. J Natl Cancer Inst. 1993;85:1580-4.

31 McKenna RJ, Sr. Clinical aspects of cancer in the elderly. Treatment decisions, treatment choices, and follow-up. Cancer. 1994;74:2107-17.

32 American Cancer Society, Support and Treatment, 2011, Available from: http://www.cancer.org

33 Evans DG, Lalloo F, Ashcroft L, Shenton A, Clancy T, Baildam AD, Brain A, Hopwood P, Howell A. Uptake of risk-reducing surgery in unaffected women at high risk of breast and ovarian cancer is risk, age, and time dependent. Cancer Epidemiol Biomarkers Prev. 2009;18:2318-24.

34 Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. AJCC American Joint Committee on Cancer: Cancer Staging Manual. New York: Springer; 2010.

35 Leo E, Belli F, Miceli R, Mariani L, Gallino G, Battaglia L, Vannelli A, Andreola S. Distal clearance margin of 1 cm or less: a safe distance in lower rectum cancer surgery. Int J Colorectal Dis. 2009;24:317-22.

36 Maly RC, Liu Y, Kwong E, Thind A, Diamant AL. Breast reconstructive surgery in medically underserved women with breast cancer: the role of patient-physician communication. Cancer. 2009;115:4819-27.

37 Cancer Research UK, 2011, Available from: http://www.cancerresearchuk.org

38 Sadeghi M, Enferadi M, Shirazi A. External and internal radiation therapy: past and future directions. J Cancer Res Ther. 2010;6:239-48.

39 Whitmyer CC, Waskowski JC, Iffland HA. Radiotherapy and oral sequelae: preventive and management protocols. J Dent Hyg. 1997;71:23-9.

40 Hortobagyi GN. Developments in chemotherapy of breast cancer. Cancer. 2000;88:3073-9.

41 Boulikas T. Introduction to Anticancer Therapeutics, in Anticancer Therapeutics (ed S. Missailidis). Chichester, UK: John Wiley & Sons, Ltd; 2008.

42 Lokich J, Anderson N. Dose intensity for bolus versus infusion chemotherapy administration: review of the literature for 27 anti-neoplastic agents. Ann Oncol. 1997;8:15-25.

43 Stavrovskaya AA. Cellular mechanisms of multidrug resistance of tumor cells. Biochemistry (Mosc). 2000;65:95-106.

44 Stringer AM, Gibson RJ, Bowen JM, Keefe DM. Chemotherapy-induced modifications to gastrointestinal microflora: evidence and implications of change. Curr Drug Metab. 2009;10:79-83.

45 Skeel RT. Handbook of cancer chemotherapy. Philadelphia: Lippincott Williams & Wilkins; 1999.

46 Xue H, Sawyer MB, Wischmeyer PE, Baracos VE. Nutrition modulation of gastrointestinal toxicity related to cancer chemotherapy: from preclinical findings to clinical strategy. JPEN J Parenter Enteral Nutr. 2011;35:74-90.

47 Kolek V, Grygarkova I, Hajduch M, Klein J, Cwiertka K, Neoral C, Langova K, Mihal V. Long term follow-up of neoadjuvant-adjuvant combination treatment of IIIA stage non-small-cell-lung cancer: results of neoadjuvant carboplatin/vinorelbine and carboplatin/paclitaxel regimens combined with selective adjuvant chemotherapy according to in-vitro chemoresistance test. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2008;152:259-66.

48 Holen JC, Hjermstad MJ, Loge JH, Fayers PM, Caraceni A, De Conno F, Forbes K, Furst CJ, Radbruch L, Kaasa S. Pain assessment tools: is the content appropriate for use in palliative care? J Pain Symptom Manage. 2006;32:567-80.

49 Qaseem A, Snow V, Shekelle P, Casey DE, Jr., Cross JT, Jr., Owens DK, Dallas P, Dolan NC, Forciea MA, et al. Evidence-based interventions to improve the palliative care of pain, dyspnea, and depression at the end of life: a clinical practice guideline from the American College of Physicians. Ann Intern Med. 2008;148:141-6.

50 Redd WH, Montgomery GH, DuHamel KN. Behavioral intervention for cancer treatment side effects. J Natl Cancer Inst. 2001;93:810-23.

51 Byar KL, Berger AM, Bakken SL, Cetak MA. Impact of adjuvant breast cancer chemotherapy on fatigue, other symptoms, and quality of life. Oncol Nurs Forum. 2006;33:E18-26.

52 Greene D, Nail LM, Fieler VK, Dudgeon D, Jones LS. A comparison of patient-reported side effects among three chemotherapy regimens for breast cancer. Cancer Pract. 1994;2:57-62.

53 Foltz AT, Gaines G, Gullatte M. Recalled side effects and self-care actions of patients receiving inpatient chemotherapy. Oncol Nurs Forum. 1996;23:679-83.

54 Taplin SC, Blanke CD, Baughman C. Nursing care strategies for the management of side effects in patients treated for colorectal cancer. Semin Oncol. 1997;24:S18-64-S18-70.

55 Semba SE, Mealey BL, Hallmon WW. The head and neck radiotherapy patient: Part 1--Oral manifestations of radiation therapy. Compendium. 1994;15:250, 2-60; quiz 61.

56 Nakamura T, Mitomi H, Ihara A, Onozato W, Sato T, Ozawa H, Hatade K, Watanabe M. Risk factors for wound infection after surgery for colorectal cancer. World J Surg. 2008;32:1138-41.

57 Karim-Kos HE, de Vries E, Soerjomataram I, Lemmens V, Siesling S, Coebergh JW. Recent trends of cancer in Europe: a combined approach of incidence, survival and mortality for 17 cancer sites since the 1990s. Eur J Cancer. 2008;44:1345-89.

58 Andreyev HJ, Norman AR, Oates J, Cunningham D. Why do patients with weight loss have a worse outcome when undergoing chemotherapy for gastrointestinal malignancies? Eur J Cancer. 1998;34:503-9.

59 Mahmoud FA, Rivera NI. The role of C-reactive protein as a prognostic indicator in advanced cancer. Curr Oncol Rep. 2002;4:250-5.

60 Blum D, Omlin A, Baracos VE, Solheim TS, Tan BH, Stone P, Kaasa S, Fearon K, Strasser F. Cancer cachexia: A systematic literature review of items and domains associated with involuntary weight loss in cancer. Crit Rev Oncol Hematol. 2011.

61 Evans WJ, Morley JE, Argiles J, Bales C, Baracos V, Guttridge D, Jatoi A, Kalantar-Zadeh K, Lochs H, et al. Cachexia: a new definition. Clin Nutr. 2008;27:793-9.

62 Ross JA, Fearon KC. Eicosanoid-dependent cancer cachexia and wasting. Curr Opin Clin Nutr Metab Care. 2002;5:241-8.

63 Van Cutsem E, Arends J. The causes and consequences of cancer-associated malnutrition. Eur J Oncol Nurs. 2005;9 Suppl 2:S51-63.

64 Argiles JM, Alvarez B, Lopez-Soriano FJ. The metabolic basis of cancer cachexia. Med Res Rev. 1997;17:477-98.

65 Fearon K, Strasser F, Anker SD, Bosaeus I, Bruera E, Fainsinger RL, Jatoi A, Loprinzi C, MacDonald N, et al. Definition and classification of cancer cachexia: an international consensus. Lancet Oncol. 2011;12:489-95.

66 Argiles JM, Anker SD, Evans WJ, Morley JE, Fearon KC, Strasser F, Muscaritoli M, Baracos VE. Consensus on cachexia definitions. J Am Med Dir Assoc. 2010;11:229-30.

67. Evans C, Dalgleish AG, Kumar D. Review article: immune suppression and colorectal cancer. Aliment Pharmacol Ther. 2006;24:1163-77.

68. Hadden JW. Immunodeficiency and cancer: prospects for correction. Int Immunopharmacol. 2003;3:1061-71.

69 McNamara MJ, Alexander HR, Norton JA. Cytokines and their role in the pathophysiology of cancer cachexia. JPEN J Parenter Enteral Nutr. 1992;16:50S-5S.

70 Argiles JM, Busquets S, Moore-Carrasco R, Figueras M, Almendro V, Lopez-Soriano FJ. Targets in clinical oncology: the metabolic environment of the patient. Front Biosci. 2007;12:3024-51.

71 Rossi Fanelli F, Cangiano C, Muscaritoli M, Conversano L, Torelli GF, Cascino A. Tumor-induced changes in host metabolism: a possible marker of neoplastic disease. Nutrition. 1995;11:595-600.

72 Dewys WD, Begg C, Lavin PT, Band PR, Bennett JM, Bertino JR, Cohen MH, Douglass HO, Jr., Engstrom PF, et al. Prognostic effect of weight loss prior to chemotherapy in cancer patients. Eastern Cooperative Oncology Group. Am J Med. 1980;69:491-7.

73 Martignoni ME, Kunze P, Friess H. Cancer cachexia. Mol Cancer. 2003;2:36.

74 Muscaritoli M, Costelli P, Aversa Z, Bonetto A, Baccino FM, Rossi Fanelli F. New strategies to overcome cancer cachexia: from molecular mechanisms to the 'Parallel Pathway'. Asia Pac J Clin Nutr. 2008;17 Suppl 1:387-90.

75 Faber J, Vos AP, Kegler D, Argiles J, Laviano A, Garssen J, Van Helvoort A. Impaired immune function: an early marker for cancer cachexia. Oncol Rep. 2009;22:1403-6.

76 Nitenberg G, Raynard B. Nutritional support of the cancer patient: issues and dilemmas. Crit Rev Oncol Hematol. 2000;34:137-68.

77 Laviano A, Meguid MM. Nutritional issues in cancer management. Nutrition. 1996;12:358-71.

78 Bozzetti F, Arends J, Lundholm K, Micklewright A, Zurcher G, Muscaritoli M. ESPEN Guidelines on Parenteral Nutrition: non-surgical oncology. Clin Nutr. 2009;28:445-54.

79 Theologides A. Cancer cachexia. Cancer. 1979;43:2004-12.

80 Langius JA, Doornaert P, Spreeuwenberg MD, Langendijk JA, Leemans CR, van Bokhorst-de van der Schueren MA. Radiotherapy on the neck nodes predicts severe weight loss in patients with early stage laryngeal cancer. Radiother Oncol. 2010;97:80-5.

81 Navari RM. Pharmacological management of chemotherapy-induced nausea and vomiting: focus on recent developments. Drugs. 2009;69:515-33.

82 Tisdale MJ. Mechanisms of cancer cachexia. Physiol Rev. 2009;89:381-410.

83 Kotler DP. Cachexia. Ann Intern Med. 2000;133:622-34.

84 Grant M, Kravits K. Symptoms and their impact on nutrition. Semin Oncol Nurs. 2000;16:113-21.

85 Tisdale MJ. Cancer anorexia and cachexia. Nutrition. 2001;17:438-42.

86 Nitenberg G, Raynard B. Nutritional support of the cancer patient: issues and dilemmas. Crit Rev Oncol Hematol. 2 000;34:137-68.

87 Ravasco P, Monteiro-Grillo I, Camilo ME. Does nutrition influence quality of life in cancer patients undergoing radiotherapy? Radiother Oncol. 2003;67:213-20.

88 Baracos VE. Cancer-associated cachexia and underlying biological mechanisms. Annu Rev Nutr. 2006.

89 Barber MD, Ross JA, Fearon KC. Cancer cachexia. Surg Oncol. 1999;8:133-41.

90 Tisdale MJ. Molecular pathways leading to cancer cachexia. Physiology (Bethesda). 2005;20:340-8.

91 Falconer JS, Fearon KC, Plester CE, Ross JA, Carter DC. Cytokines, the acute-phase response, and resting energy expenditure in cachectic patients with pancreatic cancer. Ann Surg. 1994;219:325-31.

92 Bosaeus I, Daneryd P, Svanberg E, Lundholm K. Dietary intake and resting energy expenditure in relation to weight loss in unselected cancer patients. Int J Cancer. 2001;93:380-3.

93 Bozzetti F. Nutrition support in patients with cancer. In: Payne-James J, Grimble G, Silk D, editors. Artificial Nutrition Support in Clinical Practice. London: Greenwich Medical Media Limited; 2001.

94 Holroyde CP, Skutches CL, Boden G, Reichard GA. Glucose metabolism in cachectic patients with colorectal cancer. Cancer Res. 1984;44:5910-3.

95 Barber MD. The pathophysiology and treatment of cancer cachexia. Nutr Clin Pract. 2002;17:203-9.

96 Argiles JM, Busquets S, Lopez-Soriano FJ. Metabolic interrelationships between liver and skeletal muscle in pathological states. Life Sci. 2001;69:1345-61.

97 Vissers YL, Dejong CH, Luiking YC, Fearon KC, von Meyenfeldt MF, Deutz NE. Plasma arginine concentrations are reduced in cancer patients: evidence for arginine deficiency? Am J Clin Nutr. 2005;81:1142-6.

98 Zuijdgeest-van Leeuwen SD, van den Berg JW, Wattimena JL, van der Gaast A, Swart GR, Wilson JH, Dagnelie PC. Lipolysis and lipid oxidation in weight-losing cancer patients and healthy subjects. Metabolism. 2000;49:931-6.

99 Thompson MP, Cooper ST, Parry BR, Tuckey JA. Increased expression of the mRNA for hormone-sensitive lipase in adipose tissue of cancer patients. Biochim Biophys Acta. 1993;1180:236-42.

100 Baumann H, Gauldie J. The acute phase response. Immunol Today. 1994;15:74-80.

101 Stephens NA, Skipworth RJ, Fearon KC. Cachexia, survival and the acute phase response. Curr Opin Support Palliat Care. 2008;2:267-74.

102 Russell ST, Tisdale MJ. Effect of a tumour-derived lipid-mobilising factor on glucose and lipid metabolism in vivo. Br J Cancer. 2002;87:580-4.

103 Tisdale MJ. Cancer anorexia and cachexia. Nutrition. 2001;17:438-42.

104 Groundwater P, Beck SA, Barton C, Adamson C, Ferrier IN, Tisdale MJ. Alteration of serum and urinary lipolytic activity with weight loss in cachectic cancer patients. Br J Cancer. 1990;62:816-21.

105 Toomey D, Redmond HP, Bouchier-Hayes D. Mechanisms mediating cancer cachexia. Cancer. 1995;76:2418-26.

106 Argiles JM, Busquets S, Lopez-Soriano FJ. Cytokines in the pathogenesis of cancer cachexia. Curr Opin Clin Nutr Metab Care. 2003;6:401-6.

107 Morley JE, Thomas DR, Wilson MM. Cachexia: pathophysiology and clinical relevance. Am J Clin Nutr. 2006;83:735-43.
108 Argiles JM. Cancer-associated malnutrition. Eur J Oncol Nurs. 2005;9 Suppl 2:S39-50.
109 Argiles JM, Busquets S, Toledo M, Lopez-Soriano FJ. The role of cytokines in cancer cachexia. Curr Opin Support Palliat Care. 2009;3:263-8.
110 Correia M, Cravo M, Marques-Vidal P, Grimble R, Dias-Pereira A, Faias S, Nobre-Leitao C. Serum concentrations of TNF-alpha as a surrogate marker for malnutrition and worse quality of life in patients with gastric cancer. Clin Nutr. 2007;26:728-35.
111 Warren S. The immediate cause of death in cancer. Am J Med Sci. 1932:610.
112 Inagaki J, Rodriguez V, Bodey GP. Proceedings: Causes of death in cancer patients. Cancer. 1974;33:568-73.
113 Barton BE. IL-6-like cytokines and cancer cachexia: consequences of chronic inflammation. Immunol Res. 2001;23:41-58.
114 Young MR. Eicosanoids and the immunology of cancer. Cancer Metastasis Rev. 1994;13:337-48.
115 Herber DL, Nagaraj S, Djeu JY, Gabrilovich DI. Mechanism and therapeutic reversal of immune suppression in cancer. Cancer Res. 2007;67:5067-9.
116 Tisdale MJ. Cachexia in cancer patients. Nat Rev Cancer. 2002;2:862-71.
117 Edington J, Winter PD, Coles SJ, Gale CR, Martyn CN. Outcomes of undernutrition in patients in the community with cancer or cardiovascular disease. Proc Nutr Soc. 1999;58:655-61.
118 Braunschweig C, Gomez S, Sheean PM. Impact of declines in nutritional status on outcomes in adult patients hospitalized for more than 7 days. J Am Diet Assoc. 2000;100:1316-22; quiz 23-4.
119 Edington J, Boorman J, Durrant ER, Perkins A, Giffin CV, James R, Thomson JM, Oldroyd JC, Smith JC, et al. Prevalence of malnutrition on admission to four hospitals in England. The Malnutrition Prevalence Group. Clin Nutr. 2000;19:191-5.
120 Tan BH, Fearon KC. Cachexia: prevalence and impact in medicine. Curr Opin Clin Nutr Metab Care. 2008;11:400-7.
121 Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001;357:539-45.
122 Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860-7.
123 Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140:883-99.
124 Ben-Baruch A. Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators. Semin Cancer Biol. 2006;16:38-52.
125 Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454:436-44.
126 Wang D, Dubois RN. Eicosanoids and cancer. Nat Rev Cancer. 2010;10:181-93.
127 de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer. 2006;6:24-37.
128 Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest. 2007;117:1175-83.
129 Schmid MC, Varner JA. Myeloid cells in the tumor microenvironment: modulation of tumor angiogenesis and tumor inflammation. J Oncol.2010:201026.
130 Allavena P, Sica A, Garlanda C, Mantovani A. The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. Immunol Rev. 2008;222:155-61.
131 Ostrand-Rosenberg S. Immune surveillance: a balance between protumor and antitumor immunity. Curr Opin Genet Dev. 2008;18:11-8.
132 Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest. 2007;117:1155-66.
133 Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol. 2009;182:4499-506.
134 Di Carlo E, Forni G, Lollini P, Colombo MP, Modesti A, Musiani P. The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood. 2001;97:339-45.
135 Mantovani A, Savino B, Locati M, Zammataro L, Allavena P, Bonecchi R. The chemokine system in cancer biol-ogy and therapy. Cytokine Growth Factor Rev. 2010;21:27-39.
136 Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell. 2009;16:183-94.

137 Vivier E. What is natural in natural killer cells? Immunol Lett. 2006;107:1-7.

138 Smyth MJ, Hayakawa Y, Takeda K, Yagita H. New aspects of natural-killer-cell surveillance and therapy of cancer. Nat Rev Cancer. 2002;2:850-61.

139 Cooper MA, Fehniger TA, Fuchs A, Colonna M, Caligiuri MA. NK cell and DC interactions. Trends Immunol. 2004;25:47-52.

140 DeNardo DG, Coussens LM. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. Breast Cancer Res. 2007;9:212.

141 DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. Cancer Metastasis Rev. 2010;29:309-16.

142. Rodriguez-Vita J, Lawrence T. The resolution of inflammation and cancer. Cytokine Growth Factor Rev. 2010;21:61-5.

143 Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: how hot is the link? Biochem Pharmacol. 2006;72:1605-21.

144 Saijo Y, Tanaka M, Miki M, Usui K, Suzuki T, Maemondo M, Hong X, Tazawa R, Kikuchi T, et al. Proinflammatory cytokine IL-1 beta promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: in vivo analysis of tumor-stromal interaction. J Immunol. 2002;169:469-75.

145 Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol. 2003;3:133-46.

146 Lazennec G, Richmond A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. Trends Mol Med. 2010;16:133-44.

147 O'Hayre M, Salanga CL, Handel TM, Allen SJ. Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. Biochem J. 2008;409:635-49.

148 Allavena P, Garlanda C, Borrello MG, Sica A, Mantovani A. Pathways connecting inflammation and cancer. Curr Opin Genet Dev. 2008;18:3-10.

149 Saji H, Koike M, Yamori T, Saji S, Seiki M, Matsushima K, Toi M. Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma. Cancer. 2001;92:1085-91.

150 Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, Koike M, Inadera H, Matsushima K. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clin Cancer Res. 2000;6:3282-9.

151 Serafini P. Editorial: PGE2-producing MDSC: a role in tumor progression? J Leukoc Biol. 2010;88:827-9.

152 Shaikh SR, Edidin M. Polyunsaturated fatty acids and membrane organization: elucidating mechanisms to balance immunotherapy and susceptibility to infection. Chem Phys Lipids. 2008;153:24-33.

153 Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol. 2010.

154 Johansson M, Denardo DG, Coussens LM. Polarized immune responses differentially regulate cancer development. Immunol Rev. 2008;222:145-54.

155 Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother. 2009;58:49-59.

156 Deans C, Wigmore SJ. Systemic inflammation, cachexia and prognosis in patients with cancer. Curr Opin Clin Nutr Metab Care. 2005;8:265-9.

157 Ross PJ, Ashley S, Norton A, Priest K, Waters JS, Eisen T, Smith IE, O'Brien ME. Do patients with weight loss have a worse outcome when undergoing chemotherapy for lung cancers? Br J Cancer. 2004;90:1905-11.

158 van Bokhorst-de van der Schueren MA. Nutritional support strategies for malnourished cancer patients. Eur J Oncol Nurs. 2005;9 Suppl 2:S74-83.

159 Muscaritoli M, Bossola M, Aversa Z, Bellantone R, Rossi Fanelli F. Prevention and treatment of cancer cachexia: new insights into an old problem. Eur J Cancer. 2006;42:31-41.

160 Ravasco P, Monteiro-Grillo I, Vidal PM, Camilo ME. Dietary counseling improves patient outcomes: a prospective, randomized, controlled trial in colorectal cancer patients undergoing radiotherapy. J Clin Oncol. 2005;23:1431-8.

161 Wickham RS, Rehwaldt M, Kefer C, Shott S, Abbas K, Glynn-Tucker E, Potter C, Blendowski C. Taste changes experienced by patients receiving chemotherapy. Oncol Nurs Forum. 1999;26:697-706.

162 Ovesen L, Allingstrup L. Different quantities of two commercial liquid diets consumed by weight-losing cancer patients. JPEN J Parenter Enteral Nutr. 1992;16:275-8.

163 Haffejee AA, Angorn IB. Nutritional status and the nonspecific cellular and humoral immune response in esophageal carcinoma. Ann Surg. 1979;189:475-9.

164 Bounous G, Gentile JM, Hugon J. Elemental diet in the management of the intestinal lesion produced by 5-fluorouracil in man. Can J Surg. 1971;14:312-24.

165 Barber MD, Ross JA, Voss AC, Tisdale MJ, Fearon KC. The effect of an oral nutritional supplement enriched with fish oil on weight-loss in patients with pancreatic cancer. Br J Cancer. 1999;81:80-6.

166 Potter J, Langhorne P, Roberts M. Routine protein energy supplementation in adults: systematic review. BMJ. 1998;317:495-501.

167 Barber MD, Fearon KC, Tisdale MJ, McMillan DC, Ross JA. Effect of a fish oil-enriched nutritional supplement on metabolic mediators in patients with pancreatic cancer cachexia. Nutr Cancer. 2001;40:118-24.

168 Bauer JD, Capra S. Nutrition intervention improves outcomes in patients with cancer cachexia receiving chemotherapy--a pilot study. Support Care Cancer. 2005;13:270-4.

169 Isenring EA, Bauer JD, Capra S. Nutrition support using the American Dietetic Association medical nutrition therapy protocol for radiation oncology patients improves dietary intake compared with standard practice. J Am Diet Assoc. 2007;107:404-12.

170 van der Meij BS, Langius JA, Smit EF, Spreeuwenberg MD, von Blomberg BM, Heijboer AC, Paul MA, van Leeuwen PA. Oral nutritional supplements containing (n-3) polyunsaturated fatty acids affect the nutritional status of patients with stage III non-small cell lung cancer during multimodality treatment. J Nutr. 2010;140:1774-80.

171 Pearce CB, Duncan HD. Enteral feeding. Nasogastric, nasojejunal, percutaneous endoscopic gastrostomy, or jejunostomy: its indications and limitations. Postgrad Med J. 2002;78:198-204.

172 Elia M, Ceriello A, Laube H, Sinclair AJ, Engfer M, Stratton RJ. Enteral nutritional support and use of diabetes-specific formulas for patients with diabetes: a systematic review and meta-analysis. Diabetes Care. 2005;28:2267-79

173 Klein S, Simes J, Blackburn GL. Total parenteral nutrition and cancer clinical trials. Cancer. 1986;58:1378-86.

174 Rivadeneira DE, Evoy D, Fahey TJ, 3rd, Lieberman MD, Daly JM. Nutritional support of the cancer patient. CA Cancer J Clin. 1998;48:69-80.

175 Ishizuka M, Nagata H, Takagi K, Kubota K. Total parenteral nutrition is a major risk factor for central venous catheter-related bloodstream infection in colorectal cancer patients receiving postoperative chemotherapy. Eur Surg Res. 2008;41:341-5.

176 Senesse P, Assenat E, Schneider S, Chargari C, Magne N, Azria D, Hebuterne X. Nutritional support during oncologic treatment of patients with gastrointestinal cancer: who could benefit? Cancer Treat Rev. 2008;34:568-75.

177 Pardini RS. Nutritional intervention with omega-3 fatty acids enhances tumor response to anti-neoplastic agents. Chem Biol Interact. 2006;162:89-105.

178 Biondo PD, Brindley DN, Sawyer MB, Field CJ. The potential for treatment with dietary long-chain polyunsaturated n-3 fatty acids during chemotherapy. J Nutr Biochem. 2008;19:787-96.

179 Murphy RA, Mourtzakis M, Chu QS, Baracos VE, Reiman T, Mazurak VC. Supplementation with fish oil increases first-line chemotherapy efficacy in patients with advanced nonsmall cell lung cancer. Cancer. 2011;117:3774-80.

180 van Bokhorst-De Van Der Schueren MA, Quak JJ, von Blomberg-van der Flier BM, Kuik DJ, Langendoen SI, Snow GB, Green CJ, van Leeuwen PA. Effect of perioperative nutrition, with and without arginine supplementation, on nutritional status, immune function, postoperative morbidity, and survival in severely malnourished head and neck cancer patients. Am J Clin Nutr. 2001;73:323-32.

181 Lochs H, Pichard C, Allison SP. Evidence supports nutritional support. Clin Nutr. 2006;25:177-9.

182 Arends J, Bodoky G, Bozzetti F, Fearon K, Muscaritoli M, Selga G, van Bokhorst-de van der Schueren MA, von Meyenfeldt M, Zurcher G, et al. ESPEN Guidelines on Enteral Nutrition: Non-surgical oncology. Clin Nutr. 2006;25:245-59.

183 Weimann A, Braga M, Harsanyi L, Laviano A, Ljungqvist O, Soeters P, Jauch KW, Kemen M, Hiesmayr JM, et al. ESPEN Guidelines on Enteral Nutrition: Surgery including organ transplantation. Clin Nutr. 2006;25:224-44.

184 Lee H, Havrila C, Bravo V, Shantz K, Diaz K, Larner J, Read P. Effect of oral nutritional supplementation on weight loss and percutaneous endoscopic gastrostomy tube rates in patients treated with radiotherapy for oropharyngeal carcinoma. Support Care Cancer. 2008;16:285-9.

185 Keele AM, Bray MJ, Emery PW, Duncan HD, Silk DB. Two phase randomised controlled clinical trial of postoperative oral dietary supplements in surgical patients. Gut. 1997;40:393-9.

186 Isenring EA, Capra S, Bauer JD. Nutrition intervention is beneficial in oncology outpatients receiving radio therapy to the gastrointestinal or head and neck area. Br J Cancer. 2004;91:447-52.

187 Gogos CA, Ginopoulos P, Salsa B, Apostolidou E, Zoumbos NC, Kalfarentzos F. Dietary omega-3 polyunsaturated fatty acids plus vitamin E restore immunodeficiency and prolong survival for severely ill patients with generalized malignancy: a randomized control trial. Cancer. 1998;82:395-402.

188 Wigmore SJ, Fearon KC, Maingay JP, Ross JA. Down-regulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. Clin Sci (Lond). 1997;92:215-21.

189 Furukawa K, Tashiro T, Yamamori H, Takagi K, Morishima Y, Sugiura T, Otsubo Y, Hayashi N, Itabashi T, et al. Effects of soybean oil emulsion and eicosapentaenoic acid on stress response and immune function after a severely stressful operation. Ann Surg. 1999;229:255-61.

190 Okamoto Y, Okano K, Izuishi K, Usuki H, Wakabayashi H, Suzuki Y. Attenuation of the systemic inflammatory response and infectious complications after gastrectomy with preoperative oral arginine and omega-3 fatty acids supplemented immunonutrition. World J Surg. 2009;33:1815-21.

191 Rooyackers OE, Nair KS. Hormonal regulation of human muscle protein metabolism. Annu Rev Nutr. 1997;17:457-85.

192 Kobayashi H, Kato H, Hirabayashi Y, Murakami H, Suzuki H. Modulations of muscle protein metabolism by branched-chain amino acids in normal and muscle-atrophying rats. J Nutr. 2006;136:234S-6S.

193 Long-term oral administration of branched chain amino acids after curative resection of hepatocellular carcinoma: a prospective randomized trial. The San-in Group of Liver Surgery. Br J Surg. 1997;84:1525-31.

194 Meng WC, Leung KL, Ho RL, Leung TW, Lau WY. Prospective randomized control study on the effect of branched-chain amino acids in patients with liver resection for hepatocellular carcinoma. Aust N Z J Surg. 1999;69:811-5.

195 Poon RT, Yu WC, Fan ST, Wong J. Long-term oral branched chain amino acids in patients undergoing chemoembolization for hepatocellular carcinoma: a randomized trial. Aliment Pharmacol Ther. 2004;19:779-88.

196 Deutz NE, Safar A, Schutzler S, Memelink R, Ferrando A, Spencer H, van Helvoort A, Wolfe RR. Muscle protein synthesis in cancer patients can be stimulated with a specially formulated medical food. Clin Nutr. 2011.

197 van Norren K, Kegler D, Argiles JM, Luiking Y, Gorselink M, Laviano A, Arts K, Faber J, Jansen H, et al. Dietary supplementation with a specific combination of high protein, leucine, and fish oil improves muscle function and daily activity in tumour-bearing cachectic mice. Br J Cancer. 2009;100:713-22.

198 Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr. 2006;83:1505S-19S.

199 Wu D, Meydani SN. n-3 polyunsaturated fatty acids and immune function. Proc Nutr Soc. 1998;57:503-9.

200 Garaiova I, Guschina IA, Plummer SF, Tang J, Wang D, Plummer NT. A randomised cross-over trial in healthy adults indicating improved absorption of omega-3 fatty acids by pre-emulsification. Nutr J. 2007;6:4.

201 Raatz SK, Redmon JB, Wimmergren N, Donadio JV, Bibus DM. Enhanced absorption of n-3 fatty acids from emulsified compared with encapsulated fish oil. J Am Diet Assoc. 2009;109:1076-81.

202 Fearon KC, Von Meyenfeldt MF, Moses AG, Van Geenen R, Roy A, Gouma DJ, Giacosa A, Van Gossum A, Bauer J, et al. Effect of a protein and energy dense N-3 fatty acid enriched oral supplement on loss of weight and lean tissue in cancer cachexia: a randomised double blind trial. Gut. 2003;52:1479-86.

203 Ryan AM, Reynolds JV, Healy L, Byrne M, Moore J, Brannelly N, McHugh A, McCormack D, Flood P. Enteral nutrition enriched with eicosapentaenoic acid (EPA) preserves lean body mass following esophageal cancer surgery: results of a double-blinded randomized controlled trial. Ann Surg. 2009;249:355-63.

204 Takagi K, Yamamori H, Furukawa K, Miyazaki M, Tashiro T. Perioperative supplementation of EPA reduces im

munosuppression induced by postoperative chemoradiation therapy in patients with esophageal cancer. Nutrition. 2001;17:478-9.

205 Takatsuka H, Takemoto Y, Iwata N, Suehiro A, Hamano T, Okamoto T, Kanamaru A, Kakishita E. Oral eicosapentaenoic acid for complications of bone marrow transplantation. Bone Marrow Transplant. 2001;28:769-74.

206 Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. Proc Nutr Soc. 2007;66:237-59.

207 Baracos VE, Mazurak VC, Ma DW. n-3 Polyunsaturated fatty acids throughout the cancer trajectory: influence on disease incidence, progression, response to therapy and cancer-associated cachexia. Nutr Res Rev. 2004;17:177-92.

208 Vos A, M'Rabet L, Stahl B, Boehm G, Garssen J. Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. Crit Rev Immunol. 2007;27:97-140.

209 Lomax AR, Calder PC. Prebiotics, immune function, infection and inflammation: a review of the evidence. Br J Nutr. 2009;101:633-58.

210 Worthley DL, Le Leu RK, Whitehall VL, Conlon M, Christophersen C, Belobrajdic D, Mallitt KA, Hu Y, Irahara N, et al. A human, double-blind, placebo-controlled, crossover trial of prebiotic, probiotic, and synbiotic supplementation: effects on luminal, inflammatory, epigenetic, and epithelial biomarkers of colorectal cancer. Am J Clin Nutr. 2009;90:578-86.

211 Macfarlane GT, Steed H, Macfarlane S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol. 2008;104:305-44.

212. Boehm G, Moro G. Structural and functional aspects of prebiotics used in infant nutrition. J Nutr. 2008;138:1818S-28S.

213 Vos AP, Haarman M, Buco A, Govers M, Knol J, Garssen J, Stahl B, Boehm G, M'Rabet L. A specific prebiotic oligosaccharide mixture stimulates delayed-type hypersensitivity in a murine influenza vaccination model. Int Immunopharmacol. 2006;6:1277-86.

214 Bruzzese E, Volpicelli M, Salvini F, Bisceglia M, Lionetti P, Cinquette M, Iacono G, Guarino A. Early administration of GOS/FOS prevents intestinal and respiratory infections in infants. J Pediatr Gastroenterol Nutr. 2006;42:E95.

215 Arslanoglu S, Moro GE, Boehm G. Early supplementation of prebiotic oligosaccharides protects formula-fed infants against infections during the first 6 months of life. J Nutr. 2007;137:2420-4.

216 Wollowski I, Rechkemmer G, Pool-Zobel BL. Protective role of probiotics and prebiotics in colon cancer. Am J Clin Nutr. 2001;73:451S-5S.

217 Lim CC, Ferguson LR, Tannock GW. Dietary fibres as "prebiotics": implications for colorectal cancer. Mol Nutr Food Res. 2005;49:609-19.

218 Fuller L. The acceptability of sweet and savoury sip feeds. Hum Nutr Appl Nutr. 1985;39:422-5.

219 Bauer J, Capra S, Battistutta D, Davidson W, Ash S. Compliance with nutrition prescription improves outcomes in patients with unresectable pancreatic cancer. Clin Nutr. 2005;24:998-1004.

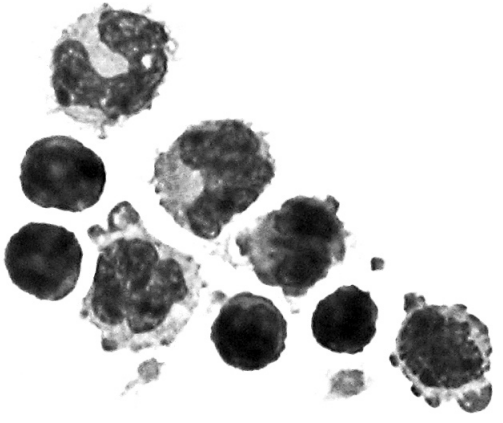
220 Ripamonti C, Zecca E, Brunelli C, Fulfaro F, Villa S, Balzarini A, Bombardieri E, De Conno F. A randomized, controlled clinical trial to evaluate the effects of zinc sulfate on cancer patients with taste alterations caused by head and neck irradiation. Cancer. 1998;82:1938-45.

221 Rahemtulla Z, Baldwin C, Spiro A, McGough C, Norman AR, Frost G, Cunningham D, Andreyev HJ. The palatability of milk-based and non-milk-based nutritional supplements in gastrointestinal cancer and the effect of chemotherapy. Clin Nutr. 2005;24:1029-37.

222 Bolze MS, Fosmire GJ, Stryker JA, Chung CK, Flipse BG. Taste acuity, plasma zinc levels, and weight loss during radiotherapy: a study of relationships. Radiology. 1982;144:163-9.

223 US Food and Drug Administration. Frequently Asked Questions About Medical Foods. In: College Park MF, Center for Food Safety and Applied Nutrition, US Department of Health and Human Services, editor.; 2007.

CHAPTER TWO



Impaired immune function: an early marker for cancer cachexia

J. Faber, A.P. Vos, D. Kegler, J. Argilés, A. Laviano, J. Garssen and A. van Helvoort

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ABSTRACT

Cachexia and chronic inflammation are major challenges for cancer patients, leading to serious consequences. Accordingly, it is of high clinical relevance to identify early risk factors for optimal treatment, as these are currently not available. The present study demonstrates a strong decline in contact hypersensitivity, a parameter for cell-mediated immunity, in tumor-bearing cachectic mice. Interestingly, a significant reduction was already observed during the pre-cachectic state, reflecting an impaired immune function prior to weight loss. Extrapolating to the human setting, reduced immune competence of cancer patients could serve as an early marker for cancer cachexia, enabling an early supportive care strategy.

INTRODUCTION

Cancer cachexia occurs in the majority of cancer patients and is a major contributor to morbidity and mortality in advanced disease (1, 2). Although there is still debate on the definition of cachexia, characteristics of this chronic condition of catabolism include, progressive, involuntary weight loss, wasting, anorexia, asthenia and fatigue (1-3). Tumor-derived factors, therapeutic strategies, but also nutritional status, age and even stress and depression are involved in this process, resulting in a chronic inflammatory state and impaired immune responsiveness (1, 4, 5).

Immune suppression is a major problem in these cancer patients leading to disease progression, increased complications and a delayed or suboptimal treatment protocol (e.g. surgery, chemotherapy, radiotherapy) resulting in a reduced quality of life and a poor prognosis (4-7). The dysfunction of the immune system involves multiple mechanisms and is in humans characterized by a reduction of monocyte-, macrophage- and dendritic cell (DC)-function and NK-cell activity, leading to an increased risk of infections and a poor clinical outcome (4, 5, 8, 9).

Prevention and treatment of cancer cachexia should be recognized as an integral part of cancer therapy. It might stop or reverse the nutritional decline and counteract dysfunction of the immune system in order to improve clinical outcome and quality of life (10, 11). However, recent findings show that significant metabolic, biochemical and molecular changes on muscle proteolysis already occur in patients before any evidence of body weight loss (12). Consequently, it is of high clinical relevance to investigate the appearance of immune suppression in the course of the cachexia process as well, in order to identify early risk factors.

A recently modified C26 tumor model was used to study the cachectic process and immune competence. Cachexia was induced by inoculation of murine colon adenocarcinoma (C26) cells in syngenic CD2F1 mice leading to several cachectic features (13-17). To measure immune competence contact hypersensitivity (CHS) towards oxazolone was measured as a validated *in vivo* parameter for Th1-mediated immune function. Th1 immunity is involved in anti-tumor immune responses, but plays a pivotal role in the defence against infections with pathogenic bacteria and viruses as well. CHS was measured at two time points during the study, to observe changes in the immune status in a pre-cachectic and a cachectic state of the mice.

METHODS

ANIMALS AND DIETS

Six to seven-week old syngenic male CD2F1 mice (BALB/c x DBA/2) were obtained from Harlan Nederland (Horst, the Netherlands). All experimental procedures were approved by the Animal Experimental Committee and complied with the principles of laboratory animal care. Animals were housed individually in a climate-controlled animal care facility with a constant room temperature and humidity. All animals had free access to food and drinking water. Upon arrival animals were acclimatized for one week and subsequently randomized on basis of bodyweight. The experiments were divided in: experiment A, designed to

investigate the effect on immune function in a pre-cachectic state and experiment B, designed to investigate the effect on immune function in a cachectic state of the mice. In both experiments A and B, mice were divided into a control group (C) and a tumor-bearing group (TB). Both groups received a control diet based on AIN93-M (Research Diet Services, Wijk bij Duurstede, the Netherlands), supplied as pellets and contained per kg food: 126 g protein (100% casein), 727 g carbohydrates and 40 g fat (100% soy oil).

EXPERIMENTAL DESIGN

Murine colon-26 adenocarcinoma cells were used to induce cachexia in mice. Shortly, on day 0 tumor cells (5 x 10^5 cells in 0.2 ml) were inoculated, under general anaesthesia (isoflurane/N_2O/O_2), subcutaneously into the right inguinal flank of CD2F1 mice in the tumor-bearing group. Animals in the control group received a sham injection with 0.2 ml HBSS. Body weight (BW), food intake and tumor size (length and width) were measured three times a week. To investigate effects on the immune system contact hypersensitivity (CHS) against oxazolone was determined, as an *in vivo* model for cellular (Th1 dependent) immunity (Figure 1). In experiment A, CHS was measured in a pre-cachectic state of the mice, while in experiment B, CHS was determined when the mice were already cachectic (Figure 1). Briefly, on day 8 (experiment A) or day 15 (experiment B) all animals were sensitized with 150 µl 3% oxazolone solution (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands, 300 mg in 7.5 ml 96% ethanol and 2.5 ml acetone) applied on their shaved breast and abdomen. Subsequently, at day 13 (experiment A) or day 19 (experiment B) ear thickness was measured under general anaesthesia and all animals were hapten challenged with 25 µl 0.8% oxazolone solution (32 mg in 3 ml 96% ethanol and 1 ml acetone) topical to the ear pinnae. At day 14 (experiment A) or day 20 (experiment B) after tumor inoculation (24 hours after the challenge), ear swelling was measured under general anaesthesia to determine the Th1 immune response. In both experiments A and B mice were sacrificed at day 20 and tumor, spleen, thymus, fat and skeletal muscles Extensor Digitorum Longus (mEDL) and m. Tibialis Anterior (mTA)) were dissected and weighed.

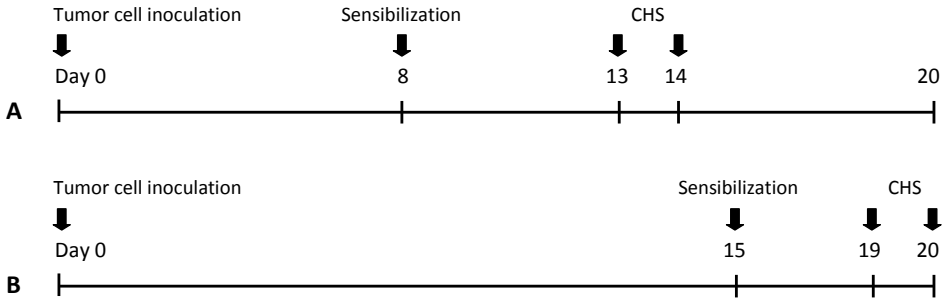

Figure 1 Experimental set-up. CHS against oxazolone is measured in a pre-cachectic state at day 13/14 (A) and in a cachectic state at day 19/20 (B).

STATISTICS

All data were expressed as means ± SEM. Statistical analysis was performed using SPSS 12.0.1 (SPSS Benelux, Gorinchem, the Netherlands). The effect of tumor inoculation was tested using a Student's T-test when data were normally distributed. A non-parametric Mann Whitney U test was performed when data were not normally distributed. Differences were considered significant at $p < 0.05$.

RESULTS

Animals in both the control (C) and tumor-bearing (TB) group demonstrated a normal growth in bodyweight (BW) until day 14 (Figure 2). Afterwards, a very strong reduction of BW was observed in the TB compared to the C group, which was statistically significant from day 17 ($p < 0.05$) to day 20 ($p < 0.001$, Figure 2). The tumor induced progressive cachectic state of the mice was confirmed by a significant reduction in carcass weight, fat weight and skeletal muscle weight at day 20 ($p < 0.001$, Table 1), while food intake was not affected. At this time point cachectic mice demonstrated a very strong decline in immune competence as measured by CHS (79.4%, $p < 0.001$, Figure 3). However, CHS responses were not affected in cachectic mice only, already during the pre-cachectic state a significant reduced ear swelling has been observed (27.0%, $p < 0.01$, Figure 3), reflecting an impaired immune function in tumor-bearing mice already prior to weight loss.

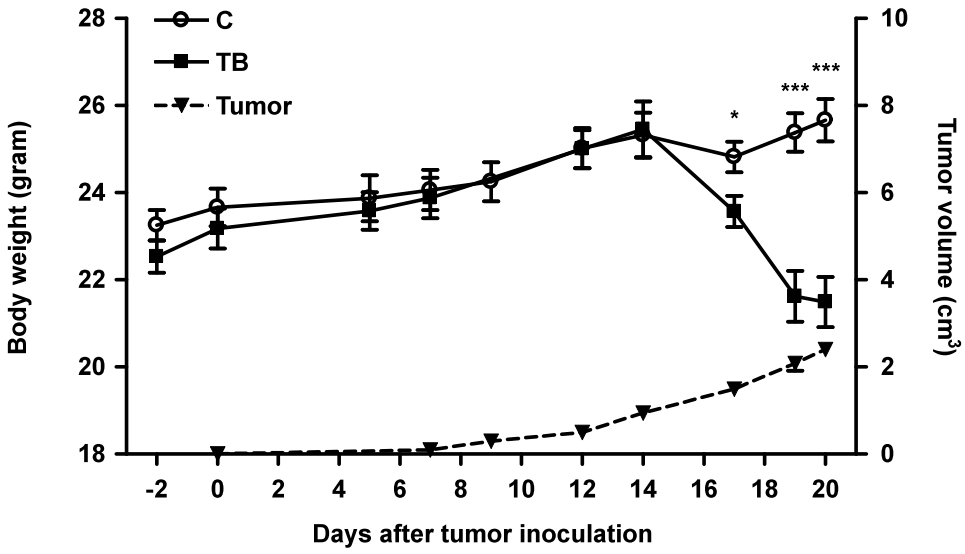

Figure 2 Effects of tumor inoculation on body weight. Left Y-axes: data represent means ± SEM of bodyweight (gram) in control (C) group (n=10) and tumor-bearing (TB) group (n=9) during the experiment. * Significantly different ($p < 0.05$) from control group (C), *** Significantly different ($p < 0.001$) from control group (C). Right Y-axes: data represent means ± SEM of tumor volume (cm^3) in the tumor-bearing (TB) group (n=9) during the experiment.

Table 1 Physiological cachexia and immune parameters at day 20.

Cachexia	C	TB
Body weight (g)	25.7 ± 0.5	21.5 ± 0.6 ***
Tumor weight (g)	0.0 ± 0.0	2.6 ± 0.2 ***
Carcass weight (g)	25.7 ± 0.5	18.9 ± 0.5 ***
Fat (g)	4.6 ± 0.3	2.5 ± 0.1 ***
m. Extensor Digitorum Longus (mg)	12.1 ± 0.5	7.8 ± 0.4 ***
m. Tibialis Anterior (mg)	46.8 ± 1.2	32.2 ± 0.9 ***
Immune	**C**	**TB**
Thymus weight (mg)	34.3 ± 2.4	18.4 ± 1.6 ***
Spleen weight (mg)	115.5 ± 4.8	225.1 ± 18.4 ***

Data represent means ± SEM of control (C) group (n=10) and tumor-bearing (TB) group (n=9). *** Significantly different ($p < 0.001$) from control group (C).

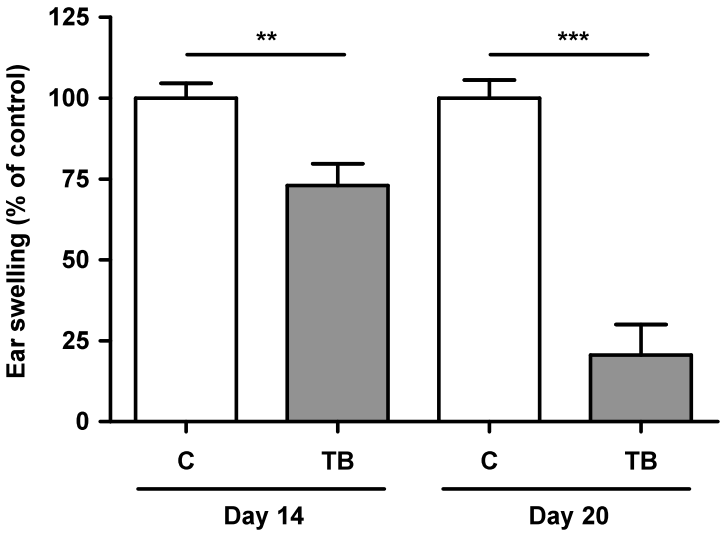

Figure 3 Effects of tumor inoculation on contact hypersensitivity in pre-cachectic and cachectic mice. Data represent means ± SEM of ear swelling in control (C) group (n=10) and tumor-bearing (TB) group (n=9) at day 14 (pre-cachectic state) and day 20 (cachectic state). ** Significantly different ($p < 0.01$) from control group (C), *** Significantly different ($p < 0.001$) from control group (C).

DISCUSSION

The reduced immune function at day 20 could be explained by the cachectic and chronic inflammatory state of the mice. The pro-inflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ and the eicosanoid PGE_2 play a major role in the impaired immune response to the tumor (1, 8), but also to pathogens leading to an increased number and severity of infections. At day 14, pro-inflammatory cytokines may already play a role in this process, but the absence of cachectic features suggests that other mechanisms are involved as well. Animal studies, but also human clinical trials de-

scribe the presence of myeloid cells involved in the initiation of the inflammatory process. Circulating monocytes are recruited to the tumor site by inflammation associated chemokines and cytokines and differentiate into tumor-associated macrophages (TAM) (8, 18). TAM exacerbate the inflammatory response at the tumor environment, further driving forward the malignancy cascade (8). Recent published data describe the relationship between inflammation and metastasis as well (19). Simultaneously, a population of suppressor cells called myeloid derived suppressor cells (MDSC) is induced by pro-inflammatory cytokines (18, 20). MDSC are found in many patients and experimental animals with cancer and cause a profound immune suppression (20).

The currently presented effects on immune function prior to weight loss may have important clinical implications when extrapolated to the human setting. Because the lack of biomarkers of a pre-cachectic state, immune function parameters, such as CHS, could serve as an early predictive marker for cancer cachexia which can be measured in cancer patients at risk for cachexia. The possibility to implement such an early marker would consequently enable an early supportive care strategy in these patients including specific nutritional interventions. The addition of immune modulatory ingredients might be an interesting opportunity for intervention, leading to a reduced inflammatory state, improved immune responsiveness and consequently, an improved performance status (10). Early provision of nutritional support might improve an effective management of cachexia related health factors as well. Every cancer patient should be regarded as a potential candidate to develop immune dysfunction and other cachexia related features. Therefore, a multi-disciplinary approach (12) is recommended, which is initiated at the moment of diagnosis and runs parallel to the pathway of cancer therapies (Figure 4).

In conclusion, the immune competence of cancer patients should be monitored in early stages of the disease, since impaired immune function could serve as an early marker for cancer cachexia. Consequently, supportive care should be provided at the earliest time point possible, to maximize the chance of reducing disease progression, reducing the frequency and severity of complications and improving treatment adherence.

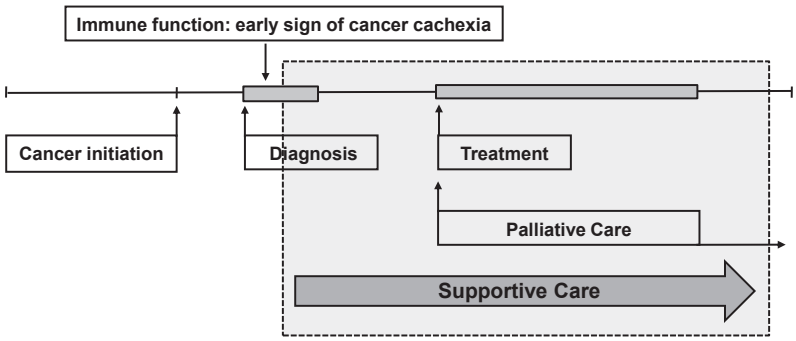

Figure 4 Supportive care strategy. Proposed multi-disciplinary supportive care strategy, which is initiated at the moment of diagnosis and runs parallel to the pathway of cancer therapies.

ACKNOWLEDGEMENTS

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REFERENCES

1 Ross JA, Fearon KC. Eicosanoid-dependent cancer cachexia and wasting. Curr Opin Clin Nutr Metab Care 2002;5: 241-8.

2 Van Cutsem E, Arends J. The causes and consequences of cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2: S51-63.

3 Argiles JM, Alvarez B, Lopez-Soriano FJ. The metabolic basis of cancer cachexia. Med Res Rev 1997;17: 477-98.

4 Evans C, Dalgleish AG, Kumar D. Review article: immune suppression and colorectal cancer. Aliment Pharmacol Ther 2006;24: 1163-77.

5 Hadden JW. Immunodeficiency and cancer: prospects for correction. Int Immunopharmacol 2003;3: 1061-71.

6 Young MR. Eicosanoids and the immunology of cancer. Cancer Metastasis Rev 1994;13: 337-48.

7 Herber DL, Nagaraj S, Djeu JY, Gabrilovich DI. Mechanism and therapeutic reversal of immune suppression in cancer. Cancer Res 2007;67: 5067-9.

8 Ben-Baruch A. Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators. Semin Cancer Biol 2006;16: 38-52.

9 Whiteside TL. Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. Semin Cancer Biol 2006;16: 3-15.

10 van Bokhorst-de van der Schueren MA. Nutritional support strategies for malnourished cancer patients. Eur J Oncol Nurs 2005;9 Suppl 2: S74-83.

11 Nitenberg G, Raynard B. Nutritional support of the cancer patient: issues and dilemmas. Crit Rev Oncol Hematol 2000;34: 137-68.

12 Muscaritoli M, Costelli P, Aversa Z, Bonetto A, Baccino FM, Rossi Fanelli F. New strategies to overcome cancer cachexia: from molecular mechanisms to the 'Parallel Pathway'. Asia Pac J Clin Nutr 2008;17 Suppl 1: 387-90.

13 Tanaka Y, Eda H, Tanaka T, Udagawa T, Ishikawa T, Horii I, et al. Experimental cancer cachexia induced by transplantable colon 26 adenocarcinoma in mice. Cancer Res 1990;50: 2290-5.

14 Strassmann G, Fong M, Kenney JS, Jacob CO. Evidence for the involvement of interleukin 6 in experimental cancer cachexia. J Clin Invest 1992;89: 1681-4.

15 Strassmann G, Jacob CO, Evans R, Beall D, Fong M. Mechanisms of experimental cancer cachexia. Interaction between mononuclear phagocytes and colon-26 carcinoma and its relevance to IL-6-mediated cancer cachexia. J Immunol 1992;148: 3674-8.

16 Tanaka M, Miyazaki H, Takeda Y, Takeo S. Detection of serum cytokine levels in experimental cancer cachexia of colon 26 adenocarcinoma-bearing mice. Cancer Lett 1993;72: 65-70.

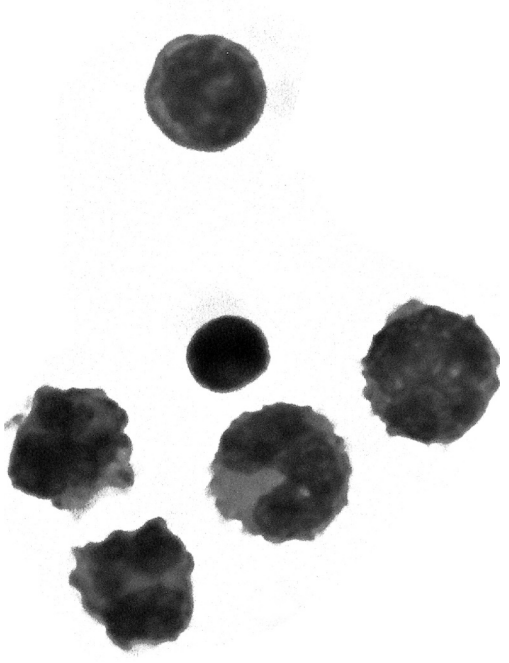
17 Soda K, Kawakami M, Kashii A, Miyata M. Characterization of mice bearing subclones of colon 26 adenocarcinoma disqualifies interleukin-6 as the sole inducer of cachexia. Jpn J Cancer Res 1994;85: 1124-30.

18 Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest 2007;117: 1155-66.

19 Mantovani A. Cancer: Inflaming metastasis. Nature 2009;457: 36-7.

20 Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res 2007;67: 10019-26.

CHAPTER THREE



Beneficial immune modulatory effects of a specific nutritional combination in a murine model for cancer cachexia

J. Faber, A.P. Vos, D. Kegler, K. van Norren, J.M. Argilés, A. Laviano, J. Garssen and A. van Helvoort

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ABSTRACT

The majority of patients with advanced cancer are recognized by impaired immune competence influenced by several factors including type and stage of the tumor and presence of cachexia. Recently, a specific nutritional combination containing fish oil, specific oligosaccharide mixture, high protein content and leucine has been developed aimed to support the immune system of cancer patients in order to reduce the frequency and severity of (infectious) complications. In a recently modified animal model cachexia is induced by inoculation of C26 tumor cells in mice. In a pre-cachectic state, no effect was observed on contact hypersensitivity, a validated *in vivo* method to measure Th1-mediated immune function, after adding the individual nutritional ingredients to the diet of tumor-bearing mice. However, the complete mixture resulted in significantly improved Th1 immunity. Moreover, in a cachectic state the complete mixture reduced plasma levels of pro-inflammatory cytokines and beneficially affected *ex vivo* immune function. Accordingly, the combination of the nutritional ingredients is required to obtain a synergistic effect leading to a reduced inflammatory state and improved immune competence. From this, it can be concluded that the specific nutritional combination has potential as immune supporting nutritional intervention to reduce the risk of (infectious) complications in cancer patients.

INTRODUCTION

Cancer patients are recognized to be hampered by serious immune failures, especially patients with tumors of the head, neck, lung, esophagus, cervix and breast (1-3). Several underlying mechanisms of immune dysfunction have been described to affect innate and adaptive immunity, leading to a poorer clinical outcome (4-7). The degree of immune dysfunction depends on type and stage of the tumor, performance status, age, anti-tumor therapies, malnutrition and presence of cachexia (1, 8-11).

Cancer cachexia occurs in the majority of patients with advanced cancer. It is inversely correlated with the survival time of the patient and it always implies a poor prognosis (12-16). Cachexia is characterized by progressive, involuntary weight loss, wasting, anorexia, asthenia, fatigue and impaired immune function (10, 12, 17-19). It has been estimated to account for 10-30% of cancer deaths, but might also contribute to deaths by other causes such as opportunistic infections (20-22).

Several mediators that are either tumor- or host-derived, such as pro-inflammatory cytokines, eicosanoids and hormones, have been implicated in the pathogenesis of cancer cachexia (10, 12, 14, 23). The pro-inflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ (also called pro-cachectic cytokines) are thought to be responsible for the metabolic changes associated with cancer cachexia via different mechanisms (12, 17, 24, 25). These cytokines each play a specific role, but it has become clear that overlapping biological activities and synergistic interactions between them lead to a progressive cachectic state (10, 15, 17). An excessive amount of these cytokines, together with the major eicosanoid prostaglandin E_2 (PGE_2), lead to impaired immune responses that have been characterized *in vivo* by a progressive decrease in delayed-type hypersensitivity to recall antigens and to dinitrochlorobenzene (DNCB) (26-28). Accordingly, a reduced *ex vivo* proliferation response of T-lymphocytes to mitogens has been reported (2, 29, 30). Functionally, this leads to a higher susceptibility to infections in cachectic patients (10, 31).

A good nutritional status is of major importance to maintain immune function in (pre-) cachectic cancer patients(10). Nutritional interventions should therefore be recognized as an integral part of cancer therapy in order to improve clinical outcomes and quality of life (32). Early provision of nutritional support can stop or even reverse the decline in the nutritional status and therefore prevent the development of malnutrition, and slow down the progression of cachexia (10, 33). This is expected to lead to a better response to therapy and fewer treatment-related complications. Recently, a specific nutritional combination (SNC) has been developed to support the immune system of catabolic cancer patients prior to the onset of weight loss or already suffering from cachexia in order to reduce the frequency and severity of (infectious) complications. The SNC is based on four active nutritional ingredients: fish oil (FO), specific oligosaccharide mixture (SOM), high protein content and leucine (high protein/leucine), in which FO and SOM have been selected for their potential effects on the immune system.

Fish oil is a generally used ingredient in immune modulating nutritional interventions, containing the conditionally essential long-chain n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These long-chain n-3 PUFAs possess a wide range of anti-inflammatory activities, including decreased production of the

inflammatory mediator PGE_2 and the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 (34). In tumor-bearing rats n-3 PUFAs prevented the fall in bodyweight due to cachexia and as a consequence survival was increased significantly (35). Clinical evidence of immune modulating activities of long-chain n-3 PUFAs exists (32), however generally without affecting systemic immune biomarkers (36). The optimal dose, formulation, relative contributions of EPA and DHA in fish oil and the target population remain yet to be defined (36, 37). The use of non-digestible carbohydrates (NDC), especially of prebiotic oligosaccharides, is based on the immune modulating activities observed in several animal experiments and clinical trials (38). In an influenza vaccination model in healthy C57BL/6 mice, a specific mixture of oligosaccharides stimulated the vaccine specific delayed-type hypersensitivity (DTH) response as a marker for T-helper 1 (Th1) immunity (39). Enhancing systemic Th1 dependent adaptive immune responses would lead in theory to better immune responses against infections that has been confirmed by applications in clinical trials (40, 41).

The addition of high protein and the branched chain amino acid (BCAA) leucine to the product focuses on alterations in protein metabolism in cancer patients and might in combination with fish oil have a positive effect on skeletal muscle function, lean body mass and daily activity (unpublished data).

In the present study, the effects of the individual nutritional ingredients FO, SOM and high protein/leucine were investigated on inflammatory status, immune function and on different parameters for cachexia. In addition, the effect of the complete mixture of these nutritional ingredients was tested and compared with the individual ingredients. The experiments were performed using a recently modified and validated animal model for cancer cachexia, based on the colon-26 tumor model, in which parameters of immune function can be quantified. In this model, cachexia is induced by inoculation of murine colon adenocacinoma (C26) cells in syngenic CD2F1 mice (42) leading to several cachectic features (43-45). The model was extended by including several immune parameters: contact hypersensitivity (CHS) against oxazolone was measured in a pre-cachectic state as an *in vivo* parameter for Th1-mediated immune function. In addition, several *ex vivo* parameters were measured at a later time point, when the animals were in a cachectic state. These preclinical experiments were performed in order to evaluate the potential benefits of specific nutritional interventions on immune function, which might lead to new applications for cancer patients.

METHODS

ANIMALS AND DIETS

Six to seven-week old syngenic male CD2F1 mice (BALB/c x DBA/2) were obtained from Harlan Nederland (Horst, The Netherlands). All experimental procedures were approved by the Animal Experimental Committee and complied with the principles of laboratory animal care. Animals were housed individually in a climate-controlled animal care facility with a constant room temperature and humidity. All animals had free access to food and drinking water. Upon arrival animals were acclimatized for one week and subsequently randomized on basis of bodyweight. The experiments were divided in: A-experiments, designed to test the effect of the individual ingredients and B-experiments, designed to test the effect of

the complete mixture of ingredients that resembles the composition of the new generation FortiCare (Nutricia Advanced Medical Nutrition, Zoetermeer, The Netherlands). In both A- and B-experiments, mice were divided into a control group (C) receiving control diet, a tumor-bearing control group (TB) receiving control diet and tumor-bearing experimental groups (TB-nutritional ingredient). Data shown are derived from the combination of several experimental runs with identical animal characteristics and experimental procedures (unless stated otherwise).

The tumor-bearing experimental group in the A-experiments received a diet based on AIN93-M (Research Diet Services, Wijk bij Duurstede, The Netherlands) with either fish oil (TB-FO), specific oligosaccharide mixture (TB-SOM) or high protein enriched with leucine (TB-HPrleu) supplied as pellets and in the B-experiments a diet with the combination of fish oil, specific oligosaccharide mixture and high protein/leucine (TB-SNC). The latter diet differed in macronutrient composition from AIN93-M to achieve a more humanized diet supplied as dough for product technical reasons.

The control diet in the A-experiments contained per kg food: 126 g protein (100% casein), 727 g carbohydrates and 40 g fat (100% soy oil). The experimental diets in the A-experiments were adapted by adding 22.1 g fish oil (providing 6.9 g EPA and 3.1 g DHA) per kg food (TB-FO), 18 g short chain galacto-oligosaccharides (Vivinal GOS, Friesland Domo Foods, Zwolle, The Netherlands) and 2 g short chain fructo-oligosaccharides (Beneo p95, Orafti, Wijchen, The Netherlands) per kg food (TB-SOM) or 151 g casein/kg and 16 g leucine/kg food (TB-HPrleu).

The control diet in the B-experiment contained more fat, although the diet is isocaloric and isonitrogenous compared to the control diet in experiments A, per kg food: 126 g protein (100% casein), 699 g carbohydrates and 52.6 g fat (100% corn oil). This control diet did not demonstrate any effect on physiological cachexia parameters and immune parameters in the used animal model (data not shown). The experimental diet in experiment B contained per kg food: 210 g protein (189 g intact protein of which 68% casein and 32% whey and 21 g free leucine), 561 g carbohydrates, 52.5 g fat (20.2 g corn oil, 10.2 g canola oil and 22.1 g fish oil (providing 6.9 g EPA and 3.1 g DHA), 18 g short chain galacto-oligosaccharides and 2 g short chain fructo-oligosaccharides.

EXPERIMENTAL DESIGN

Murine colon-26 adenocarcinoma cells were used to induce cachexia in mice (42, 43, 46). In short, on day 0 tumor cells (5 x 10^5 cells in 0.2 ml) were inoculated, under general anesthesia (isoflurane/N_2O/O_2), subcutaneously into the right inguinal flank of CD2F1 mice in the tumor-bearing groups. Animals in the control group received a sham injection with 0.2 ml HBSS. Body weight (BW), food intake and tumor size (length and width) were measured three times a week. To investigate effects on the immune system contact hypersensitivity (CHS) against oxazolone was determined, as an *in vivo* model for cellular (Th1 dependent) immunity. Briefly, on day 8 all animals were sensitized with 150 µl 3% oxazolone solution (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands, 300 mg in 7.5 ml 96% ethanol and 2.5 ml acetone) applied on their shaved breast and abdomen. Subsequently, at day 13 ear thickness was measured under general anesthesia and all animals were hapten challenged with 25 µl 0.8% oxazolone solution (32

mg in 3 ml 96% ethanol and 1 ml acetone) topical to the ear pinnae. At day 14 after tumor inoculation (24 hours after the challenge), ear swelling was measured under general anesthesia to determine the Th1 immune response.

At day 20, blood was collected by cardiac puncture and sampled in heparin tubes. After sacrification, spleens were dissected, weighed and stored in cold culture medium (RPMI-1640 containing 25 mM HEPES and 2 mM L-glutamine, Life-Technologies, Merelbeke, Belgium, enriched with 100 U/ml penicillin/streptomycin) with 10 % heat-inactivated fetal calf serum (FCShi) for immunological analysis. Skeletal muscles (m. Tibialis Anterior (mTA), m. Extensor Digitorum Longus (mEDL), m. Soleus (mS) and m. Gastrocnemius (mG)), tumor, epididymus fat, thymus, were dissected, weighted and frozen at -80°C (skeletal muscles).

IMMUNOLOGICAL ANALYSIS

All *ex vivo* incubations of cells were performed at 37°C in a humidified environment containing 5% CO_2.

Whole blood assay

Blood was added in 50 µl/well to 100 µl/well culture medium in a 96-well plate and was subsequently incubated with 50 µl/well LPS (Fc 1 µg/ml, *E.coli*, B55:O55, Sigma-Aldrich Chemie) or culture medium (control) for 20 hours or with ConA (Fc 40 µg/ml, Concanavalin A from canavalia ensiformis Type IV, Sigma-Aldrich Chemie) or culture medium (control) for 44 hours. Afterwards, supernatants were harvested and stored at –80°C until analysis. Plasma was obtained from the residual blood and stored at –80°C until analysis.

Splenocyte assay

Splenocytes were isolated by pressing spleens through cell strainers (40 µm) into a 50 ml tube. After erythrocyte lysis, splenocytes were plated in 20 µl/well (Fc 2 x 10^5 cells/well) in a 96-well plate and 80 µl/well culture medium with 10% FCS^{hi} was added. Thereafter, cells were incubated with 100 µl/well LPS (Fc 1 µg/ml) or culture medium (control) for 20 hours for PGE_2 and cytokine production or with 100 µl/well ConA (Fc 3 µg/ml) or culture medium (control) for 44 hours both for proliferation and cytokine production. After 28 hours incubation, cells for proliferation were labeled with 0,4 µCi/well tritiated thymidine (^3H thymidine, Perkin Elmer, Zaventem, Belgium) and incubated for another 16 hours. Subsequently, cells were harvested on Packard GF/C filter plates (Perkin Elmer) and dried on air. Afterwards 25 µl scintillation fluid (Packard Ultima Gold, Perkin Elmer) was added to the wells and plates were counted in a scintillation counter (Wallac MicroBeta radioactivity plate counter, Perkin Elmer). For PGE_2 and cytokine production, plates were centrifuged and supernatants were harvested and stored at –80°C until analysis.

PGE_2 and cytokine measurement

PGE_2 was measured using a commercial anti-PGE_2 rabbit polyclonal antibody-based direct enzyme immunoassay (Oxford Biomedical Research, Oxford, MI, USA) according to the manufacturer's protocol. Cytokines in plasma were measured using a commercial mouse cytokine 10-plex bead immunoassay (Biosource, Etten-Leur, The Netherlands) according to the manufacturer's protocol and cytokines in supernatants were measured

using a commercial mouse cytokine Th1/Th2 bead immunoassay or a commercial mouse cytokine inflammatory bead immunoassay (both from Biosource) according to the manufacturer's protocol.

Flowcytometric analysis

Splenocytes were added in 100 µl to a 96 well plate (Fc 1 x 10^6 cells/well), centrifuged and resuspended in cold PBS with 1% FCS^{hi} and 0.1% sodiumazide. Cells were incubated with fluorescent labeled antibodies for 30 minutes on ice in a total volume of 50 µl. After washing, cells were analyzed on an Epics XL flowcytometer (Beckman Coulter, Mijdrecht, The Netherlands). The following monoclonal antibody combinations were used: (1) GR-1-FITC (BD Pharmingen, Alphen aan den Rijn, The Netherlands), F4/80-PE (Serotec Ltd., Oxford, United Kingdom) and 7-AAD (Coulter Immunotech, Beckman Coulter), (2) CD3-FITC, CD4-PE-CY5 (both from BD Pharmingen) and CD8-PE (Beckman Coulter), (3) TCR-alpha-FITC, DX5-PE and CD19-PE-CY5 (all from Beckman Coulter), (4) unlabeled cells. Results were analyzed using Expo32 software (Beckman Coulter, Mijdrecht, The Netherlands). The method for determining positive cells using these antibodies was validated using isotype control antibodies in previous experiments. Dead 7-AAD^+ cells were excluded in antibody combination (1). The forward- and side-scatter profile from 7-AAD negative cells in (1) was used to identify living cells and exclude dead cells in (2), (3) and (4).

Determination of phospholipid fatty acids in blood cells and splenocytes

Residual heparin blood and 1 x 10^7 splenocytes were centrifuged and cellpellets were stored at –80°C until analysis. Phospholipids were separated from total cellular lipids using Bond-Elut® solid phase extraction columns and the Vac-Elut SPS 24TM system. Phospholipid extracts were converted into methyl esters by using 10% BF_3 in methanol at 100°C for 60 min. After hexane extraction derivatized phospholipids were dissolved in iso-octane and the fatty acid composition was analyzed by gas chromatography (GC) using a capillary column (50 m x 0.25 mm, CP-SIL88-fame). Peaks were identified by commercial reference standards.

STATISTICS

All data were expressed as means ± SEM. Statistical analysis was performed using SPSS 12.0.1 (SPSS Benelux, Gorinchem, The Netherlands). In the A-experiments different batches of animals were used. For that reason, it was examined for all parameters, by using ANOVA, if combination of the data was allowed and if no interaction between the groups and experiments existed. The effect of treatment was tested using a one-way ANOVA, followed by LSD post-hoc analysis when data were normally distributed and showed equal variances. When equal variances were not assumed, post-hoc a dunnett's T3 test was performed. A non-parametric Mann Whitney U test was performed when data were not normally distributed. Differences were considered significant at $p < 0.0125$ (Table 1 and Figure 1) and $p < 0.025$ (Tables 2 and 3 and Figures 2 and 3) based on α/k, in which $\alpha = 0.05$ and k the number of comparisons.

RESULTS

FUNCTIONAL PARAMETERS MEASURED IN BOTH EXPERIMENTS A AND B

Physiological cachexia and immune parameters

At day 20 after tumor inoculation mice were sacrificed and both cachexia and immune parameters were measured. Data from the different experiments with the individual nutritional ingredients (A-experiments) were combined and displayed in Table 1 and data from the experiment to test the efficacy of the complete mixture of FO, SOM and high protein/leucine (B-experiment) were presented in Table 2.

Table 1 Effect of oral administration of fish oil, specific oligosaccharide mixture or high protein/leucine on physiological cachexia parameters and immune parameters.

Cachexia	C	TB	TB-FO	TB-SOM	TB-HPrleu
Body weight (g)	24.4 ± 0.3*	22.8 ± 0.4	23.0 ± 0.8	23.8 ± 0.8	21.8 ± 0.6
Tumor weight (g)	0.0 ± 0.0*	2.2 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	1.8 ± 0.1
Carcass weight (g)	24.4 ± 0.3*	20.7 ± 0.4	20.9 ± 0.8	21.5 ± 0.8	20.0 ± 0.6
Immune	**C**	**TB**	**TB-FO**	**TB-SOM**	**TB-HPrleu**
Thymus weight (mg)	35.9 ± 1.2*	18.7 ± 1.0	21.1 ± 2.1*	20.2 ± 2.1	14.5 ± 1.8
Spleen weight (mg)	98.7 ± 2.9*	267.7 ± 8.1	231.1 ± 9.8	284.1 ± 20.5	232.9± 15.8

Data from different experiments were combined and represent means ± SEM of control (C) group (n=40), tumor-bearing control (TB) group (n=40) and tumor-bearing groups after supplementation with fish oil (TB-FO, n=10), specific oligosaccharide mixture (TB-SOM, n=10) or high protein/leucine (TB-HPrleu, n=10). * Significantly different ($p < 0.0125$) from the TB group.

In experiments A, bodyweight (BW) and carcass weight (CW = BW minus tumor weight (TW)) were significantly decreased from 24.4 gram (both) in the control (C) group to 22.8 and 20.7 gram respectively in the tumor bearing control (TB) group, while in experiment B a decrease was observed from 25.7 gram (both) in the C group to 20.1 and 18.0 gram respectively in the TB group. This reduction could be caused by the significant weight loss of fat and skeletal muscles in the TB group. Food intake has been controlled and was not affected in both experiments A and B (data not shown).

Addition of one of the individual nutritional ingredients to the diet did not result in any significant effect on BW or CW compared with animals in the TB group. However, a diet containing the complete mixture of FO, SOM and high protein/leucine improved both BW and CW significantly from 20.1 and 18.0 gram respectively in the TB group to 21.9 and 20.3 gram respectively in the TB-SNC group (Table 2), indicating a less cachectic state of the mice. This was emphasized by a positive effect on other cachectic features such as a significant inhibition of weight loss of epididymus fat and skeletal muscles, which was absent after feeding a diet with the individual nutritional ingredients.

In both experiments A and B thymus weight was significantly decreased after tumor inoculation with 47.9% and 61.7% respectively, whereas spleen weight was more than twice as high in the TB group compared to the C group. After the addition of FO or the complete mixture of FO, SOM and high protein/leucine to the diet, a significant inhibition of thymus weight loss was observed, while none of the individual nutritional ingredients affected spleen weight (Table 2).

Table 2 Effects of oral administration of the complete mixture of fish oil, specific oligosaccharide mixture and high protein/leucine on physiological cachexia parameters and immune parameters.

Cachexia	**C**	**TB**	**TB-SNC**
Body weight (g)	25.7 ± 0.5*	20.1 ± 0.4	21.9 ± 0.5*
Tumor weight (g)	0.0 ± 0.0	2.1 ± 0.1	1.8 ± 0.1 *
Carcass weight (g)	25.7 ± 0.5*	18.0 ± 0.3	20.3 ± 0.5*
Epididymus fat (mg)	230.3 ± 17.4*	40.9 ± 10.9	88.2 ± 10.9*
m. Tibialis Anterior (mg)	44.7 ± 1.0*	33.6 ± 0.7	38.5 ± 0.8*
m. EDL (mg)	8.9 ± 0.2*	6.7 ± 0.2	7.6 ± 0.2*
m. Soleus (mg)	6.4 ± 0.2*	4.8 ± 0.1	5.4 ± 0.2*
m. Gastrocnemius (mg)	132.1 ± 2.4*	99.5 ± 2.2	110.7 ± 2.9*
Immune	**C**	**TB**	**TB-SNC**
Thymus weight (mg)	36.8 ± 1.8*	14.1 ± 1.1	20.7 ± 1.8*
Spleen weight (mg)	95.2 ± 4.3*	210.5 ± 14.3	209.8 ± 9.3
Spleen cells (1 x 10^7cells/ml)	2.7 ± 0.1*	5.6 ± 0.5	5.6 ± 0.3
Granulocytes[a] (%)	4.6 ± 0.6*	28.2 ± 1.9	28.2 ± 1.4
Granulocytes[a] (cells/spleen)	50.8 ± 7.8*	598.9 ± 44.1	613.4 ± 29.2
Monocytes[b] (%)	2.7 ± 0.2*	5.8 ± 0.2	6.2 ± 0.3
Monocytes[b] (cells/spleen)	29.1 ± 2.2*	126.0 ± 8.3	139.6 ± 11.0
Macrophages[c] (%)	5.0 ± 0.2	5.0 ± 0.3	4.0 ± 0.2*
Marophages[c] (cells/spleen)	54.9 ± 4.2*	110.7 ± 10.9	87.3 ± 5.2
CD3+CD4+ T-cells (%)	6.9 ± 0.4*	3.6 ± 0.2	4.2 ± 0.3
CD3+CD4+ T-cells (cells/spleen)	75.7 ± 5.8	78.7 ± 6.3	90.8 ± 6.2
CD3+CD8+ T-cells (%)	3.2 ± 0.2*	1.7 ± 0.1	1.7 ± 0.1
CD3+CD8+ T-cells (cells/spleen)	35.4 ± 3.2	34.8 ± 2.0	36.9 ± 2.6

Data represent means ± SEM of control (C) group (n=10), tumor-bearing control (TB) group (n=19) and tumor-bearing group after oral administration of the specific nutritional combination (TB-SNC) (n=20). * Significantly different ($p < 0.025$) from the TB group. [a] defined as GR-1high cells, [b] defined on the base of forward- and side-scatter profile, F4/80dull and GR-1low to dull, [c] defined as F4/80high cells.

Contact Hypersensitivity

A contact hypersensitivity (CHS) test was performed at day 13/14 to determine *in vivo* immune function prior to weight loss. CHS responses were significantly reduced in the TB group compared to the C group in experiments A (28.1%, Figure 1) and B (31.0%, Figure 2A) indicating an impaired Th1 immune response in tumor bearing mice. After adding one of the individual nutritional ingredients to the diet of tumor-bearing mice no effect was observed on this immune biomarker (Figure 1). However, after administration of the complete mixture of FO, SOM and high protein/leucine to the tumor-bearing mice (TB-SNC), immune responsiveness was increased significantly by 20.7% compared to the TB mice, demonstrating a better Th1 mediated immune response (Figure 2A).

ADDITIONAL IMMUNE PARAMETERS MEASURED IN EXPERIMENT B

To obtain more information on immune competence and inflammatory status of tumor bearing mice in a cachectic state and to determine the effect of adding the complete mixture of FO, SOM and high protein/leucine to the diet of these mice, several additional immune parameters were measured after sacrificing the mice at day 20.

Flowcytometric analysis of splenocytes and determination of phospholipid fatty acids. Spleen weight was increased significantly in tumor bearing control animals due to an increase in the number of spleen cells caused by an enormous expansion of granulocytes

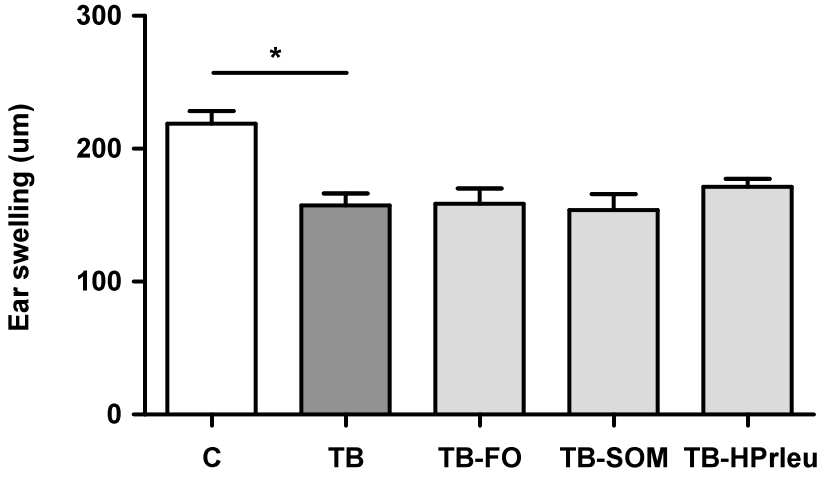

Figure 1 Effects of oral administration of fish oil, specific oligosaccharide mixture or high protein/leucine on contact hypersensitivity. Data represent means (µm) ± SEM of control (C) group (n=20), tumor-bearing control (TB) group (n=20) and tumor-bearing groups after supplementation with fish oil (TB-FO, n=10), specific oligosaccharide mixture (TB-SOM, n=10) or high protein/leucine (TB-HPrleu, n=10). * Significantly different ($p < 0.0125$) from the TB group.

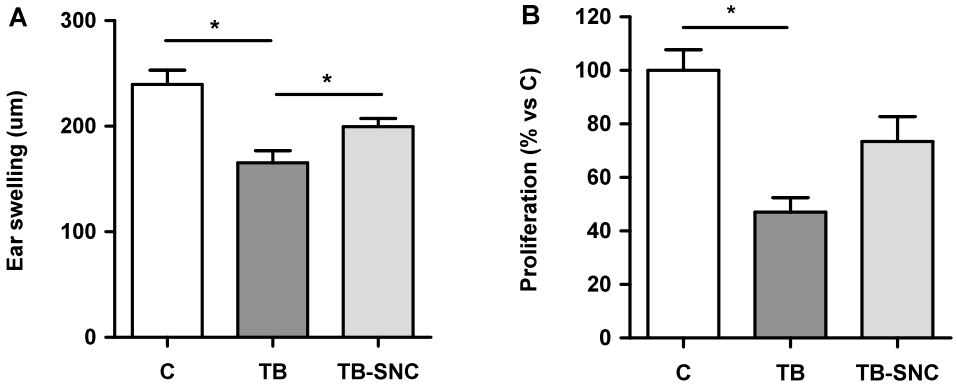

Figure 2 Effects of oral administration of the complete mixture of fish oil, specific oligosaccharide mixture and high protein/leucine on contact hypersensitivity (A) and ConA-stimulated T-lymphocyte proliferation by splenocytes (B). Data represent means ± SEM of control (C) group (n=10), tumor-bearing control (TB) group (n=19) and tumor-bearing group after oral administration of the specific nutritional combination (TB-SNC) (n=20). For ConA-stimulated T-lymphocyte proliferation all values were calculated as percentage of the control group, which is set at 100%. * Significantly different ($p < 0.025$) from the TB group.

from 50.8 x 10^7 cells/spleen in the C group to 598.9 x 10^7 cells/spleen in the TB group (Table 2). Also monocytes showed a significant increase in the number of cells/spleen, while the total number of $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells was not affected. Relatively, the percentages $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells were almost two times lower in the TB group compared to the C group, probably due to the high increase of granulocytes. After adding the complete mixture of nutritional ingredients to the diet of tumor-bearing mice no effect was observed on the total number of splenocytes, neither on the absolute or relative number of granulocytes, monocytes and $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells.

In cell membranes of the isolated splenocytes percentages n-6 and n-3 fatty acids of total phospholipid fatty acids were measured (data not shown). No effect was observed on total n-6 fatty acids content after tumor inoculation, whereas the percentage of total n-3 was increased significantly in the TB group, probably due to the significant increase in DHA. After feeding a diet with the total combination of the nutritional ingredients total n-6 content in cell membranes of splenocytes was decreased significantly compared to the TB group due to a strong reduction of AA from 19.6% in the TB group to 8.9% in the TB-SNC group. The percentages of the n-3 fatty acids EPA and DHA were increased significantly from 0.4% and 2.5% respectively in the TB group to 3.5% and 4.4% respectively in the TB-SNC group leading to a significantly higher n-3 content.

Plasma cytokine and PGE_2 concentrations

Pro-inflammatory cytokines and PGE_2 were measured in plasma obtained from blood at day 20. IL-6, TNF-α, as well as PGE_2 were increased significantly from 14.8, 0.3 and 9488 pg/ml respectively in the C group to 152.1, 12.3 and 49096 pg/ml respectively in TB group (Figure 3A-3D), whereas IL-1β and IFN-γ levels were below the detection limit of the assay. A strong decline in the production of the pro-inflammatory cytokines IL-6, TNF-α and PGE_2 was observed to levels of 111.8 ($p = 0.038$), 7.0 ($p = 0.017$) and 19601 pg/ml ($p < 0.001$) (all one-tailed tested), respectively after the addition of the complete mixture of FO, SOM and high protein/leucine to the diet of tumor-bearing mice, leading to a lower inflammatory state.

Ex vivo ConA-stimulated T-lymphocyte proliferation in splenocytes

In addition to CHS, which was measured prior to weight loss, *ex vivo* ConA-stimulated T-lymphocyte proliferation by splenocytes was measured in cachectic tumor-bearing mice. A significant decrease was observed from 100% in the C group (all values were calculated as percentage of the control group, which is set at 100%), to 47.0 % in the TB group (Figure 2B). After adding the complete mixture of nutritional ingredients to the tumor bearing mice a strong trend to an improved immune response was observed, although this effect was not significant ($p = 0.031$).

Ex vivo ConA and LPS-stimulated cytokine production in whole blood and splenocytes.

In ConA-stimulated whole blood and splenocytes both Th1 and Th2 cytokines were measured, whereas in LPS-stimulated whole blood and splenocytes pro-inflammatory cytokines and PGE_2 were measured. All values were calculated as percentage of the con-

trol group, which is set at 100%. In ConA-stimulated whole blood, IL-4 (Th2 cytokine) production was reduced significantly from 100% in the C group to 11.3% in the TB group (Table 3). Moreover, in ConA-stimulated splenocytes IL-4 (Th2) as well as IL-2 and IFN-γ (Th1) were decreased significantly in the TB group. After administration of the complete mixture of FO, SOM and high protein/leucine to the tumor-bearing mice, IL-4 production in both ConA-stimulated whole blood and splenocytes demonstrated a significant increase ($p < 0.025$).

In LPS-stimulated whole blood both IL-1β and TNF-α were decreased significantly from 100% in the C group to 17.5% and 18.5% respectively in the TB group (Table 3). In addition, a trend to decreased IL-6 levels was observed, while PGE_2 showed a trend to enhanced levels in the TB group. In LPS-stimulated splenocytes IL-6 was reduced significantly in the TB compared to the C group. By contrast, PGE_2 production showed a significant increase from 100% in the C group to 178% in the TB group. After feeding a diet with the complete mixture of nutritional ingredients, PGE_2 production in LPS-stimulated whole blood was decreased significantly from 137.9% in the TB group to 43.1% in the TB-SNC group, whereas PGE_2 production in LPS-stimulated splenocytes was not affected.

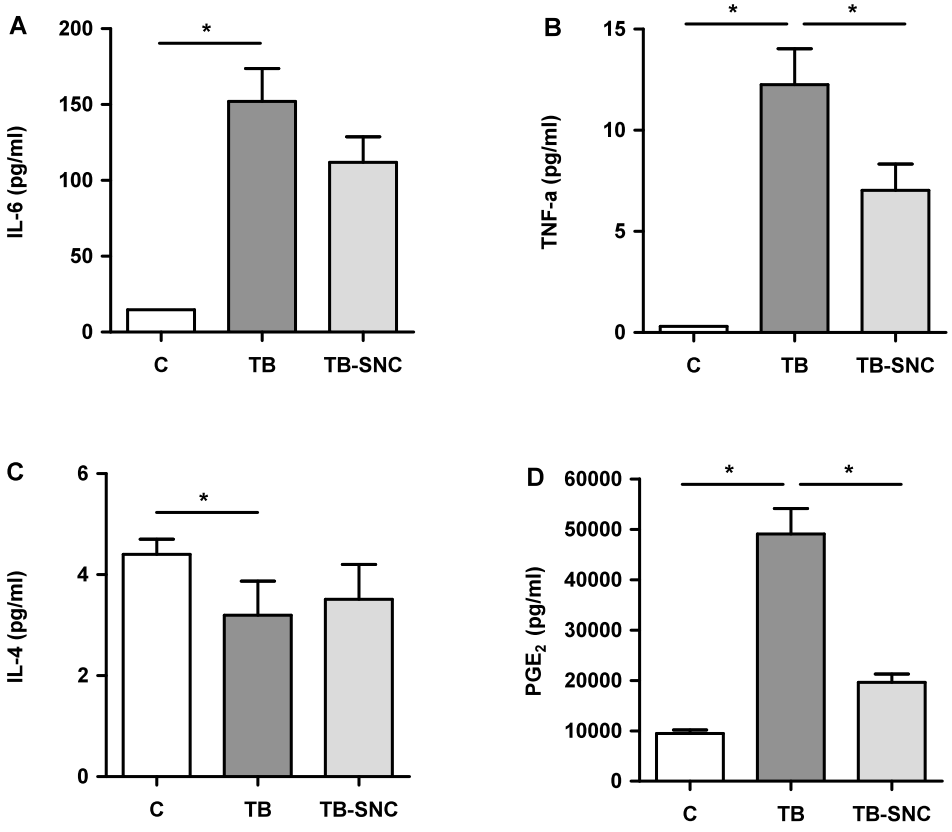

Figure 3 Effects of oral administration of the complete mixture of fish oil, specific oligosaccharide mixture and high protein/leucine on plasma cytokine IL-6 (A), TNF-α (B), IL-4 (C) and PGE_2 (D) concentrations. Data represent means (pg/ml) ± SEM of control (C) group (n=10), tumor-bearing control (TB) group (n=19) and tumor-bearing group after oral administration of the specific nutritional combination (TB-SNC) (n=20). * Significantly different ($p < 0.025$) from the TB group.

Table 3 Effects of oral administration of the complete mixture of fish oil, specific oligosaccharide mixture and high protein/leucine on ConA-stimulated cytokine production in whole blood and splenocytes and on LPS-stimulated cytokine and PGE_2 production in whole blood and splenocytes.

ConA-stimulated cytokine production	C	TB	TB-SNC
Whole blood			
IL-2 (% vs C)	100.0 ± 14.0	65.0 ± 7.2	96.3 ± 15.3
IL-4 (% vs C)	100.0 ± 10.5*	11.3 ± 3.1	69.9 ± 23.6*
IL-12 (% vs C)	100.0 ± 8.0	84.7 ± 14.8	75.4 ± 16.1
Splenocytes (FCS^{hi})			
IL-2 (% vs C)	100.0 ± 11.1*	34.4 ± 2.3	42.3 ± 3.4
IL-4 (% vs C)	100.0 ± 13.3*	45.9 ± 4.9	70.9 ± 11.5*
IFN-γ (% vs C)	100.0 ± 10.5*	34.6 ± 8.7	80.0 ± 27.6
LPS-stimulated cytokine production	**C**	**TB**	**TB-SNC**
Whole blood			
IL-1β (% vs C)	100.0 ± 8.1*	17.5 ± 2.7	38.5 ± 15.5
IL-6 (% vs C)	100.0 ± 21.2	72.3 ± 13.3	126.9 ± 40.3
TNF-α (% vs C)	100.0 ± 8.3*	18.5 ± 2.4	38.2 ± 15.0
PGE_2 (% vs C)	100.0 ± 8.9	137.9 ± 20.4	43.1 ± 3.2*
Splenocytes (FCS^{hi})			
IL-1β (% vs C)	100.0 ± 4.3	107.5 ± 11.6	97.0 ± 13.6
IL-6 (% vs C)	100.0 ± 8.6*	76.5 ± 12.7	107.7 ± 14.5
TNF-α (% vs C)	100.0 ± 4.7	102.7 ± 8.0	102.1 ± 10.4
PGE_2 (% vs C)	100.0 ± 2.1*	178.0 ± 12.4	196.6 ± 11.4

Data represent means ± SEM of control (C) group (ConA/LPS whole blood n=10, ConA/LPS splenocytes n=7), tumor-bearing control (TB) group (ConA/LPS whole blood n=10, ConA/LPS splenocytes n=13) and tumor-bearing group after oral administration of the specific nutritional combination (TB-SNC) (ConA/LPS whole blood n=10, ConA/LPS splenocytes n=12). All values were calculated as percentage of the control group, which is set at 100%,
* Significant differently ($p < 0.025$) from the TB group.

DISCUSSION

The present study demonstrated a significant improved Th1 immune response after feeding a diet containing the complete mixture of nutritional ingredients FO, SOM and high protein/leucine in tumor-bearing animals in a pre-cachectic state. In addition, in mice already suffering from cachexia the complete mixture of ingredients affected several physiological and immune parameters, representing a lower inflammatory state, better immune responses and less wasting of protein and lipid stores, leading to less severe cachexia. The cachectic features of the C26 mouse model have been described earlier by other authors, who attributed a pivotal role to the pro-inflammatory cytokine IL-6 in the induction of cachexia (42-45). In the current study, parameters of immune competence were measured in parallel to cachexia- and inflammation-related parameters, to study cachexia-related immune dysfunction in this model as well. To this end, *in vivo* Th1-related immune function was quantified using oxazolone-induced CHS responses. In addition, several *ex vivo* parameters were measured in whole blood and isolated splenocytes cultures.

In both experiments A and B, body weight and carcass weight were reduced significantly in the TB group, which is in accordance with a significant reduction in the weight of epididymus fat and skeletal muscles and argues for a cachectic status of the mice. In experiment B,

the pro-inflammatory cytokines IL-6 and TNF-α were measured. These cytokines have been described as pro-cachectic cytokines involved in different metabolic changes associated with wasting during cancer cachexia (10, 17, 25). In literature, TNF-α levels measured in the C26 model were below the detection limit (43, 45). In the present study however, TNF-α levels can be measured, in spite of the low levels, and differences between groups were determined. Both IL-6 and TNF-α were increased significantly in the TB group, which confirmed the role of TNF-α besides IL-6, in the pathogenesis of cancer cachexia in the C26 model. By contrast, levels of IL-4, in literature stated as an anti-cachectic cytokine (25), showed a significant decrease in the TB group, which is in agreement with the cachectic state of the animals.

CHS responses were measured in a pre-cachectic state and were reduced significantly in the TB group, indicating an impaired Th1 immune response in tumor bearing mice before the onset of weight loss. In previous experiments CHS was also determined in mice already suffering from cachexia, demonstrating an even stronger reduction (unpublished data). It should be realized that this reduction was severe because of the dramatic health status of the mice at this time point, therefore the effect of the nutritional ingredients was only determined in a pre-cachectic state. Nevertheless, in cachectic mice *ex vivo* parameters were measured to assess immune competence.

To evaluate the potential benefits of specific nutritional interventions on cachexia features and immune function, FO, SOM and high protein/leucine were added to the diet of tumor bearing mice. No effect of the individual ingredients was demonstrated on BW, CW, epididymus fat weight and weight of skeletal muscles, indicating no advances in the poor cachectic state of the mice. This was confirmed by the absence of potential effects on immune function, measured by contact hypersensitivity in a pre-cachectic state. When FO in combination with high protein/leucine was added to the diet, BW, CW and weight of epididymus fat and the skeletal muscle mTA were improved significantly (unpublished data). Moreover, CHS responses seems to show a small increase, but this was far from significant ($p=0.716$) compared to animals in the TB group (data not shown). However, after the addition of the complete mixture of FO, SOM and high protein/leucine to the diet of tumor-bearing animals, a significant increase in ear swelling was observed compared to animals in the TB group, indicating an improved Th1 immune response leading to a better resistance against infections. In addition, in mice already suffering from cachexia, physiological parameters as body weight, carcass weight and weight of epididymus fat and skeletal muscles demonstrated a significant increase after adding the complete mixture of nutritional ingredients to the diet compared to the reduction observed in TB mice (Table 2). Consequently, the cachectic state of the mice was reduced significantly and muscle function and daily activity, important contributors to the quality of life, were improved significantly (unpublished data). Taken together, the results indicate that the combination of the nutritional ingredients FO, SOM and high protein/leucine is able to induce a synergistic effect leading to less severe cachexia and improved immune responses.

Thymus weight was decreased after tumor-inoculation and demonstrated a significant recovery after adding the complete mixture of FO, SOM and high protein/leucine to the diet. The thymus is one of the central primary lymphoid organs and plays an important role

in cellular immunity by generating circulating T-lymphocytes (47). Therefore, the recovery of thymus weight is of major importance for an efficient working and balanced immune system. In contrast to thymus weight, spleen weight was elevated in the TB group. This is mainly caused by a strong increase in both percentage and total number of granulocytes among the total splenocytes population. In literature this is known as a leukemoid reaction which is previously described in tumor-bearing mice and in different types of human cancers (48, 49). When the complete mixture of FO, SOM and high protein/leucine was added to the diet of tumor-bearing animals, the leukemoid reaction was not reduced. Therefore, the beneficial effect of nutritional intervention on immune function is argued to be due to other effects on the immune system.

In addition to measurement of CHS, several *ex vivo* assays were performed to determine immune function in cachectic mice. In ConA-stimulated splenocytes cultures from TB mice a significant reduction of T-lymphocyte proliferation and both Th1 and Th2 cytokine production was observed, probably caused by a relative decrease in the number of T-lymphocytes and by a lower activity. Moreover, also in ConA-stimulated whole blood Th1 and Th2 cytokines were affected, which is probably the consequence of a general reduction in T-cell activity. These results are consistent with the immune suppression that was observed *in vivo* by the measurement of CHS. In LPS-stimulated whole blood a significant reduction was observed on IL-1β and TNF-α production in the TB group, representing a reduced capacity of immune cells to react to an infection *ex vivo*. IL-6 showed a trend to a decrease and PGE_2 even tended to an increase, possibly due to the high serum levels in plasma that were present in whole blood also.

After adding the complete mixture of FO, SOM and high protein/leucine to the diet of TB mice, ConA-stimulated T-cell responses were positively affected both in splenocytes as well as in whole blood. In addition, LPS-stimulated IL-1β, TNF-α and IL-6 production by whole blood demonstrated a trend to an increase compared to TB mice, indicating an enhanced capacity of immune cells to mount acute infection-like responses *ex vivo*. By contrast, LPS-stimulated PGE_2 production was decreased significantly in the TB-SNS group, which might be explained by the absence of high PGE_2 levels in plasma or by the inhibitory effect the complete mixture in the *ex vivo* situation.

As mentioned before plasma levels of the pro-inflammatory cytokines IL-6 and TNF-α were increased significantly in cachectic TB mice leading to a chronic inflammatory state. Together with PGE_2, another inflammatory mediator that is enhanced significantly in TB mice, these cytokines play a central role in the induction of the immune suppression observed in these tumor bearing mice (5). After feeding a diet with the complete mixture of FO, SOM and high protein/leucine, IL-6 levels in plasma showed a tendency to a decrease and TNF-α levels were decreased significantly compared to that in the TB group.

Inhibition of IL-6 production in this cachexia model is established to prevent the development of cachexia as shown after treatment with a murine antibody to IL-6 (43), while TNF-α is thought to play an important role in wasting of fat and to induce IL-6 secretion by many cell types (46). Nevertheless, IL-6 is not only produced by TNF-α signaling, it is thought that in animals carrying C26 tumors, the majority of IL-6 is produced by the tumor cells in response to macrophages residing in these tumors leading to the severe inflammatory state inducing different catabolic processes involving cachexia (46). Accordingly,

the observed reduction of IL-6 and TNF-α in the TB-SNC group might partly account for the reduced inflammatory state and the improved functional aspects of the catabolism associated immune suppression. Also PGE_2 might be involved in this process. PGE_2 can be secreted by many cell types, including monocytes, macrophages and tumor cells, and suppresses multiple immune functions as T-lymphocyte proliferation and macrophage activation (31). After adding the complete mixture of nutritional ingredients, PGE_2 levels in plasma of tumor-bearing animals were reduced significantly compared to that in the TB group. This might be the result of the incorporation of the n-3 PUFA EPA (present in FO) in cell membrane phospholipids that leads, by a reduction of the percentage AA, to a decreased production of PGE_2 by the cyclooxygenase (COX) enzyme (14, 50). In literature FO has been established to possess anti-cachectic, anti-inflammatory and immune modulating properties, both *in vivo* and *ex vivo*, but dose and ratio of the n-3 PUFAs EPA and DHA are described as important factors that determine these properties (10, 32, 35, 36). The FO dose of 22.1 g (providing 6.9 g EPA and 3.1 g DHA) per kg food described in the experiments with the individual nutritional ingredients (experiments A) reported nevertheless no effect on both physiological and immunological parameters, whereas the double dose tested in this model demonstrated significant improved cachectic features and a trend to enhanced immune function measured by CHS (data not shown). Additionally, no effect of adding the specific oligosaccharide mixture was observed on cachexia and immune parameters (experiments A) in tumor-bearing animals, whereas a mixture of 2% (w/w) specific oligosaccharides in a 9:1 ratio induced Th1 immunity in an influenza vaccination model in healthy C57BL/6 mice (39). However, when the FO dose of 22.1 g (providing 6.9 g EPA and 3.1 g DHA) is combined with the SOM and high protein/leucine less severe cachexia and significant improved immune responses were demonstrated. To this end, it seems that besides inhibition of the chronic inflammatory state present in the cachectic mice, it is also required to support the nutritional state of the mice with the combination of ingredients, to obtain positive effects on immune function.

In conclusion, the present study demonstrated the beneficial immune modulatory effects of the specific nutritional combination leading to a multi-target approach. No effect was observed after feeding a diet containing the individual nutritional ingredients FO, SOM or high protein/leucine on contact hypersensitivity in tumor-bearing animals in a pre-cachectic state. In contrast, the complete mixture of nutritional ingredients reported a significant improved Th1 immune response in tumor-bearing mice prior to weight loss. In mice already suffering from cachexia the complete mixture of ingredients affected several physiological and immune parameters, representing a lower inflammatory state, better immune responses and less wasting of protein and lipid stores, leading to less severe cachexia. Accordingly, the combination of the nutritional ingredients FO, SOM and high protein/leucine is able to induce a synergistic effect leading to an improved health status of the mice related to immune competence, weight gain and multiple inflammatory indices. From this, it can be concluded that the specific nutritional combination has potential as immune supporting nutritional intervention. As demonstrated in the present study, immune function in tumor-bearing mice is already affected before the onset of weight loss. Therefore, it is very important to provide nutritional support with immune modulating properties

as early as possible in order to stop or reverse the nutritional decline, slowing down the progression of cachexia and counteract dysfunction of the immune system to reduce the risk of (infectious) complications. Currently, clinical studies with cancer patients are being performed to study whether mentioned effects can be extrapolated to the human setting.

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REFERENCES

1 Pak AS, Wright MA, Matthews JP, Collins SL, Petruzzelli GJ, Young MR. Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34(+) cells which suppress immune functions within cancers that secrete granulocyte-macrophage colony-stimulating factor. Clin Cancer Res 1995;1: 95-103.

2 Hadden JW. Immunodeficiency and cancer: prospects for correction. Int Immunopharmacol 2003;3: 1061-71.

3 Mafune K, Tanaka Y. Influence of multimodality therapy on the cellular immunity of patients with esophageal cancer. Ann Surg Oncol 2000;7: 609-16.

4 Herber DL, Nagaraj S, Djeu JY, Gabrilovich DI. Mechanism and therapeutic reversal of immune suppression in cancer. Cancer Res 2007;67: 5067-9.

5 Young MR. Eicosanoids and the immunology of cancer. Cancer Metastasis Rev 1994;13: 337-48.

6 Young MR, Wright MA, Lozano Y, Matthews JP, Benefield J, Prechel MM. Mechanisms of immune suppression in patients with head and neck cancer: influence on the immune infiltrate of the cancer. Int J Cancer 1996;67: 333-8.

7 Heimdal JH, Aarstad HJ, Klementsen B, Olofsson J. Peripheral blood mononuclear cell (PBMC) responsiveness in patients with head and neck cancer in relation to tumour stage and prognosis. Acta Otolaryngol 1999;119: 281-4.

8 Whiteside TL. Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. Semin Cancer Biol 2006;16: 3-15.

9 Evans C, Dalgleish AG, Kumar D. Review article: immune suppression and colorectal cancer. Aliment Pharmacol Ther 2006;24: 1163-77.

10 Argiles JM. Cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2: S39-50.

11 Young MR, Kolesiak K, Achille NJ, Meisinger J, Gonzalez E, Liu SW, et al. Impact of aging on immune modulation by tumor. Cancer Immunol Immunother 2001;50: 315-20.

12 Van Cutsem E, Arends J. The causes and consequences of cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2: S51-63.

13 Zhou W, Jiang ZW, Tian J, Jiang J, Li N, Li JS. Role of NF-kappaB and cytokine in experimental cancer cachexia. World J Gastroenterol 2003;9: 1567-70.

14 Ross JA, Fearon KC. Eicosanoid-dependent cancer cachexia and wasting. Curr Opin Clin Nutr Metab Care 2002;5: 241-8.

15 McNamara MJ, Alexander HR, Norton JA. Cytokines and their role in the pathophysiology of cancer cachexia. JPEN J Parenter Enteral Nutr 1992;16: 50S-55S.

16 Argiles JM, Busquets S, Moore-Carrasco R, Figueras M, Almendro V, Lopez-Soriano FJ. Targets in clinical oncology: the metabolic environment of the patient. Front Biosci 2007;12: 3024-51.

17 Toomey D, Redmond HP, Bouchier-Hayes D. Mechanisms mediating cancer cachexia. Cancer 1995;76: 2418-26.

18 Puccio M, Nathanson L. The cancer cachexia syndrome. Semin Oncol 1997;24: 277-87.

19 Finley JP. Management of cancer cachexia. AACN Clin Issues 2000;11: 590-603.

20 Barton BE. IL-6-like cytokines and cancer cachexia: consequences of chronic inflammation. Immunol Res 2001;23: 41-58.

21 Inagaki J, Rodriguez V, Bodey GP. Proceedings: Causes of death in cancer patients. Cancer 1974;33: 568-73.

22 Warren S. The immediate cause of death in cancer. Am J Med Sci 1932: 610.

23 Tisdale MJ. Biology of cachexia. J Natl Cancer Inst 1997;89: 1763-73.

24 Morley JE, Thomas DR, Wilson MM. Cachexia: pathophysiology and clinical relevance. Am J Clin Nutr 2006;83: 735-43.

25 Argiles JM, Busquets S, Lopez-Soriano FJ. Cytokines in the pathogenesis of cancer cachexia. Curr Opin Clin Nutr Metab Care 2003;6: 401-6.

26 Haffejee AA, Angorn IB. Nutritional status and the nonspecific cellular and humoral immune response in esophageal carcinoma. Ann Surg 1979;189: 475-9.

27 Singh SN, Agrawal BM, Shanker R, Rajvanshi VS. A study of T-lymphocytes and delayed cutaneous hypersensitivity reaction in patients with squamous cell carcinoma of head and neck region. Indian J Cancer 1979;16: 53-8.

28 Stein JA, Adler A, Efraim SB, Maor M. Immunocompetence, immunosuppression, and human breast cancer. I. An analysis of their relationship by known parameters of cell-mediated immunity in well-defined clinical stages of disease. Cancer 1976;38: 1171-87.

29 Wustrow TP, Issing WJ. Immune defects in patients with head and neck cancer. Anticancer Res 1993;13: 2507-19.

30 Anderson TC, Jones SE, Soehnlen BJ, Moon TE, Griffith K, Stanley P. Immunocompetence and malignant lymphoma: immunologic status before therapy. Cancer 1981;48: 2702-9.

31 Ben-Baruch A. Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators. Semin Cancer Biol 2006;16: 38-52.

32 van Bokhorst-de van der Schueren MA. Nutritional support strategies for malnourished cancer patients. Eur J Oncol Nurs 2005;9 Suppl 2: S74-83.

33 Correia M, Cravo M, Marques-Vidal P, Grimble R, Dias-Pereira A, Faias S, et al. Serum concentrations of TNF-alpha as a surrogate marker for malnutrition and worse quality of life in patients with gastric cancer. Clin Nutr 2007;26: 728-35.

34 Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006;83: 1505S-19S.

35 Pizato N, Bonatto S, Piconcelli M, de Souza LM, Sassaki GL, Naliwaiko K, et al. Fish oil alters T-lymphocyte proliferation and macrophage responses in Walker 256 tumor-bearing rats. Nutrition 2006;22: 425-32.

36 Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. Proc Nutr Soc 2007;66: 237-59.

37 Fearon KC, Barber MD, Moses AG, Ahmedzai SH, Taylor GS, Tisdale MJ, et al. Double-blind, placebo-controlled, randomized study of eicosapentaenoic acid diester in patients with cancer cachexia. J Clin Oncol 2006;24: 3401-7.

38 Vos A, M'Rabet L, Stahl B, Boehm G, Garssen J. Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. Crit Rev Immunol 2007;27: 97-140.

39 Vos AP, Haarman M, Buco A, Govers M, Knol J, Garssen J, et al. A specific prebiotic oligosaccharide mixture stimulates delayed-type hypersensitivity in a murine influenza vaccination model. Int Immunopharmacol 2006;6: 1277-86.

40 Bruzzese E, Volpicelli M, Salvini F, Bisceglia M, Lionetti P, Cinquette M, et al. Early administration of GOS/FOS prevents intestinal and respiratory infections in infants. J Pediatr Gastroenterol Nutr 2006;42: E95.

41 Arslanoglu S, Moro GE, Boehm G. Early supplementation of prebiotic oligosaccharides protects formula-fed infants against infections during the first 6 months of life. J Nutr 2007;137: 2420-4.

42 Tanaka Y, Eda H, Tanaka T, Udagawa T, Ishikawa T, Horii I, et al. Experimental cancer cachexia induced by transplantable colon 26 adenocarcinoma in mice. Cancer Res 1990;50: 2290-5.

43 Strassmann G, Fong M, Kenney JS, Jacob CO. Evidence for the involvement of interleukin 6 in experimental cancer cachexia. J Clin Invest 1992;89: 1681-4.

44 Tanaka M, Miyazaki H, Takeda Y, Takeo S. Detection of serum cytokine levels in experimental cancer cachexia of colon 26 adenocarcinoma-bearing mice. Cancer Lett 1993;72: 65-70.

45 Soda K, Kawakami M, Kashii A, Miyata M. Characterization of mice bearing subclones of colon 26 adenocarcinoma disqualifies interleukin-6 as the sole inducer of cachexia. Jpn J Cancer Res 1994;85: 1124-30.

46 Strassmann G, Jacob CO, Evans R, Beall D, Fong M. Mechanisms of experimental cancer cachexia. Interaction between mononuclear phagocytes and colon-26 carcinoma and its relevance to IL-6-mediated cancer cachexia. J Immunol 1992;148: 3674-8.

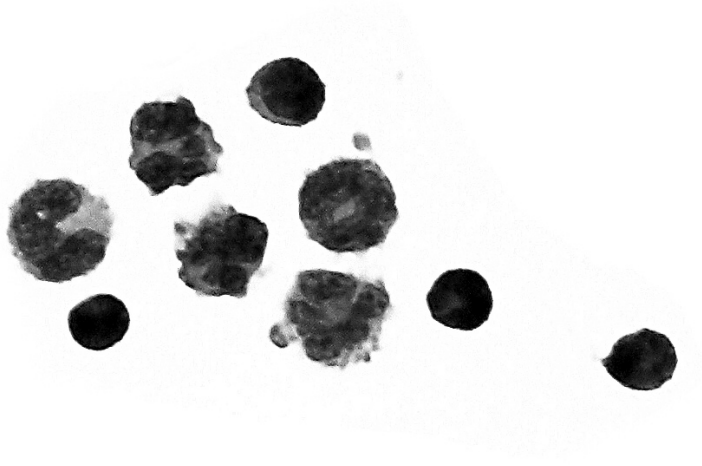
47 Delves PJ, Roitt IM. The immune system. First of two parts. N Engl J Med 2000;343: 37-49.

48 DuPre SA, Hunter KW, Jr. Murine mammary carcinoma 4T1 induces a leukemoid reaction with splenomegaly: association with tumor-derived growth factors. Exp Mol Pathol 2007;82: 12-24.

49 Nimieri HS, Makoni SN, Madziwa FH, Nemiary DS. Leukemoid reaction response to chemotherapy and radiotherapy in a patient with cervical carcinoma. Ann Hematol 2003;82: 316-7.

50 Trebble TM, Wootton SA, Miles EA, Mullee M, Arden NK, Ballinger AB, et al. Prostaglandin E_2 production and T cell function after fish-oil supplementation: response to antioxidant cosupplementation. Am J Clin Nutr 2003;78: 376-82.

CHAPTER FOUR



Bacterial translocation is reduced by a specific nutritional combination in mice with chemo-therapy-induced neutropenia

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ABSTRACT

In many cancer patients immune function is compromised leading to an increased risk of (infectious) complications. Chemotherapy-induced neutropenia is a common cause of treatment-induced immune suppression. In the present study, the effect of a specific nutritional combination (SNC) on bacterial translocation was studied in a model of chemotherapy-induced neutropenia in C3H/HeN mice colonized with *Pseudomonas aeruginosa* PAO-1. Dietary intervention started after stable colonization with *P. aeruginosa* to compare the SNC containing high protein, L-leucine, fish oil and specific oligosaccharides, to an iso-energetic control diet. After three weeks, the mice were treated with cyclophosphamide to induce neutropenia. This rendered the mice susceptible to *Pseudomonas* translocation, which was quantified five days later.

Intervention with the SNC resulted in a significantly reduced incidence and intensity of bacterial translocation to the liver ($p < 0.05$) and a similar trend in the lungs ($p = 0.053$, $p = 0.057$, respectively). In addition, the SNC reduced the fecal pH ($p < 0.05$) and decreased *P. aeruginosa* counts in fecal samples ($p < 0.05$). Moreover, plasma levels of pro-inflammatory cytokines showed a strong correlation with the reduced bacterial translocation to the liver ($\rho > 0.78$, $p < 0.001$).

In conclusion, dietary intervention with the SNC significantly reduced the incidence and severity of *P. aeruginosa* translocation in a mouse model of chemotherapy-induced immune suppression. Several mechanisms might have played a role, including the modulation of the intestinal microbiota, an improved gut barrier function, immune function and a reduced inflammatory state. These results may represent an opportunity to develop new applications in cancer patients, with the aim to reduce infectious and other complications.

INTRODUCTION

In many cancer patients immune function is compromised due to host-, tumor- and treatment-related factors, leading to an increased risk of (infectious) complications. In addition, age, stress, depression and nutritional status are important factors that may contribute to the immune deficiency (1, 2). A systemic inflammatory state accounts for the production of chemokines, cytokines, prostaglandins and reactive oxygen/nitrogen species inducing profound immune suppression, which facilitates the escape of tumor cells from immune surveillance (3-5). Similar inflammatory mediators may be produced by the tumor itself as well, which in turn can facilitate angiogenesis, tumor cell growth and the recruitment of myeloid derived suppressor cells (MDSC) (3, 5-7). MDSC are a population of $CD11b^+$/$GR\text{-}1^+$ cells which contribute to tumor escape and immune suppression and they are a potential link between inflammation and tumor progression. The risk of immune deficiency-induced complications is even higher after cancer treatment. Surgery, radiotherapy and chemotherapy are associated with suppression of the cellular immune system and lead, in combination with malnutrition, to a reduced treatment efficacy and a higher frequency and severity of (infectious) complications (1, 8-11). In addition, cancer treatment can induce a change in patients' intestinal microbiota and encourage damage of the gastrointestinal (GI) mucosa leading to severe inflammation and a diminished barrier function (12-15). To reduce the risk of (infectious) complications and to support the performance state of cancer patients, a multi-targeted approach should be applied including nutritional support. Every effort should be made to prevent involuntary weight loss and delayed treatment schedules. In malnourished patients, pre-operative nutritional support is associated with a 50% reduction of post-operative complications (11), but benefits of oral supplementation including decreased gastro-intestinal toxicity, improved performance status and increased immune responses were described as well (16). Therefore, nutritional interventions have been recommended as an integral part of cancer therapy to improve clinical outcomes and quality of life (8, 11, 16).

In daily practice, chemotherapy often accounts for the development of severe neutropenia, which is defined as an absolute neutrophil count < 500 cells/mm^3 and is an important risk factor for the development of bacterial infections (9, 17). Infections by Gram-positive as well as Gram-negative bacteria were observed in neutropenic cancer patients, in which coagulase-negative staphylococci and *Staphylococcus aureus* are the most common Gram-positive bacteria. *Escherichia coli*, Klebsiella species, and *Pseudomonas aeruginosa* are the most common Gram-negative pathogens isolated from neutropenic patients and account for 60-65% of documented bacterial infections. Moreover, Gram-negative infections have been associated with a higher morbidity and mortality in advanced disease (18, 19). In neutropenic cancer patients, the opportunistic pathogen *P. aeruginosa* can cause severe infections of the respiratory tract which may lead to sepsis. Other mucosal sites including the GI tract, the urinary tract and the eyes can be colonized by *P. aeruginosa* as well. Moreover, the bacterium can easily translocate from the GI tract into the bloodstream as a consequence of chemotherapy-induced GI mucosal damage (20-22).

In previous studies in tumor-bearing mice, a recently developed specific nutritional combination (SNC) containing high protein, L-leucine, fish oil and specific oligosaccharides was shown to ameliorate tumor-induced suppression of Th1-mediated immunity and to re-

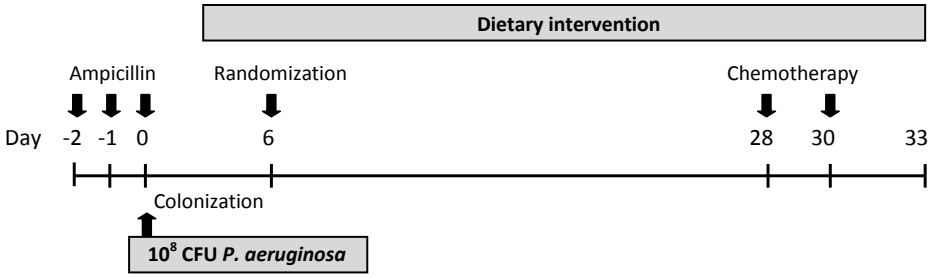
duce the inflammatory state. The complete SNC was necessary for this effect, since no effects were observed with the individual nutritional ingredients (23). Similarly, the SNC, but not the individual ingredients, beneficially affected the tumor-induced catabolic state and preserved muscle mass and function (24). In the current proof of concept study, functional effects of the SNC were studied in an immune compromised situation in chemotherapy-induced neutropenic mice, as a model for anti-cancer treatment-induced infectious complications. Translocation of a clinically relevant pathogen, *P. aeruginosa*, was used as the main functional outcome in this study.

METHODS

STUDY DESIGN

In order to establish a stable colonization with *P. aeruginosa*, female C3H/HeN mice were pre-treated with the broad-spectrum antibiotic Ampicillin, (i.p. injection, 200 mg/kg dissolved in 0.2 ml saline, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) for three consecutive days (day -2, -1 and 0, Figure 1).

The mice in the *P. aeruginosa* groups were infected with 0.2 ml ampicillin resistant *Pseudomonas aeruginosa* strain PAO-1 (ATTC BAA-47: 10^9 CFU/ml in PBS + 3% bicarbonate) administered by oral gavage (infection on day 0) as described earlier (20,



Groups	Short	n	*P. aeruginosa*	Chemotherapy	Diet
Control diet	**C**	**10**	**-**	**-**	**Control**
Control diet & *P.aeruginosa*	C-P	10	+	-	Control
Control diet & chemotherapy	C-T	10	-	+	Control
SNC diet & chemotherapy	SNC-T	10	-	+	SNC
Control diet & *P.aeruginosa* &	**C-PT**	**20**	**+**	**+**	**Control**
SNC diet & *P.aeruginosa* & chemotherapy	**SNC-PT**	**20**	**+**	**+**	**SNC**

Figure 1 Experimental set-up and group arrangement of the mice receiving a Control or SNC diet and a treatment with or without *P. aeruginosa* and/or chemotherapy. The study described in the paper focused on the main three groups, which were bold printed in the table. The C group is added as a control group without any treatment. The C-P group and C-T groups are added to examine the effect of either *P. aeruginosa* colonization or chemotherapy treatment and as negative control groups for chemotherapy-induced translocation by *P. aeruginosa*. The SNC-T group is added to exclude potential interactions between the chemotherapy and the SNC diet. The C-PT and SNC-PT groups were added to determine the effects of the SNC on translocation of *P. aeruginosa* in chemotherapy-induced neutropenic mice.

25, 26). The other groups received an oral gavage with 0.2 ml saline. The colonization of *P. aeruginosa* was measured in fecal samples and a stable gut colonization was obtained after five days, as observed in previous validation experiments. Colonization of the GI tract with *P. aeruginosa* remained stable after antibiotic treatment and restoration of the otherwise normal microbiota. On day 6, the mice in the *P. aeruginosa* groups were randomized on the basis of their colonization level and body weight, before the start of the dietary intervention with either Control diet or SNC diet. The diets were refreshed every day and the food intake per cage was recorded. During the experiment, weight development was measured three times per week and several times per week fresh feces samples were obtained from individual mice to follow the colonization of the gut with *P. aeruginosa*.

After three weeks of dietary intervention, neutropenia and mucosal damage were induced by chemotherapy. On day 28 and day 30, the mice in the chemotherapy groups received i.p. 100 mg cyclophosphamide (in sterile saline, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) per kilogram body weight leading to *P. aeruginosa* dissemination and systemic translocation. The other groups received an i.p. injection with only saline solution. After chemotherapy, blood was drawn daily from different mice in selected experimental groups by submandibular puncture to determine the degree of neutropenia. This was performed in such a way that each of the mice in these groups provided one blood sample after chemotherapy. In addition, after chemotherapy body weight, food intake and health score of the mice were monitored daily and fresh feces samples were collected to determine colonization levels of *P. aeruginosa*. In contrast to studies described in literature, the validated model used in this experiment only examines morbidity of the mice rather than mortality. At day 33, five days after the first chemotherapy dose, blood of the mice was collected by cardiac puncture and sampled in heparin tubes to measure pro-inflammatory cytokines to establish the inflammatory state of the mice and neutropenia was assessed by hematological analysis. After the mice were killed, liver and lungs were removed aseptically to determine translocation of *P. aeruginosa* to these organs (25, 27, 28). In addition, spleen, brain, thymus and the skeletal muscles were dissected. Intestinal content samples were obtained by ileum lavage (using 1 ml sterile demi water) and removal of the cecum content. The pH of these samples was measured and they were frozen at -80°C.

MICE AND DIETS

Seven to eight-week-old female C3H/HeN mice were obtained from Charles River (Maastricht, the Netherlands). All experimental procedures were approved by the Animal Experimental Committee and complied with the principles of laboratory animal care. Mice were housed in groups of five in individual ventilated cages (IVC) to prevent contamination among groups and all handlings were performed in a laminar flow cabinet. The IVC-cages were placed in a climate-controlled animal care facility (12:12 dark-light cycle with a constant room temperature of 21 °C and humidity of 50%). All mice had free access to food and sterilized drinking water. Upon arrival, mice were acclimatized for one week. During this acclimatization period and before the start of the dietary intervention, the mice received a maintenance diet (AIN-93M (29), Research Diet Services, Wijk bij Duurstede, the Netherlands). The presented results are representative of two separate experiments. To confirm the results of the first experiment, it was repeated with a similar setup. In both experiments

mice were divided in different groups receiving either a Control diet supplied as pellets or an experimental diet enriched with a specific nutritional combination (SNC) containing high protein, L-leucine, fish oil and a specific mixture of prebiotic oligosaccharides. Both diets are iso-energetic and are based on semi-synthetic AIN-93M diet with a modified fat source. In the control diet, the soy oil was replaced by corn oil, which was used as the base oil in the SNC diet as well. The control diet contained per kg food: 125.8 g protein (100% casein), 697.7 g carbohydrates, 52.6 g fat (100% corn oil) with a polyunsaturated: saturated (P/S) fat ratio of 4.4 and a (n-3):(n-6) ratio of 0.014, and 50.0 g fibre (cellulose). The SNC diet contained per kg food: 209.6 g protein (188.6 g intact protein of which 68% was casein and 32% was whey and 21 g was free L-leucine), 540.3 g carbohydrates, 52.5 g fat (20.2 g corn oil, 10.2 g canola oil and 22.1 g fish oil (providing 6.9 g Eicosapentaenoic acid (EPA) and 3.1 g Docosahexaenoic acid (DHA))) with a P/S fat ratio of 4.0 and a (n-3): (n-6) ratio of 0.9, 50.0 g fibre of which 22.0 g cellulose, 19.8 g short-chain galacto-oligo saccharides

Table 1 Diet compositions based on AIN-93M diet

Nutrients, *per kg food*	**Control diet**	**SNC diet**
Total energy, *kJ (kcal)*	15857 (3790)	15862 (3791)
Polyunsaturated : saturated fat ratio	4.4	4.0
(n-3) : (n-6) ratio	0.014	0.9
Total fat, *g*	52.6	52.5
- **corn oil**	52.6	20.2
- **canola oil**	0.0	10.2
- **fish oil**	0.0	22.1
- **EPA**	0.0	~ 6.9
- **DHA**	0.0	~ 3.1
Total carbohydrates, *g*	697.7	540.3
Total intact protein, *g*	125.8	188.6
- **whey**	0.0	62.2
- **casein**	125.8	126.4
- **Free L-leucine**	0.0	21.0
Fibers, *g*	50.0	50.0
- **cellulose**	50.0	22.0
- **GOS**	0.0	19.8
- **lactose from GOS syrup**	0.0	6.0
- **scFOS**	0.0	2.2
Mineral mix, *g*	35.0	35.0
Vitamin mix, *g*	10.0	10.0
L-Cystine, *g*	1.8	1.8
Choline bitartate (41.1% choline), *g*	2.5	2.5
tert-Butylhydroquinone (TBHQ), *mg*	3.0	3.0

Values represent the nutrients per kg food of the Control diet and SNC diet.

(GOS, Vivinal GOS, Friesland Domo Foods, Zwolle, the Netherlands), 6.0 g lactose from GOS syrup and 2.2 g short-chain fruto-oligosaccharides (FOS, Beneo p95, Orafti, Wijchen, the Netherlands) (Table 1).

The study focused on the three main experimental groups: the group receiving the Control diet without any treatment (C, n=10), the group receiving Control diet and *P. aeruginosa* and chemotherapy (C-PT, n=20) and the group receiving SNC diet and *P. aeruginosa* and chemotherapy (SNC-PT, n=20). Since colonization and bacterial translocation of *P. aeruginosa* could not be measured in group C, this group is not shown in colonization and translocation graphs (Figures 3 and 5). Moreover, to control the effects of *P. aeruginosa* and chemotherapy treatment, and to exclude potential interactions between the chemotherapy and the SNC diet, three additional reference groups were added to the study (Figure 1). Results from these groups didn't show bacterial translocation or any other unexpected findings and are, for that reason, not mentioned in the paper.

PSEUDOMONAS AERUGINOSA

Culture

P. aeruginosa strain PAO-1 (ATTC BAA-47) was sub-cultured on Nutrient Agar and inoculated into Trypticase Soy Broth (500 ml). The overnight culture was washed, concentrated and diluted to a concentration of approximately 1 x 10^9 CFU/mL in PBS (D-PBS, Invitrogen, Merelbeke, Belgium) + 3% bicarbonate (for neutralization of gastric acid) as determined by spectrophotometry (OD600). The number of bacteria was confirmed by plating dilutions (in PBS) of bacteria on *P. aeruginosa* C-N selective supplement agar (Oxoid, Badhoevedorp, the Netherlands).

Determination of colonization

At regular time-intervals fresh feces samples were collected from each mouse by placing the mice individually in a clean and empty cage for 5 minutes. The samples were weighted, diluted and homogenized. Subsequently, the pH of the samples was measured and they were diluted in Peptone Physiological Salt solution before plating on C-N agar with 20 mg/L ampicillin. After overnight incubation at 37°C, the number of *P. aeruginosa* (CFU) per gram feces was determined.

Bacterial translocation to liver and lungs

Aseptically removed organs were collected in 0.5 ml Buffered Peptone Water, weighted and homogenized (4°C) by using an ultra-turrax (IKA, autoclavable disposable turrax). Tenfold dilution series were prepared in PBS and plated on Nutrient Agar and C-N agar plates. After overnight incubation at 37°C, the number of specific *P. aeruginosa* colonies and non-specific colonies were determined.

HEMATOLOGY

To determine white- and red blood cell count and differential heparinized blood was diluted 2 x with sterile physiological salt solution and was measured on the ADVIA120TM Hematology System (Siemens, Mijdrecht, the Netherlands).

PGE_2 AND CYTOKINE MEASUREMENT

Prostaglandin E_2 (PGE_2) in plasma was measured using a commercial anti-PGE_2 rabbit polyclonal antibody-based direct enzyme immunoassay (Oxford Biomedical Research, Oxford, MI, USA) according to the manufacturer's protocol. Cytokines in plasma were measured using a commercial mouse cytokine 10-plex bead immunoassay (Biosource, Etten-Leur, the Netherlands) according to the manufacturer's protocol.

STATISTICAL ANALYSIS

All data were expressed as means ± SEM or as medians (IQR; 25th-75th percentiles). Statistical analysis was performed using SPSS 15.0 (SPSS Benelux, Gorinchem, the Netherlands). Comparisons between the groups were made using a one-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc analysis when data were normally distributed. A non-parametric Mann-Whitney U-test or Kruskal-Wallis Test was performed when data were not normally distributed. For statistical analysis of correlations on data that were not normally distributed the Spearman's correlation test was performed. Differences were considered significant at $p < 0.05$.

RESULTS

BODY WEIGHT AND FOOD INTAKE

In the first part of the study a normal bodyweight curve was observed without any differences between the three groups (Figure 2). From day 20 onwards, the increase in body weight was more pronounced in the SNC-PT group compared to the C and C-PT group. After the chemotherapy treatment of both the C-PT and SNC-PT groups, body weight declined compared to the C group. However, the bodyweight in the SNC-PT group was still significantly higher than in the C-PT group ($p < 0.05$). After the chemotherapy treatment food intake was monitored daily, but no significant differences between the groups were observed.

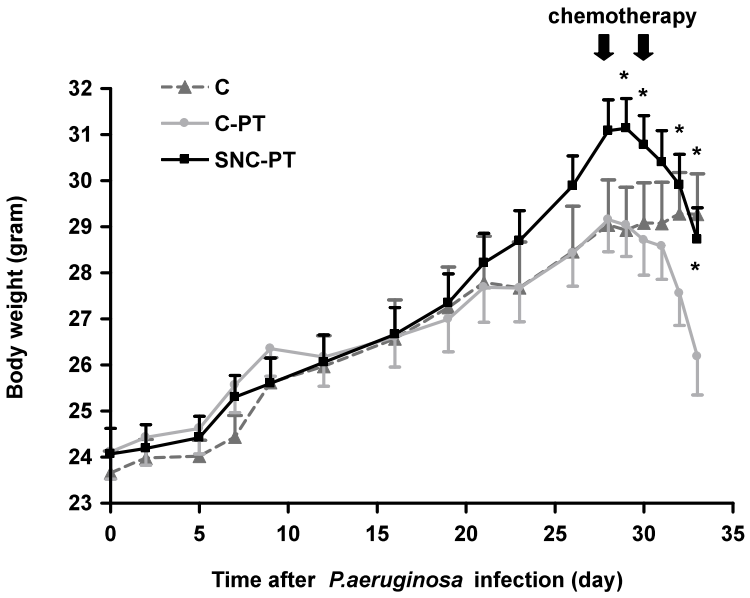


Figure 2 Body weights of mice in the C, C-PT, and SNC-PT groups. Data are mean ± SEM, n = 18 or 10 (C). *Different from C-PT, $p < 0.05$ (least significant difference test).

P. AERUGINOSA COLONIZATION, PH AND BENEFICIAL BACTERIA

Five days after the *P. aeruginosa* infection a stable colonization was obtained, as observed in previous validation experiments (data not shown). On day 6, the mice in the *P. aeruginosa* groups were randomized on the basis of their colonization level (5.7 ± 0.2 $\log^{10}$ CFU/g feces in the C-PT group vs 5.6 ± 0.2 $\log^{10}$ CFU/g feces in the SNC-PT group) and body weight. Subsequently, several times per week fresh feces samples were collected from individual mice to follow the colonization of the gut with *P. aeruginosa* (Figure 3). At day 13, the colonization of *P. aeruginosa* in the SNC-PT group decreased significantly compared to the C-PT group ($p < 0.05$). Afterwards both the levels in the C-PT and SNC-PT group stabilized but remained significantly different ($p < 0.05$). After the chemotherapy treatment, *P. aeruginosa* levels in fecal samples of both the C-PT and SNC-PT group decreased, but at day 30 and day 31 the colonization in the SNC-PT group was still significantly lower compared to the C-PT group ($p < 0.05$).

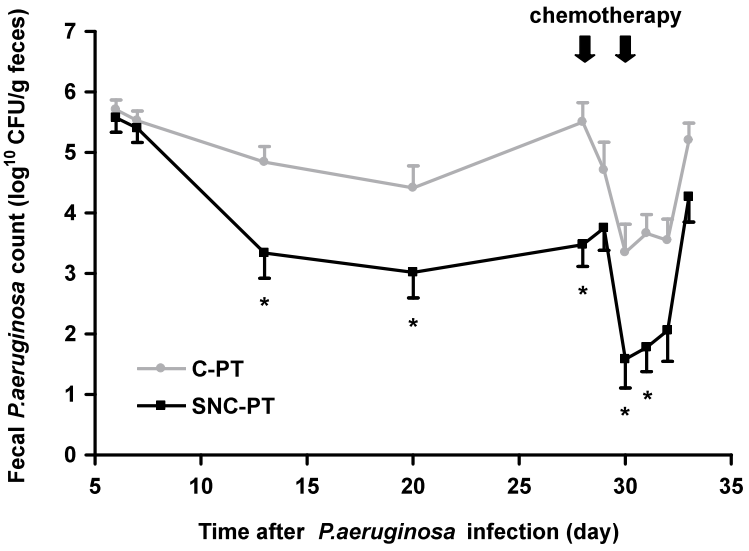


Figure 3 *P. aeruginosa* colonization levels in individual fresh feces samples of the mice in the C-PT and SNC-PT groups. Data are means ± SEM, n = 18. *Different from C-PT, $p < 0.05$ (Mann-Whitney U test).

In addition, the fecal pH of the mice in the SNC-PT group were significantly lower at day 7 and day 31 (pH 7.4 and 8.1, respectively) compared to the fecal pH in the C-PT group (pH 8.1 and 8.5, respectively at day 7 and day 31, $p < 0.05$). Besides the colonization of *P. aeruginosa,* the presence of beneficial bacteria was measured at several time points. At day 28 and day 33, significant higher numbers of lactobacilli were observed in the SNC-PT group (10.9 ± 0.13 $\log^{10}$ CFU/g feces and 11.1 ± 0.35 $\log^{10}$ CFU/g feces, respectively, $p < 0.05$) compared to the C-PT group (10.3 ± 0.16 $\log^{10}$ CFU/g feces and 10.4 ± 0.35 $\log^{10}$ CFU/g feces, respectively). Bifidobacteria could hardly be detected in the feces of the mice in the different groups and showed a large variation.

CHEMOTHERAPY-INDUCED NEUTROPENIA

After the chemotherapy treatment, the mice in the C-PT and SNC-PT group became severely neutropenic compared to the mice in the C group ($p < 0.05$, Figure 4A). Moreover, the absolute number of lymphocytes (Figure 4B) and the total white blood cell count (data not shown) decreased significantly ($p < 0.05$). No differences were observed between the C-PT and SNC-PT groups with respect to the numbers of the different cell types.

P. AERUGINOSA TRANSLOCATION

At day 33, after the mice were killed, bacterial translocation of *P. aeruginosa* to liver and lungs was measured. The translocation incidence to the liver was significantly lower in the SNC-PT group compared to the C-PT group ($p < 0.05$, Figure 5A), while the other experimental groups did not show any translocation.

In addition, the incidence of *P. aeruginosa* translocation to the lung tended to be lower in the SNC-PT group compared to the C-PT group ($p = 0.053$). The mice that died of infection

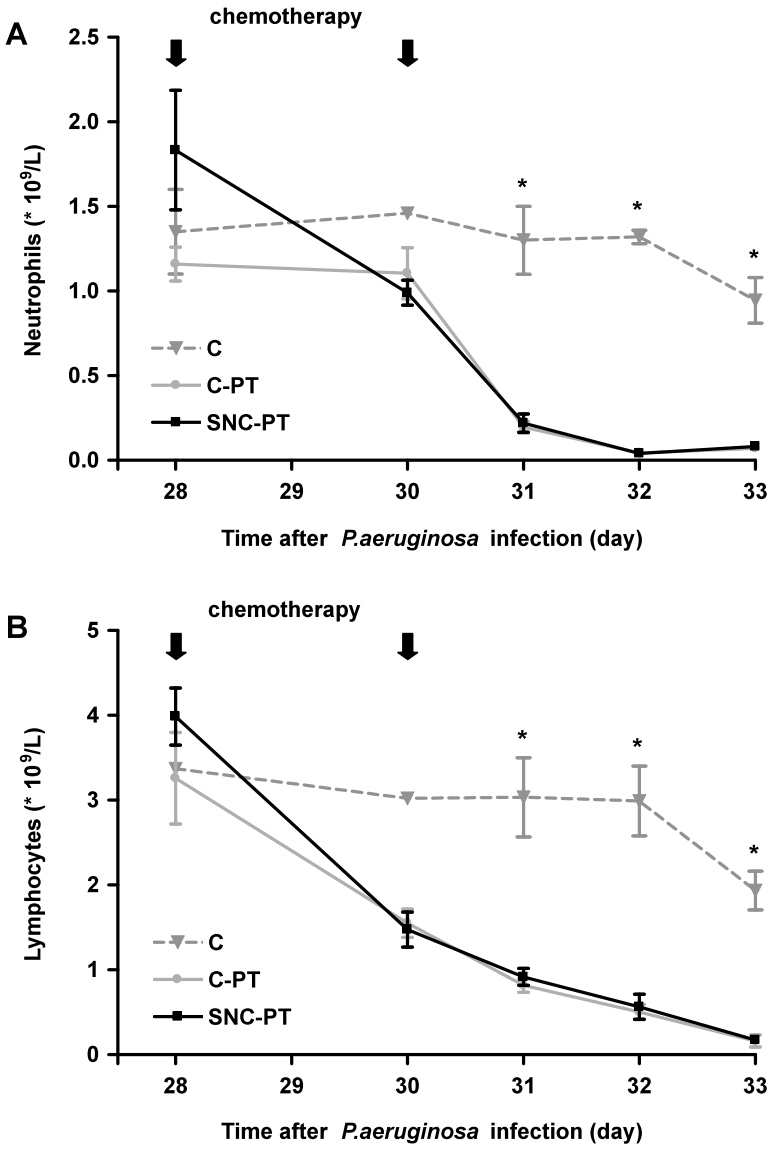


Figure 4 Absolute neutrophil (A) and lymphocyte (B) counts in a subgroup of mice in the C, C-PT, and SNC-PT groups. Data are means ± SEM, n = 9 (C), 13 (C-PT), or 10 (SNC-PT). *Different from C-PT, $p < 0.05$ (Kruskal-Wallis test).

were included in the translocation analysis by giving them the highest score of translocation, as translocation can be expected in these mice. Accordingly, there may be an effect on survival in the SNC-PT group, since only 1 mouse died due to infection compared to 3 mice in the C-PT group. Besides the translocation incidence, the *P. aeruginosa* counts in the liver were also significantly decreased in the SNC-PT group compared to the C-PT group ($p < 0.05$, Figure 5B) and a trend was observed in translocation intensity to the lung ($p = 0.057$).

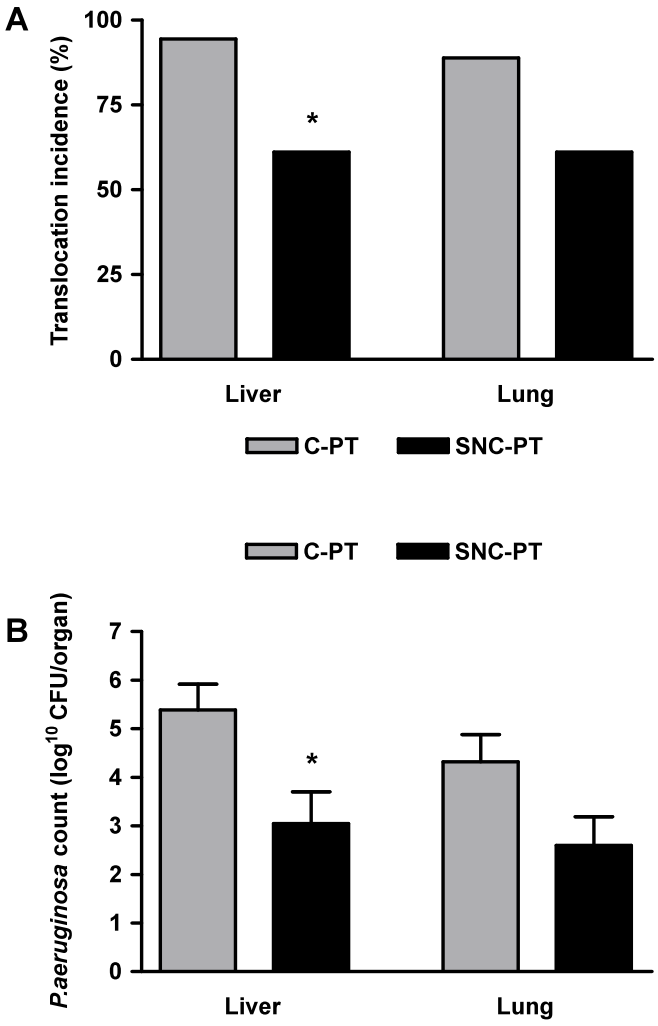


Figure 5 *P. aeruginosa* translocation incidence (A) and intensity (B) to the liver and lungs of the mice in the C-PT and SNC-PT groups. Data are percent of total number of mice (A) or means ± SEM, n = 18 (B). *Different from C-PT, $p < 0.05$ (Mann-Whitney U test).

PLASMA CYTOKINES AND PGE_2

The pro-inflammatory cytokines Interleukin (IL)-1β, IL-6, Tumor Necrosis Factor (TNF)-α, Interferon (IFN)-γ and the inflammatory mediator PGE_2 were measured in plasma after the mice were killed at day 33 (Table 2). For all the cytokines a significant increase was observed in the C-PT group compared to the C group ($p < 0.001$). In the SNC-PT group the levels of all the cytokines tended to be decreased compared to the C-PT group ($p = 0.19$, $p = 0.28$, $p = 0.097$, $p = 0.38$ for IL-1β, IL-6, TNF-α and IFN-γ, respectively), but due to the high variance these effects were not significant. However, these pro-inflammatory cytokines correlated highly with bacterial translocation ($\rho = 0.78$-0.82; $p < 0.001$).

Table 2 Plasma levels of pro-inflammatory cytokines and PGE_2 of mice in the C group, the C-PT group and the SNC-PT group

	n	IL-1β *ng/L*	IL-6 *ng/L*	TNF-α *ng/L*	IFN-γ *ng/L*	PGE_2 *µg/L*
C	10	1.5 (1.5-1.5)[A]	0.1 (0.1-0.1)[A]	2.8 (2.8-2.8)[A]	1.4 (1.4-1.4)[A]	5.81 (4.65-8.02)
C-PT	12	21.7 (6.0-86.2)	1830 (891-9030)	56.4 (43.0-252)	107 (29.9-180)	6.17 (5.90-7.40)
SNC-PT	17	6.0 (1.5-42.0)	912 (4.30-5540)	10.0 (2.8-114)	54.3 (1.4-253)	5.75 (4.60-8.13)
Correlation translocation intensity to the liver		ρ = 0.82[B]	ρ = 0.82[B]	ρ = 0.78[B]	ρ = 0.80[B]	ρ = 0.26

Data are medians and IQR (25th-75th percentiles) and ρ (rho) for the correlation coefficient with bacterial translocation to the liver. [A]Different from C-PT, $p < 0.001$ (Kruskal-Wallis Test), [B]Correlation between the specific cytokines and the translocation intensity to the liver, $p < 0.001$ (Spearman's correlation 2-tailed).

DISCUSSION

The present study demonstrates a significant reduction of bacterial translocation after dietary intervention with the SNC in a mouse model for chemotherapy-induced neutropenia. Previous studies demonstrated that the complete SNC, but not the individual ingredients, induced beneficial immune modulatory effects in tumor-bearing mice (23), suggesting that the SNC might support the resistance to infections in a compromised host. In the current proof of concept study, functional effects of the SNC were investigated in an immune compromised mouse model of chemotherapy-induced neutropenia, as a clinically relevant model for anti-cancer treatment-induced infectious complications, in which the translocation of *P. aeruginosa* was measured as the primary outcome parameter. Several mechanisms might have played a role in the effects observed in this study, including modulation of the intestinal microbiota, beneficial effects on gut barrier function, immune function and a reduced inflammatory state.

As expected, chemotherapy treatment induced a strong reduction in the number of neutrophils, lymphocytes (Figure 4) and consequently in the number of total white blood cells (data not shown). However, no differences were observed after the intervention with the SNC on either neutrophil or lymphocyte count. In general, chemotherapy-induced neutropenia and lymphopenia result in suppression of the innate immune system, as well as the adaptive immune system, resulting in a high risk of bacterial infections (30). However, the chemotherapy-induced elimination of neutrophils and lymphocytes is not the only risk factor for bacterial translocation. The fact that the SNC significantly reduced translocation of *P. aeruginosa* to liver and lungs can also be explained by a beneficial effect on the integrity of the intestinal mucosa, by decreasing the number of colonized *P. aeruginosa* in the intestine (Figure 3) or by improving immune cell function, rather than the number of immune cells, comparable with results observed in previous experimental mouse models (23). Although this study focused on functional outcomes rather than mechanistic evidence, the current findings and previous data provide suggestions about the mechanisms involved. In the present study, mice were treated with a short course of antibiotics to enable the

GI colonization with *P. aeruginosa* that remained stable after the subsequent restoration of the normal GI microbiota. Therefore, modulation of the metabolism or composition of the microbiota may have played a role in the observed effects, especially since the specific oligosaccharides (GOS)/FOS) are present in the SNC. These non-digestible oligosaccharides are fermentable fibers with prebiotic properties, that have been associated with immune modulatory effects and other health benefits including an improved gut barrier function (31). Although the present study provides no data on the intestinal integrity, the present study showed a significant reduction of fecal pH after dietary intervention with the SNC. This might be the result of intestinal short-chain fatty acid (SCFA) production, including butyrate, acetate and lactate, formed by the fermentation of the prebiotic oligosaccharides by the colonic microbiota, which can contribute to a restoration of the intestinal barrier function (31-33). Accordingly, the reduced pH in the intestinal lumen can lead to the inhibition of pathogen growth and adhesion. Furthermore, the fermentation of the specific oligosaccharides can stimulate the growth of beneficial bacteria as bifidobacteria and lactobacilli, which in turn can also inhibit pathogens by the production of antimicrobial substances (31, 34). The percentage of these bacteria tended to be higher at multiple time points in the group receiving the SNC diet compared to the groups receiving Control diet ($p = 0.06$-0.6). At day 33, significant higher numbers of lactobacilli were detected, which might have contributed to the reduced *P. aeruginosa* levels in the GI tract. Since intestinal colonization of *P. aeruginosa* is a predictor of systemic infections (28, 35), the observed reduction in colonization might contribute to the beneficial effect of the SNC on *P. aeruginosa* translocation. Consequently, in severely immune compromised subjects a reduction in bacterial translocation to extra-intestinal sites might similarly lead to a reduction of septic morbidity (28).

Another explanation for the beneficial reduction of *P. aeruginosa* translocation by the SNC is its potential effect on the reduced immune function of the host (27). As mentioned before, the specific oligosaccharides can modulate the immune system via a microbiota-dependent (prebiotic) mechanism, but they may also affect immune function directly by blocking or activating specific receptors on immune cells leading to improved immune responses and eventually to enhanced resistance to systemic infections. Moreover, the specific oligosaccharides might reduce the binding of *P. aeruginosa* on specific receptors leading to a decreased capability to infect (31). Nevertheless, in previous studies in tumor-bearing mice it was demonstrated that only the complete mixture of high protein, L-leucine, fish oil and specific oligosaccharides was able to enhance Th1-mediated immunity and to reduce the inflammatory state (23). In the present study, the beneficial effects might be due to the combination of fish oil and oligosaccharides affecting the above mentioned risk factors as well, since also fish oil is associated with decreased levels of bacterial translocation and a reduced systemic inflammatory state (36-39). Fish oil contains high amounts of the (n-3) long-chain polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) playing a major role in the regulation of immune responses and inflammation (40-42). In the present study, the severe inflammatory state of the mice after chemotherapy-induced bacterial translocation is confirmed by the increase of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ in plasma. In addition, a significant correlation was observed between the pro-inflammatory cytokines and the bacterial translocation intensity to the liver ($p < 0.001$), which is regarded by the authors as a marker

for systemic infection due to bacterial translocation from the GI tract. After dietary intervention with the SNC, all the cytokines tended to decrease compared to the C-PT group (p = 0.097-0.38). This might be the result of both the anti-inflammatory effect of fish oil and the immune modulatory and prebiotic effect of the specific oligosaccharides.

The SNC, containing high levels of protein and the branched chain amino acid L-leucine, is hypothesized to modulate metabolism, as it beneficially affected the tumor-induced catabolic state, preserved muscle mass and function and significantly reduced loss of body weight in previous experiments (24). In the present study, body weights were higher in mice fed the SNC diet compared to mice fed the control diet from day 20 onwards, although food intake was comparable between groups. Therefore this effect is attributed to differences in diet compositions and the associated modulation of the metabolism. In addition, after chemotherapy treatment, body weight in the SNC-PT group was significantly higher than in the C-PT group, which was associated with a smaller loss of muscle and fat mass (data not shown) and could be due to a reduced inflammatory state and/or the high levels of protein and L-leucine in the SNC.

In this and previous studies, the choice was made to investigate the effects of n-3 PUFA enrichment, addition of the oligosaccharides and high levels of protein and L-Leucine in a test diet with a normal macronutrient composition for mice, based on the AIN-93M definition (43). Since the envisaged application of the SNC concept in humans is an oral nutritional supplement aimed at increasing total protein intake and increasing the relative n-3 PUFA intake, the test and control diets were designed to be iso-energetic but not iso-nitrogenous. Since the total dietary intake of the mice was not expected to be different between groups, this setup allowed to test a relative increase in protein and n-3 PUFA intake. In order to study a good contrast between the test and control diets with regard to fatty acid composition, the AIN-93M-based diets were modified with regard to the base fat source, replacing soy with corn oil. Corn oil, containing a (n-3):(n-6) ratio of 0.014, was used as a base fat source that is highly predominant in n-6 PUFAs, similar to westernized types of diet although more outspoken. Other parameters of the fat source, such as the total amount of fat and the P/S ratio were kept similar to the normal mouse diet. It is recognized that all aspects of the fat content of the diet play a role in the functional effects of dietary PUFA interventions (44, 45), but the choice was made to focus on a change in the (n-3):(n-6) PUFA intake ratio. The relatively high levels of (n-6) PUFA in the control diet might be argued to induce inflammation (40). However, in mice receiving the control diet, levels of pro-inflammatory cytokines were very low compared to the mice in the C-PT group (Table 2), showing no spontaneous induction of inflammation. Moreover, in previous studies in tumor-bearing mice, no differences on inflammation were observed between the use of low levels of soy oil compared to corn oil (23). In the present proof of concept study, a model without the presence of a tumor was used, since it would complicate the model further, making it difficult to control well. For applications in human cancer patients, it is important to get insight into the relation between the tumor, the chemotherapy treatment and the nutritional ingredients, both for safety reasons, to explore the potential benefits and to optimize the nutritional intervention with regard to dose, composition, schedule of administration and target populations (44, 46). As mentioned before, this study focused on functional outcome parameters after nutritional intervention with the SNC in a mouse model featuring a clinically relevant pathogen. Therefore, the mechanistic evidence is limited in this study and no direct parameters of

intestinal inflammation, intestinal permeability or intestinal immune cell depletion were measured. Future studies using histological techniques and functional analysis of intestinal permeability could provide more insight into the mechanisms that underlie the observed effects. The current model, using a relatively low dose of chemotherapy, was set-up to study morbidity, rather than mortality, in a sensitive way. Therefore, this model is thought to be most relevant for prevention of septic infections, rather than treating and ameliorating the course of existing septic infections. In this sensitive model, we regarded translocation of *P. aeruginosa*, combined with elevated and highly correlated levels of serum inflammatory cytokines, as a marker for infection (25, 27, 28). Other clinical symptoms of infections (e.g. fever, illness score) were not analyzed in these mice. However, since translocation is a necessary step in sepsis with pathogens of intestinal origin, it is argued that the protective effect on translocation is of potential clinical relevance. Although the induction of *P. aeruginosa* colonization does not correspond to the normal clinical exposure to the pathogen, the functional situation after colonization is clinically relevant. In this regard, the model resembles the clinical situation in humans suffering from a quiescent intestinal infection that, e.g. after chemotherapy, surgery or transplantation, leads to bacterial translocation and results in a severe sepsis (28).

In conclusion, dietary intervention with the SNC significantly reduced the incidence and severity of *P. aeruginosa* translocation in chemotherapy-induced neutropenic mice by reducing the translocation to liver and a similar trend in the lungs. In addition, the SNC reduced the fecal pH which may at least partly explain the lower *P. aeruginosa* counts in fecal samples during the nutritional intervention phase of the experiment. Plasma levels of pro-inflammatory cytokines tended to be reduced and a strong correlation was observed with bacterial translocation to the liver. With the presence of high protein, L-leucine, fish oil and specific oligosaccharides in the SNC, the underlying mechanism may involve a prebiotic as well as an immune modulatory effect. Together with previous results showing beneficial effects on cellular immunity, muscle function and body composition in tumor-bearing mice, these results might represent a new opportunity for applications in cancer patients to reduce (infectious) complications.

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REFERENCES

1 **Hadden JW.** Immunodeficiency and cancer: prospects for correction. Int Immunopharmacol 2003;3: 1061-71.

2 **Evans C, Dalgleish AG, Kumar** D. Review article: immune suppression and colorectal cancer. Aliment Pharmacol Ther 2006;24: 1163-77.

3 **Ben-Baruch A**. Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators. Semin Cancer Biol 2006;16: 38-52.

4 **Allavena P, Sica A, Garlanda C, Mantovani A**. The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. Immunol Rev 2008;222: 155-61.

5 **Dolcetti L, Marigo I, Mantelli B, Peranzoni E, Zanovello P, Bronte V.** Myeloid-derived suppressor cell role in tumor-related inflammation. Cancer Lett 2008;267: 216-25.

6 **Allavena P, Garlanda C, Borrello MG, Sica A, Mantovani A**. Pathways connecting inflammation and cancer. Curr Opin Genet Dev 2008;18: 3-10.

7 **Mantovani A.** Cancer: Inflaming metastasis. Nature 2009;457: 36-7.

8 **Van Cutsem E, Arends J.** The causes and consequences of cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2: S51-63.

9 **Kuderer NM, Dale DC, Crawford J, Cosler LE, Lyman GH**. Mortality, morbidity, and cost associated with febrile neutropenia in adult cancer patients. Cancer 2006;106: 2258-66.

10 **Whimbey E, Englund JA, Couch RB**. Community respiratory virus infections in immunocompromised patients with cancer. Am J Med 1997;102: 10-8; discussion 25-6.

11 **Senesse P, Assenat E, Schneider S, Chargari C, Magne N, Azria D, et al**. Nutritional support during oncologic treatment of patients with gastrointestinal cancer: who could benefit? Cancer Treat Rev 2008;34: 568-75.

12 **Stringer AM, Gibson RJ, Bowen JM, Keefe DM.** Chemotherapy-induced modifications to gastrointestinal microflora: evidence and implications of change. Curr Drug Metab 2009;10: 79-83.

13 **Keefe DM, Cummins AG, Dale BM, Kotasek D, Robb TA, Sage RE.** Effect of high-dose chemotherapy on intestinal permeability in humans. Clin Sci (Lond) 1997;92: 385-9.

14 **Gibson RJ, Stringer AM.** Chemotherapy-induced diarrhoea. Curr Opin Support Palliat Care 2009;3: 31-5.

15 **Wessner B, Strasser EM, Koitz N, Schmuckenschlager C, Unger-Manhart N, Roth E**. Green tea polyphenol administration partly ameliorates chemotherapy-induced side effects in the small intestine of mice. J Nutr 2007;137: 634-40.

16 **van Bokhorst-de van der Schueren MA**. Nutritional support strategies for malnourished cancer patients. Eur J Oncol Nurs 2005;9 Suppl 2: S74-83.

17 **del Giglio A, Eniu A, Ganea-Motan D, Topuzov E, Lubenau H**. XM02 is superior to placebo and equivalent to Neupo gen in reducing the duration of severe neutropenia and the incidence of febrile neutropenia in cycle 1 in breast cancer patients receiving docetaxel/doxorubicin chemotherapy. BMC Cancer 2008;8: 332.

18 **Rolston KVI.** Bacterial infection in neutropenic cancer patients: an overview. Iranian Journal of Clinical Infectious Diseases 2009;4: 8.

19 **Klastersky J, Ameye L, Maertens J, Georgala A, Muanza F, Aoun M, et al.** Bacteraemia in febrile neutropenic cancer patients. Int J Antimicrob Agents 2007;30 Suppl 1: S51-9.

20 **Koh AY, Priebe GP, Pier GB.** Virulence of Pseudomonas aeruginosa in a murine model of gastrointestinal colonization and dissemination in neutropenia. Infect Immun 2005;73: 2262-72.

21 **Mutlu GM, Wunderink RG.** Severe pseudomonal infections. Curr Opin Crit Care 2006;12: 458-63.

22 **Tetaert D, Pierre M, Demeyer D, Husson MO, Beghin L, Galabert C, et al.** Dietary n-3 fatty acids have suppressive effects on mucin upregulation in mice infected with Pseudomonas aeruginosa. Respir Res 2007;8: 39.

23 **Faber J, Vos P, Kegler D, van Norren K, Argiles JM, Laviano A, et al.** Beneficial immune modulatory effects of a specific nutritional combination in a murine model for cancer cachexia. Br J Cancer 2008;99: 2029-36.

24 **van Norren K, Kegler D, Argiles JM, Luiking Y, Gorselink M, Laviano A, et al.** Dietary supplementation with a specific combination of high protein, leucine, and fish oil improves muscle function and daily activity in tumour-bearing cachectic mice. Br J Cancer 2009;100: 713-22.

25 **Matsumoto T, Tateda K, Miyazaki S, Furuya N, Ohno A, Ishii Y, et al.** Adverse effects of tumour necrosis factor in cyclophosphamide-treated mice subjected to gut-derived Pseudomonas aeruginosa sepsis. Cytokine 1997;9: 763-9.

26 **Urano T, Maejima K.** Provocation of pseudomoniasis with cyclophosphamide in mice. Lab Anim 1978;12: 159-61.

27 **Van Leeuwen PA, Boermeester MA, Houdijk AP, Ferwerda CC, Cuesta MA, Meyer S, et al.** Clinical significance of translocation. Gut 1994;35: S28-34.

28 **MacFie J, O'Boyle C, Mitchell CJ, Buckley PM, Johnstone D, Sudworth P.** Gut origin of sepsis: a prospective study investigating associations between bacterial translocation, gastric microflora, and septic morbidity. Gut 1999;45: 223-8.

29 **Reeves PG**. Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr 1997;127: 838S-41S.

30 **Steele TA**. Chemotherapy-induced immunosuppression and reconstitution of immune function. Leuk Res 2002;26: 411-4.

31 **Vos A, M'Rabet L, Stahl B, Boehm G, Garssen J.** Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. Crit Rev Immunol 2007;27: 97-140.

32 **Macfarlane GT, Steed H, Macfarlane S.** Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol 2008;104: 305-44.

33 **Worthley DL, Le Leu RK, Whitehall VL, Conlon M, Christophersen C, Belobrajdic D, et al**. A human, double-blind, placebo-controlled, crossover trial of prebiotic, probiotic, and synbiotic supplementation: effects on luminal, inflammatory, epigenetic, and epithelial biomarkers of colorectal cancer. Am J Clin Nutr 2009;90: 578-86.

34 **Boehm G, Moro G.** Structural and functional aspects of prebiotics used in infant nutrition. J Nutr 2008;138: 1818S-28S.

35 **Andremont A, Marang B, Tancrede C, Baume D, Hill C.** Antibiotic treatment and intestinal colonization by Pseudomonas aeruginosa in cancer patients. Antimicrob Agents Chemother 1989;33: 1400-2.

36 **Alexander JW**. Bacterial translocation during enteral and parenteral nutrition. Proc Nutr Soc 1998;57: 389-93.

37 **Pscheidl E, Schywalsky M, Tschaikowsky K, Boke-Prols T.** Fish oil-supplemented parenteral diets normalize splanchnic blood flow and improve killing of translocated bacteria in a low-dose endotoxin rat model. Crit Care Med 2000;28: 1489-96.

38 **Calder PC.** Use of fish oil in parenteral nutrition: Rationale and reality. Proc Nutr Soc 2006;65: 264-77.

39 **Bjornsson S, Hardardottir I, Gunnarsson E, Haraldsson A.** Dietary fish oil supplementation increases survival in mice following Klebsiella pneumoniae infection. Scand J Infect Dis 1997;29: 491-3.

40 **Calder PC.** n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006;83: 1505S-19S.

41 **Wu D**, Meydani SN. n-3 polyunsaturated fatty acids and immune function. Proc Nutr Soc 1998;57: 503-9.

42 **Tiesset H, Pierre M, Desseyn JL, Guery B, Beermann C, Galabert C, et al.** Dietary (n-3) polyunsaturated fatty acids affect the kinetics of pro- and antiinflammatory responses in mice with Pseudomonas aeruginosa lung infection. J Nutr 2009;139: 82-9.

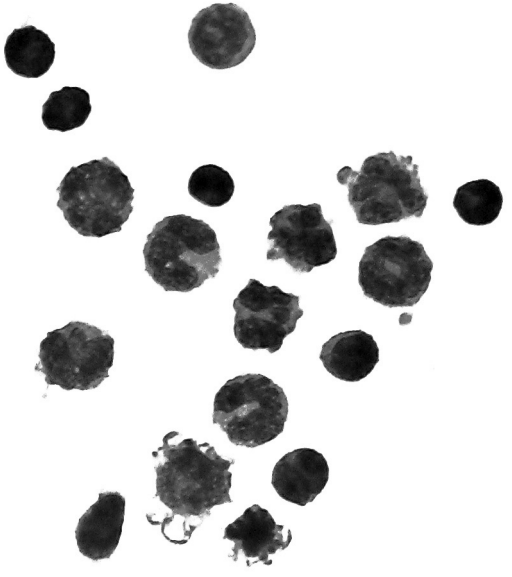
43 **Reeves PG, Nielsen FH, Fahey GC,** Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123: 1939-51.

44 **Xue H, Sawyer MB, Wischmeyer PE, Baracos VE.** Nutrition modulation of gastrointestinal toxicity related to cancer chemotherapy: from preclinical findings to clinical strategy. JPEN J Parenter Enteral Nutr;35: 74-90.

45 **Robinson LE, Clandinin MT, Field CJ.** The role of dietary long-chain n-3 fatty acids in anti-cancer immune defense and R3230AC mammary tumor growth in rats: influence of diet fat composition. Breast Cancer Res Treat 2002;73: 145-60.

46 **Baracos VE, Mazurak VC, Ma DW.** n-3 Polyunsaturated fatty acids throughout the cancer trajectory: influence on disease incidence, progression, response to therapy and cancer-associated cachexia. Nutr Res Rev 2004;17: 177-92.

CHAPTER FIVE



Supplementation with a fish oil-enriched high-protein medical food leads to rapid incorporation of EPA into white blood cells and modulates immune responses within one week in healthy men and women

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ABSTRACT

Immune modulatory effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are well described. However, these fatty acids must be incorporated effectively into cell membrane phospholipids to modify cell function. In order to address the absence of human data regarding short term incorporation, the present study investigated the incorporation of EPA and DHA into white blood cells (WBC) at different time points during one week of supplementation with a medical food, which is high in protein and leucine and enriched with fish oil and specific oligosaccharides. Additionally, the effects on *ex vivo* immune function were determined.

In a single-arm, open label study, twelve healthy men and women consumed 2 x 200 ml of medical food providing 2.4 g EPA, 1.2 g DHA, 39.7 g protein (including 4.4 g leucine) and 5.6 g oligosaccharides daily. Blood samples were taken at day 0 (baseline), 1, 2, 4 and 7. Already after one day of nutritional intervention, the percentage of EPA in phospholipids of white blood cells was increased from 0.5% at baseline to 1.3% ($p < 0.001$). After one week the percentage of EPA rose to 2.8% ($p < 0.001$). Additionally, the production of pro-inflammatory cytokines in LPS-stimulated whole blood cultures was significantly increased within one week.

Nutritional supplementation with a fish-oil enriched medical food significantly increased the percentage of EPA in phospholipids of white blood cells within one week. Simultaneously, *ex vivo* immune responsiveness to LPS was increased significantly. These results hold promise for novel applications, such as fast-acting nutritional interventions in cancer patients, which should be investigated in future studies.

INTRODUCTION

Immune modulatory effects of long-chain polyunsaturated fatty acids (PUFA) are well described. Both (n-6) and (n-3) PUFA are recognized to play a major role in immune regulation and the balance between them may affect the development and severity of inflammatory diseases (1, 2). Immune cell function and interactions are regulated by several membrane-associated events, including the production of different protein and lipid mediators that are essential in managing a successful immune response (3-5). PUFA are precursors for the production of different series of eicosanoids (4), in which prostaglandin E_2 (PGE_2) is a major metabolite of the (n-6) fatty acid pathway. PGE_2 has pro-inflammatory properties and is able to suppress different immunological effector mechanisms (6-8). By contrast, eicosanoids derived from (n-3) PUFA differ in structure and function and are generally considered less inflammatory than (n-6) PUFA derived eicosanoids (5).

To modify cell function and to obtain beneficial immune modulatory effects, EPA and DHA have to be incorporated effectively into cell membrane phospholipids. In the literature, the majority of the studies providing EPA and DHA examine fatty acid incorporation and immune modulatory activities after 4, 8 or 12 weeks of supplementation (9-12). In human immune cells, near plateau levels were reached after 4 weeks of nutritional intake, followed by a small rise towards week 8 and week 12 (11, 13-15). Gibney et al. examined the incorporation of fatty acids after 2 weeks of intervention with 15 g/d (n-3) PUFA rich marine oil (providing 4.2 g/d EPA and 1.4 g/d DHA) and demonstrated a significant incorporation of EPA but not DHA into the membrane phospholipids of platelets, neutrophils, monocytes, T-lymphocytes and B-lymphocytes (16). To the best of our knowledge, no faster incorporation rates have been described using oral administered PUFA.

It is anticipated that effects of oral (n-3) PUFA intake may be obtained much faster than 4 or even 2 weeks. EPA and DHA incorporation into cell membranes can occur very rapidly, since white blood cells have a high turnover and EPA can exchange rapidly from plasma to immune cells (16). Furthermore, there is a continuous biogenesis of new membrane lipids in white blood cells and a high turnover of membrane phospholipids by re-acylation after activation (17, 18). Finally, the absorption of fish oil and consequently the incorporation of EPA and DHA can be improved and will be more efficient by use of pre-emulsified fish oil (19, 20).

In order to address the absence of human data regarding short term incorporation after oral nutritional intervention in the literature, the present study investigates the incorporation of EPA and DHA into white blood cell (WBC) phospholipids at different time points during one week of supplementation with a recently developed medical food* in healthy men and women. This medical food is high in protein and leucine and enriched with emulsified fish oil (containing EPA and DHA) and a specific oligosaccharide mixture. This concept was developed for application in cancer patients, aiming to reduce complications and to provide optimal treatment support by reducing the inflammatory state, supporting immune function and preserving muscle mass and function. These effects have been demonstrated in previous preclinical studies using an animal model of tumor-induced cachexia (21, 22).
To support the functional effects of the fatty acid incorporation in combination with the immune modulatory oligosaccharides present in the specific medical food, the effects on

ex vivo immune responsiveness were investigated in lipopolysaccharide (LPS)-stimulated whole blood cultures. The present study was performed in healthy volunteers and designed as a proof of concept study, in preparation for a randomized, controlled efficacy study in cancer patients.

*A medical food is in USA defined in 21 U.S.C. § 360ee(b)(3) as ''a food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognizable scientific principles, are established by medical evaluation'' (23). A comparable definition exists in the harmonized legislation of the European Union (cf. Article 1, 2(b) of Commission Directive 1999/21/EC of 25 March 1999 on dietary foods for special medical purposes).

METHODS

PARTICIPANTS AND STUDY DESIGN

Twelve healthy Caucasian volunteers with an age above 55 years and a BMI between 18.5 and 30 kg/m^2 were recruited for the study from the database of the Clinical Research Unit of Ampha (Nijmegen, the Netherlands). At this age women are most probably in a post-menopausal phase and therefore no gender differences were expected. All participants were willing and able to abstain from alcohol, smoking, fish, fish oil-containing supplements, vitamin supplements or other oil supplements during the study. Excluded from the study were participants who were intolerant of or allergic to ingredients of the study products, participants who had used fish oil-containing supplements, vitamin supplements or other oil supplements during the previous 4 weeks, participants who had smoked within 6 months before the study, participants with an altered immune function (e.g. active infection or active allergy), participants who used immunosuppressive or immune modulatory medication and participants with any condition that might interfere with the definition "healthy volunteer" according to the investigator's judgment.

After initial screening, subject characteristics, relevant medical history and fish and alcohol consumption habits were recorded. Before visit 1, one unit of Basic medical food (200 ml oral nutritional supplement) was consumed by the participants to minimize differences at baseline. At visit 1 (baseline, day 0) blood samples (40 ml) were collected in the morning within 2 hours of intake of the Basic medical food. An additional breakfast before blood sampling was not allowed. Active medical food products were dispensed to the participants with the request to take 2 units (2 x 200 ml) of Active medical food a day. At visit 2 (day 1), 3 (day 2), 4 (day 4) and 5 (day 7) blood samples (40 ml) were collected in the morning within 2 hours of intake of one unit of the Active medical food. An additional breakfast before blood sampling was not allowed. The study was conducted in compliance with the principles of the 'Declaration of Helsinki' (59th WMA General Assembly, Seoul, October 2008) according to the ICH-GCP guidelines. Both the selection procedure and visits were performed by the Clinical Research Unit of Ampha. All participants gave their written informed consent before the start of the study. The study was approved by the Independent Review Board Nijmegen, Nijmegen, the Netherlands.

NUTRITIONAL INTERVENTION

During the study all participants consumed 2 units (2 x 200 ml) of the Active medical food (FortiCare containing high protein, leucine, fish oil and specific oligosaccharides, Nutricia, Zoetermeer, the Netherlands, Table 1) providing 2.4 g EPA and 1.2 g DHA daily. The Active medical food is an energy dense (682 kJ per 100 ml/163 kcal per 100 ml), nutritionally complete oral supplement, high in protein and leucine (9.9 g protein per 100 ml of which 3.2 g whey protein per 100 ml, 5.6 g casein per 100 ml and 1.1 g free leucine per 100 ml) and enriched with emulsified fish oil (0.6 g EPA and 0.3 g DHA per 100 ml) and specific oligosaccharides (1.2 g galactooligosaccharides (GOS) and 0.2 g fructooligosaccharides (FOS) per 100 ml). Before visit 1 all participants consumed one unit of Basic medical food (200 ml oral nutritional supplement, Nutricia, Zoetermeer, the Netherlands).

Table 1 Nutritional composition of the Actice medical food (FortiCare) in grams per 100 ml.

Ingredients	Active medical food
Macronutrients	
Energy, kJ (*kcal*)	**682 (163)**
Carbohydrates, *g*	**17.4**
Protein, *g*	**9.9**
- Whey, *g*	3.2
- Casein, *g*	5.6
- Added amino acids: free leucine, *g*	1.1
Total fat, g	**5.3**
- EPA, *g*	0.6
- DHA, *g*	0.3
Oligosaccharides, *g*	**1.4**
- GOS, *g*	1.2
- FOS, *g*	0.2
Minerals & trace elements	
Sodium, *mg*	**110**
Potassium, *mg*	**215**
Chloride, *mg*	**140**
Calcium, *mg*	**147**
Phosphorus, *mg*	**115**
Magnesium, *mg*	**28.2**
Iron, *mg*	**1.9**
Zinc, *mg*	**2.1**
Copper, *µg*	**288**
Manganese, *mg*	**0.7**
Fluoride, *mg*	**0.2**
Molybdenum, *µg*	**16.0**
Selenium, *µg*	**13.5**
Chromium, *µg*	**11.0**
Iodine, *µg*	**21.0**
Vitamins	
Vitamin A (retinol), *µg*	**130**
Vitamin D3 (cholecalciferol), *µg*	**1.1**
Vitamin E (α-tocopherol*), mg*	**3.2**
Vitamin K, *µg*	**8.5**
Vitamin B1 (Thiamin), *mg*	**0.2**
Vitamin B2 (Riboflavin), *mg*	**0.3**
Vitamin B3 (Niacin), *mg*	**2.9**
Vitamin B5 (Pantothenic acid), *mg*	**0.9**
Vitamin B6, *mg*	**0.6**
Vitamin B9 (Folic acid), *µg*	**53**
Vitamin B12 (Cobalamin), *µg*	**0.6**
Vitamin H (B7, Biotin), *µg*	**6.4**
Vitamin C (L-ascorbic acid), *mg*	**21**
Carotenoids, *mg*	**0.3**
Other	
L-Carnitine, *mg*	**10.9**
Choline, *mg*	**59**
Taurine, *mg*	**13.3**

The Basic medical food is an iso-energetic oral supplement without fish oil enrichment or specific oligosaccharides. Besides the restrictions mentioned above, no other diet restrictions applied for the healthy volunteers.

INCORPORATION OF FATTY ACIDS INTO PHOSPHOLIPIDS OF WBC, RBC AND PLASMA

WBC and RBC isolation

WBC were isolated using sterile Leucosep tubes (Greiner Bio-One B.V., Alphen aan den Rijn, the Netherlands). In short, 30 ml heparinized blood was diluted with 10 ml PBS (Gibco BRL, Life Technologies, Merelbeke, Belgium) + 2% heat-inactivated fetal calf serum (FCS^{hi}) (Hyclone, Perbio Science, Etten-Leur, the Netherlands) and divided over two Leucosep tubes pre-filled with Ficoll-Isopaque. Tubes were centrifuged for 10 min at 1000 g (RT, no brake). After centrifugation, WBC were collected and washed with PBS + 2% FCS^{hi}. WBC were resuspended in cold culture medium (RPMI-1640 containing 25 mmol/L HEPES and 2 mmol/L L-glutamine; Life-Technologies, enriched with 100 kU/L penicillin/streptomycin) with 10% FCS^{hi} and counted with a Coulter Counter (Beckman Coulter, Mijdrecht, the Netherlands). Subsequently, WBC were transferred to an Eppendorf tube and centrifuged for 10 min. at 17000g (RT). The supernatant was discarded and the pellet was stored at -80°C until analysis.

RBC were collected after the centrifugation step. One ml of the RBC pellet was pipetted into an Eppendorf tube and RBC were stored at -80°C until analysis.

Plasma was obtained by centrifugation of 5 ml heparin blood for 5 min at 1300 g (RT). Plasma was aliquoted and stored at –80°C until analysis.

Phospholipid fatty acid analysis

Phospholipid fatty acids were analyzed by gas chromatography as described before (21).

IMMUNOLOGICAL ANALYSIS

Whole blood assay

Blood (100 µl/well) was added to 50 µl/well culture medium in a 96-well plate (flat-bottom, polystyrene, BD Falcon Erembodegem Aalst, Belgium) and was subsequently incubated with 50 µl/well LPS (final concentration 100 µg/L, *E.coli*, B55:O55, Sigma-Aldrich Chemie, Steinheim, Germany) or culture medium (control) for 20 h at 37°C in a humidified environment containing 5% CO_2. Afterwards, plates were centrifuged for 5 min at 250 g (RT) and supernatants were harvested and stored at –80°C until analysis.

Serum isolation

Five ml blood was collected in serum tubes (clotting tubes) and incubated for a minimum of 2 h at RT. Afterwards, blood was centrifuged for 10 min at 1300 g (RT). Serum was harvested, aliquoted into Eppendorf tubes and stored at -80°C until analyses.

PGE_2 and cytokine measurement

PGE_2 was measured using a commercial enzyme immunoassay (Biotrak Amersham, Buckinghamshire, UK) according to the manufacturer's protocol. Cytokines (IL-1β, IL-6, IL-8, IFN-γ and TNF-α) were measured using a commercial custom-made human Bio-

Plex Cytokine bead immunoassay (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's protocol.

STATISTICAL ANALYSIS

Comparisons between the different visits and baseline were made on an intention-to-threat basis with ANOVA using sex as a covariate (SPSS version 15.0, SPSS Benelux, Gorinchem, the Netherlands). If the variables were not normally distributed (analyzed with the Kolmogorov-Smirnov test with Lilliefors Significance Correction), the Wilcoxon signed rank test was used. Because four comparisons were made between the different visits and baseline, Bonferroni post-hoc analysis was applied to correct for multiple testing. A p-value of 0.0125 was considered to be statistically significant, based on α/k, in which $\alpha = 0.05$ and k is the number of comparisons. In all other cases, a value of $p < 0.05$ was considered to be statistically significant. Data presented in the text were given as means ± SEM.

RESULTS

PARTICIPANTS

A total of 12 Caucasian healthy volunteers (6 men and 6 women) were included in the study with a mean age of 62.0 ± 4.5 years and a BMI of 25.6 ± 3.2 kg/m^2 (Table 2). All 12 men and women completed the study with 9 participants consuming all units of the Active

Table 2 Baseline charactertistics of the healthy Caucasian perticipants

Variables	Participants (n=12)
Sex, *n (%)*	
Female	6 (50.0%)
Male	6 (50.0%)
Age, *years*	62.0 ± 4.5
BMI, *kg/m^2*	25.6 ± 3.2
Fish consumption, *n (%)*	
None	1 (8.3%)
White fish	2 (16.7%)
Fatty fish	3 (25.0%)
Both	6 (50.0%)
Fatty fish consumption, *n (%)*	
Every day	-
Three times a week	-
Two times a week	2 (22.2%)
Once a week	1 (11.1%)
Two times a month	2 (22.2%)
Once a month	4 (44.4%)
Once in two months	-
Alcohol consumption (per week), *n (%)*	
None	4 (33.3%)
1-10 glasses[1]	8 (66.7%)
11-20 glasses	-
> 20 glasses	-

Data are means ± SD or n (%), n = 12,[1] One glass of alcohol ≈ 10 g ethanol.

medical food (100% compliance) and one unit of the Basic medical food. Two participants missed one unit (94% compliance) and one subject missed two units (88% compliance) of the Active medical food. There was no difference in the BMI of the participants before (25.6 ± 3.2 kg/m^2) and after the study (25.5 ± 3.0 kg/m^2). No Serious Adverse Events were reported during the study and no unexpected Adverse Events were observed.

FATTY ACID COMPOSITION OF WBC, RBC AND PLASMA

The incorporation of different PUFA into phospholipids of WBC, RBC and plasma was measured (Figure 1). In addition, the percentages of (n-3) and (n-6) fatty acids and the ratio of (n-6)/(n-3) fatty acids were determined (Table 3). After one day of nutritional supplementation the percentage of EPA of total fatty acids in phospholipids of WBC was increased significantly from 0.5% at day 0 (baseline) to 1.3% ($p < 0.001$)(Figure 1A). After one week the percentage of EPA rose to 2.8% ($p < 0.001$, compared to baseline). No effect was observed on the percentage of DHA in phospholipids of WBC after one week of nutritional

Table 3 Percentage of (n-3) and (n-6) fatty acids and the (n-6)/(n-3) fatty acids ratio of total phospholipid fatty acids in WBC and RBC and plasma of healthy men and women during 1 week of nutritional intervention with the fish oil-enriched, high-protein medical food.

	WBC			RBC			Plasma		
Day	n-6 PUFA	n-3 PUFA	n-6 / n-3	n-6 PUFA	n-3 PUFA	n-6 / n-3	n-6 PUFA	n-3 PUFA	n-6 / n-3
0	30.8 ± 1.4	5.6 ± 1.0	5.7 ± 1.3	27.7 ± 1.6	6.6 ± 1.1	4.3 ± 0.9	36.2 ± 2.2	5.6 ± 1.4	6.9 ± 2.0
1	30.1 ± 1.0	6.8 ± 0.9^A	4.5 ± 0.7^A	27.4 ± 1.4^C	6.7 ± 1.1^B	4.2 ± 0.8^A	32.9 ± 2.9^A	7.9 ± 1.3^A	4.3 ± 1.0^A
2	29.4 ± 1.1^A	7.6 ± 0.6^A	3.9 ± 0.4^A	26.8 ± 1.3^A	7.1 ± 1.1^A	3.9 ± 0.7^A	32.5 ± 1.4^A	9.2 ± 1.0^A	3.6 ± 0.5^A
4	28.6 ± 1.1^A	8.6 ± 0.6^A	3.4 ± 0.3^A	26.4 ± 1.2^A	7.6 ± 1.1^A	3.5 ± 0.6^A	31.3 ± 1.1^A	10.7 ± 0.9^A	2.9 ± 0.3^A
7	27.5 ± 0.8^A	9.4 ± 0.7^A	3.0 ± 0.3^A	26.0 ± 1.1^A	8.4 ± 1.0^A	3.2 ± 0.5^A	30.0 ± 1.5^A	11.8 ± 1.0^A	2.6 ± 0.3^A

Data are means ± SD, n = 12. Letters indicate different from d 0 (baseline): $^A p < 0.001$, $^B p = 0.009$, $^C p = 0.004$ (ANOVA).

supplementation, whereas the percentage of docosapentaenoic acid (DPA, (n-3)) increased significantly within one week ($p < 0.001$). The percentage of AA was reduced from day 2 onward ($p < 0.001$). After one day of nutritional supplementation the percentage of (n-3) fatty acids was increased from 5.6% at day 0 (baseline) to 6.8% ($p < 0.001$) and at day 7 the percentage of (n-3) fatty acids was increased further to 9.4% ($p < 0.001$, compared to baseline) (Table 3). Correspondingly, the percentage of (n-6) fatty acids in phospholipids of WBC was significantly decreased after one week ($p < 0.001$), as was the ratio of (n-6)/(n-3) fatty acids.

In RBC, the percentage of EPA was significantly increased after one day of nutritional supplementation and increased further to day 7 ($p < 0.001$)(Figure 1B). Furthermore, in RBC an increase in the percentage of DHA was observed. DPA (n-3) showed a small but significant increase after one week of nutritional intervention ($p < 0.001$). The (n-6) fatty acid AA demonstrated a significant reduction at day 2 ($p = 0.001$), although at day 4 and

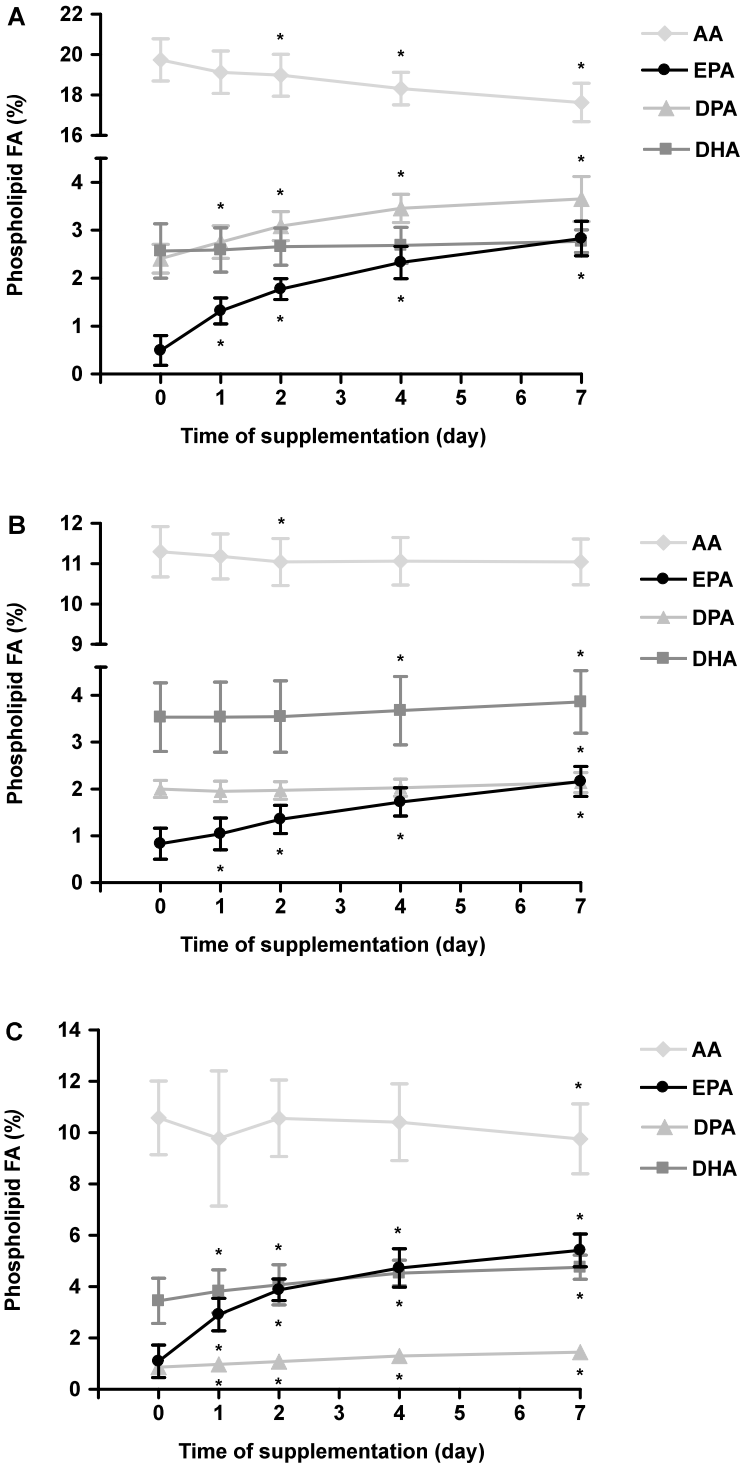

Figure 1 Percentages of the (n-6) PUFA AA and the (n-3) PUFA EPA, DPA, and DHA in total phospholipid fatty acids in WBC (A), RBC (B), and plasma (C) of healthy men and women during 1 week of nutritional intervention with the fish oil-enriched, high-protein medical food. Data are means (%) ± SD, n = 12. *Different from day 0 (baseline) $p \leq 0.001$ (ANOVA).

day 7 no significant differences could be detected. The percentage of (n-3) fatty acids was significantly increased and the percentage of (n-6) fatty acids and the ratio of (n-6)/(n-3) fatty acids were significantly decreased already after one day of nutritional intervention ($p = 0.009$, $p = 0.004$ and $p < 0.001$, respectively).

In the plasma phospholipids, the percentages of EPA, DHA and DPA (n-3) all increased from baseline at day 1, 2, 4 and 7 ($p < 0.001$)(Figure 1C). AA only demonstrated a significant reduction at day 7 ($p < 0.001$). The percentage of (n-3) fatty acids was significantly increased and the percentage of (n-6) fatty acids and the ratio of (n-6)/(n-3) fatty acids were significantly decreased already after one day of nutritional intervention (all $p < 0.001$).

IMMUNOLOGICAL ANALYSIS

Whole blood assay

The production of the pro-inflammatory mediators was measured in LPS-stimulated whole blood (Figure 2). A good stimulation response was observed for all the pro-inflammatory mediators at the different time points. The production of IL-1β and TNF-α increased significantly in response to LPS after 4 days of nutritional intervention ($p = 0.009$ and $p < 0.001$, for IL-1β and TNF-α respectively, Figure 2A). The production of IL-6 and IL-8, showed the same pattern as for IL-1β and TNF-α, and was significantly increased within one week of nutritional intervention ($p < 0.001$ and $p = 0.001$ for IL-6 and IL-8, respectively, Figure 2B). The effect on LPS-stimulated IFN-γ production was even more pronounced. Already within one day of nutritional intervention a significant increase was observed on the production of IFN-γ in response to LPS ($p < 0.001$, Figure 2C). By contrast, the LPS-stimulated PGE_2 production was not altered after nutritional intervention (Figure 2C). Levels of PGE_2 tended to be lower compared to baseline at day 4 ($p = 0.09$), but this was not statistically significant.

Serum cytokines

Serum cytokines were difficult to measure in healthy volunteers, since concentrations were mostly around the lower detection limits of the assays used (Table 4). The nutritional intervention did not affect serum IL-1β and IL-6 concentrations, however these concentrations were very low. Serum IL-8 concentrations however, were significantly increased after one week ($p = 0.008$). No differences were observed on serum concentrations of TNF-α and IFN-γ, whereas serum PGE_2 concentrations were significantly decreased at day 2. From day 3 onwards, PGE_2 concentrations reverted back to the baseline values.

DISCUSSION

The present study demonstrated the rapid incorporation of EPA and DHA into phospholipids of WBC, RBC and plasma within one week of nutritional supplementation with a medical food enriched with emulsified fish oil. In addition, immune responsiveness to LPS in *ex vivo* whole blood cultures was modulated as a result of the nutritional intervention.

At baseline, the percentages of the (n-6) PUFA AA and the (n-3) PUFA EPA, DPA and DHA of total phospholipid fatty acids in WBC and plasma were comparable with percentages measured in previous studies (11, 12, 24). After nutritional supplementation with the fish oil enriched medical food, the percentage of EPA in phospholipids of WBC increased

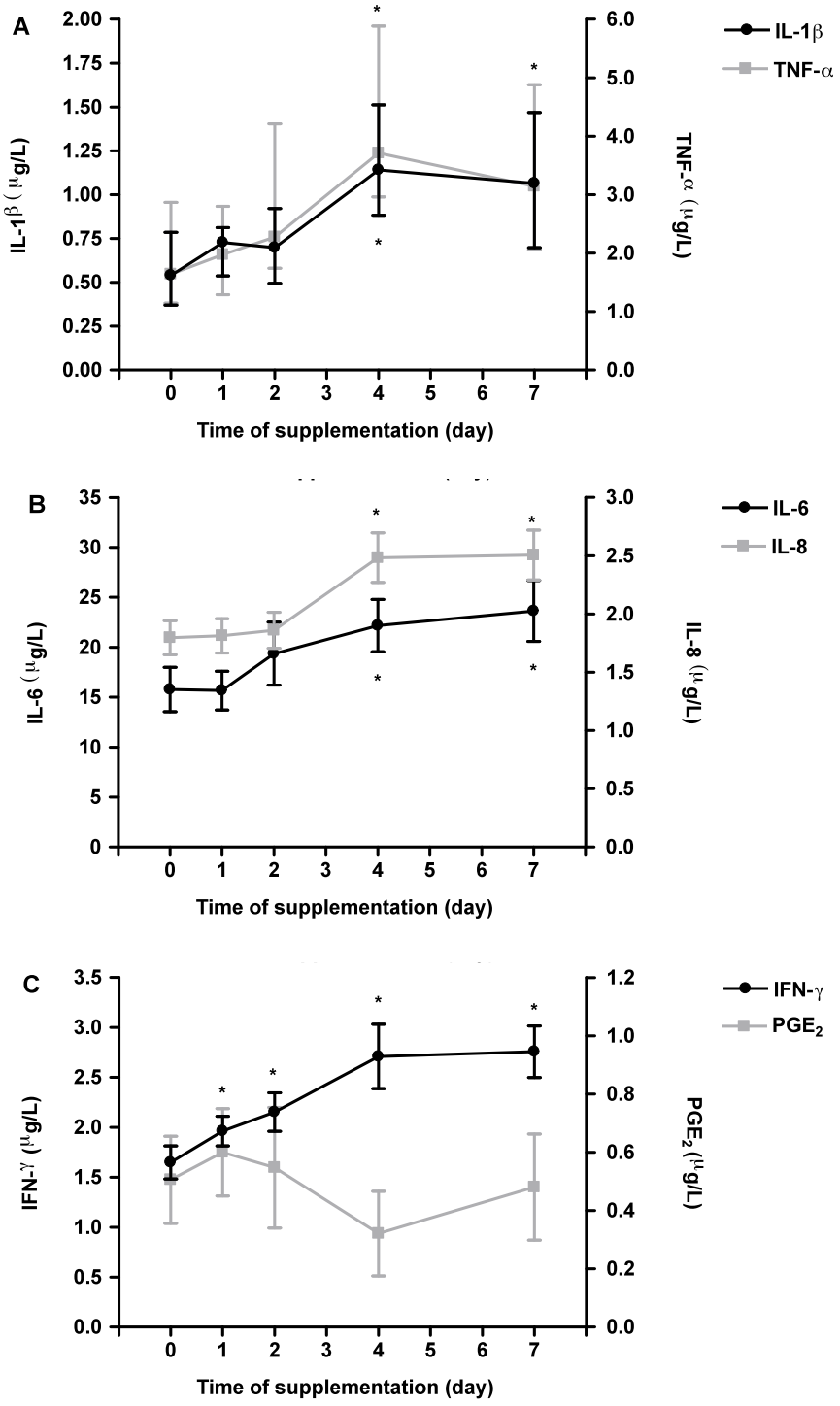

Figure 2 Concentrations (μg/L) of IL-1β and TNF-α (A), IL-6 and IL-8 (B), and IFN-γ and PGE_2 (C) in LPS-stimulated whole blood cultures of healthy men and women during 1 wk of nutritional intervention with the fish oil-enriched, high-protein medical food. Data are medians (μg/L) ± IQR (25th–75th percentiles), n = 12 for A and means (μg/L) ± SEM, n = 12 for B and C. *Different from d 0 (baseline), $P < 0.010$ (ANOVA).

Table 4 Serum concentrations of pro-inflammatory cytokines and PGE_2 (ng/L) of healthy men and women during one week of nutritional intervention with the fish oil-enriched, high-protein medical food

Day	IL-1β *ng/L*	IL-6 *ng/L*	IL-8 *ng/L*	IFN-γ *ng/L*	TNF-α *ng/L*	PGE_2 *ng/L*
0	1.5 (1.5-1.5)	0.8 (0.8-0.9)	7.8 (6.0-11.4)	5.3 (4.6-10.3)	2.6 (2.6-10.0)	623 (332-1190)
1	1.5 (1.5-1.5)	0.8 (0.8-2.1)	7.6 (6.4-9.3)	5.6 (4.6-10.3)	3.8 (2.6-10.2)	400 (343-880)
2	1.5 (1.5-1.5)	0.8 (0.8-3.2)	5.9 (5.6-7.1)	4.6 (4.6-7.5)	4.6 (2.6-11.0)	265 (199-412)^{B}
4	1.5 (1.5-1.5)	0.8 (0.8-1.0)	7.6 (6.4-8.9)	4.9 (4.6-9.1)	3.2 (2.6-6.8)	444 (264-1000)
7	1.5 (1.5-1.5)	0.8 (0.8-1.1)	14.5 (9.0-25.7)^{A}	5.9 (4.6-11.0)	3.0 (2.6-6.3)	626 (401-1060)

Data are medians ± IQR, n = 12. Letters indicate different from d 0 (baseline): $^{A}p = 0.008$, $^{B}p = 0.014$ (Wilcoxon signed rank-test).

significantly within one week. Surprisingly, this increase was already significant after one day. In addition, the EPA levels that were reached within one week (2.8 ± 0.4 %), are comparable with the levels described in the literature after a 4-, 8- or even 12-week intervention period (13). Yaqoob et al. described EPA incorporation in blood mononuclear cells from 0.8% of total lipids at baseline to 2.5% after 4 weeks and 2.8% after 8 weeks of supplementation with fish oil providing 2.1 g EPA and 1.1 g DHA per day (15). Moreover, Rees et al. demonstrated EPA incorporation in blood mononuclear cells from 0.6% of total phospholipids at baseline to 2.7% after 12 weeks of supplementation with fish oil providing 2.7 g EPA and 0.6 g DHA per day (12). In this respect, the results from the current study, obtaining comparable EPA levels within one week of nutritional intervention (providing 2.4 g EPA and 1.2 g DHA daily) are surprising and suggest the attainment near a plateau already within one week. Rapid incorporation of EPA into platelet phospholipids has already been shown after intravenous administration of fish oil (25), but the current study demonstrates that such fast effects can also be obtained by oral administration of a specific nutritional intervention containing pre-emulsified fish oil.

No effects were observed on the incorporation of DHA into WBC within one week. Similar observations were described before, demonstrating a significant increase in EPA incorporation in plasma, platelets, neutrophils, monocytes, T and B cells after a 2-week intervention period, while no effects on DHA were observed, (16). The authors stated that changes in plasma phospholipid fatty acid composition do not allow a prediction of the probable composition of the immune cells, with the notable exception of EPA. It has been suggested that the turnover of DHA in membranes is slower than that of EPA, with the hypothesis that DHA is located in the inner leaflet of the phospholipid bilayer and does not exchange with plasma phospholipids (26). Another factor may be that DHA levels in phospholipids of WBC at baseline are already much higher than EPA levels (Figure 1A) and therefore more refractory to change. In addition, the body might conserve DHA better than EPA, since the conversion from α-linolenic acid (ALA), an essential fatty acid in the diet, to EPA is more efficient than the conversion to DHA. Therefore, because the body can obtain more EPA from ALA it may be more protective of DHA (27-29).

Whereas no effects on DHA were observed in WBC, DPA levels showed the same pattern as EPA and were already significantly increased after one day of nutritional intervention. It is unlikely that this increase is only caused by the DPA from the product, since these levels are relatively low (68.4 mg DPA per 100 ml in the Active medical food). Therefore, it is likely that EPA is readily elongated to DPA, leading to an accumulation of DPA, as the conversion from DPA to DHA is inefficient (12, 29). The present study demonstrated that the incorporation of EPA in RBC is slower than in WBC and doesn't reach such high levels within one week. Possible explanations are the long lifespan of RBC (120 days vs approximately 4 days for WBC) and the slow exchange of fatty acids from plasma to membrane phospholipids (30). Another factor may be a difference in fatty acid or phospholipid metabolism of the cells.

In the present study, the increased percentage of (n-3) fatty acids in WBC, RBC and plasma phospholipids was partly at the expense of the (n-6) PUFA AA (Figure 1A, 1B and 1C). Although the production of the AA-derived eicosanoid PGE_2 in LPS-stimulated whole blood was not reduced significantly, the observed tendency may be explained by a decreased availability of AA as substrate and/or by the inhibition of AA metabolism by (n-3) PUFA (5, 12, 31). Dooper et al. described the ability of PGE_2 to inhibit TNF-α and IFN-γ production in *ex vivo* stimulated mononuclear cells (32). Indeed, in this study, the tendency of reduced LPS-stimulated PGE_2 production was accompanied by an increased production of the cytokines IL-1β, TNF-α, IL-6, IL-8 and IFN-γ, indicating immune modulatory effects already within one week of nutritional intervention.

Many earlier studies, reviewed by Sijben et al., have examined the effects of long-chain (n-3) PUFA on LPS-stimulated cytokine production (33). The outcomes of these studies show a large variance, probably due to differences in the culture conditions (e.g. purified mononuclear cells or whole blood; use of FCS or autologous serum; LPS concentration used; duration of culture). Nevertheless, such rapid immune modulatory effects as seen in the current study have not been described earlier to the best of our knowledge. The observed increased production of LPS-stimulated pro-inflammatory cytokines could be beneficial for an individual's ability to react to acute infectious triggers. Moreover, specific situations have been described in which a positive inflammatory response is protective against septic complications and death in critically ill humans (34). On the other hand, others have interpreted enhanced LPS-stimulated cytokine responses to be indicative of an elevated (chronic) inflammatory state (35, 36). To determine whether the observed changes in immune function translate into clinical benefits, specific studies need to be performed in the proper target population.

Cancer patients are recognized to be hampered by serious immune failures due to disease and treatment-related factors (37-40). Moreover, cancer patients often start with a treatment regimen (e.g. chemotherapy, radiotherapy, surgery) soon after diagnosis. Therefore, it may be beneficial to supply a fast acting product to these patients to reduce complications and to provide optimal treatment support. The combination of previous and current results are promising for short term effects in cancer patients, potentially supporting protective immunity and reducing systemic levels of pro-inflammatory cytokines, but as mentioned above, this has to be investigated in future studies. In healthy volunteers, it is difficult to measure systemic pro-inflammatory cytokines, since the concentrations are around the detection limits of the assays used. For IL-8 a significant increase was observed

at day 7, but these concentrations are still within normal healthy control ranges compared with other studies (14.5 vs 13.4 ng/L, respectively), while concentrations of IL-8 in head and neck cancer patients are reported to be higher (41). Moreover, in previous animal studies in severe cachectic mice, nutritional intervention with a specific nutritional combination containing the same product features as the described medical food (i.e. high protein, leucine, fish oil and specific oligosaccharides) resulted in reduced plasma concentrations of IL-6, TNF-α and PGE_2 and moreover, in improved *in vivo* Th1 immune responses (21).

While the present study indicated that the cancer specific medical food induced a rapid incorporation of EPA in WBC and consequently induced immune modulatory effects within one week, the lack of a control group limits the ability to attribute the changes exclusively to the study product. The observed rapid incorporation of fatty acids in plasma and membrane phospholipids can only be explained by the intake of fish oil supplied in the medical food. However, the immune modulatory effects cannot be attributed solely to the EPA incorporation, since previous animal studies have shown that only nutritional intervention with the complete combination of high protein, leucine, fish oil and specific oligosaccharides, induced anti-inflammatory and immune modulatory effects (21). Moreover, the study population recruited for the study was aged above 55 years. Since EPA and DHA incorporation might be more efficient in older people compared to young people (3), the findings in this study cannot be generalized to all age groups.

No unexpected related adverse events were observed; the majority of adverse events were of gastrointestinal origin and were considered as being mild. No additional safety parameters were measured since the duration of the study was only one week and in a previous 4-week intervention study using the same nutritional intervention (manuscript in preparation), analyzed safety parameters were all within reference ranges and no clinically relevant changes on liver function, kidney function or prothrombin time were observed.

In conclusion, nutritional intervention of healthy volunteers with a cancer specific medical food, which is high in protein and leucine and enriched with emulsified fish oil and specific oligosaccharides, significantly increased the percentage of EPA in phospholipids of WBC within one week. In addition, the *ex vivo* immune responsiveness to LPS was increased significantly. The current results are promising for achieving rapid results with nutritional intervention a clinical setting. Whether this approach can reduce the inflammatory state and support immune function, with the aim to provide optimal treatment support, is currently being investigated in cancer patients.

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REFERENCES

1 Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006;83: 1505S-19S.

2 Song C, Manku MS, Horrobin DF. Long-chain polyunsaturated fatty acids modulate interleukin-1beta-induced changes in behavior, monoaminergic neurotransmitters, and brain inflammation in rats. J Nutr 2008;138: 954-63.

3 Wu D, Meydani SN. n-3 polyunsaturated fatty acids and immune function. Proc Nutr Soc 1998;57: 503-9.

4 Shaikh SR, Edidin M. Polyunsaturated fatty acids and membrane organization: elucidating mechanisms to balance immunotherapy and susceptibility to infection. Chem Phys Lipids 2008;153: 24-33.

5 Calder PC. The relationship between the fatty acid composition of immune cells and their function. Prostaglandins Leukot Essent Fatty Acids 2008;79: 101-8.

6 Hilkens CM, Vermeulen H, van Neerven RJ, Snijdewint FG, Wierenga EA, Kapsenberg ML. Differential modulation of T helper type 1 (Th1) and T helper type 2 (Th2) cytokine secretion by prostaglandin E2 critically depends on interleukin-2. Eur J Immunol 1995;25: 59-63.

7 Betz M, Fox BS. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J Immunol 1991;146: 108-13.

8 Wang D, Dubois RN. Eicosanoids and cancer. Nat Rev Cancer 2010;10: 181-93.

9 Wallace FA, Miles EA, Calder PC. Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. Br J Nutr 2003;89: 679-89.

10 Harris WS, Pottala JV, Sands SA, Jones PG. Comparison of the effects of fish and fish-oil capsules on the n 3 fatty acid content of blood cells and plasma phospholipids. Am J Clin Nutr 2007;86: 1621-5.

11 Calder PC. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids 2007;77: 327-35.

12 Rees D, Miles EA, Banerjee T, Wells SJ, Roynette CE, Wahle KW, et al. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. Am J Clin Nutr 2006;83: 331-42.

13 Thies F, Nebe-von-Caron G, Powell JR, Yaqoob P, Newsholme EA, Calder PC. Dietary supplementation with gamma-linolenic acid or fish oil decreases T lymphocyte proliferation in healthy older humans. J Nutr 2001;131: 1918-27.

14 Healy DA, Wallace FA, Miles EA, Calder PC, Newsholm P. Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. Lipids 2000;35: 763-8.

15 Yaqoob P, Pala HS, Cortina-Borja M, Newsholme EA, Calder PC. Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. Eur J Clin Invest 2000;30: 260-74.

16 Gibney MJ, Hunter B. The effects of short- and long-term supplementation with fish oil on the incorporation of n-3 polyunsaturated fatty acids into cells of the immune system in healthy volunteers. Eur J Clin Nutr 1993;47: 255-9.

17 Anel A, Naval J, Gonzalez B, Torres JM, Mishal Z, Uriel J, et al. Fatty acid metabolism in human lymphocytes. I. Time-course changes in fatty acid composition and membrane fluidity during blastic transformation of peripheral blood lymphocytes. Biochim Biophys Acta 1990;1044: 323-31.

18 Valette L, Croset M, Prigent AF, Meskini N, Lagarde M. Dietary polyunsaturated fatty acids modulate fatty acid composition and early activation steps of concanavalin A-stimulated rat thymocytes. J Nutr 1991;121: 1844-59.

19 Garaiova I, Guschina IA, Plummer SF, Tang J, Wang D, Plummer NT. A randomised cross-over trial in healthy adults indicating improved absorption of omega-3 fatty acids by pre-emulsification. Nutr J 2007;6: 4.

20 Raatz SK, Redmon JB, Wimmergren N, Donadio JV, Bibus DM. Enhanced absorption of n-3 fatty acids from emulsified compared with encapsulated fish oil. J Am Diet Assoc 2009;109: 1076-81.

21 Faber J, Vos P, Kegler D, van Norren K, Argiles JM, Laviano A, et al. Beneficial immune modulatory effects of a specific nutritional combination in a murine model for cancer cachexia. Br J Cancer 2008;99: 2029-36.

22 van Norren K, Kegler D, Argiles JM, Luiking Y, Gorselink M, Laviano A, et al. Dietary supplementation with a specific combination of high protein, leucine, and fish oil improves muscle function and daily activity in tumour-bearing cachectic mice. Br J Cancer 2009;100: 713-22.

23 US Food and Drug Administration. Frequently Asked Questions About Medical Foods. In: College Park MF, Center for Food Safety and Applied Nutrition, US Department of Health and Human Services, editor.; 2007.

24 Kew S, Banerjee T, Minihane AM, Finnegan YE, Williams CM, Calder PC. Relation between the fatty acid composition of peripheral blood mononuclear cells and measures of immune cell function in healthy, free-living subjects aged 25-72 y. Am J Clin Nutr 2003;77: 1278-86.

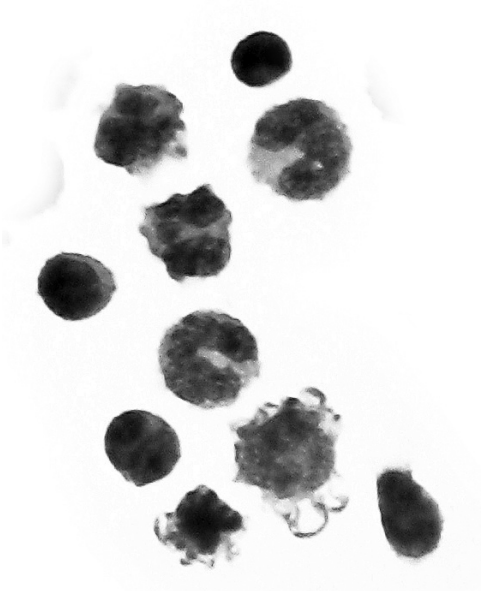
25 Pittet YK, Berger MM, Pluess TT, Voirol P, Revelly JP, Tappy L, et al. Blunting the response to endotoxin in healthy subjects: effects of various doses of intravenous fish oil. Intensive Care Med 2009.

26 Brown AJ, Pang E, Roberts DC. Persistent changes in the fatty acid composition of erythrocyte membranes after moderate intake of n-3 polyunsaturated fatty acids: study design implications. Am J Clin Nutr 1991;54: 668-73.

27 Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N, Jr. Physiological compartmental analysis of alpha-linolenic

acidmetabolism in adult humans. J Lipid Res 2001;42: 1257-65.
28 Hussein N, Ah-Sing E, Wilkinson P, Leach C, Griffin BA, Millward DJ. Long-chain conversion of [13C]linoleic acid and alpha-linolenic acid in response to marked changes in their dietary intake in men. J Lipid Res 2005;46: 269-80.
29 Burdge GC, Calder PC. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. Reprod Nutr Dev 2005;45: 581-97.
30 Reed CF. Phospholipid exchange between plasma and erythrocytes in man and the dog. J Clin Invest 1968;47: 749-60.
31 Calder PC. Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale. Biochimie 2009;91: 791-5.
32 Dooper MM, Wassink L, M'Rabet L, Graus YM. The modulatory effects of prostaglandin-E on cytokine production by human peripheral blood mononuclear cells are independent of the prostaglandin subtype. Immunology 2002;107: 152-9.
33 Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. Proc Nutr Soc 2007;66: 237-59.
34 Hall MW, Gavrilin MA, Knatz NL, Duncan MD, Fernandez SA, Wewers MD. Monocyte mRNA phenotype and adverse outcomes from pediatric multiple organ dysfunction syndrome. Pediatr Res 2007;62: 597-603.
35 Wigmore SJ, Fearon KC, Maingay JP, Ross JA. Down-regulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. Clin Sci (Lond) 1997;92: 215-21.
36 Heimdal JH, Aarstad HJ, Klementsen B, Olofsson J. Ex vivo interleukin (IL)-1 beta, IL-6, IL-12 and tumor necrosis factor-alpha responsiveness with monocytes from patients with head and neck carcinoma. Eur Arch Otorhinolaryngol 1999;256: 250-6.
37 Herber DL, Nagaraj S, Djeu JY, Gabrilovich DI. Mechanism and therapeutic reversal of immune suppression in cancer. Cancer Res 2007;67: 5067-9.
38 Young MR. Eicosanoids and the immunology of cancer. Cancer Metastasis Rev 1994;13: 337-48.
39 Young MR, Wright MA, Lozano Y, Matthews JP, Benefield J, Prechel MM. Mechanisms of immune suppression in patients with head and neck cancer: influence on the immune infiltrate of the cancer. Int J Cancer 1996;67: 333-8.
40 Heimdal JH, Aarstad HJ, Klementsen B, Olofsson J. Peripheral blood mononuclear cell (PBMC) responsiveness in patients with head and neck cancer in relation to tumour stage and prognosis. Acta Otolaryngol 1999;119: 281-4.
41 Gokhale AS, Haddad RI, Cavacini LA, Wirth L, Weeks L, Hallar M, et al. Serum concentrations of interleukin-8, vascular endothelial growth factor, and epidermal growth factor receptor in patients with squamous cell cancer of the head and neck. Oral Oncol 2005;41: 70-6.

CHAPTER SIX



Reduced serum PGE_2 levels and improved body weight and performance status after nutritional intervention with a specific medical food in newly diagnosed esophageal cancer patients

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ABSTRACT

In cancer patients, metabolic alterations and reduced immune competence lead to wasting and an increased risk of (infectious) complications. In the present study, the effect of a nutritionally complete medical food, which is high in protein and leucine and enriched with fish oil and specific oligosaccharides, was investigated on immune function in patients with esophageal cancer.

In an explorative, randomized, controlled, double-blind study, 64 newly diagnosed esophageal cancer patients consumed 400 ml of a medical food or routine nutritional support daily for 4 weeks before the start of anti-cancer therapy. Blood samples were taken at day 1 (baseline) and day 28 to measure several immune parameters. Additionally, 40 age-matched healthy volunteers were included for baseline comparisons.

At baseline, no differences between healthy volunteers and the patient population were observed on *ex vivo* stimulations of blood mononuclear cells and subsequently, no effect of the nutritional intervention could be detected. Several inflammatory serum markers were significantly higher in patients compared to volunteers at baseline. After nutritional intervention, serum Prostaglandin E_2 (PGE_2) levels were significantly decreased in the medical food group and increased in the routine group ($p = 0.002$). In addition, body weight increased significantly ($p < 0.05$) and ECOG performance status was significantly improved after intervention with the medical food ($p < 0.05$).

Nutritional intervention with the specific medical food significantly reduced serum PGE_2 levels in newly diagnosed esophageal cancer patients. This effect was accompanied by a significant increase in body weight and an improved performance status.

INTRODUCTION

The majority of cancer patients becomes malnourished and loses weight during the course of their disease. Worldwide, the incidence of malnutrition during cancer ranges from 30 to 90%, being most prevalent in patients with esophageal, pancreatic, lung, prostate or colon cancer (1-5). The incidence and severity of malnutrition is affected by the type, location, grade and stage of the tumor, as well as by anti-cancer treatments, patient characteristics and individual susceptibility (2, 4). Severe and prolonged malnutrition can lead to cancer cachexia, which is a major contributor to morbidity and mortality, especially in advanced disease. Characteristics of this chronic condition of catabolism include progressive, involuntary weight loss, anorexia, asthenia, fatigue, depletion of lipid stores and severe loss of skeletal muscle proteins (6, 7). Other important features of the cachexia syndrome include the presence of a chronic inflammatory state and, paradoxically, a state of impaired immune responsiveness (6, 8, 9). Several mediators that are either tumor- or host-derived (e.g. pro-inflammatory cytokines, chemokines and prostaglandins) induce a cascade of events leading to a suppressed immune function, thereby reducing the acute response to infectious triggers (6, 10-12). This compromised immune competence may lead to increased complications, delayed or suboptimal anti-cancer treatment and even to accelerated disease progression, resulting ultimately in a decreased quality of life and reduced survival rates in patients (13-17).

To reduce the risk of (infectious) complications and to support the performance status of cancer patients, a multidisciplinary approach should be applied in which nutritional intervention is recommended as an integral part of anti-cancer therapy to prevent involuntary weight loss and delayed treatment schedules, and to improve clinical outcomes and quality of life (3, 6, 18, 19). In malnourished patients, pre-operative nutritional support is associated with a 50% reduction of post-operative complications (18), including decreased gastro-intestinal toxicity, improved performance status and increased immune responses (3). However, recent findings show that impaired immune responsiveness and muscle protein degradation may already occur before the onset of weight loss. Consequently, it is of clinical relevance to provide the optimal treatment support as early as possible, preferably starting at diagnosis and running parallel to the pathway of anti-cancer therapies (19, 20). Recently, a specific medical food* has been developed for application in cancer patients. This medical food is high in protein and leucine and is enriched with emulsified fish oil (containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) and a specific oligosaccharide mixture and is designed to reduce complications and to provide optimal treatment support by reducing the inflammatory state, supporting immune function, and preserving muscle mass and function. These effects have been demonstrated in previous pre-clinical studies using an animal model of tumor-induced cachexia (21, 22).The aim of this exploratory study was to investigate the effects of this medical food specifically on immune function in an early phase in a group of newly diagnosed esophageal cancer patients before the start of anti-cancer therapy.

*A medical food is in USA defined in 21 U.S.C. § 360ee(b)(3) as ¨a food which is formulated to be consumed or administered enterally under the supervision of a physician and which

is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognizable scientific principles, are established by medical evaluation" (23). A comparable definition exists in the harmonized legislation of the European Union (cf. Article 1, 2(b) of Commission Directive 1999/21/EC of 25 March 1999 on dietary foods for special medical purposes).

METHODS

An explorative, randomized, controlled, double-blind study with parallel groups was conducted in order to determine the effects of a 4-week nutritional intervention with a specific medical food on immune function in newly diagnosed esophageal cancer patients before the start of anti-cancer therapy, compared with the effects of routine nutritional support. The secondary objective was to assess the effects of this medical food on inflammation and nutritional status. Data on immune function, inflammation and nutritional state of healthy volunteers were obtained to compare baseline values and allow an adequate interpretation of the data.

SUBJECTS

In the period between August 2007 and February 2009, sixty-four newly diagnosed patients with histologically confirmed carcinoma located in the esophagus or gastro-esophageal junction (Siewert-Stein classification type I-III) (24) planned for esophageal cancer treatment were recruited from the Erasmus Medical Center, Rotterdam, the Netherlands. Patients had an age of 18 years and above and were included in the study after informed consent was obtained. Exclusion criteria were life expectancy <3 months, planned start of anti-cancer treatment within 3 weeks, Eastern Cooperative Oncology Group (ECOG) performance status ≥2, esophagus related surgery after diagnosis before inclusion, chemotherapy and/or radiotherapy in the past 5 years, altered immune function, dysphagia score of 4, dependency on tube feed or parenteral nutrition during previous 4 weeks, use of fish oil-containing supplements during previous 4 weeks, intolerance or allergy to dairy products, fish or other ingredients of the study products, dependency on fibre-free diet, pregnancy or lactation, dementia or altered mental status that would prohibit the understanding and giving of informed consent, any other medical condition that may interfere with the safety of the patient or the outcome parameters or uncertainty about the willingness or ability of the patient to comply with the protocol requirements, according to the investigator's judgement. In addition to the patients, a reference group of 40 healthy volunteers was recruited for the study from the database of Vaxinostics BV, University Vaccine Center Rotterdam Nijmegen, Rotterdam, the Netherlands. Subjects in the reference group were age- and sex-matched with the Dutch esophageal cancer population, had a BMI between 18.5 and 30 kg/m^2 and were included in the study after signing informed consent. Exclusion criteria for subjects in the reference group were significant involuntary weight loss in the past year, smoking, acute or chronic disease, altered immune function, pregnant or lactating and any other condition that may interfere with the definition "healthy volunteer" according to the investigator's judgement.

STUDY DESIGN

The study was conducted in compliance with the principles of the 'Declaration of Helsinki' (52nd WMA General Assembly, Edinburgh, Scotland, October 2000 including the Notes of Clarification as added in 2002, Washington, and in 2004, Tokyo) according to the ICH-GCP guidelines and was approved by the Ethics Committee of the Erasmus MC, Rotterdam, the Netherlands. After initial screening of the patients, subject characteristics, relevant medical history, dysphagia score and anthropometrics were determined at visit 1 (baseline). Patients were randomized to the Active group receiving the specific medical food or to the Control group receiving routine nutritional support using a computerized randomization program after stratification based on their nutritional status. Patients with 0 to <5% weights loss in the past 3 months and dysphagia score of 0 or 1 (group 1) were assigned to the Active group receiving the medical food or to the Control group receiving a Placebo product. Patients with ≥5% weight loss in the past 3 months and/or dysphagia score 2 or 3 and/or prescribed sip feed in the last 4 weeks (group 2) were assigned to the Active group receiving the medical food or to the Control group receiving an iso-caloric control product. Patients were asked to complete the quality of life questionnaires and the required study parameters as body weight and performance status were recorded at baseline (visit 1) and after 4 weeks of nutritional intervention (visit 3). In addition, blood was drawn for the measurement of several immune-, nutritional- and safety-parameters. Two weeks after the start of the study patients visited the clinic (visit 2) to monitor the use of concomitant medication, body weight and product palatability. The amount of study product taken was recorded daily in a diary by the patient. Patients with an intake of <75% of the minimum amount of 2x200 ml Active or Control product per day were considered as noncompliant.
The subjects in the reference group were pre-screened and eligible subjects visited the clinic once. Subject characteristics, relevant medical history and anthropometrics were determined and the required study parameters were recorded. In addition, blood was drawn for the measurement of several immune- and nutritional-parameters. Subjects in the reference group did not receive any intervention with a study product.

NUTRITIONAL INTERVENTION

All patients received dietary counselling in addition to the nutritional intervention. The prescribed product intake during the study was 2 doses (2x200 ml sip feed) of either the Active medical food or routine nutritional support (Control product) daily for patients in group 1 and at least 2 doses for patients in group 2. The Active medical food is an energy dense (163 kcal/100 ml), nutritionally complete oral supplement (FortiCare) that is high in protein and leucine (9.9 g protein /100 mL of which 3.2 g whey protein /100 mL, 5.6 g casein /100 mL and 1.1 g free leucine /100 mL) and is enriched with emulsified fish oil (0.6 g EPA and 0.3 g DHA /100 mL), specific oligosaccharides (1.2 g galactooligosaccharides (GOS) and 0.2 g fructooligosaccharides (FOS) /100 mL) and a balanced mix of vitamins, minerals and trace elements (Table 1, Nutricia NV, Zoetermeer, the Netherlands). The Control product is for group 1 a non-caloric Placebo product and for group 2 an energy dense (163 kcal/100 ml) iso-caloric standard nutritional product.

Table 1 Nutritional composition of the Active medical food (FortiCare), Placebo product and Iso-caloric control product in grams per 100 ml

Ingredients	Active medical food	Placebo product	Iso-caloric control product
Macronutrients			
Energy (kJ/kcal)	**683/163**	**0**	**669/160**
Carbohydrates (g)	**17.4**	**0**	**21.0**
Protein (g)	**9.9**	**0**	**6.0**
- Whey (g)	3.2	0	0
- Casein (g)	5.6	0	6.0
- Added amino acids: free leucine (g)	1.1	0	0
Total fat (g)	**5.3**	**0**	**5.8**
- EPA (g)	0.6	0	0
- DHA (g)	0.3	0	0
Oligosaccharides (g)	**1.4**	**0**	**0**
- GOS (g)	1.2	0	0
- FOS (g)	0.2	0	0
Minerals & trace elements			
Sodium (mg)	**110**	**12**	**90**
Potassium (mg)	**215**	**33**	**159**
Chloride (mg)	**140**	**0**	**87**
Calcium (mg)	**147**	**2**	**91**
Phosphorus (mg)	**115**	**6**	**78**
Magnesium (mg)	**28.2**	**2.4**	**23**
Iron (mg)	**1.9**	**0**	**2.4**
Zinc (mg)	**2.1**	**0**	**1.8**
Copper (µg)	**288**	**0**	**270**
Manganese (mg)	**0.7**	**0**	**0.5**
Fluoride (mg)	**0.2**	**0**	**0.15**
Molybdenum (µg)	**16.0**	**0**	**15.0**
Selenium (µg)	**13.5**	**0**	**8.6**
Chromium (µg)	**11.0**	**0**	**10.0**
Iodine (µg)	**21.0**	**0**	**20.0**
Vitamins			
Vitamin A (µg-RE)	**130**	**0**	**123**
Vitamin D3 (µg)	**1.1**	**0**	**1.1**
Vitamin E (mg-α-TE)	**3.2**	**0**	**1.9**
Vitamin K (µg)	**8.5**	**0**	**8.0**
Thiamin (B1) (mg)	**0.2**	**0**	**0.2**
Riboflavin (B2) (mg)	**0.3**	**0**	**0.2**
Niacin (B3) (mg-NE)	**2.9**	**0**	**2.7**
Pantothenic acid (B5) (mg)	**0.9**	**0**	**0.8**
Vitamin B6 (mg)	**0.6**	**0**	**0.3**
Folic acid (µg)	**53**	**0**	**40**
Vitamin B12 (µg)	**0.6**	**0**	**0.3**
Biotin (µg)	**6.4**	**0**	**6.0**
Vitamin C (mg)	**21**	**0**	**15**
Carotenoids (mg)	**0.3**	**0**	**0.3**
Other			
L-Carnitine (mg)	**10.9**	**0**	**0**
Choline (mg)	**59**	**0**	**55**
Taurine (mg)	**13.3**	**0**	**0**

Values represent the amount of ingredients of the medical food in grams per 100 ml. Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoid acid; GOS, galactooligosaccharides; FOS, fructooligosaccharides.

STUDY OUTCOME

The primary outcome parameters of the study were the *ex vivo* Concanavalin (Con)A-stimulated T-lymphocyte proliferation and cytokine (Interleukin (IL)-2, IL-4, IL-5, IL-10,

IL-12 and Interferon (IFN)-γ) production by Blood Peripheral Mononuclear Cells (PBMC) as markers for immune function (25). PBMC were isolated from heparin blood using density-gradient centrifugation and stored in liquid-nitrogen. PBMC were thawed and stimulated with 2.5 and 10 µg/ml ConA (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) in medium with 10% autologous serum for 44 hours at 37^{0}C and 5% CO_2. T-lymphocyte proliferation was measured by the addition of tritiated thymidine (H-TdR) 16 hours prior to harvesting the cells. In addition, PBMC were stimulated with 10 ng/ml Lipopolysaccharide (LPS, *Escherichia coli*, B55:O55, Sigma-Aldrich Chemie) in medium with 10% autologous serum for 44 hours at 37ºC and 5% CO_2 to measure *ex vivo* B-lymphocyte proliferation as described above and for 20 hours to measure cytokine (IL-1β, IL-6, IL-8, IL-10 and Tumor Necrosis Factor (TNF)-α) and PGE_2 production by PBMC. Serum samples were assayed for levels of inflammatory mediators (IL-1β, IL-6, IL-8, IL-10, TNF-α, PGE_2 and C-reactive protein (CRP)). Cytokine levels in culture supernatants and serum were measured using a Bio-Plex Cytokine bead immunoassay (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's protocol and PGE_2 was measured using a commercial enzyme immunoassay (Biotrak Amersham, Buckinghamshire, UK) according to the manufacturer's protocol.

Natural Killer (NK)-cell activity was determined using three different assays, i.e. classic NK-cell activity against K562 target cells, lymphokine-activated killer (LAK) activity using Daudi cells and antibody dependent cell-mediated cytotoxicity (ADCC) against P815 target cells using a standard 4 hours ^{51}Chromium release assay (26).

During the visits body weight and BMI were recorded and blood was collected to determine white blood cell count and differential , the lymphocyte subset count (27),pre-albumin and albumin, but also safety parameters for liver function (ALAT and γ-GT), kidney function (creatinine) and prothrombin time were measured at the Clinical Chemistry Laboratory, Erasmus Medical Center, Rotterdam, the Netherlands. Moreover, the phospholipid fatty acid profile of plasma was measured (gas chromatography) (21), ECOG performance status was assessed (28), quality of life was recorded (QLQ-C30, OES18, EuroQoL-5D), dysphagia was assessed (29) and study product intake and palatability were scored.

STATISTICAL ANALYSIS

The study was considered an exploratory study; the primary parameters have not been reported in newly diagnosed cancer patients before. Therefore, the expected difference between the Active and Control group and its variance was estimated. Based on two studies it was assumed that a sample size of 40 for each of the two groups was sufficient to detect a statistically significant result between the groups (30, 31). A blinded interim analysis on primary efficacy and safety was performed after 64 patients. The results were reviewed to check whether the calculated sample size was adequate and that no safety concerns had arisen. From this interim analysis it was concluded that in order to find differences on the primary outcome, the sample size had to be adjusted to an unrealistically high number of patients. Therefore, it was decided to stop the study and perform the final analysis on the available 64 patients. All subjects that received the study products were included in the intention-to-treat (ITT) analysis. For baseline comparisons the differences between healthy

volunteers and total patients, group 1 and group 2 and between the Active and Control group were determined. Moreover, the differences between visit 1 and 3 were compared between the Active and Control group and between group 1 and group 2.

The results of the ConA and LPS stimulations were corrected for the un-stimulated cultures by subtraction of the latter. ANOVA with treatment and stratification for group 1 and group 2 as covariates was used to analyze the measurement of the study parameters. When the data were not normally distributed, the Mann Whitney U test adjusted for stratification (group 1 and group 2) was used and correlations were made using the Spearman's Rank test. NK cell activity was measured at four different E:T ratios and the WMSL (Weighted Mean of Specific Lysis) was calculated (26, 32). For the ordinal variables performance status (ECOG) and dysphagia score, Visit 1 and 3 were compared between the groups using the Mann Whitney U test adjusted for stratification (group 1 and group 2).

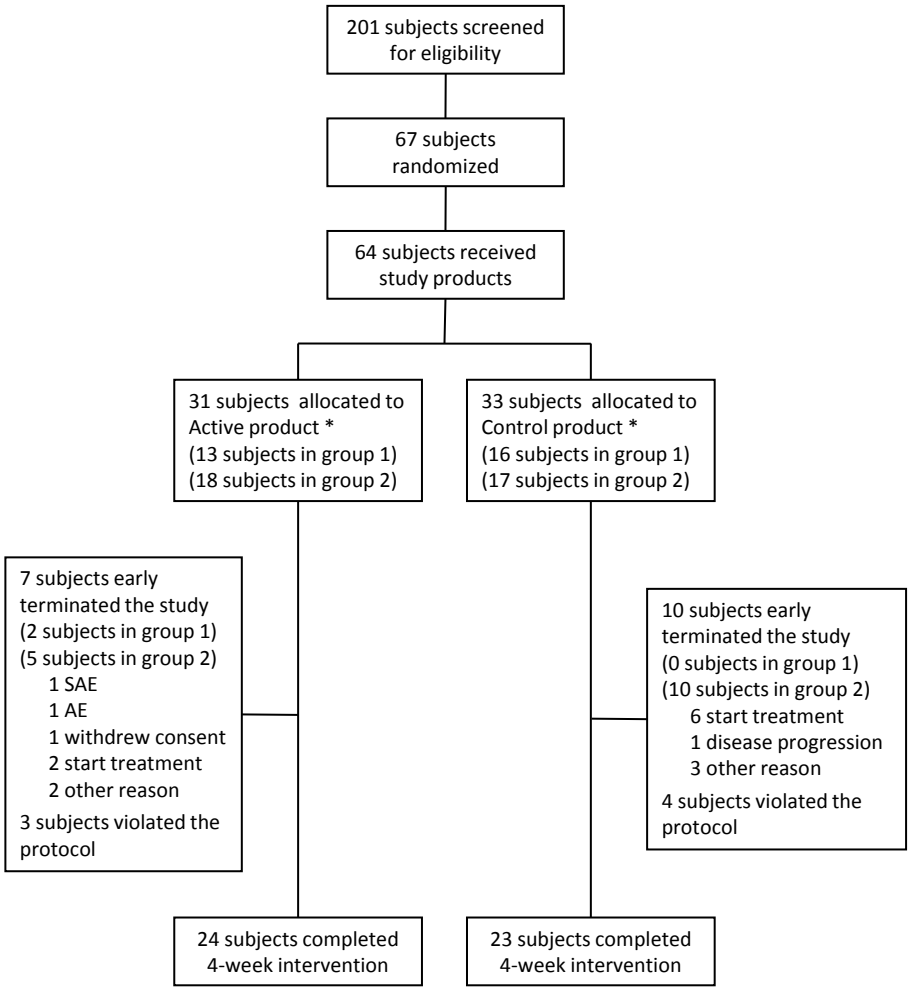

Figure 1 Trial profile; screening, randomization and study completion. Patients with 0 to <5% weights loss in the past 3 months and a dysphagia score of 0 or 1 were assigned to group 1 and patients with ≥5% weight loss in the past 3 months and/or dysphagia score 2 or 3 and/or prescribed sip feed in the last 4 weeks were as signed to group 2. * Included in ITT analysis.

All adverse events were assessed and medical history and medication use were checked individually for subjects having AEs. The statistical analyses were performed using SPSS for Windows Release 15.0.0.

RESULTS

STUDY POPULATION AND COMPLIANCE

Of the 201 subjects that were screened in the study, 67 subjects were randomized and 64 subjects received the study products (Figure 1). Subjects considered a screening failure did not fulfill the inclusion criteria or received an anti-cancer treatment within 3 weeks. Of the 64 subjects that received the study products, 31 subjects were allocated to the Active product (13 subjects in group 1 and 18 in group 2) and 33 were allocated to the Control product (16 subjects in group 1 and 17 in group 2) and all these subjects were included in the ITT analysis. A total of 17 subjects terminated the study early (7 subjects in the Active group and 10 in the Control group), with most of the patients coming out of group 2. Product compliance was not significantly different between the groups with 89% for the Active product and 87% for the Control products, respectively.

BASELINE CHARACTERISTICS

At baseline, BMI of the total patient group significantly differed from the healthy volunteer (HV) group ($p < 0.01$, Table 2). By definition, patients in group 2 had lost significantly more weight in the past three months than patients in group 1 ($p \leq 0.001$). Furthermore, patients in group 2 scored lower on the quality life scales (EQ-VAS and EQ-5D) ($p \leq 0.001$), had a higher tumor length ($p \leq 0.02$) and a higher dysphagia score than patients in group 1 ($p \leq 0.001$). Control and Active groups matched very well with regard to baseline characteristics, except for smoking history (patients in the Active group had smoked longer than patients in the Control group ($p=0.001$)). In more than fifty percent of the patients the tumor was located in the esophagus and in the remaining patients the tumor was located at the gastro-intestinal junction. Clinical stage ranged from I-IV, and was equally distributed.

Table 2a General baseline characteristics of the study groups

Variable	Presented as	Healthy volunteers (n=40)	Total patients (n=64)	Active (n=31)	Control (n=33)
Sex	n (%)				
Female		8 (20.0%)	14 (21.9%)	7 (22.6%)	7 (21.2%)
Male		32 (80.0%)	50 (78.1%)	24 (77.4%)	26(78.8%)
Age (years)	mean ± SD	63.6 ± 10.2	61.4 ± 9.2	61.1 ± 9.2	61.6 ± 9.4
BMI (kg/m^2)	mean ± SD	27.0 ± 2.1	25.4 ± 4.1[A]	25.5 ± 4.6	25.4 ± 3.6

Table 2b Disease specific baseline characteristics of the study groups

Variable	Presented as	Patients group 1 (n=29)	Patients group 2 (n=35)	Active (n=31)	Control (n=33)
Body weight change in past 3 months (%)	mean ± SD	0.8 ± 2.7	-8.0 ± 4.5[B]	-4.2 ± 6.0	-3.8 ± 5.7
Days since diagnosis	median (IQR)	0 (-2-19)	4 (-4-14)	0 (-1-18)	0 (-6-14)
Score on EQ-VAS (mm)	median (IQR)	80 (70-90)	60 (50-72.5)[B]	60 (50-70)	77.5 (60-87.5)
Score on EQ-5D index	median (IQR)	0.81 (0.81-1.0)	0.69 (0.37-0.81)[B]	0.81 (0.68-0.84)	0.81 (0.69-1.0)
Tumor length (cm)	median (IQR)	3.8(2.0-5.0)	5.4 (3.0-7.8)[C]	4.0 (3.0-5.5)	4.0 (3.0-6.0)
Years smoked	mean ± SD	30.0 ± 12.1	37.8 ± 17.3[C]	40.9 ± 11.9[D]	27.9 ± 16.1
Tumor location	n (%)				
Esophagus		20 (69.0%)	18 (54.5%)	20 (64.5%)	18 (58.1%)
Gastro-esophageal junction: I, II, III		9 (31.0%): 7, 2, 2	15 (45.5%): 5, 4, 6	11 (35.5%) 6, 3, 3	13 (41.9%) 6, 3, 5
Histology	n (%)				
Adenocarcinoma		22 (75.9%)	29 (82.9%)	25 (80.6%)	26 (78.8%)
Squamous carcinoma		6 (20.7%)	5 (14.3%)	6 (19.4%)	5 (15.2%)
Other		1 (3.4%)	1 (2.9%)	0 (0%)	2 (6.1%)
TNM stage	n (%)				
I		2 (6.9%)	0 (0%)	2 (6.5%)	0 (0%)
IIA		6 (20.7%)	6 (17.1%)	4 (12.9%)	8 (24.2%)
IIB		5 (17.2%)	1 (2.9%)	3 (9.7%)	3 (9.1%)
III		4 (13.8%)	9 (25.7%)	8 (25.8%)	5 (15.2%)
IV		1 (3.4%)	2 (5.7%)	1 (3.2%)	2 (6.1%)
IVA		5 (17.2%)	5 (14.3%)	5 (16.1%)	5 (15.2%)
IVB		0 (0%)	1 (2.9%)	0 (0%)	1 (3.0%)
Unknown		6 (20.7%)	11 (31.4%)	8 (25.8%)	9 (27.3%)
Dysphagia score	n (%)				
Score 0		13 (44.8%)	4 (11.%)[E]	6 (19.4%)	11 (33.3%)
Score 1		13 (44.8%)	13 (37.1%)	14 (45.2%)	12 (36.4%)
Score 2		3 (10.3%)	11 (31.4%)	7 (22.6%)	7 (21.2%)
Score 3		0 (0%)	7 (20.0%)	4 (12.9%)	3 (9.1%)

Data represent the baseline characteristics as the number of subjects (n) and percentages or means ± SD of the healthy volunteers group (n=40), the total patient group (n=64), the Active medical food group (n=31) and the Control group (n=33) (Table 1a) and the baseline disease specific characteristics as the number of subjects (n) and percentages, means ± SD or medians and interquartile ranges (IQR, 25th-75th percentiles) of patient group 1 (n=29), patient group 2 (n=35), the Active medical food group (n=31) and the Control group (n=33) (Table 1b). Patients with 0 to <5% weights loss in the past 3 months and a dysphagia score of 0 or 1 were assigned to group 1 and patients with ≥5% weight loss in the past 3 months and/or dysphagia score 2 or 3 and/or prescribed sip feed in the last 4 weeks were assigned to group 2. [A] Significantly different from healthy volunteers group, $p < 0.01$ (Mann-Whitney), [B] Significantly different from group 1, $p \leq 0.001$ (Mann-Whitney), [C] Significantly different from group 1, $p \leq 0.02$ (Mann-Whitney), [D] Significantly different from the

Control group, p = 0.001 (Mann-Whitney), [E] The distribution over the different dysphagia scores is significantly different from group 1, p ≤ 0.001 (Mann-Whitney), the lower the score, the better. Abbreviations: BMI, body mass index; EQ-VAS, EuroQol-Visual Analogue Scale, a standard vertical 20 cm visual analogue scale for recording an individual's rating for their current health-related quality of life state in which the higher the score, the better; EQ-5D, EuroQol 5-dimension, descriptive system of health-related quality of life states consisting of five dimensions (mobility, self-care, usual activities, pain/discomfort, anxiety/depression) each of which can take one of three responses. The responses record three levels of severity (no problems/some or moderate problems/extreme problems) in which the higher the score, the better; TNM, tumor, node, metastasis.

EFFICACY

At baseline, the primary parameter, ConA-stimulated T-lymphocyte proliferation and cytokine production in PBMC, was not significantly different between the total patient group and HV (Table 3a), with the exception of IFN-γ production, which was significantly lower in the total patient group (p = 0.005). Also no baseline differences between group 1 and 2 were observed. Furthermore, after four weeks of nutritional intervention no differences between the Active and Control groups were observed on the change from baseline (as the delta of visit 3 – visit 1) regarding the primary parameter. Similarly, no differences on LPS-stimulated B-lymphocyte proliferation or on cytokine production by monocytes in PBMC were observed between the total patient group and HV at baseline or after the nutritional intervention.

At baseline, absolute levels of leucocytes were significantly higher in the total patient group compared to HV (p ≤ 0.001), which is mainly caused by the higher number of neutrophils (p ≤ 0.001), but also monocytes were significantly higher in the total patient group compared to HV (p ≤ 0.001) (Table 3b). By contrast, NK-lymphocytes were significantly lower in the total patient group compared to HV (p = 0.002), but nevertheless, levels of all mentioned cell types were within the normal range.

Moreover, no differences in cell types were observed between the Active and Control group after the four week intervention period. In addition to the number of NK-lymphocytes, NK-cell activity was measured as a parameter of innate immune function (Table 3b). After the four week nutritional intervention period, no differences between the Active and Control group were observed.

Serum concentrations of inflammatory cytokines were relatively low in both patients and HV, e.g. most levels were just above the detection limit of the assay (Table 4). However at baseline, serum IL-6, IL-1β and CRP levels were significantly higher in the total patient group compared to HV (all p ≤ 0.001). Additionally, patients in group 2 had significantly higher CRP levels than patients in group 1 (p = 0.005). No differences were detected on the change from baseline between the Active and Control group after four weeks of nutritional intervention. Serum concentrations of PGE_2, were not different at baseline between the total patient group and the HV. However, patients in group 2 showed higher PGE_2 levels (953 ± 1229 pg/ml, means ± SD) compared to patients in group 1 (414 ± 474 pg/ml, means ± SD, p = 0.05). After the nutritional intervention, serum PGE_2 levels (as the delta of visit 3 – visit 1) in the Active group were decreased significantly compared to increased levels observed in the Control group (p = 0.01, Figure 2A). Analyzing group 1 and group 2 separately, the differences between Active and Control were more pronounced in group 2 (p = 0.01, Figure 2C) compared to group 1 (p = 0.05, Figure 2B).

Table 3a Proliferation response and cytokine and PGE_2 production in ConA- and LPS-stimulated PBMC

	Baseline (visit 1)			Δ (visit 3-visit 1)	
	Healthy volunteers	Total patients	Patients group 1 Patients group 2	Active	Control
Leucocytes (*10^9/L)	5.78 ± 1.21	8.06 ± 2.24[B]	7.63 ± 1.99 8.42 ± 2.40	0.99 ± 2.21	-0.15 ± 1.87
Neutrophils (*10^9/L)	3.38 ± 1.00	5.56 ± 2.24[B]	5.27 ± 1.79 5.81 ± 2.56	0.33 ± 7.91	-3.61 ± 9.81
Monocytes (*10^9/L)	0.47 ± 0.14	0.59 ± 0.18[B]	0.55 ± 0.14 0.63 ± 0.21	-0.14 ± 1.85	0.96 ± 2.34
Lymphocytes (*10^9/L)	1.73 ± 0.51	1.69 ± 0.52	1.65 ± 0.51 1.73 ± 0.53	-0.67 ± 6.17	2.00 ± 6.93
B-Lymphocytes (*10^9/L)	0.16 ± 0.08	0.16 ± 0.08	0.15 ± 0.08 0.16 ± 0.08	0.03 ± 0.09	0.01 ± 0.05
T-Lymphocytes (*10^9/L)	1.02 ± 0.39	1.12 ± 0.43	1.08 ± 0.41 1.15 ± 0.45	0.20 ± 0.51	0.08 ± 0.22
NK-Lymphocytes (*10^9/L)	0.23 ± 0.13	0.16 ± 0.09[C]	0.17 ± 0.11 0.16 ± 0.07	0.04 ± 0.09	0.04 ± 0.10
NK activity (K562) (%)	13.36 ± 7.92	11.23 ± 7.35	11.97 ± 8.53 10.36 ± 5.80	-0.37 ± 3.74	0.56 ± 6.17
LAK activity (Daudi) (%)	3.37 ± 5.18	2.95 ± 2.64	3.11 ± 3.24 2.76 ± 1.76	0.04 ± 1.46	-0.87 ± 2.38
ADCC activity (P815) (%)	19.01 ± 8.48	18.42 ± 8.03	19.15 ± 8.04 17.55 ± 8.18	-0.44 ± 5.51	-0.32 ± 5.78

Table 3b White blood cell counts, differential and NK cell activity

	Baseline (visit 1)			Δ (visit 3-visit 1)	
	Healthy volunteers	Total patients	Patients group 1 Patients group 2	Active	Control
ConA-stimulated PBMC					
Proliferation (cpm)	3229 (2199-8507)	4478 (2947-6369)	3684 (2398-6603) 5170 (4038-6316)	-546 (-2232-291)	-464 (-1281-235)
IL-2 (pg/ml)	164 (91.2-289)	162 (96.6-230)	164 (96.6-305) 162 (84.1-217)	-10.0 (-39.5-39.6)	-15.2 (-66.5-27.4)
IFN-γ (pg/ml)	398 (261-775)	214 (119-446)[A]	233 (89.9-662) 198 (131-420)	-52.0 (-250-64.3)	5.5 (-157-104)
LPS-stimulated PBMC					
Proliferation (cpm)	276 (98.2-706)	182 (78.9-486)	222 (82.9-774) 149 (59.9-224)	-43.1 (-97.8-25.2)	-6.1 (-97.9-90.0)
IL-6 (pg/ml)	3084 (2185-4664)	3102 (1906-5392)	2706 (1749-3407) 3682 (2215-7402)	-141 (-1037-945)	-24.3 (-956-676)
TNF-α (pg/ml)	239 (162-380)	227 (166-509)	224 (175-447) 336 (143-728)	-65.2 (-257-9.8)	11.5 (-73.0-54.0)
PGE_2 (pg/ml)	4744 (2583-11808)	6245 (2903-9286)	6693 (2903-9921) 5202 (2696-8779)	-840 (-3243-656)	79.3 (-3932-4774)

Data represent medians and interquartile ranges (25th-75th percentiles) of the proliferation response and cytokine and PGE_2 production in ConA-stimulated (10 µg/ml) and LPS-stimulated (10 ng/ml) PBMC of the healthy volunteers group (n=40), the total patients group (n=46) and the patient groups 1 (n=26) and 2 (n=20) at baseline. For the comparisons of the Active medical food group (n=24) and the Control group (n=22), the deltas between visit 1 and visit 3 are presented (Table 3a). Data represent means ± SD of the white blood cell counts, differential and NK-cell activity of the healthy volunteers group (n=40), the total patients group (n=64) and the patient groups 1 (n=29) and 2 (n=35) at baseline. For the comparisons of the Active medical food group (n=24) and the Control group (n=23), the deltas between visit 1 and visit

3 are presented (Table 3b). NK-cell activity was measured using three different assays (classic NK-cell activity against K562 target cells, lymphokine-activated killer (LAK) activity using Daudi cells and antibody dependent cell-mediated cytotoxicity (ADCC) against P815 target cells). All three assays were measured with four effector:target cell ratio's, whereas % WMSL of the ratio's is presented in the table. [A] Significantly different from healthy volunteers group, p = 0.005 (Mann-Whitney). [B] Significantly different from healthy volunteers group, p ≤ 0.001 (ANOVA), [C] Significantly different from healthy volunteers group, p = 0.002 (ANOVA). Abbreviations: ADCC, antibody dependent cell-mediated cytotoxicity; ConA, Concanavalin A; cpm, counts per minute; IL, interleukin; IFN-γ, interferon-gamma; LAK, lymphokine-activated killer; LPS, lipopolysaccharide; TNF-Ð, tumor necrosis factor-alpha; NK, natural killer; PGE_2, prostaglandin E_2; WMSL, weighted mean of specific cytolysis.

To determine the uptake of fatty acids from the product, the percentage phospholipid fatty acids was measured in plasma (Table 5). The only differences at baseline were the lower percentage total n-3 polyunsaturated fatty acids (PUFAs) (p = 0.006) and EPA (p = 0.001) in the total patients group compared to HV. After the four week nutritional intervention period, a significant higher increase was observed in the Active group for total n-3 PUFAs, EPA, DPA and DHA (p ≤ 0.001) compared to the Control group and a significant higher decrease for total n-6 PUFAs, AA and the ratio n-6/n-3 PUFAs (p ≤ 0.001) compared to the Control group.

Table 4 Serum levels of pro-inflammatory cytokines and CRP

	Baseline (visit 1)			Δ (visit 3-visit 1)	
	Healthy volunteers	Total patients	Patients group 1 Patients group 2	Active	Control
IL-6 (pg/ml)	1.9 (1.2-2.0)	2.7 (1.2-9.1)[A]	2.4 (1.2-6.6) 3.6 (1.4-17.8)	0.0 (-0.5-1.2)	0.0 (-1.5-0.1)
TNF-α (pg/ml)	1.1 (1.1-1.7)	1.7 (1.1-1.8)	1.7 (1.1-1.8) 1.6 (1.1-1.7)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
IL-8 (pg/ml)	10.4 (5.1-21.0)	15.9 (8.4-37.8)	11.3 (4.7-28.9) 16.7 (12.6-38.9)	-0.1 (-13.2-1.5)	-0.4 (-9.8-18.2)
IL-1β (pg/ml)	2.3 (1.1-2.5)	3.6 (2.3-9.4)[A]	3.7 (3.1-11.1) 3.2 (1.9-3.6)	0.0 (-1.2-0.0)	0.0 (-1.5-0.0)
CRP (mg/l)	2.0 (1.0-2.75)	4.0 (2.0-8.75)[A]	2.0 (1.5-4.0) 5.0 (3.0-11.0)[B]	0.5 (0.0-3.3)	0.0 (-2.3-1.5)

Data represent medians and interquartile ranges (25th-75th percentiles) of the serum levels of pro-inflammatory mediators of the healthy volunteers group (n=40), the total patients group (n=47) and the patient groups 1 (n=27) and 2 (n=20) at baseline. For the comparisons of the Active medical food group (n=24) and the Control group (n=23), the deltas between visit 1 and visit 3 are presented. [A] Significantly different from healthy volunteers group, p ≤ 0.001 (Mann-Whitney), [B] Significantly different from group 1, p = 0.005 (Mann-Whitney). Abbreviations: IL, interleukin; TNF-α, tumor necrosis factor-alpha; CRP, C-reactive protein.

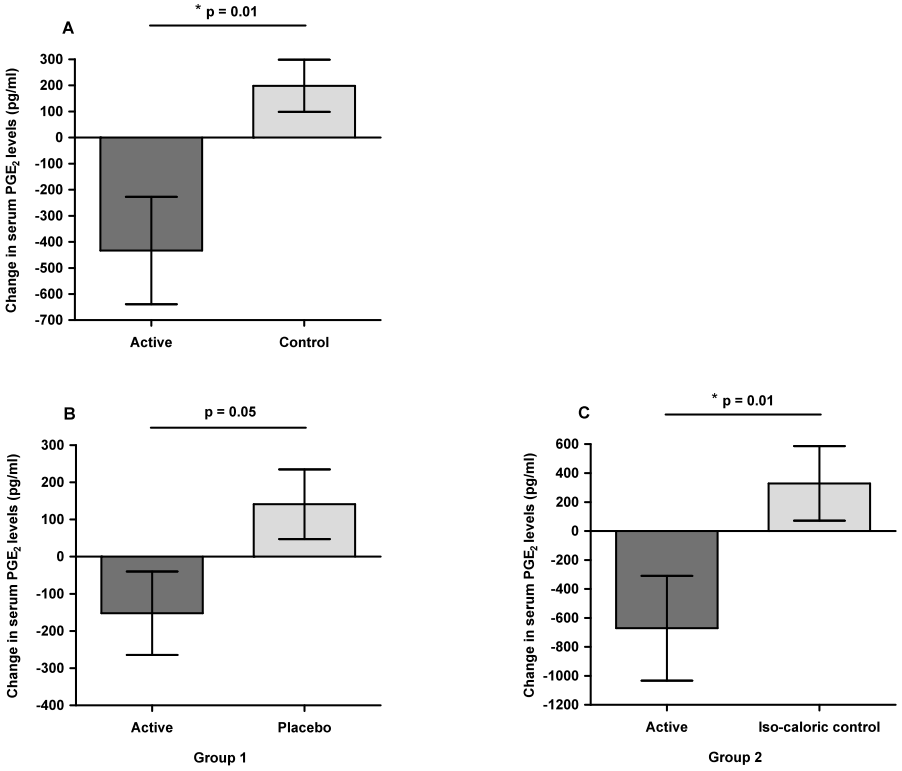

Figure 2 Change in serum PGE_2 levels (pg/ml) in the Active medical food group (n=24) and the Control group (n=23) in the total patient group (A), in the Active medical food group (n=11) and the Placebo group (n=16) in group 1 (B) and in the Active medical food group (n=13) and the Iso-caloric control group (n=7) in group 2 (C) after a four week nutritional intervention period. Data are presented as the delta between visit 1 (baseline) and 3 in means ± SEM. * Significantly different from visit 1 (baseline), p = 0.01 (ANOVA).

Table 5 Percentages phospholipid fatty acids in plasma

	Baseline (visit 1)			Δ (visit 3-visit 1)	
	Healthy volunteers	Total patients	Patients group 1 Patients group 2	Active	Control
Total n-6 LC PUFAs (%)	32.76 ± 2.80	32.97 ± 2.41	33.53 ± 2.41 32.49 ± 2.33	-7.34 ± 3.63[C]	0.21 ± 2.21
AA (%)	9.93 ± 1.97	10.72 ± 2.18	10.89 ± 2.09 10.58 ± 2.28	-1.94 ± 1.41[C]	-0.47 ± 1.58
Total n-3 LC PUFAs (%)	7.19 ± 2.57	6.02 ± 1.68[A]	6.01 ± 1.56 6.02 ± 1.80	8.96 ± 3.87[C]	0.12 ± 1.15
EPA (%)	1.89 ± 1.38	1.20 ± 0.68[B]	1.31 ± 0.62 1.11 ± 0.71	5.61 ± 2.26[C]	0.01 ± 0.65
DPA (%)	0.98 ± 0.16	0.96 ± 0.21	0.96 ± 0.22 0.97 ± 0.20	0.98 ± 0.48[C]	0.02 ± 0.13
DHA (%)	4.11 ± 1.34	3.66 ± 1.12	3.56 ± 0.99 3.74 ± 1.23	2.44 ± 1.52[C]	0.03 ± 0.51
Ratio n-6/n-3 LC PUFAs	5.20 ± 2.07	5.99 ± 2.05	6.10 ± 2.21 5.89 ± 1.93	-4.03 ± 2.41[C]	0.08 ± 1.09

Data represent means ± SD of the percentage phospholipid fatty acids in plasma of the healthy volunteers group (n=40), the total patient group (n=63) and the patient groups 1 (n=29) and 2 (n=34) at baseline. For the comparisons of the Active medical food group (n=24) and the Control group (n=23), the deltas between visit 1 and visit 3 are presented.

[A] Significantly different from healthy volunteers group, p = 0.006 (ANOVA), [B] Significantly different from healthy volunteers group, p = 0.001 (ANOVA), [C] Significantly different from the Control group, p ≤ 0.001 (ANOVA). Abbreviations: PUFAs, poly-unsaturated fatty acids; AA, arachidonic acid; EPA, eicosapentaenoid acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

To determine the uptake of fatty acids from the product, the percentage phospholipid fatty acids was measured in plasma (Table 5). The only differences at baseline were the lower percentage total n-3 polyunsaturated fatty acids (PUFAs) (p = 0.006) and EPA (p = 0.001) in the total patients group compared to HV. After the four week nutritional intervention period, a significant higher increase was observed in the Active group for total n-3 PUFAs, EPA, DPA and DHA (p ≤ 0.001) compared to the Control group and a significant higher decrease for total n-6 PUFAs, AA and the ratio n-6/n-3 PUFAs (p ≤ 0.001) compared to the Control group.

As already mentioned, at baseline the BMI of the total patient group differed significantly from the HV (p < 0.01), which is partly due to a trend in lower body weights in the total patient group (79.1 ± 15.6, mean ± SD) compared to HV (84.4 ± 8.9, mean ± SD, p = 0.05). After the four week nutritional intervention period, a significant higher weight gain was observed in the Active group compared to the Control group (p < 0.05, Figure 3A). Analyzing group 1 and group 2 separately, the weight gain in Active group 1 was more pronounced compared to the total group (Figure 3B).

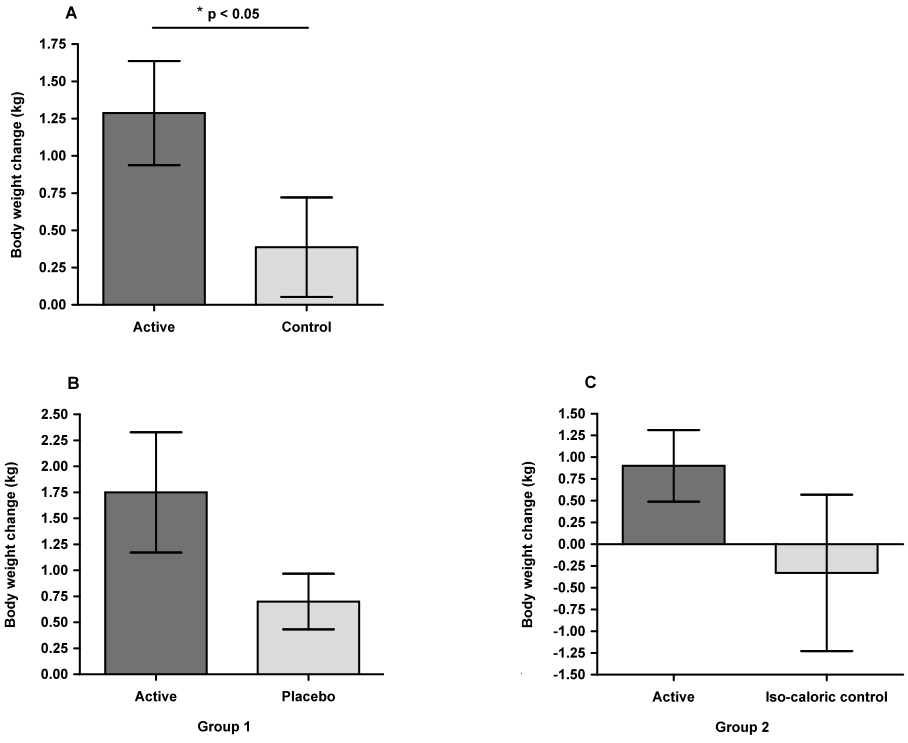

Figure 3 Body weight change (kg) in the Active medical food group (n=24) and the Control group (n=23) in the total patient group (A), in the Active medical food group (n=11) and the Placebo group (n=16) in group 1 (B) and in the Active medical food group (n=13) and the Iso-caloric control group (n=7) in group 2 (C) after a four week nutritional intervention period. Data are presented as the delta between visit 1 (baseline) and 3 in means ± SEM. * Significantly different from visit 1 (baseline), p <0.05 (ANOVA).

In contrast to the Active groups, patients in Control group 2 appeared to lose body weight despite receiving an iso-caloric control product (Figure 3C).

The performance status of the patients was assessed by ECOG score. At baseline, no differences were observed between the Active and Control group, whereas patients in group 2 had a significant worse performance status compared to patients in group 1 ($p < 0.01$, data not shown). After the four weeks nutritional intervention, the performance status was significantly different between Active and Control group ($p < 0.05$). ECOG improved with 1 score in 17.4% of the patients in the Active group compared to 0% in the Control group, was stable in 65.2% of the patients in the Active group compared to 72.7% in the Control group and was worsened in 17.4% of the patients in the Active group compared to 27.3% in the Control group (Figure 4).

After the nutritional intervention, no significant differences between the Active and Control group were observed on the nutritional parameters pre-albumin and albumin, on quality of life (QoL) or on dysphagia score (data not shown).

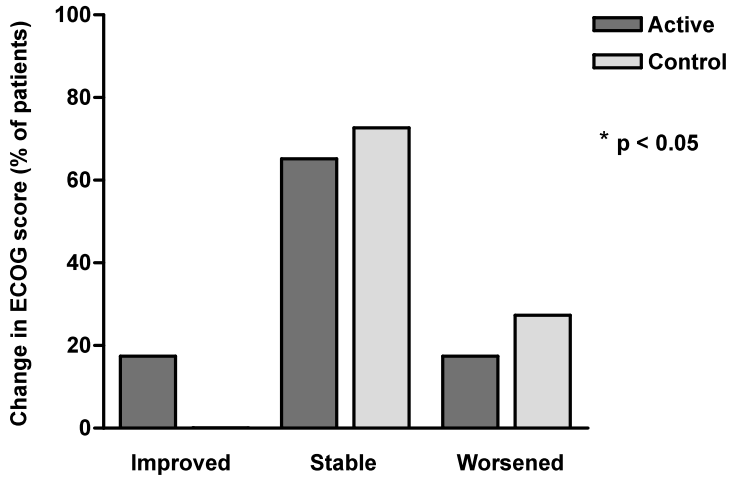

Figure 4 Change in ECOG score (% of patients) in the Active medical food group (n=24) and the Control group (n=23) after a four week nutritional intervention period. Improved means ECOG score improved with 1 score, stable means ECOG score did not change, worsened means ECOG score worsened with 1 score. Data are presented as the delta between visit 1 (baseline) and 3 as the percentage of patients. * Significantly different from visit 1 (baseline), $p < 0.05$ (Mann Whitney U).

SAFETY AND TOLERABILITY

A total of 79 adverse events (AEs) were reported, 44 in the Active group (occurring in 37 patients) and 35 in the Control group (occurring in 24 patients), including 2 product-unrelated serious adverse events. However, the number of patients with at least one (s)AE was not different between the Active and Control group. Most adverse events were gastrointestinal related with full feeling, nausea and constipation most frequently observed in the Active group and diarrhea and constipation most frequently observed in the Control group. Blood safety parameter means were all within reference ranges and no clinically relevant changes on liver and kidney function and prothrombin time were observed.

DISCUSSION

In this double-blind, randomized, placebo-controlled study, a significant reduction of serum PGE_2 levels was observed in newly-diagnosed esophageal cancer patients after a four week nutritional intervention with the Active medical food compared to routine nutritional support. This effect was accompanied by a significant increase in body weight and an improved ECOG performance status of the patients.

PGE_2 is one of the best studied eicosanoids that contributes to the inflammatory state and immune suppression during the course of cancer (13, 33). It is involved in several human malignancies including colon, lung, breast and head and neck cancer and is produced during the course of inflammation in response to growth factors, hormones and inflammatory cytokines (33-35). PGE_2 is produced by various types of cancer cells and their surrounding cells, leading to a range of oncogenic effects including stimulation of cell proliferation, protection against apoptosis, and induction of migration and invasion (12, 36). In addition, it can induce epithelial cells to secrete growth factors, pro-inflammatory mediators and angiogenic factors, switching a normal microenvironment to a tumor-supporting environment (33, 34, 37). PGE_2 contributes to the shift of the tumor microenvironment from an anti-tumor Th1 response to an immunosuppressive Th2 response by down-regulating Th1 cytokines (IFN-γ, TNF-α and IL-2) and up-regulating Th2 cytokines (IL-4, IL-6 and IL-10) and has a clear role in the regulation of immune suppression (13, 34). Another mechanism by which PGE_2 is involved in the inflammation promoting tumor progression is through the induction of Myeloid Derived Suppressor Cells (MDSC) (34, 35). MDSC have been demonstrated to inhibit immune surveillance and to be potent suppressors of anti-tumor immunity. PGE_2 can act as a chemotactic factor for MDSC and therefore regulate the recruitment of these cells to the tumor (37, 38). In turn, MDSC can produce PGE_2 by themselves, thereby maintaining the inflammatory vicious circle (33).

A reduction of PGE_2 might be important to reduce the inflammatory state and to improve immune responsiveness in cancer patients. Clinically, this may lead to an improved acute response to infectious triggers and may beneficially affect tumor-immunity. For that reason, the reduction of serum PGE_2 levels in the present study, observed after the nutritional intervention of the medical food, could be beneficial for these cancer patients. Each of the product features, being fish oil, specific oligosaccharides, high protein and leucine, might play a specific role in this process, but overlapping biological activities and synergistic interactions between them eventually lead to the overall effect (21, 22). Fish oil contains high amounts of the n-3 PUFAs EPA and DHA, playing a major role in the regulation of immune responses and inflammation (39, 40). After intervention with the Active medical food, both the percentages EPA and DHA, as well as the total percentage n-3 PUFAs of plasma phospholipids were significantly increased (Table 5). This is partly due to the high compliance to the study product, inducing comparable effects as observed in a previous study in healthy volunteers (41). The increase in n-3 PUFAs was partly at the expense of the n-6 PUFA AA, but also the total percentage n-6 PUFAs was decreased significantly. Since AA can be used as a substrate for the COX-enzyme to produce PGE_2, a reduction in AA may explain the decrease in PGE_2, but also other factors were involved. The specific oligosaccharides (GOS/FOS) may affect the process of PGE_2 production as well. These non-digestible oligosac-

charides are fermentable fibers that have been associated with a reduced production of PGE_2 and pro-inflammatory cytokines in different parts of the gut (42). In addition, immune modulatory effects and other health benefits as an improved gut barrier function have been described, which may be related to their prebiotic properties (43). Besides the direct effect of fish oil and the oligosaccharides on PGE_2 metabolism, these ingredients were also described to reduce the systemic inflammatory state by decreasing the production of several inflammatory mediators (39).

In the present study, besides the effects on PGE_2, no effects were observed on other inflammatory mediators as IL-6, TNF-α, IL-8, IL-1β and CRP (Table 4), but levels of these markers were very low. Nevertheless, baseline levels of IL-6, IL-1β and CRP were significantly higher in the total patient group compared to healthy volunteers, even though a more severe inflammatory state of these patients was expected before the start of the study. In the patient group, baseline CRP levels were significantly higher in group 2, compared to group 1, indicating a more severe inflammatory state of the patients in group 2.

Patients in group 2 had lost significantly more weight in the past 3 months than patients in group 1, which might contribute to the induction of a (pre-) cachectic state and consequently into more inflammation. In the present study, this was confirmed by higher baseline levels of PGE_2 in group 2 compared to group 1. After the nutritional intervention with the Active medical food, both body weight loss and PGE_2 levels were reduced, demonstrating most pronounced effects in group 2. Accordingly, a relation between the improved bodyweight and reduced serum PGE_2 levels may exist via the reduction of (pre-) cachexia and inflammation, since bodyweight show a significant inverse correlation with serum PGE_2 levels in group 2 ($p=0.02$). However, also the presence of high protein and leucine in the Active medical food may have contributed to the preservation of body weight. Leucine has been added to the Active product to provide an anabolic trigger for muscle protein synthesis and high levels of protein were added in order to provide sufficient amounts of protein building blocks. The acute effect of this composition on muscle protein synthesis has recently been shown in a clinical study in catabolic cancer patients with involuntary weight loss (44) and the effects on muscle mass and function have been demonstrated in previous pre-clinical studies using an animal model of tumor induced cachexia (22). In the present study the effects of the medical food on muscle function are reflected by a significantly improved ECOG performance status after the intervention with the Active medical food compared to routine nutritional support. Cachectic cancer patients often suffer from a reduced activity and decreased performance status, which is related to the increase of weight loss in these patients affecting the muscle compartments as well (5, 18). Moreover, weight loss and a decreased performance status may negatively affect immune competence leading to an increased risk of (infectious) complications (5). In relationship with the observed weight loss, McMillan described the link between weight loss, poor performance status, poor response to treatment and poor prognosis, which is probably due to loss of skeletal muscle. Although, the loss of adipose tissue accounts for the majority of the weight loss, the loss of muscle accounts for most of the morbidity and mortality (45). PGE_2 might also be involved, since a rise in PGE_2 is suggested to be associated with muscle protein degradation in cancer cachexia (46). Consequently, reduced PGE_2 levels might diminish the loss of muscle function and thereby improve ECOG performance status.

Despite the above mentioned effects on PGE_2, no effects of the nutritional intervention were observed on proliferation responses or cytokine production in PBMC. This could partly be explained by the fact that the total patient group did not differ from the healthy volunteers in respect to their immune status at baseline. In contrast to these findings, esophageal cancer patients are frequently described to be at high risk for malnutrition and reduced immune responsiveness, especially around major surgery (47, 48). However, these studies were primarily performed in Japan, where the incidence of squamous cell carcinoma of the esophagus is higher compared to adenocarcinoma (49), whereas in the present Dutch study adenocarcinoma of the esophagus is mostly observed.

It appears that the patients included in this study had a better immune function than expected, possibly due to the type of cancer or the early phase of patient inclusion, just after diagnose. Goto demonstrated a reduced production of IL-2, IFN-γ and IL-10 in PHA-stimulated PBMC and a reduction of IL-12 and TNF-α in LPS-stimulated PBMC of cancer patients compared to healthy controls (50). However, these patients suffered from different types of advanced cancer which were irresectable with multiple metastases. Other explanations for the lack of effects on these immunological parameters were the high number of differences between Active and Control already at baseline and the higher drop-out of patients in group 2 in the Control group, leading to a skewed distribution of patients with milder and more advanced disease between Active and Control groups, possibly obscuring treatment effects. Another limitation is the high variance on the various immune parameters in combination with a relatively small group size.

In conclusion, the present exploratory study demonstrates a significant reduction of serum PGE_2 levels in newly-diagnosed esophageal cancer patients after a 4-week nutritional intervention with a medical food, which is high in protein and leucine and enriched with emulsified fish oil (containing EPA and DHA) and a specific oligosaccharide mixture compared to routine nutritional support. This effect is accompanied by a significant increase in body weight, an improved ECOG performance status of the patients and an efficient incorporation of n-3 PUFAs. Moreover, the medical food is well-appreciated with a high compliance rate of study product intake. No clinically relevant safety concerns were reported and no changes in blood safety parameters were measured. Consequently, these results show that nutritional intervention with the specific medical food may represent a new opportunity for applications in cancer patients being an integral part of disease management to provide optimal treatment support. However, additional research is recommended to elucidate the potential immunological effects in different types and stages of cancer.

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REFERENCES

1 Barber MD, Ross JA, Fearon KC. Cancer cachexia. Surg Oncol 1999;8: 133-41.
2 Nitenberg G, Raynard B. Nutritional support of the cancer patient: issues and dilemmas. Crit Rev Oncol Hematol 2000;34: 137-68.
3 van Bokhorst-de van der Schueren MA. Nutritional support strategies for malnourished cancer patients. Eur J Oncol Nurs 2005;9 Suppl 2: S74-83.
4 Argiles JM. Cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2: S39-50.
5 Dewys WD, Begg C, Lavin PT, Band PR, Bennett JM, Bertino JR, et al. Prognostic effect of weight loss prior to chemotherapy in cancer patients. Eastern Cooperative Oncology Group. Am J Med 1980;69: 491-7.
6. Van Cutsem E, Arends J. The causes and consequences of cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2: S51-63.
7 Muscaritoli M, Bossola M, Aversa Z, Bellantone R, Rossi Fanelli F. Prevention and treatment of cancer cachexia: new insights into an old problem. Eur J Cancer 2006;42: 31-41.
8 Evans C, Dalgleish AG, Kumar D. Review article: immune suppression and colorectal cancer. Aliment Pharmacol Ther 2006;24: 1163-77.
9 Ross JA, Fearon KC. Eicosanoid-dependent cancer cachexia and wasting. Curr Opin Clin Nutr Metab Care 2002;5: 241-8.
10 Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420: 860-7.
11 Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454: 436-44.
12 Ben-Baruch A. Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators. Semin Cancer Biol 2006;16: 38-52.
13 Young MR. Eicosanoids and the immunology of cancer. Cancer Metastasis Rev 1994;13: 337-48.
14 Laviano A, Meguid MM. Nutritional issues in cancer management. Nutrition 1996;12: 358-71.
15 Hadden JW. Immunodeficiency and cancer: prospects for correction. Int Immunopharmacol 2003;3: 1061-71.
16 Herber DL, Nagaraj S, Djeu JY, Gabrilovich DI. Mechanism and therapeutic reversal of immune suppression in cancer. Cancer Res 2007;67: 5067-9.
17 Kotler DP. Cachexia. Ann Intern Med 2000;133: 622-34.
18 Senesse P, Assenat E, Schneider S, Chargari C, Magne N, Azria D, et al. Nutritional support during oncologic treatment of patients with gastrointestinal cancer: who could benefit? Cancer Treat Rev 2008;34: 568-75.
19 Muscaritoli M, Costelli P, Aversa Z, Bonetto A, Baccino FM, Rossi Fanelli F. New strategies to overcome cancer cachexia: from molecular mechanisms to the 'Parallel Pathway'. Asia Pac J Clin Nutr 2008;17 Suppl 1: 387-90.
20 Faber J, Vos AP, Kegler D, Argiles J, Laviano A, Garssen J, et al. Impaired immune function: an early marker for cancer cachexia. Oncol Rep 2009;22: 1403-6.
21 Faber J, Vos P, Kegler D, van Norren K, Argiles JM, Laviano A, et al. Beneficial immune modulatory effects of a specific nutritional combination in a murine model for cancer cachexia. Br J Cancer 2008;99: 2029-36.
22 van Norren K, Kegler D, Argiles JM, Luiking Y, Gorselink M, Laviano A, et al. Dietary supplementation with a specific combination of high protein, leucine, and fish oil improves muscle function and daily activity in tumour-bearing cachectic mice. Br J Cancer 2009;100: 713-22.
23 US Food and Drug Administration. Frequently Asked Questions About Medical Foods. In: College Park MF, Center for Food Safety and Applied Nutrition, US Department of Health and Human Services, editor.; 2007.
24 Siewert JR, Stein HJ. Classification of adenocarcinoma of the oesophagogastric junction. Br J Surg 1998;85: 1457-9.
25 Albers R, Antoine JM, Bourdet-Sicard R, Calder PC, Gleeson M, Lesourd B, et al. Markers to measure immunomodulation in human nutrition intervention studies. Br J Nutr 2005;94: 452-81.
26 Lamers HJ, Gratama JW, van Putten WL, Stoter G, Bolhuis RL. Exogenous interleukin 2 recruits in vitro lymphokine-activated killer activity by in vivo activated lymphocytes. Cancer Res 1991;51: 2324-8.
27 Lamers CH, Gratama JW, Pouw NM, Langeveld SC, Krimpen BA, Kraan J, et al. Parallel detection of transduced T lymphocytes after immunogene therapy of renal cell cancer by flow cytometry and real-time polymerase chain reaction: implications for loss of transgene expression. Hum Gene Ther 2005;16: 1452-62.
28 Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol 1982;5: 649-55.
29 Knyrim K, Wagner HJ, Bethge N, Keymling M, Vakil N. A controlled trial of an expansile metal stent for palliation of esophageal obstruction due to inoperable cancer. N Engl J Med 1993;329: 1302-7.
30 Takagi K, Yamamori H, Furukawa K, Miyazaki M, Tashiro T. Perioperative supplementation of EPA reduces immunosuppression induced by postoperative chemoradiation therapy in patients with esophageal cancer. Nutrition 2001;17: 478-9.
31 Furukawa K, Tashiro T, Yamamori H, Takagi K, Morishima Y, Sugiura T, et al. Effects of soybean oil emulsion and eicosapentaenoic acid on stress response and immune function after a severely stressful operation. Ann Surg 1999;229: 255-61.
32 Sephton SE, Kraemer HC, Neri E, Stites DP, Weissbecker I, Spiegel D. Improving methods of assessing natural killer cell cytotoxicity. Int J Methods Psychiatr Res 2006;15: 12-21.

33 Serafini P. Editorial: PGE2-producing MDSC: a role in tumor progression? J Leukoc Biol;88: 827-9.

34 Wang D, Dubois RN. Eicosanoids and cancer. Nat Rev Cancer;10: 181-93.

35 Rodriguez-Vita J, Lawrence T. The resolution of inflammation and cancer. Cytokine Growth Factor Rev 2010;21: 61-5.

36 Shaikh SR, Edidin M. Polyunsaturated fatty acids and membrane organization: elucidating mechanisms to balance immunotherapy and susceptibility to infection. Chem Phys Lipids 2008;153: 24-33.

37 Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 2009;182: 4499-506.

38 Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol.

39 Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006;83: 1505S-19S.

40 Wu D, Meydani SN. n-3 polyunsaturated fatty acids and immune function. Proc Nutr Soc 1998;57: 503-9.

41 Faber J, Berkhout M, Vos AP, Sijben JW, Calder PC, Garssen J, et al. Supplementation with a Fish Oil-Enriched, High-Protein Medical Food Leads to Rapid Incorporation of EPA into White Blood Cells and Modulates Immune Responses within One Week in Healthy Men and Women. J Nutr 2011;141: 964-70.

42 Lomax AR, Calder PC. Prebiotics, immune function, infection and inflammation: a review of the evidence. Br J Nutr 2009;101: 633-58.

43 Vos A, M'Rabet L, Stahl B, Boehm G, Garssen J. Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. Crit Rev Immunol 2007;27: 97-140.

44 Deutz NE, Safar A, Schutzler S, Memelink R, Ferrando A, Spencer H, et al. Muscle protein synthesis in cancer patients can be stimulated with a specially formulated medical food. Clin Nutr.

45 McMillan DC. Systemic inflammation, nutritional status and survival in patients with cancer. Curr Opin Clin Nutr Metab Care 2009;12: 223-6.

46 Smith KL, Tisdale MJ. Mechanism of muscle protein degradation in cancer cachexia. Br J Cancer 1993;68: 314-8.

47 Saito T, Kuwahara A, Shigemitsu Y, Kinoshita T, Shimoda K, Miyahara M, et al. Factors related to malnutrition in patients with esophageal cancer. Nutrition 1991;7: 117-21.

48 Tashiro T, Yamamori H, Takagi K, Hayashi N, Furukawa K, Nitta H, et al. Changes in immune function following surgery for esophageal carcinoma. Nutrition 1999;15: 760-6.

49 Hori H, Kawano T, Endo M, Yuasa Y. Genetic polymorphisms of tobacco- and alcohol-related metabolizing enzymes and human esophageal squamous cell carcinoma susceptibility. J Clin Gastroenterol 1997;25: 568-75.

50 Goto S, Sato M, Kaneko R, Itoh M, Sato S, Takeuchi S. Analysis of Th1 and Th2 cytokine production by peripheral blood mononuclear cells as a parameter of immunological dysfunction in advanced cancer patients. Cancer Immunol Immunother 1999;48: 435-42.

CHAPTER SEVEN

Rapid incorporation of EPA and DHA into white blood cells and reduced serum PGE_2 levels after one week of nutritional intervention with a medical food in cancer patients receiving radiotherapy

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ABSTRACT

In cancer patients, metabolic alterations, reduced immune competence and anti-cancer treatment can increase the risk of (infectious) complications. A rapid-acting nutritional intervention might reduce this risk and support overall treatment. The present study investigated whether one week of intervention with a specific medical food led to rapid fatty acid incorporation and functional immunological changes in cancer patients.

In a randomized, double-blind, controlled study, 38 cancer patients receiving radiotherapy consumed daily for one week 400 ml of specific medical food, which is high in protein and leucine, and enriched with fish oil (providing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) and specific oligosaccharides (Active group), or iso-caloric/iso-nitrogenous product (Control group). The incorporation of phospholipid fatty acids and serum levels of inflammatory mediators were analyzed at day 0 and 7. The production of cytokines was measured in LPS-stimulated whole blood cultures.

The baseline percentage of EPA in white blood cells was comparable between the Active (0.4%) and Control group (0.6%); it increased significantly after one week in the Active (to 2.6%) compared to the Control group (to 1.0%, $p < 0.001$). For DPA and DHA, a similar increase in the Active group was observed ($p < 0.05$). Serum PGE_2 levels decreased in the Active group and increased in the Control group ($p < 0.01$). No differences were observed in LPS-stimulated cytokine production.

In cancer patients receiving radiotherapy, nutritional intervention with a specific medical food rapidly increased the percentage EPA and DHA in white blood cell phospholipids and reduced serum levels of the inflammatory mediator PGE_2 within one week.

INTRODUCTION

In many cancer patients, metabolic alterations lead to a chronic condition of catabolism, including involuntary weight loss, wasting, anorexia, asthenia and fatigue, resulting in a poor performance status and reduced quality of life (1-3). Other important features include the presence of a chronic inflammatory state and, paradoxically, a state of impaired immune responsiveness (4-7). Several mediators that are either tumor- or host-derived (e.g. pro-inflammatory cytokines, chemokines and prostaglandins) induce a cascade of events leading to a suppressed immune function, thereby reducing the acute response to infectious triggers and facilitating the escape of tumor cells from immune surveillance (8-10). The risk of immune deficiency is even higher after anti-cancer treatment. Surgery, radiotherapy and chemotherapy are associated with suppression of the cellular immune system and lead, in combination with malnutrition, to a reduced treatment efficacy and a higher frequency and severity of infectious and other complications (5, 11-16). In addition, anti-cancer treatment can lead to damage of the gastrointestinal (GI) mucosa, a diminished barrier function and a change in the intestinal microbiota contributing to local and systemic inflammation (17-20).

To reduce the risk of (infectious) complications and to support the performance status of cancer patients, a multi-targeted approach should be applied of which nutritional support is an integral part. Every effort should be made to prevent involuntary weight loss and delayed treatment schedules. In malnourished patients, pre-operative nutritional support is associated with a 50% reduction of post-operative complications (14). In patients receiving radiotherapy, nutritional supplementation resulted in reduced weight loss and fewer treatment interruptions due to a reduction in acute mucositis and/or maintenance of performance status (14, 21). Nutritional interventions have been recommended as an integral part of anti-cancer therapy to improve clinical outcomes and quality of life (5, 14, 22, 23). Cancer patients often start with an anti-cancer treatment soon after diagnosis and it is therefore of obvious clinical relevance to provide the optimal nutritional support as early as possible.

Immune modulatory effects of long-chain PUFA are well described. Both (n-6) and (n-3) PUFA play a major role in immune regulation and the balance between them may affect the development and severity of inflammatory diseases (24, 25). However, to modify cell function and obtain beneficial immune modulatory effects, EPA and DHA must be effectively incorporated into cell membrane phospholipids. In the literature, the majority of studies providing EPA and DHA examine fatty acid incorporation and immune modulatory activities after 4, 8 or 12 weeks of supplementation (26-29). In a previous study in healthy volunteers, however, nutritional intervention with a recently developed specific medical food* significantly increased the percentage of EPA into white blood cell (WBC) phospholipids within one week. Additionally, *ex vivo* immune responsiveness to LPS was increased significantly (30).The aim of the present study was, to investigate the rapid-acting effects of the medical food on the fatty acid incorporation into WBC phospholipids and the subsequent changes on parameters of immune function in cancer patients receiving radiotherapy. This medical food is high in protein and leucine and is enriched with emulsified fish oil (containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) and a specific oligosaccharide

mixture, to reduce the inflammatory state and support immune function in cancer patients, aiming to reduce complications and to provide optimal treatment support.

*A medical food is in the USA defined in 21 U.S.C. § 360ee(b)(3) as ''a food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognizable scientific principles, are established by medical evaluation'' (31). A comparable definition exists in the harmonized legislation of the European Union (cf. Article 1, 2(b) of Commission Directive 1999/21/EC of 25 March 1999 on dietary foods for special medical purposes).

METHODS

A randomized, controlled, double-blind study was conducted in order to investigate the incorporation of EPA and DHA into WBC phospholipids and to determine the effects on functional immune parameters after one week of nutritional intervention with a medical food in cancer patients receiving radiotherapy, compared with the effects of an iso-caloric and iso-nitrogenous control product. Secondary study objectives were to investigate the incorporation of EPA and DHA into red blood cell (RBC) phospholipids and plasma and to assess the effects of the medical food on inflammatory status. Moreover, nutritional status, safety and compliance were determined in these patients as well.

SUBJECTS

In the period between October 2009 and January 2010, 39 patients with histologically confirmed solid tumor(s), receiving radiotherapy, were recruited from the Department of Radiology, Division of Radiooncology, Freiburg University Hospital, Freiburg, Germany. Patients had an age of 18 years and above, a pathologically confirmed diagnosis of a solid tumor, a body mass index (BMI) between 18.5-30 kg/m^2, were willing and able to abstain from alcohol use, smoking, fish or fish oil-, vitamin-, herbal -or other oil-containing supplements and were included in the study after written informed consent was obtained. Exclusion criteria were a life expectancy less than 3 months, surgery, chemotherapy and/or hormone therapy in the previous 6 weeks, radiotherapy (before the current treatment cycles) in the previous 6 weeks, an Eastern Cooperative Oncology Group (ECOG) performance status of higher than 2, an altered immune function (e.g. caused by a major active infection, autoimmune disease, active allergy, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, or by use of medication such as immunosuppressive drugs, immunomodulators or systemic corticosteroids), a dependency on tube feed or parenteral nutrition during the previous 4 weeks, use of fish oil-, herbal -, or other oil-containing supplements during the previous 4 weeks, currently smoking or having smoked in the past 6 months, intolerance of or allergy to dairy products, fish or other ingredients of the study products, pregnancy or lactation, dementia or an altered mental status that would prohibit the understanding and giving of informed consent, any other medical condition that might interfere with the safety of the patient or the outcome parameters or uncertainty about the willingness or ability of the patient to comply with the protocol requirements, according to

the investigator's judgement. Patients were advised to use paracetamol if analgesics were required during the study period and not to use NSAIDs (including aspirin) in the 48 hours before their visits.

STUDY DESIGN

The study was conducted in compliance with the principles of the 'Declaration of Helsinki' (59th WMA General Assembly, Seoul, October 2008) according to the ICH-GCP guidelines and was approved by the Ethics Committee of the Albert Ludwigs University, Freiburg, Germany. After an initial pre-screening of patients undergoing out-patient radiotherapy by reviewing their electronic records, the remaining group was interviewed and patients fulfilling the selection criteria were screened and asked to participate in the study. Subject characteristics, relevant medical history and anthropometrics were determined at visit 1 (baseline). Patients were randomized to the Active group receiving the medical food or to the Control group receiving an iso-caloric Control product using a computerized randomization program. All subjects were asked to consume the study products for seven successive days in addition to their normal food, starting with one dose in the afternoon of day 0, 2 doses on days 2 to 6 and one dose in the morning of day 7.

The morning of day 0, before visit 1, all patients consumed one unit of iso-caloric Control product to minimize differences at baseline. At visit 1, before the first dose of the Active medical food or Control product and at visit 2 (day 7), after the morning dose of the Active medical food or Control product, body weight was measured and blood was drawn for the measurement of several immune, nutritional and safety parameters. The amount of study product taken was recorded daily in a diary by the patient. Additionally, compliance, the use of concomitant medication or nutritional supplements, the occurrence of adverse events and diary completion were monitored at day 7. Patients with an intake of <85% (less than 12 doses) of the minimum amount of 2 x 200 ml Active or Control product per day, or an intake of <2 of the 3 products on days 6 and 7, were considered as noncompliant and therefore as a protocol violation.

NUTRITIONAL INTERVENTION

The prescribed product intake during the study was 2 doses (2 x 200 ml sip feed) of either the Active medical food or the Control product daily. The Active medical food is an energy dense (163 kcal/100 ml), nutritionally complete oral supplement that is high in protein and leucine and is enriched with emulsified fish oil (providing 2.4 g EPA and 1.2 g DHA daily), specific oligosaccharides and a balanced mix of vitamins, minerals and trace elements (Table 1, Nutricia N.V., Zoetermeer, the Netherlands). The Control product is an energy dense (160 kcal/100 ml), iso-caloric and iso-nitrogenous, commercially available, standard nutritional product (Table 1, Fortisip Extra, Nutricia N.V., Zoetermeer, the Netherlands). All products were provided in white cartons (in two different flavours) to ensure blinding.

STUDY OUTCOME

The primary outcome parameters of the study were the percentages of EPA and DHA of total phospholipid fatty acids of WBC and the *ex vivo* LPS-stimulated cytokine and prostaglandin E_2 (PGE_2) production in whole blood after one week of intervention, as markers

Table 1 Nutritional composition of the Active medical food and Iso-caloric control product (Fortisip Extra) in grams per 100 ml

Ingredients	Active medical food	Iso-caloric control product
Macronutrients		
Energy (kJ/kcal)	**683/163**	**675/160**
Carbohydrates (g)	**17.4**	**18.1**
Protein (g)	**9.9**	**10.0**
- Whey (g)	3.2	2.0
- Casein (g)	5.6	8.0
- Added amino acids: free leucine (g)	1.1	0
Total fat (g)	**5.3**	**5.3**
- EPA (g)	0.6	0
- DHA (g)	0.3	0
Oligosaccharides (g)	**1.4**	**0**
- GOS (g)	1.2	0
- FOS (g)	0.2	0
Minerals & trace elements		
Sodium (mg)	**110**	**50.0**
Potassium (mg)	**215**	**220**
Chloride (mg)	**140**	**81.0**
Calcium (mg)	**147**	**280**
Phosphorus (mg)	**115**	**197**
Magnesium (mg)	**28.2**	**40.0**
Iron (mg)	**1.9**	**2.2**
Zinc (mg)	**2.1**	**2.4**
Copper (µg)	**288**	**338**
Manganese (mg)	**0.7**	**0.6**
Fluoride (mg)	**0.2**	**0.2**
Molybdenum (µg)	**16.0**	**19.0**
Selenium (µg)	**13.5**	**19.0**
Chromium (µg)	**11.0**	**13.0**
Iodine (µg)	**21.0**	**35.0**
Vitamins		
Vitamin A (µg-RE)	**130**	**188**
Vitamin D3 (µg)	**1.1**	**2.5**
Vitamin E (mg-α-TE)	**3.2**	**2.3**
Vitamin K (µg)	**8.5**	**10.0**
Thiamin (B1) (mg)	**0.2**	**0.3**
Riboflavin (B2) (mg)	**0.3**	**0.3**
Niacin (B3) (mg-NE)	**2.9**	**4.0**
Pantothenic acid (B5) (mg)	**0.9**	**1.0**
Vitamin B6 (mg)	**0.6**	**0.4**
Folic acid (µg)	**53.0**	**50.0**
Vitamin B12 (µg)	**0.6**	**0.7**
Biotin (µg)	**6.4**	**7.5**
Vitamin C (mg)	**21.0**	**19.0**
Carotenoids (mg)	**0.3**	**0**
Other		
L-Carnitine (mg)	**10.9**	**0**
Choline (mg)	**59.0**	**69.0**
Taurine (mg)	**13.3**	**0**

Values represent the amount of ingredients of the medical food in grams per 100 ml. Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoid acid; GOS, galactooligosaccharides; FOS, fructooligosaccharides.

for immune function (32). Secondary parameters were the percentages of other n-3 and n-6 poly-unsaturated fatty acids (PUFA) of total phospholipid fatty acids of WBC and RBC and plasma and serum levels of pro-inflammatory cytokines, C-reactive protein (CRP) and PGE_2 as markers for the inflammatory state of the patients.

WBC and RBC were isolated from heparinized blood using a density gradient and stored

at -80°C until use. In short, 30 ml heparin blood was diluted with 10 ml PBS (Gibco BRL, Life Technologies, Merelbeke, Belgium) + 2% heat-inactivated fetal calf serum (FCS^{hi}) (Hyclone, Perbio Science, Etten-Leur, the Netherlands) and divided over two Leucosep tubes pre-filled with Ficoll-Isopaque (Greiner Bio-One B.V., Alphen aan den Rijn, the Netherlands). Tubes were centrifuged for 10 min at 1000 g (RT, no brake), WBC were collected and washed with PBS + 2% FCS^{hi}. WBC were resuspended in cold culture medium (RPMI-1640 containing 25 mM HEPES and 2 mM L-glutamine; Life-Technologies, enriched with 100 kU/l penicillin/streptomycin) with 10 % FCS^{hi}, counted and centrifuged for 10 min. at 17000 g (RT). WBC pellets were stored at -80°C until analysis. RBC were collected after gradient centrifugation and stored at -80°C until analysis. Plasma was obtained by centrifugation of 5 ml heparinized blood for 5 min at 1300 g (RT) and stored at -80°C until analysis. Phospholipid fatty acids of WBC, RBC and plasma were analyzed by gas chromatography as described before (33).

The whole blood assay was performed by adding 100 µl/well blood to 50 µl/well culture medium in a 96-well plate (flat-bottom, polystyrene, BD Falcon Erembodegem Aalst, Belgium). Blood was subsequently incubated with 50 µl/well LPS (final concentration 100 µg/L, *E.coli*, B55:O55, Sigma-Aldrich Chemie, Steinheim, Germany) or culture medium (control) for 20 h at 37°C in a humidified environment containing 5% CO_2. Afterwards, plates were centrifuged for 5 min at 250 g (RT) and supernatants were harvested and stored at –80°C until analysis. Serum was obtained from blood collected in serum tubes (clotting tubes), which were incubated for 2 hours at RT. Afterwards, blood was centrifuged for 10 min at 1300 g (RT) and serum was stored at –80°C until analysis. Cytokine production (interleukin (IL)-8, IL-1β, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and IL-10) was measured using a Bio-Plex Cytokine bead immunoassay (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's protocol. PGE_2 was measured using a commercial enzyme immunoassay (Biotrak Amersham, Buckinghamshire, UK) according to the manufacturer's protocol.

In addition, the following parameters were determined: white and red blood cell count and differential, hemoglobin, pre-albumin, albumin, and calcium and also safety parameters for liver function (ALAT and ASAT), kidney function (creatinine) and partial thromboplastin time were measured at the Tumor Biology Center, Freiburg, Germany.

STATISTICAL ANALYSIS

The primary parameters have not been reported in cancer patients receiving radiotherapy before. Therefore, the expected difference in the percentage of EPA in plasma phospholipids between the Active and Control group and its variance was estimated based on three studies in healthy volunteers (29, 30, 34). Group size was based on the expected difference of the LPS-stimulated cytokine production in whole blood cultures between the Active and Control group. Its variance was estimated based on one study in healthy volunteers (30). With a significance level (α) of 0.05, a standard deviation of 24% and a power of 80%, a sample size of 13 per group would allow for a statistically significant difference after one week of nutritional intervention. Taking into account a drop-out rate of 20% and the possible effects of the Control sip feed, a sample size of 18 for each of the two groups would be sufficient to detect a statistically significant result between the groups.

All subjects who received the study products were included in the intention-to-treat (ITT) analysis. For baseline comparisons and efficacy analysis, the differences between the Active and Control group were determined. The LPS-stimulated data were corrected for the non-stimulated measurements by subtraction of the latter (resulting negative values were cut off at zero). ANOVA with treatment as covariate was used to analyze the measurement of the study parameters. When the data were not normally distributed, the Mann Whitney U test was used. For the nominal variables a Fisher's exact test was used and for the ordinal variables the difference between the Active and Control group were compared with the Mann Whitney U test. All adverse events were assessed and for affected patients medical history and medication use were checked. The statistical analyses were performed using SAS version 9.1.3.

RESULTS

STUDY POPULATION AND COMPLIANCE

In total 534 subjects, scheduled to undergo out-patient radiotherapy, were pre-screened in the study by reviewing their electronic records. Afterwards, 349 patients were interviewed and of the 45 subjects that were screened in the study, 39 subjects were randomized and 38 subjects received the study products (Figure 1), since 1 subject withdrew shortly after signing the informed consent form. Patients failed to be included in the study due to disinterest (n=108), appointment time for radiotherapy unsuitable to process blood samples (n=81), smoking (n=57), cancer diagnosis within last 3 months (n=54), NSAID use (n=46), concomitant chemotherapy (n=29), obesity (n=21), surgery within past 6 weeks (n=20), no solid tumor (n=20), inability to swallow (n=19), use of oral nutritional supplements (n=17), language difficulties (n=16), radiotherapy completed within less than 5 days (n=16), and other causes (n=37).

Of the 38 subjects that received the study products, 20 were allocated to the Active product and 18 to the Control product. These subjects were all included in the ITT analysis. A total of 2 subjects (1 subject in the Active group and 1 subject in the Control group) terminated the study early due to the development of nausea, resulting in no follow-up data. Of the remaining 36 subjects, 3 subjects violated the protocol (2 subjects in the Active group and 1 subject in the Control group) by non-compliance or the use of corticosteroids. The average compliance with consumption of the products was similar for both groups (92% for the Active product and 93% for the Control product).

BASELINE CHARACTERISTICS

The baseline characteristics of the patients are presented in Table 2. At baseline, the mean age of the total patient group was 62.7 ± 11.0 years with a body weight of 70.8 ± 12.6 kg. The Active and Control group matched well, with the only baseline difference being that patients in the Active group had lost weight in the past three months (-1.8% ± 4.8%) while patients in the Control group had gained in weight (1.5% ± 6.3%, $p < 0.01$). In 50% of the patients the tumor was located in the breast and in 26% of the patients the tumor was located in the prostate. Other locations of the tumor included the gynaecologic area (5.3%), urinary tract (5.3%), lungs (2.6%), head and neck (2.6%), esophagus (2.6%) and others (5.3%).

TNM stage ranged from I-IV, and was equally distributed among the study groups. Before radiotherapy, most patients had already received one or more previous treatments, including surgery (n=29), radiotherapy (n=1), chemotherapy (n=12) and hormone therapy (n=11), but no differences between the Active and the Control group were detected. The planned radiotherapy schedule of the patients was 6.3 ± 1.4 weeks with an average planned dose of 53.4 ± 16.2 Gy divided over a total of 28.7 ± 9.8 fractions, which was not different between the groups.

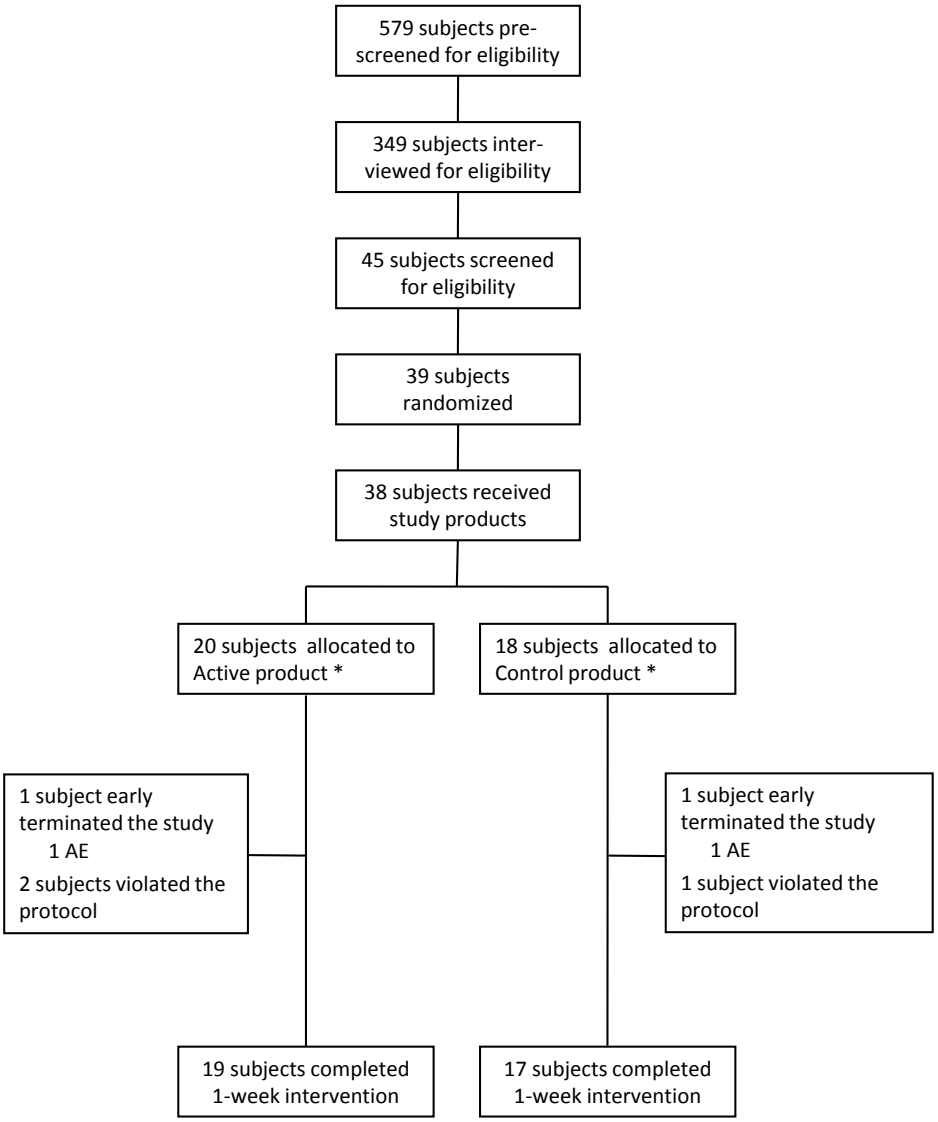

Figure 1 Trial profile; screening, randomization and study completion. * Included in ITT analysis.

Table 2 Baseline characteristics of the study groups

Variable	Presented as	Active (n=20)	Control (n=18)	Total (n=38)
Sex	n (%)			
Female		6 (30%)	8 (44%)	14 (37%)
Male		14 (70%)	10 (56%)	24 (63%)
Age (years)	mean ± SD	61.4 ± 13.1	64.3 ± 8.1	62.7 ± 11.0
BMI (kg/m^2)	mean ± SD	25.1 ± 3.3	24.6 ± 3.7	24.8 ± 3.5
Bodyweight (kg)	mean ± SD	70.8 ± 13.2	70.8 ± 12.3	70.8 ± 12.6
Body weight change in past 3 months (%)	mean ± SD	-1.8 ± 4.8[a]	1.5 ± 6.3	-0.2 ± 5.7
Time since diagnosis (months)	mean ± SD	8.0 ± 11.5	15.6 ± 40.9	11.6 ± 29.2
Tumor location (primary)	n (%)			
Lung		0 (0%)	1 (5.6%)	1 (2.6%)
Head and neck		1 (5.0%)	0 (0%)	1 (2.6%)
Gynaecologic		1 (5.0%)	1 (5.6%)	2 (5.3%)
Breast		11 (55%)	8 (44%)	19 (50%)
Prostate		5 (25%)	5 (28%)	10 (26%)
Urinary tract		1 (5.0%)	1 (5.6%)	2 (5.3%)
Oesophagus		0 (0%)	1 (5.6%)	1 (2.6%)
Other		1 (5.0%)	1 (5.6%)	2 (5.3%)
Staging (TNM stage)	n (%)			
I		8 (40%)	4 (22%)	12 (32%)
II		3 (15%)	5 (28%)	8 (21%)
III		4 (20%)	3 (17%)	7 (18%)
IV		4 (20%)	5 (28%)	9 (24%)
Other		1 (5.0%)	1 (5.6%)	2 (5.3%)
Previous treatment	n (%)			
Surgery		17 (85%)	12 (67%)	29 (76%)
Radiotherapy		0 (0%)	1 (5.6%)	1 (2.6%)
Chemotherapy		5 (25%)	7 (39%)	12 (32%)
Hormone therapy		7 (35%)	4 (22%)	11 (29%)
No treatment		1 (5.0%)	3 (17%)	4 (11%)
Curative		19 (100%)	12 (80%)	31 (91%)
Palliative		0 (0%)	3 (20.0%)	3 (8.8%)
Radiotherapy schedule	mean ± SD			
Number of weeks		6.4 ± 1.0	6.3 ± 1.7	6.3 ± 1.4
Planned dose (Gy)		54.0 ± 16.9	52.7 ± 15.9	53.4 ± 16.2
Number of fractions		31.0 ± 9.0	26.1 ± 10.3	28.7 ± 9.8
Dose per fraction (Gy)		1.9 ± 0.1	2.1 ± 0.4	2.0 ± 0.3
Curative	n (%)	20 (100%)[b]	14 (78%)	34 (89%)
Palliative		0 (0%)	4 (22%)	4 (11%)

Data represent the baseline characteristics as the number of subjects (n) and percentages or means ± SD of the Active group (n=20), the Control group (n=18) and the total patient group (n=38). a Significantly different from the Control group, $p < 0.01$ (Mann Whitney) b The distribution of patients over Curative and Palliative treatment is significantly different from the Control group, $p < 0.05$ (Fisher's exact). Abbreviations: BMI, body mass index; Gy, gray (unit of radiotherapy); TNM, tumor, node, metastasis.

EFFICACY

After one week of intervention, the incorporation of EPA, DPA and DHA into WBC phospholipids was significantly higher in the Active group compared to the Control group ($p < 0.001$, $p < 0.01$ and $p < 0.05$ for EPA, DPA and DHA, respectively, Figure 2). By contrast, the incorporation of arachidonic acid (AA) was significantly decreased in the Active group compared to an increase in the Control group ($p < 0.001$). The percentage of total n-3 PUFA showed a significantly higher increase in the Active group compared to the Control group, whereas the percentage of total n-6 PUFA in WBC phospholipids was decreased in the

Active group compared to the Control group (Table 3, $p < 0.001$), as was also observed for the ratio n-6/n-3 PUFA ($p < 0.001$). In RBC phospholipids, the EPA and DHA incorporation were increased in the Active group compared to a decrease in the Control group after the intervention ($p < 0.001$ and $p < 0.05$ for EPA and DHA, respectively, Table 3) but no effect of the intervention on DPA and AA was observed. In plasma, the percentages of EPA, DPA and DHA were significantly increased in the Active group compared to the Control group ($p < 0.001$), but no effect on AA was observed.

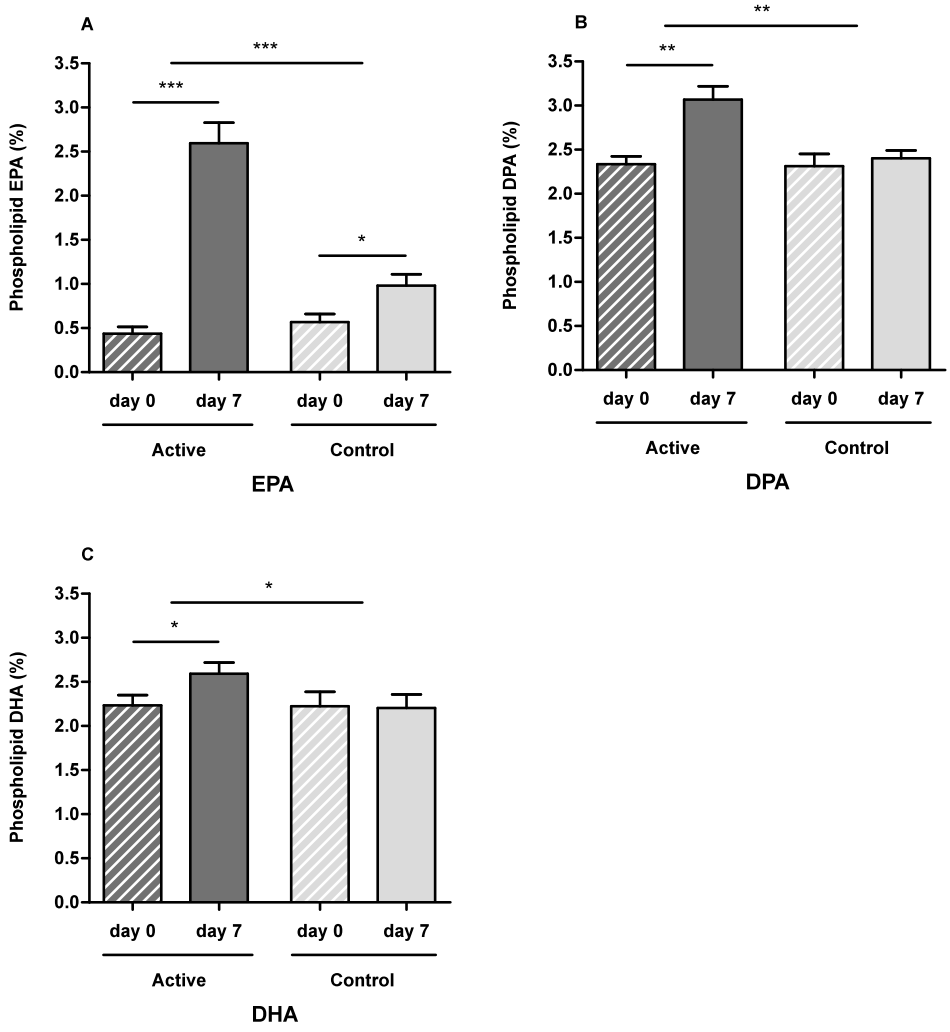

Figure 2 Incorporation of the (n-3) PUFAs EPA (A), DPA (B) and DHA (C) in WBC phospholipids of patients in the Active group (n=20) and Control group (n=18) presented as the percentage of total phospholipid fatty acids at day 0 and day 7. Data represent means (%) ± SEM. *** Incorporation at day 7 is significantly different from day 0 or the delta of d7-d0 in the Active group is significantly different from the Control group, $p < 0.001$ (ANOVA), ** Incorporation at day 7 is significantly different from day 0 or the delta of d7-d0 in the Active group is significantly different from the Control group, $p < 0.01$ (ANOVA),* Incorporation at day 7 is significantly different from day 0 or the delta of d7-d0 in the Active group is significantly different from the Control group, $p < 0.05$ (ANOVA). Abbreviations: FA, fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Table 3 Incorporation of EPA, DPA, DHA, AA, total n-3 fatty acids, total n-6 fatty acids and ratio total n-6/n-3 fatty acids of total phospholipid fatty acids in WBC, RBC and in plasma of patients in the Active group (n=20) and the Control group (n=18) presented as percentages at day 0 and day 7 or as the Δd7-d0.

WBC	Group	EPA	DPA	DHA	AA	Total n-3	Total n-6	n-6/n-3
Day 0	**Active**	0.4 ± 0.3	2.3 ± 0.4	2.2 ± 0.5	20.4 ± 1.0	5.2 ± 1.0	30.8 ± 1.9	6.1 ± 1.1
	Control	0.6 ± 0.4	2.3 ± 0.6	2.2 ± 0.7	19.3 ± 2.0	5.3 ± 1.1	29.4 ± 2.4	5.8 ± 1.0
Day 7	**Active**	2.6 ± 1.0^{A}	3.1 ± 0.7^{B}	2.6 ± 0.5^{C}	18.0 ± 1.9^{B}	8.4 ± 1.7^{A}	27.1 ± 2.2^{A}	3.4 ± 0.9^{A}
	Control	1.0 ± 0.5	2.4 ± 0.4	2.2 ± 0.6	19.8 ± 1.9	5.7 ± 1.0	30.3 ± 1.7	5.4 ± 0.9
Δd7-d0	**Active**	2.1 ± 1.0^{A}	0.7 ± 0.6^{B}	0.4 ± 0.4^{C}	-2.5 ± 2.2^{A}	3.1 ± 1.5^{A}	-3.8 ± 2.5^{A}	-2.7 ± 1.0^{A}
	Control	0.5 ± 0.7	0.0 ± 0.5	0.0 ± 0.5	0.3 ± 1.6	0.5 ± 1.2	0.8 ± 2.5	-0.4 ± 1.2

RBC	Group	EPA	DPA	DHA	AA	Total n-3	Total n-6	n-6/n-3
Day 0	**Active**	1.0 ± 0.3	2.3 ± 0.3	3.8 ± 0.7	11.8 ± 0.8	7.3 ± 1.1	28.9 ± 1.5^{D}	4.1 ± 0.7
	Control	1.1 ± 0.4	2.3 ± 0.4	3.7 ± 1.1	11.4 ± 1.0	7.3 ± 1.5	27.8 ± 1.4	4.0 ± 1.0
Day 7	**Active**	2.3 ± 0.5^{A}	2.3 ± 0.5	3.8 ± 1.0	11.0 ± 1.1	8.6 ± 1.7^{E}	26.6 ± 1.8	3.2 ± 0.6^{E}
	Control	0.9 ± 0.4	2.2 ± 0.6	3.5 ± 1.2	10.8 ± 2.5	6.7 ± 2.0	26.9 ± 5.5	4.4 ± 1.3
Δd7-d0	**Active**	1.3 ± 0.3^{A}	0.0 ± 0.4	0.1 ± 0.6^{D}	-0.8 ± 1.0	1.3 ± 1.2^{E}	-2.4 ± 1.9^{E}	-0.9 ± 0.4^{E}
	Control	-0.2 ± 0.2	-0.1 ± 0.5	-0.2 ± 0.6	-0.6 ± 2.5	-0.6 ± 1.3	-0.9 ± 5.7	0.4 ± 0.7

Plasma	Group	EPA	DPA	DHA	AA	Total n-3	Total n-6	n-6/n-3
Day 0	**Active**	1.1 ± 0.4	1.0 ± 0.2	3.3 ± 0.9	9.7 ± 1.0	5.6 ± 1.2	33.7 ± 2.2	6.3 ± 1.5
	Control	1.0 ± 0.4	1.0 ± 0.2	3.1 ± 0.9	9.6 ± 1.6	5.5 ± 1.2	32.7 ± 1.5	6.3 ± 1.8
Day 7	**Active**	5.6 ± 0.9^{A}	1.7 ± 0.3^{A}	4.9 ± 0.7^{A}	9.2 ± 1.2	12.5 ± 1.4^{A}	28.0 ± 1.6^{A}	2.3 ± 0.3^{A}
	Control	1.0 ± 0.4	1.0 ± 0.2	3.2 ± 1.0	9.4 ± 1.7	5.5 ± 1.3	33.8 ± 2.3	6.6 ± 2.2
Δd7-d0	**Active**	4.6 ± 0.8^{A}	0.7 ± 0.2^{A}	1.7 ± 0.5^{A}	-0.4 ± 0.8	6.9 ± 1.1^{A}	-5.9 ± 2.0^{A}	-4.1 ± 1.4^{A}
	Control	0.0 ± 0.4	-0.0 ± 0.1	0.0 ± 0.7	-0.3 ± 0.9	0.0 ± 1.0	1.0 ± 2.0	0.3 ± 1.1

Data represent means (%) ± SD. ^{A} Significantly different from Control $p < 0.001$ (ANOVA), ^{B} significantly different from Control $p < 0.01$ (ANOVA), ^{C} significantly different from Control $p < 0.05$ (ANOVA). D significantly different from Control $p < 0.05$ (Mann-Whitney). E significantly different from Control $p < 0.001$ (Mann-Whitney). Abbreviations: EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid.

The production of pro-inflammatory cytokines (IL-8, IL-1β, IL-6, TNF-α, IFN-γ and IL-10) and PGE_2 was measured in LPS-stimulated whole blood (Table 4). A good stimulation response to LPS was observed for all the mediators at the different time points. However, levels of IL-6 were above the detection limit of the assay and therefore not measurable. After one week of nutritional intervention no significant differences between the Active group and Control group were observed for any of the pro-inflammatory mediators.

Table 4 Production of cytokines and PGE_2 in LPS-stimulated whole blood of patients in the Active group (n=19) and the Control group (n=17) at day 0 and day 7 and calculated as the Δd7-d0.

	Group	IL-8	IL-1β	TNF-α	IFN-γ	IL-10	PGE_2
Day 0	Active	2121 (1378-2578)	1256 (943-2120)	3166 (2479-5940)	822 (636-965)	531 (334-700)	1422 (815-4915)
	Control	2271 (1692-2964)	1277 (821-1759)	3550 (2637-4909)	816 (689-1049)	395 (303-630)	761 (388-1186)
Day 7	Active	1640 (1179-2341)	1408 (957-1906)	3996 (2644-5084)	679 (533-831)	638 (435-689)	1107 (608-3482)
	Control	2324 (1800-3065)	1263 (672-1784)	3616 (1850-6100)	696 (335-1187)	545 (289-655)	742 (395-1575)
Δd7-d0	Active	-196 (-719-264)	83.8 (-162-292)	339 (-749-921)	-45.5 (-284-48.2)	75.3 (-43.0-164)	-206 (-2000-636)
	Control	68.5 (-505-487)	88.2 (-277-354)	-282 (-892-380)	0.2 (-191-213)	35.1 (-54.0-214)	-43.6 (-350-606)

Data represent medians (pg/ml) and interquartile ranges (25th-75th percentiles). Abbreviations: IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; PGE_2, prostaglandin E_2.

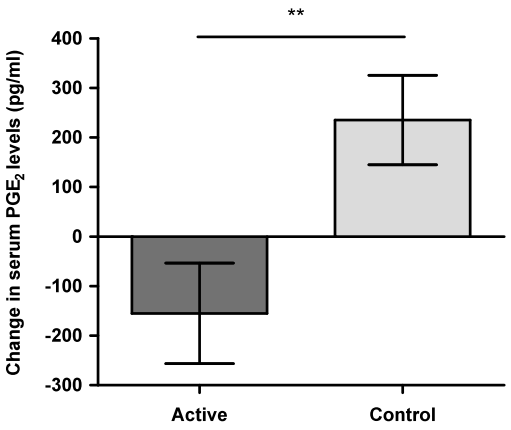

Figure 3 Change in serum PGE_2 levels (pg/ml) of patients in the Active group (n=19) and Control group (n=16). Data are presented as the delta of d7-d0 in means ± SEM. ** Significant difference between the Active and the Control group, $p < 0.01$ (ANOVA).

Serum concentrations of pro-inflammatory cytokines were relatively low, with most levels below or just above the detection limit of the assay. Serum levels of only IL-6 and IL-8 could be measured properly, with levels of 0.99 pg/ml and 4.23 pg/ml in the Active group and 0.75 pg/ml and 3.47 pg/ml in the Control group at day 0 and levels of 0.90 pg/ml and 4.33 pg/ml in the Active group and 1.01 pg/ml and 4.77 pg/ml in the Control group at day 7. No significant differences were observed between the Active and Control group. By contrast, serum PGE_2 levels were decreased from 627 pg/ml at day 0 to 463 pg/ml at day 7 in the Active group and increased from 433 pg/ml at day 0 to 736 pg/ml at day 7 in the Control group. The change (delta d7-d0) was significantly different in the Active group (-78 pg/ml) and the Control group (202 pg/ml, $p < 0.01$, Figure 3). By contrast, serum CRP levels were relatively low and no differences were observed between the Active and Control group.

No differences were observed between the Active group and Control group in total and differential blood cell count (leukocytes, neutrophils, monocytes, lymphocytes, basophils and eosinophils) at baseline and after one week (Table 5).

Table 5 White blood cell counts in blood of patients in the Active group (n=20) and the Control group (n=18) at day 0 and day 7 and calculated as the Δd7-d0.

	Group	Leucocytes	Neutrophils	Monocytes	Lymphocytes	Basophils	Eosinophils
Day 0	**Active**	6.80 ± 2.12	4.83 ± 2.05	0.64 ± 0.20	1.03 ± 0.52	0.05 ± 0.04	0.24 ± 0.23
	Control	5.66 ± 1.24	3.73 ± 1.19	0.54 ± 0.16	1.13 ± 0.65	0.05 ± 0.03	0.23 ± 0.19
Day 7	**Active**	5.83 ± 1.84	3.95 ± 1.49	0.62 ± 0.23	0.98 ± 0.45	0.07 ± 0.04	0.23 ± 0.13
	Control	5.62 ± 1.23	3.71 ± 1.18	0.56 ± 0.14	1.08 ± 0.56	0.06 ± 0.03	0.21 ± 0.19
Δd7-d0	**Active**	-0.86 ± 1.88	-0.84 ± 1.80	-0.01 ± 0.17	-0.05 ± 0.22	0.01 ± 0.04	0.03 ± 0.12
	Control	-0.04 ± 0.58	-0.01 ± 0.65	0.04 ± 0.13	-0.09 ± 0.28	0.01 ± 0.03	-0.01 ± 0.08

Data represent means (x 10^9 cells/L) ± SD.

As already mentioned, the body weight change in the previous 3 months was significantly different between the Active and Control group, however body weight at baseline was not different between the groups. After one week of nutritional intervention, body weight increased from 73.4 kg at day 0 to 73.9 kg at day 7 in the Active group and decreased from 72.8 kg at day 0 to 71.7 kg at day 7 in the Control group, but there was no significant difference between groups. Furthermore, no significant differences between the Active and Control group were observed in serum concentrations of pre-albumin, albumin and total calcium.

SAFETY AND TOLERABILITY

A total of 32 adverse events (AEs) were reported, 19 in the Active group (in 13 patients) and 13 in the Control group (in 10 patients). No serious adverse events were reported. The number of patients with at least one AE was not different between the Active and Control group. Most adverse events were gastrointestinal with flatulence, constipation and eructation most frequently observed in the Active group and diarrhea, flatulence, nausea and gastroesophageal reflux most frequently observed in the Control group. Blood safety parameters all remained within the respective reference ranges and no clinically relevant changes in liver and kidney function or in partial thromboplastin time were observed (data not shown).

DISCUSSION

In the literature, incorporation of EPA into WBC is generally described after a 4-, 8-, or even 12-week intervention period (35), reaching levels of 2.5% of total lipids after 4 weeks of supplementation with fish oil providing 2.1 g EPA and 1.1 g DHA per day (36).

Interestingly, in a previous study in healthy volunteers, incorporation of EPA into WBC phospholipids was demonstrated within one week of nutritional intervention with the medical food (30). Additionally, immune modulatory effects were observed by the increased production of pro-inflammatory cytokines in LPS-stimulated whole blood cultures within one week of nutritional intervention. Comparably, in this double-blind, randomized, controlled study in cancer patients receiving radiotherapy, a rapid incorporation of EPA and DHA into phospholipids of WBC, RBC and plasma was observed after one week of intervention with the fish oil-enriched medical food compared to an iso-caloric and iso-nitrogenous Control product. Moreover, serum levels of the inflammatory mediator PGE_2 were significantly decreased in the Active medical food group compared to an increase in the Control group, whereas no effects were observed on the production of pro-inflammatory cytokines in LPS-stimulated whole blood cultures.

Cancer patients often suffer from a severe systemic inflammatory state that accounts for the production of chemokines, pro-inflammatory cytokines, prostaglandins and reactive oxygen/nitrogen species, inducing a cascade of events leading to a suppressed immune function (5, 10, 37, 38). Prostaglandin E_2 (PGE_2) is a major inflammatory and immune suppressive mediator and is described to play a role in various human malignancies, including colon, lung, breast and head and neck cancer, and is often associated with a poor prognosis (8). It is produced by different types of cancer cells and their surrounding cells during the course of inflammation in response to growth factors, hormones and inflammatory cytokines (8, 39, 40). PGE_2 can act by regulating cell proliferation, apoptosis, migration and invasion of tumor cells, by secretion of growth factors, pro-inflammatory mediators and angiogenic factors or it can act as a chemotactic factor for myeloid derived suppressor cells (MDSCs), which have been demonstrated to inhibit immune surveillance and to be potent suppressors of anti-tumor immunity (8, 9, 39, 41).

In cancer patients receiving anti-cancer treatment, inflammatory and immune suppressive effects were even more pronounced. Surgery, radiotherapy and chemotherapy are associated with suppression of the cellular immune system and lead, in combination with malnutrition, to a reduced treatment efficacy and a higher frequency and severity of (infectious) complications (5, 11-14). Moreover, in patients receiving radiotherapy, the radiation exposure is associated with an increase in eicosanoid production, in which PGE_2 is predominantly detected. Moreover, inhibition of these prostaglandins can potentiate the anti-tumor effects of radiotherapy, with a main role for COX-2 being described (42). On that account a reduction in PGE_2 levels, as observed in the present study, might be beneficial for patients receiving radiotherapy, to reduce the inflammatory state, improve immune responsiveness and reduce infections, and possibly potentiate the anti-tumor effects of the radiation. Each of the product features (high protein, leucine, fish oil and specific oligosaccharides) may have played a role in this process, but based on preclinical studies, overlapping biological activities and synergistic interactions between these ingredients are hypothesized to lead to the overall effect (33, 43, 44).

In contrast to serum levels of PGE_2, levels of other inflammatory markers were very low and around the detection limit of the assay, whilst before the start of the study a more severe inflammatory state of these patients was expected (38, 45). It appears that the

condition of the patients in this study is better than expected, which might also be expressed by a good immune responsiveness. A possible explanation for the low inflammatory state of the patients might be the fact that in total 76% of the patients had had a previous operation, which might indicate that the tumor is dissected and less inflammatory cytokines become systemically available. Moreover, the low levels of inflammatory mediators suggest the absence of a true catabolic and cachectic state of the patients, which is confirmed by the low amount of weight loss in the Active group (-1.8 ± 4.8 %) and even weight gain in the Control group (1.5 ± 6.3 %) in the previous 3 months. Weight changes did not reach significance after the short term nutritional intervention with the Active medical food, while in a previous study in esophageal cancer patients body weight was significantly increased after a period of 4 weeks of nutritional intervention with the Active medical food, compared to a Control product (submitted for publication).

In a study comparable to the present one, the effect of the Active medical food was investigated in cancer patients without any treatment, after one week of nutritional intervention. In the period between September 2009 and May 2010 31 patients were recruited from the Meander Medical Centre, Amersfoort, the Netherlands and from the Gelderse Vallei Hospital, Ede, the Netherlands. Patients in this study suffered from an advanced disease (mainly stage III and IV), being mainly lower GI cancer (n=13 of 31) and breast cancer (n=10 of 31) and were randomized to the Active (n= 16) and Control (n= 15) group. In total 13 of 31 patients received no treatment before the study, but with the exception of one patient, the rest of the patients (n=17) were treated with a palliative intention. This might indicate a severe illness, but even in these patients, serum levels of pro-inflammatory cytokines were very low and consequently, no differences between the Active and Control group were observed. In this patients group suffering from advanced cancer, a severe inflammatory state would be expected (38). However, only CRP levels were slightly higher (5 (2-13) mg/L, median (IQR)) compared to patients receiving radiotherapy in the previously described study (2 (1-6) mg/L, median (IQR)), which might be correlated with the palliative phase of these cancer patients (46). By contrast, body weight and BMI of patients without any treatment were even higher compared to the patients receiving radiotherapy in the previously described study. Moreover, serum levels of PGE_2 in the patients without any treatment were lower compared to the baseline levels of PGE_2 in patients receiving radiotherapy, which might be explained by the inclusion of mainly breast and prostate cancer patients in the latter, which is associated with a high COX-2 expression (47, 48). However, also in colorectal cancer patients enhanced levels of PGE_2 were demonstrated (49). Another explanation could be the radiotherapy treatment of the patients, which might lead to high serum levels of PGE_2 as well (50). In contrast to the study in patients receiving radiotherapy, no effects of the nutritional intervention were observed on serum PGE_2 levels after one week. This might be due to the relatively low levels in the patients without any treatment, since the rapid incorporation of EPA and DHA into phospholipids of WBC, RBC and plasma after the intervention was similar as in patients receiving radiotherapy. However, also the large variance within the relatively small patient groups in both studies might limit the ability to detect differences in the various immune parameters.

In conclusion, the present study demonstrates a rapid incorporation of EPA and DHA into WBC and a significant reduction of serum PGE_2 levels in cancer patients receiving

radiotherapy after a one week of nutritional intervention with a medical food, which is high in protein and leucine and enriched with emulsified fish oil (containing EPA and DHA) and a specific oligosaccharide mixture compared with the effects of an iso-caloric and iso-nitrogenous control product. Moreover, the medical food is well-appreciated with a high compliance rate of study product intake. No clinically relevant safety concerns were reported and no changes in blood safety parameters were measured. Consequently, these results show that nutritional intervention with the specific medical food may represent a new opportunity for applications in cancer patients being an integral part of disease management to provide optimal treatment support. Additional research is recommended to elucidate the potential immunological effects in different types and stages of cancer.

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REFERENCES

1 Nitenberg G, Raynard B. Nutritional support of the cancer patient: issues and dilemmas. Crit Rev Oncol Hematol 2000;34: 137-68.
2 Fearon KC. Cancer cachexia: developing multimodal therapy for a multidimensional problem. Eur J Cancer 2008;44: 1124-32.
3 Argiles JM. Cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2: S39-50.
4 Evans C, Dalgleish AG, Kumar D. Review article: immune suppression and colorectal cancer. Aliment Pharmacol Ther 2006;24: 1163-77.
5 Van Cutsem E, Arends J. The causes and consequences of cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2: S51-63.
6 Tan BH, Fearon KC. Cachexia: prevalence and impact in medicine. Curr Opin Clin Nutr Metab Care 2008;11: 400-7.
7 Ross JA, Fearon KC. Eicosanoid-dependent cancer cachexia and wasting. Curr Opin Clin Nutr Metab Care 2002;5: 241-8.
8 Wang D, Dubois RN. Eicosanoids and cancer. Nat Rev Cancer;10: 181-93.
9 Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 2009;182: 4499-506.
10 Ben-Baruch A. Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators. Semin Cancer Biol 2006;16: 38-52.
11 Kuderer NM, Dale DC, Crawford J, Cosler LE, Lyman GH. Mortality, morbidity, and cost associated with febrile neutropenia in adult cancer patients. Cancer 2006;106: 2258-66.
12 Whimbey E, Englund JA, Couch RB. Community respiratory virus infections in immunocompromised patients with cancer. Am J Med 1997;102: 10-8; discussion 25-6.
13 Hadden JW. Immunodeficiency and cancer: prospects for correction. Int Immunopharmacol 2003;3: 1061-71.
14 Senesse P, Assenat E, Schneider S, Chargari C, Magne N, Azria D, et al. Nutritional support during oncologic treatment of patients with gastrointestinal cancer: who could benefit? Cancer Treat Rev 2008;34: 568-75.
15 Steele TA. Chemotherapy-induced immunosuppression and reconstitution of immune function. Leuk Res 2002;26: 411-4.
16 Arends J. Metabolism in cancer patients. Anticancer Res 2010;30: 1863-8.
17 Stringer AM, Gibson RJ, Bowen JM, Keefe DM. Chemotherapy-induced modifications to gastrointestinal microflora: evidence and implications of change. Curr Drug Metab 2009;10: 79-83.
18 Keefe DM, Cummins AG, Dale BM, Kotasek D, Robb TA, Sage RE. Effect of high-dose chemotherapy on intestinal permeability in humans. Clin Sci (Lond) 1997;92: 385-9.
19 Gibson RJ, Stringer AM. Chemotherapy-induced diarrhoea. Curr Opin Support Palliat Care 2009;3: 31-5.
20 Wessner B, Strasser EM, Koitz N, Schmuckenschlager C, Unger-Manhart N, Roth E. Green tea polyphenol administration partly ameliorates chemotherapy-induced side effects in the small intestine of mice. J Nutr 2007;137: 634-40.
21 Garg S, Yoo J, Winquist E. Nutritional support for head and neck cancer patients receiving radiotherapy: a systematic review. Support Care Cancer;18: 667-77.
22 van Bokhorst-de van der Schueren MA. Nutritional support strategies for malnourished cancer patients. Eur J Oncol Nurs 2005;9 Suppl 2: S74-83.
23 Arends J, Bodoky G, Bozzetti F, Fearon K, Muscaritoli M, Selga G, et al. ESPEN Guidelines on Enteral Nutrition: Non-surgical oncology. Clin Nutr 2006;25: 245-59.
24 Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006;83: 1505S-19S.
25 Song C, Manku MS, Horrobin DF. Long-chain polyunsaturated fatty acids modulate interleukin-1beta-induced changes in behavior, monoaminergic neurotransmitters, and brain inflammation in rats. J Nutr 2008;138: 954-63.
26 Wallace FA, Miles EA, Calder PC. Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. Br J Nutr 2003;89: 679-89.
27 Harris WS, Pottala JV, Sands SA, Jones PG. Comparison of the effects of fish and fish-oil capsules on the n 3 fatty acid content of blood cells and plasma phospholipids. Am J Clin Nutr 2007;86: 1621-5.
28 Calder PC. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids 2007;77: 327-35.
29 Rees D, Miles EA, Banerjee T, Wells SJ, Roynette CE, Wahle KW, et al. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. Am J Clin Nutr 2006;83: 331-42.
30 Faber J, Berkhout M, Vos AP, Sijben JW, Calder PC, Garssen J, et al. Supplementation with a Fish Oil-Enriched, High-Protein Medical Food Leads to Rapid Incorporation of EPA into White Blood Cells and Modulates Immune Responses within One Week in Healthy Men and Women. J Nutr 2011;141: 964-70.
31 US Food and Drug Administration. Frequently Asked Questions About Medical Foods. In: College Park MF, Center for Food Safety and Applied Nutrition, US Department of Health and Human Services, editor.; 2007.

32 Albers R, Antoine JM, Bourdet-Sicard R, Calder PC, Gleeson M, Lesourd B, et al. Markers to measure immunomodulation in human nutrition intervention studies. Br J Nutr 2005;94: 452-81.

33 Faber J, Vos P, Kegler D, van Norren K, Argiles JM, Laviano A, et al. Beneficial immune modulatory effects of a specific nutritional combination in a murine model for cancer cachexia. Br J Cancer 2008;99: 2029-36.

34. Cao J, Schwichtenberg KA, Hanson NQ, Tsai MY. Incorporation and clearance of omega-3 fatty acids in erythrocyte membranes and plasma phospholipids. Clin Chem 2006;52: 2265-72.

35 Thies F, Nebe-von-Caron G, Powell JR, Yaqoob P, Newsholme EA, Calder PC. Dietary supplementation with gamma-linolenic acid or fish oil decreases T lymphocyte proliferation in healthy older humans. J Nutr 2001;131: 1918-27.

36 Yaqoob P, Pala HS, Cortina-Borja M, Newsholme EA, Calder PC. Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. Eur J Clin Invest 2000;30: 260-74.

37 Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420: 860-7.

38 Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454: 436-44.

39 Serafini P. Editorial: PGE2-producing MDSC: a role in tumor progression? J Leukoc Biol;88: 827-9.

40 Rodriguez-Vita J, Lawrence T. The resolution of inflammation and cancer. Cytokine Growth Factor Rev 2010;21: 61-5.

41 Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol.

42 Choy H, Milas L. Enhancing radiotherapy with cyclooxygenase-2 enzyme inhibitors: a rational advance? J Natl Cancer Inst 2003;95: 1440-52.

43 van Norren K, Kegler D, Argiles JM, Luiking Y, Gorselink M, Laviano A, et al. Dietary supplementation with a specific combination of high protein, leucine, and fish oil improves muscle function and daily activity in tumour-bearing cachectic mice. Br J Cancer 2009;100: 713-22.

44 Faber J, van Limpt K, Kegler D, Luiking Y, Garssen J, van Helvoort A, et al. Bacterial Translocation Is Reduced by a Specific Nutritional Combination in Mice with Chemotherapy-Induced Neutropenia. J Nutr 2011.

45 Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell;140: 883-99.

46 McMillan DC. Systemic inflammation, nutritional status and survival in patients with cancer. Curr Opin Clin Nutr Metab Care 2009;12: 223-6.

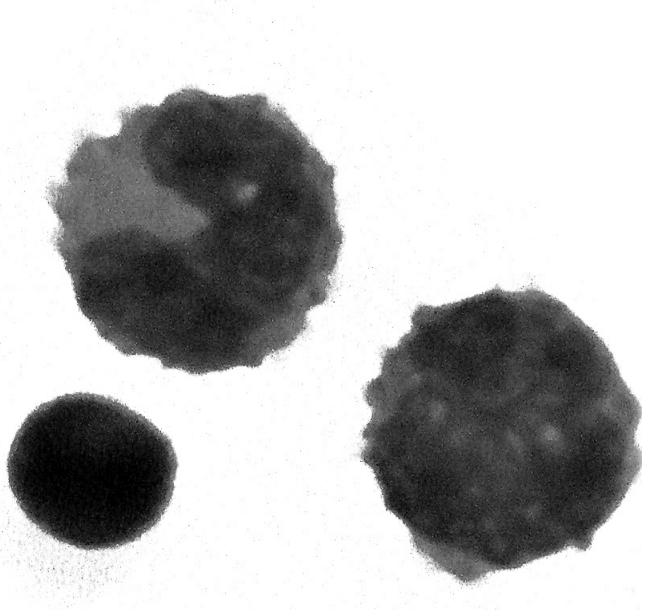
47 Dossus L, Kaaks R, Canzian F, Albanes D, Berndt SI, Boeing H, et al. PTGS2 and IL6 genetic variation and risk of breast and prostate cancer: results from the Breast and Prostate Cancer Cohort Consortium (BPC3). Carcinogenesis;31: 455-61.

48 Wild PJ, Kunz-Schughart LA, Bataille F, Simon R, Sauter G, Mihatsch M, et al. Strong COX-2 overexpression in breast and prostate cancer - a potential therapeutic target. Proceedings of the American Association for Cancer Research 2004;45.

49 Greenhough A, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, et al. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis 2009;30: 377-86.

50 Khayyal MT, El-Ghazaly MA, El-Hazek RM, Nada AS. The effects of celecoxib, a COX-2 selective inhibitor, on acute inflammation induced in irradiated rats. Inflammopharmacology 2009;17: 255-66.

CHAPTER EIGHT



Discussion and future perspectives

DISCUSSION

The results described in this thesis, obtained with the medical food concept in the preclinical experiments provide insights into the role of nutrition and are consistent and promising for translation to the human setting. In the colon-26 tumor model, immune function was already suppressed before the onset of weight loss (Chapter 2). This could be the result of a chronic inflammatory state recruiting different types of inflammatory cells including TAM and MDSC as has been shown by the $GR1^+$ and $F4/80^+$ cells in the spleens presented in Chapter 3. It might be that these GR^1+ and $F4/80^+$ cells, in Chapter 3 named granulocytes and macrophages/monocytes, are the immune suppressive TAM and MDSC, since they have the same receptors, however, this could not be confirmed since no other more specific markers of TAM and MDSC were measured.

Both TAM and MDSC exacerbate the inflammatory response at the tumor environment by the production of pro-inflammatory mediators as IL-6, TNF-α and PGE_2, as was also observed in Chapter 3, further driving forward the malignancy cascade. Moreover, MDSC are associated with a profound immune suppression in animals and many cancer patients (1, 2).

At this early stage of the disease, before weight loss is apparent, the specific nutritional combination significantly restored Th1 immune responses that were suppressed in the presence of the colon-26 tumor (Chapter 3). In cachectic mice, these effects on immune function were supported by the beneficial effects of the SNC on *ex vivo* ConA-stimulated T-cell responses in splenocytes and whole blood combined with a trend to an increased IL-1β, TNF-α and IL-6 production in LPS-stimulated whole blood. These findings indicate an enhanced capacity of immune cells to mount acute infection-like responses *ex vivo*. More-

over, the SNC reduced the plasma levels of pro-inflammatory mediators as IL-6, TNF-α and PGE_2 and inhibited wasting of protein and lipid stores, leading to less severe cachexia.

However, no effects of the SNC were observed on the $GR1^+$ and $F4/80^+$ cells, making the role of TAM and MDSC in the cachexia-induced inflammation and immune suppression less clear. Nevertheless, it can be concluded that the SNC has potential as immune-supporting nutritional intervention. Since immune function in tumor-bearing mice is already affected before the onset of weight loss, and with the assumption that this is also the case in the human setting, it is important to consider nutritional support with immune-modulating properties as early as possible in order to stop or reverse the nutritional decline, slow down the progression of cachexia and counteract dysfunction of the immune system to reduce the risk of (infectious) complications.

Translating the beneficial effects on immune function to a setting investigating the resistance to infections with a relevant living pathogen, the SNC significantly reduced the incidence and severity of *Pseudomonas aeruginosa* infections in a model for chemotherapy-induced neutropenia, dealing with severe immune suppression (Chapter 4)(3). No effects of the SNC on either neutrophil or lymphocyte count could be observed, similar to what was observed in the C26 model in Chapter 3. Nevertheless, the beneficial effects of the SNC on the immune system in both studies can be explained by a beneficial effect on the integrity of the intestinal mucosa, by decreasing the number of pathogenic bacteria and/or by improving immune cell function rather than the number of immune cells. This might be the result of the combination of specific oligosaccharides and fish oil, present in the SNC.

The non-digestible oligosaccharides are fermentable fibers with prebiotic properties that have been associated with immune modulatory effects and other health benefits, including an improved gut barrier function. They can modulate the immune system via a microbiota-dependent (prebiotic) mechanism, but they may also directly affect immune function by blocking or activating specific receptors on immune cells, leading to improved immune responses. Moreover, fish oil contains high amounts of the (n-3) PUFA EPA and DHA playing a major role in the regulation of immune responses and inflammation as well. Treatment with the SNC also improved body weight in Chapter 4. Although this may be related to the reduction in translocation and inflammatory mediators, the high levels of protein and the BCAA leucine may be involved as well, since these ingredients have contributed to the preservation of muscle mass and function, and significantly reduced weight loss in previous experiments, when combined with fish oil (4).

In Chapter 4, a model without the presence of a tumor was used, since adding this would complicate the model too much, making it difficult to control well. Nevertheless, the reduced incidence and severity of infections after intervention with the SNC seems promising for applications in human cancer patients, who are vulnerable for infections due to chemotherapy treatment or other reasons. It is important to gain insight into the relation between the tumor, the anti-cancer treatment (chemotherapy) and the nutritional ingredients both for safety reasons and efficacy reasons and to optimize the nutritional intervention with regard to dose, composition, schedule of administration, and target populations.

Before investigating the effects of the specific nutritional combination in cancer patients, the concept was tested in healthy volunteers, with the additional goal to investigate

the incorporation kinetics of EPA and DHA into white blood cell phospholipids within one week of intervention (Chapter 5). In literature, the majority of studies providing EPA and DHA examined fatty acid incorporation and immune modulatory activities after 4, 8 or 12 weeks of supplementation (5-8). For that reason, the results of the present study, demonstrating a very rapid incorporation of EPA into white blood cell phospholipids within one week of intervention with the medical food, may be beneficial for applications in cancer patients starting a treatment regimen soon after diagnosis, in order to reduce complications and provide optimal treatment support. Moreover, the rapid immune modulatory effects that were observed in the study have not, to the best of our knowledge, been described previously. The observed increased production of LPS-stimulated pro-inflammatory cytokines could be beneficial for an individual's ability to react to acute infectious triggers. However, others have interpreted enhanced LPS-stimulated cytokine responses as indicative of an elevated (chronic) inflammatory state (9, 10). Therefore, further translational research in cancer patients is recommended, to determine whether these immunological changes lead to functional benefits.

A next step in this process was described in Chapter 6, focusing on the effects of the medical food in cancer patients, frequently suffering from a severe inflammatory state and a reduced immune competence, factors that have been associated with the presence of cachexia (11-13). Malnutrition before the start of treatment has even been related to shorter survival in patients suffering from gastrointestinal cancers and head and neck cancers (14-16). Moreover, in malnourished patients, pre-operative nutritional support is associated with a 50% reduction of post-operative complications (17), including decreased gastrointestinal toxicity, improved performance status and increased immune responses (18).

For that reason, the selection of esophageal cancer patients in Chapter 6, was based on the fact that 57%-69% of these patients generally suffer from weight loss, depending on the stage of their disease. Moreover, recent data showed that 32% of the patients who had an esophagectomy experienced more than 10% weight loss preoperatively (19, 20). Accordingly, dietary intake, serum CRP concentrations and stage of the disease have been demonstrated as independent variables which have been associated with the degree of weight loss with an estimated effect of 38%, 34% and 28%, respectively (20-22). In addition to the high prevalence of malnutrition, esophageal cancer patients are frequently described to suffer from a suppressed immune function preoperatively (23-25). Nutritional intervention with an EPA-enriched concept might induce beneficial effects on parameters of immune function and inflammation around surgery (26-28). Moreover, the time period in esophageal cancer patients without other treatments as chemo-or radiotherapy before surgery is relatively long, enabling an intervention period of 4 weeks before treatment.

In the study described in Chapter 6, already at baseline, hardly any differences could be detected between healthy volunteers and the patient population on immune function or inflammation related parameters. It appears that the patients included in this study had a better immune function than expected, possibly due to the type of cancer or the early phase of patient inclusion, just after diagnosis. Moreover, the studies in esophageal cancer patients demonstrating severe malnutrition and reduced immune responsiveness, especially around major surgery were primarily performed in Japan, where the incidence

of squamous cell carcinoma of the esophagus is higher compared to adenocarcinoma (29), whereas in the present Dutch study adenocarcinoma of the esophagus is mostly observed. Accordingly, immune function of patients in the Dutch study might be less compromised compared to patients in the studies performed in Japan.

Nevertheless, baseline levels of IL-6, IL-1β and CRP were significantly higher in the total patient group compared to healthy volunteers. In the patient group, baseline CRP levels were significantly higher in group 2, compared to group 1, indicating a more severe inflammatory state of the patients in group 2, probably related to a higher weight loss in this group. However, after nutritional intervention, serum PGE_2 levels were significantly decreased in the medical food group and increased in the Control group. In addition, body weight increased significantly and ECOG performance status was significantly improved after intervention with the medical food. Accordingly, a relation between the improved body weight and reduced serum PGE_2 levels may exist via the reduction of (pre-) cachexia and inflammation, since body weight show a significant inverse correlation with serum PGE_2 levels in group 2 (p=0.02).

In cancer patients receiving anti-cancer treatment, the inflammatory and immune suppressive effects were expected to be even more pronounced, since surgery, radiotherapy and chemotherapy are associated with suppression of the cellular immune system and lead, in combination with malnutrition, to a reduced treatment efficacy and a higher frequency and severity of (infectious) complications (12, 17, 30-32). Moreover, in patients receiving radiotherapy (Chapter 7), the radiation exposure is associated with an increase in eicosanoid production, in which PGE_2 is predominantly detected (33). However, in contrast to serum levels of PGE_2, levels of other inflammatory markers were mostly below the detection limit of the assays used, whilst before the start of the study a more severe inflammatory state of these patients was expected (34, 35).

It appears that the condition of the patients in this study is better than expected, which might also be associated with a good immune responsiveness. A possible explanation for the low inflammatory state of the patients might be the fact that in total 76% of the patients had a previous operation, which might indicate that the tumor was resected, leading to lower levels of (tumor-induced) systemic inflammatory cytokines. Moreover, the low levels of inflammatory mediators suggest the absence of a true catabolic and cachectic state of the patients, which is confirmed by the minimal amount of weight loss in the Active group (-1.8 ± 4.8 %) and even weight gain in the Control group (1.5 ± 6.3 %) in the past 3 months. However, in a comparable study in patients without any treatment, patients suffered from advanced disease (mainly stage III and IV) and more than 50% of the patients were treated with a palliative intention. In this group of more severe patients, a major inflammatory state would be expected (34), but even in these patients, serum levels of pro-inflammatory cytokines were very low. Only serum CRP levels were slightly higher compared to patients receiving radiotherapy, which might be correlated to the palliative phase of the cancer patients without any treatment (36). However, serum levels of PGE_2 in patients without any treatment were lower compared to the baseline levels of PGE_2 in patients receiving radiotherapy and body weight and BMI of patients without any treatment were even higher compared to the patients receiving radiotherapy, which might be explained by the inclusion of

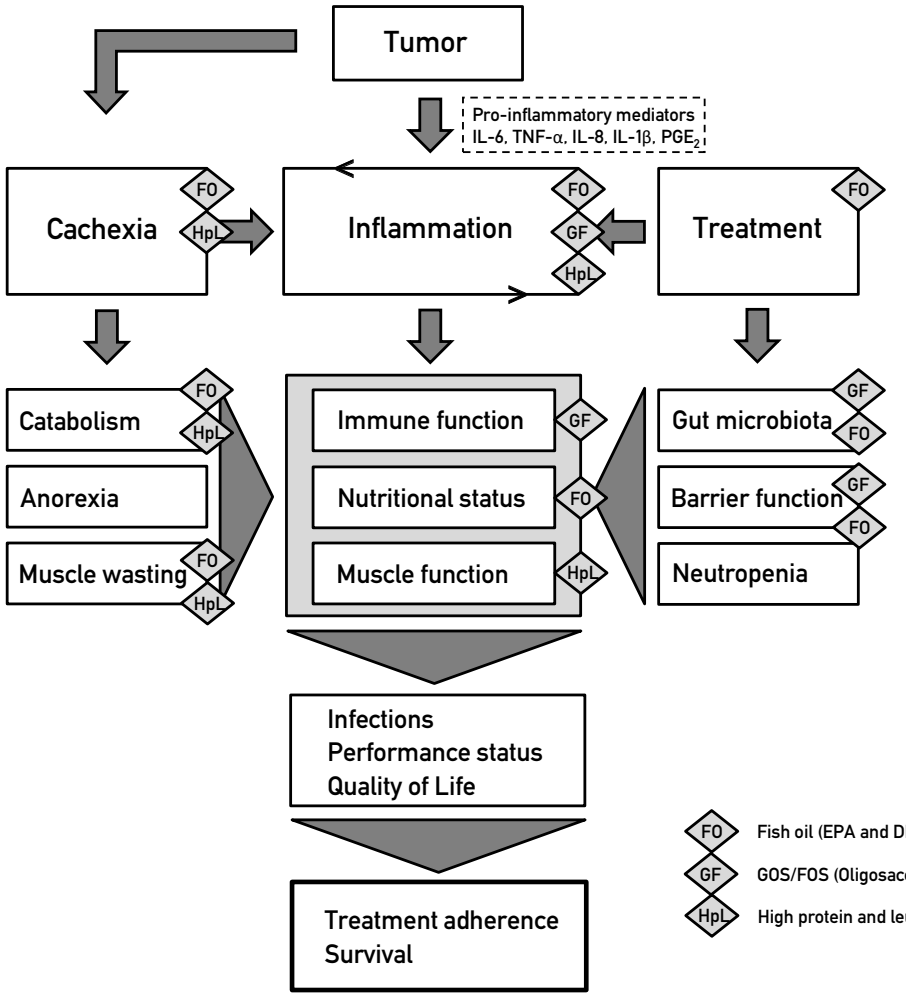


Figure 1 Schematic overview of several tumor-induced health aspects, in which inflammation play a central role leading to more infections, a reduced performance status and decreased QoL and eventually to a reduced treatment adherence and even to reduced survival of cancer patients. Moreover, targets and mechanisms of action of the ingredients of the specific medical food were presented.

mainly breast and prostate cancer patients in the latter (37, 38). Another explanation might be the radiotherapy treatment of the patients (39). However, the large variance within the relatively small patient groups in both studies limited the power of the studies to detect differences on the various immune parameters.

In general, it is known that inflammation plays a central role in the process of tumor-induced cachexia and treatment-related disorders. Each of the ingredients in the specific nutritional combination might play a specific role in this process, but overlapping biological activities and synergistic interactions between them eventually lead to a multi-target approach (Figure 1).

However, the beneficial effects of the specific nutritional combination on immune function and inflammation observed in the pre-clinical studies have only partly been demonstrated in a clinical setting in cancer patients. From the three clinical studies in several

types of cancer patients, it appears that these patients are in a better state, have a better immune competence and less inflammation than expected, based on several reports in literature. Serum levels of pro-inflammatory cytokines and CRP were very low and *ex vivo* ConA- or LPS-stimulated proliferation and cytokine production in PBMC was not changed between patients and healthy volunteers. Even when patients have lost 5-10% of their weight in the past 3 months due to esophageal cancer, as described in Chapter 6, their inflammatory state was not as high as expected and no apparent compromise in immune function was observed.

For that reason, the window for the nutritional intervention to lower inflammation or restore immune function was limited. These findings stress the need for a critical reevaluation of the role and timing of nutritional support during cancer in specific patient groups. Nevertheless, in both the study in esophageal cancer patients and the study in cancer patients receiving radiotherapy, serum PGE_2 levels were significantly reduced. Although the inflammatory state was not severe in these patient groups, this finding may still indicate a beneficial effect on the inflammatory state, difficult to quantify as it may be, that may lead to a clinical benefit. It is described that in patients receiving radiotherapy, the radiation exposure is associated with an increase in eicosanoid production, in which PGE_2 is predominantly detected (33).

On that account, inhibition of these prostaglandins can potentiate the anti-tumor effects of radiotherapy, with a main role for COX-2 being described (33). The effect on PGE_2 was accompanied by a significant increase in body weight and an improved ECOG performance status of esophageal cancer patients, which are important targets in cancer patients that may contribute to a better quality of life. In cancer patients receiving radiotherapy, the reduction in PGE_2 was accompanied by a rapid incorporation of EPA and DHA into white blood cells after one week of nutritional intervention. Since the medical food was well-appreciated and no clinically relevant safety concerns were reported, these results indicate that an approach to provide rapid nutritional support in cancer patients is feasible and safe.

FUTURE PERSPECTIVES

As a first step, it is essential to get more insight into the prevalence of cancer-specific problems in the different types of cancer. It appears that some cancer types have a higher incidence of specific characteristics, including weight loss, cachexia, inflammation, reduced immune function and performance status and decreased quality of life, than others. The stage of the disease, presence of a tumor and anti-cancer treatments may influence these conditions as well. For that reason, we have to look critically where to give nutritional support to which patient group and in what time frame. We have to think about the benefits we would like to achieve, the outcomes we have to measure and the patient groups we would like to target. Weight loss (especially lean body mass), infectious complications, treatment-related toxicities and reduced quality of life are interesting targets in patients suffering, for example, from head and neck cancers and colorectal cancers, whereas in patient suffering from breast cancer weight loss is less pronounced. Before as well as during chemotherapy and radiotherapy specific nutritional support can be beneficial providing optimal treatment support leading to improved clinical outcomes.

The timing of nutritional support, including the start of nutritional support as well as the duration of the intervention, is also a major factor, since the need for nutritional support is not the same on different time points during the course of cancer. For that reason we have to think about specific nutritional concepts for specific target groups, e.g. during chemotherapy, before or after surgery, or about a concept for a specific cancer type. We have to define a target population, map out their problems, adapt the medical food concept and make it attractive to achieve a high compliance and to obtain beneficial effects.
Small proof of concept studies in specific cancer types can be performed to gain insight into mechanisms, to clearly define target groups and to validate measurements before the implementation into larger studies. Moreover, these proof of concept studies could be used to scope small immune modulatory effects of a nutritional concept in this specific setting. Ideally, immune markers should correlate with clinically relevant outcomes and predict the resistance to infections and other illnesses associated with the dysregulation of the immune system. Moreover, it is important to know the association between changes in any given immune marker and predisposition to, or presence of a disease (40).

For that reason, it would be valuable to measure immune function by a kind of CHS or DTH like *in vivo* response to an immune challenge of some sort, as indicated a primary recommended marker for immune function. Moreover, vaccine-specific serum antibody production would be of high value as well. However, in cancer patients, it is very difficult to design a study in which a vaccination protocol is safe, ethical, well-tolerated and suiting in the treatment schedule of the patients, without interfering the study outcome. *Ex vivo* innate parameters, such as phagocyte function, NK-cell activity and APC function, would be of interest as well, but the variation in different groups of cancer patients is very high as was also observed in our studies.

To collect more evidence on the support of the immune system in severe affected and immune compromised cancer patients, upon the subtle effects found in the pilot studies, it is pivotal to perform future studies with a high number of patients in order to confirm the clinical benefits. Studies have to be randomized, controlled, multi-centered with clinical endpoint parameters such as (infectious) complications, antibiotic usage, treatment adherence and toxicities, since these endpoints can really affect patients outcome, recovery, and most importantly for the patients themselves, quality of life. In addition, health economics have to be determined in these studies as well, in order to find out whether the benefits of the concept will compensate for the potential higher costs. Moreover, besides effects on immune function, studies that focus on body weight and the loss of lean body mass can provide important insights, since significant effects on body weight and performance status have been observed with the specific nutritional combination. Loss of lean tissue, specifically skeletal muscle, may be the result of the tumor or a side-effect of chemotherapy or other drugs. Consequently, lean tissue loss in turn has important adverse implications for toxicity of anti-neoplastic therapy and, hence, cancer prognosis (41). Inflammatory markers may be coupled to body weight, lean tissue and performance status of the patients as well, since the improved body weight in esophageal cancer patients correlated to the reduction of the inflammatory markers PGE_2, as presented in this thesis.

Another option would be to offer a complete treatment support including physical activity

(PA). PA can be combined with a nutritional intervention in cancer patients, since PA is an important health behavior for the prevention and management of many acute and chronic diseases (42). PA is defined as any bodily movement produced by the skeletal muscles that results in a substantial increase in energy expenditure. Research to date suggest that PA may reduce the risk of developing cancer, help cancer patients to cope with and recover from treatments, improve the health of cancer patients, and possibly even reduce the risk of recurrence and extended survival in some cancer groups. Many mechanisms may be involved including insulin resistance, sex hormones, inflammation, immune function and vitamin D, and combined with the specific nutritional ingredients the effects might be even more pronounced (42). However, to elucidate the effect of PA in combination with a specific nutritional intervention, additional research is needed to investigate timing, dose and benefits for specific patient groups.

In conclusion, this thesis shows that nutritional intervention with a unique specific medical food may represent a new opportunity for applications in cancer patients being an integral part of disease management and enabling an early supportive care strategy. However, to demonstrate that these effects could maximize the chances of reducing disease progression, reducing the frequency and severity of complications and improving treatment adherence and quality of life, additional steps have to be taken to translate the preclinical findings to clinical practice. For that reason, additional research is necessary to elucidate the potential immunological effects of nutritional concepts in different types and stages of cancer, measured by clinically relevant endpoints, in order to optimize a powerful supportive care strategy in the future that leads to clinical benefits in cancer patients.

REFERENCES

1 Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res 2007;67: 10019-26.

2 Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest 2007;117: 1155-66.

3 Faber J, van Limpt K, Kegler D, Luiking Y, Garssen J, van Helvoort A, et al. Bacterial translocation is reduced by a specific nutritional combination in mice with chemotherapy-induced neutropenia. J Nutr 2011;141: 1292-8.

4 van Norren K, Kegler D, Argiles JM, Luiking Y, Gorselink M, Laviano A, et al. Dietary supplementation with a specific combination of high protein, leucine, and fish oil improves muscle function and daily activity in tumour-bearing cachectic mice. Br J Cancer 2009;100: 713-22.

5 Wallace FA, Miles EA, Calder PC. Comparison of the effects of linseed oil and different doses of fish oil on mono nuclear cell function in healthy human subjects. Br J Nutr 2003;89: 679-89.

6 Harris WS, Pottala JV, Sands SA, Jones PG. Comparison of the effects of fish and fish-oil capsules on the n 3 fatty acid content of blood cells and plasma phospholipids. Am J Clin Nutr 2007;86: 1621-5.

7 Calder PC. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids 2007;77: 327-35.

8 Rees D, Miles EA, Banerjee T, Wells SJ, Roynette CE, Wahle KW, et al. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. Am J Clin Nutr 2006;83: 331-42.

9 Wigmore SJ, Fearon KC, Maingay JP, Ross JA. Down-regulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. Clin Sci (Lond) 1997;92: 215-21.

10 Heimdal JH, Aarstad HJ, Klementsen B, Olofsson J. Ex vivo interleukin (IL)-1 beta, IL-6, IL-12 and tumor necrosis factor-alpha responsiveness with monocytes from patients with head and neck carcinoma. Eur Arch Otorhinolaryngol 1999;256: 250-6.

11 Ross JA, Fearon KC. Eicosanoid-dependent cancer cachexia and wasting. Curr Opin Clin Nutr Metab Care 2002;5: 241-8.

12 Van Cutsem E, Arends J. The causes and consequences of cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2: S51-63.

13 Evans C, Dalgleish AG, Kumar D. Review article: immune suppression and colorectal cancer. Aliment Pharmacol Ther 2006;24: 1163-77.

14 Andreyev HJ, Norman AR, Oates J, Cunningham D. Why do patients with weight loss have a worse outcome when undergoing chemotherapy for gastrointestinal malignancies? Eur J Cancer 1998;34: 503-9.

15 Rey-Ferro M, Castano R, Orozco O, Serna A, Moreno A. Nutritional and immunologic evaluation of patients with gastric cancer before and after surgery. Nutrition 1997;13: 878-81.

16 van Bokhorst-de van der S, van Leeuwen PA, Kuik DJ, Klop WM, Sauerwein HP, Snow GB, et al. The impact of nutritional status on the prognoses of patients with advanced head and neck cancer. Cancer 1999;86: 519-27.

17 Senesse P, Assenat E, Schneider S, Chargari C, Magne N, Azria D, et al. Nutritional support during oncologic treatment of patients with gastrointestinal cancer: who could benefit? Cancer Treat Rev 2008;34: 568-75.

18 van Bokhorst-de van der Schueren MA. Nutritional support strategies for malnourished cancer patients. Eur J Oncol Nurs 2005;9 Suppl 2: S74-83.

19 Bailey SH, Bull DA, Harpole DH, Rentz JJ, Neumayer LA, Pappas TN, et al. Outcomes after esophagectomy: a ten-year prospective cohort. Ann Thorac Surg 2003;75: 217-22; discussion 22.

20 Bozzetti F. Nutritional support in patients with oesophageal cancer. Supportive Care Cancer 2010;18: S41-S50.

21 Deans DA, Tan BH, Ross JA, Rose-Zerilli M, Wigmore SJ, Howell WM, et al. Cancer cachexia is associated with the IL10 -1082 gene promoter polymorphism in patients with gastroesophageal malignancy. Am J Clin Nutr 2009;89: 1164-72.

22 Deans DA, Tan BH, Wigmore SJ, Ross JA, de Beaux AC, Paterson-Brown S, et al. The influence of systemic in flammation, dietary intake and stage of disease on rate of weight loss in patients with gastro-oesophageal cancer. Br J Cancer 2009;100: 63-9.

23 Tsutsui S, Sonoda K, Sumiyoshi K, Kitamura K, Toh Y, Kitamura M, et al. Prognostic significance of immunological parameters in patients with esophageal cancer. Hepatogastroenterology 1996;43: 501-9.

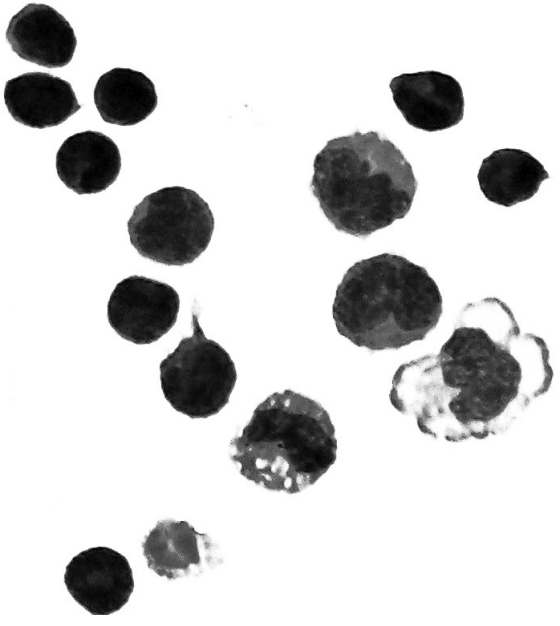
24 Takagi K, Yamamori H, Morishima Y, Toyoda Y, Nakajima N, Tashiro T. Preoperative immunosuppression: its relationship with high morbidity and mortality in patients receiving thoracic esophagectomy. Nutrition 2001;17: 13-7.

25 Tsutsui S, Morita M, Kuwano H, Matsuda H, Mori M, Okamura S, et al. Influence of preoperative treatment and surgical operation on immune function of patients with esophageal carcinoma. J Surg Oncol 1992;49: 176-81.

26 Ryan AM, Reynolds JV, Healy L, Byrne M, Moore J, Brannelly N, et al. Enteral nutrition enriched with eicosapentaenoic acid (EPA) preserves lean body mass following esophageal cancer surgery: results of a double-blinded randomized controlled trial. Ann Surg 2009;249: 355-63.

27 Aiko S, Yoshizumi Y, Tsuwano S, Shimanouchi M, Sugiura Y, Maehara T. The effects of immediate enteral feeding with a formula containing high levels of omega-3 fatty acids in patients after surgery for esophageal cancer. JPEN J Parenter Enteral Nutr 2005;29: 141-7.
28 Takagi K, Yamamori H, Furukawa K, Miyazaki M, Tashiro T. Perioperative supplementation of EPA reduces immunosuppression induced by postoperative chemoradiation therapy in patients with esophageal cancer. Nutrition 2001;17: 478-9.
29 Hori H, Kawano T, Endo M, Yuasa Y. Genetic polymorphisms of tobacco- and alcohol-related metabolizing enzymes and human esophageal squamous cell carcinoma susceptibility. J Clin Gastroenterol 1997;25: 568-75.
30 Kuderer NM, Dale DC, Crawford J, Cosler LE, Lyman GH. Mortality, morbidity, and cost associated with febrile neutropenia in adult cancer patients. Cancer 2006;106: 2258-66.
31 Whimbey E, Englund JA, Couch RB. Community respiratory virus infections in immunocompromised patients with cancer. Am J Med 1997;102: 10-8; discussion 25-6.
32 Hadden JW. Immunodeficiency and cancer: prospects for correction. Int Immunopharmacol 2003;3: 1061-71.
33 Choy H, Milas L. Enhancing radiotherapy with cyclooxygenase-2 enzyme inhibitors: a rational advance? J Natl Cancer Inst 2003;95: 1440-52.
34 Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454: 436-44.
35 Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140: 883-99.
36 McMillan DC. Systemic inflammation, nutritional status and survival in patients with cancer. Curr Opin Clin Nutr Metab Care 2009;12: 223-6.
37 Dossus L, Kaaks R, Canzian F, Albanes D, Berndt SI, Boeing H, et al. PTGS2 and IL6 genetic variation and risk of breast and prostate cancer: results from the Breast and Prostate Cancer Cohort Consortium (BPC3). Carcinogenesis 2010;31: 455-61.
38 Wild PJ, Kunz-Schughart LA, Bataille F, Simon R, Sauter G, Mihatsch M, et al. Strong COX-2 overexpression in breast and prostate cancer - a potential therapeutic target. Proceedings of the American Association for Cancer Research 2004;45.
39 Khayyal MT, El-Ghazaly MA, El-Hazek RM, Nada AS. The effects of celecoxib, a COX-2 selective inhibitor, on acute inflammation induced in irradiated rats. Inflammopharmacology 2009;17: 255-66.
40 Albers R, Antoine JM, Bourdet-Sicard R, Calder PC, Gleeson M, Lesourd B, et al. Markers to measure immuno modulation in human nutrition intervention studies. Br J Nutr 2005;94: 452-81.
41 Prado CM, Antoun S, Sawyer MB, Baracos VE. Two faces of drug therapy in cancer: drug-related lean tissue loss and its adverse consequences to survival and toxicity. Curr Opin Clin Nutr Metab Care 2011;14: 250-4.
42 Courneya KS, Friedenreich CM. Physical Activity and Cancer. Berlin Heidelberg: Springer; 2011.

CHAPTER NINE



Summary
Samenvatting
Affiliations
Curriculum Vitae
List of publications
Dankwoord

SUMMARY

Cancer is a collective name for more than a hundred distinct diseases and is an important cause of morbidity and mortality worldwide (Chapter 1). The development of cancer is a multi-factorial process. Genetic factors, as well as lifestyle factors are involved in this process. Ageing is another fundamental factor in the development of cancer. Cancer generates a complex set of conditions in which many aspects can affect the clinical course and prognosis of the disease, including the type and stage of the tumor, individual characteristics, anti-cancer treatment and presence of cancer cachexia.

Cachexia is a major problem in many cancer patients with a global incidence of malnutrition ranging from 30% to 90% during the course of cancer. The main characteristics of this chronic condition of catabolism include progressive weight loss, anorexia, wasting of muscle and adipose tissue, muscle weakness (asthenia) and fatigue. Tumor-derived factors, therapeutic strategies, but also nutritional status, age and even stress and depression are involved in this process, resulting in a chronic inflammatory state and paradoxically impaired immune responsiveness. Together, these factors may lead to disease progression, increased (infectious) complications and a delayed or suboptimal treatment regimen resulting in a reduced quality of life and a poor prognosis.

Accordingly, a multi-disciplinary approach, including nutritional support is recommended which is initiated at the moment of diagnosis and runs parallel to the pathway of anti-cancer therapies. Moreover, guidelines are published by the European Society for Clinical Nutrition and Metabolism (ESPEN) providing evidence-based information on nutritional support relating to specific problems like timing, dosing, composition and route of application. A first key step is to identify patients who might benefit by routine screening

using a thorough nutritional assessment by a dietician. Accordingly, nutritional support can be provided, tailored to patient's individual needs, medical condition and the current and expected side effects of treatment in order to prevent involuntary weight loss and delayed treatment schedules, and to improve clinical outcomes and quality of life.

For that reason, a specific nutritional combination has been developed for application in cancer patients. This concept is high in protein and leucine and is enriched with emulsified fish oil (containing EPA and DHA) and a specific oligosaccharide mixture and is designed to reduce complications and to provide optimal treatment support by reducing the inflammatory state, supporting immune function, and preserving muscle mass and function. In order to get insights into the efficacy of nutritional ingredients, a recently modified and validated animal model for cancer cachexia was used, based on the colon-26 tumor model, in which amongst others parameters of immune function were measured.

In Chapter 2, the appearance of immune suppression during the course of the cachexia process was studied by the measurement of contact hypersensitivity (CHS) towards oxazolone as a validated *in vivo* parameter for Th1-mediated immune function. CHS was measured at two time-points during the study to observe changes in the immune status in a pre-cachectic and a cachectic state of the mice. The results indicated that immune function was already suppressed before weight loss was apparent. For that reason, immune suppression was identified as a potential early risk factor for cachexia. This would argue for an early supportive care strategy in cancer patients including specific nutritional support. The consideration to provide nutritional support as early as possible, preferably starting at diagnosis and running parallel to the pathway of anti-cancer therapies might therefore be of high importance to support immune function and to reduce inflammation and other cachexia features.

To evaluate the potential benefits of specific nutritional support for immune competence, the effects of the individual ingredients as well as of the complete mixture of ingredients of the specific nutritional combination (SNC) were investigated on inflammatory status, immune function and on different biomarkers for cachexia in the colon-26 tumor model (Chapter 3). The addition of one of the individual nutritional ingredients to the diet did not result in any effect on bodyweight compared with animals in the tumor-bearing group. However, a diet containing the SNC improved bodyweight significantly, indicating a less cachectic state of the mice. This was emphasized by a positive effect on other cachectic features, such as a significant inhibition of weight loss of epididymus fat and skeletal muscles. Moreover, no effect of the individual ingredients on CHS was observed in tumor-bearing mice in a pre-cachectic state.

However, after administration of the complete mixture (SNC), immune responsiveness was increased significantly, indicating a better Th1-mediated immune response prior to weight loss. In addition, in mice already suffering from cachexia, the SNC reduced plasma levels of pro-inflammatory cytokines and beneficially affected *ex vivo* immune function representing a lower inflammatory state and better immune responses. Apparently, the combination of the nutritional ingredients is required to obtain a synergistic effect, leading to a reduced inflammatory state and improved immune competence in this early phase compared to an iso-caloric control diet.

To translate these beneficial immune modulatory effects to a setting investigating the resistance to infections with a relevant living pathogen, the effect of the SNC on *Pseudomonas aeruginosa* infections was studied in a murine model for chemotherapy-induced neutropenia, dealing with severe immune suppression (Chapter 4). This resembles the situation in patients suffering from chemotherapy-induced infectious complications, which have been associated with a higher morbidity and mortality during advanced disease. Infections were measured by the incidence and severity of bacterial translocation to liver and lungs and confirmed by plasma levels of pro-inflammatory cytokines.

After the chemotherapy treatment, the mice became severely neutropenic. However, the SNC did not affect the levels of the different blood cell types. Nevertheless, bodyweight growth in mice that received the SNC diet was already higher before the chemotherapy treatment compared to mice that received a Control diet, whereas food intake was not affected. After chemotherapy treatment, bodyweight declined, but was still significantly higher in the SNC group. Moreover, dietary intervention with the SNC significantly reduced the incidence and severity of *P. aeruginosa* infection by reducing the translocation to the liver. A similar trend was observed in the lungs.

In addition, the SNC reduced the fecal pH, which may at least partly explain the lower *P. aeruginosa* counts in fecal samples during the nutritional intervention phase of the experiment. Plasma levels of pro-inflammatory cytokines tended to be reduced and a strong correlation was observed with bacterial translocation to the liver. Several mechanisms might have played a role, including modulation of the intestinal microbiota, an improved gut barrier function, immune function, and a reduced inflammatory state. Translated to the human situation these results might be beneficial for patients suffering from a quiescent intestinal infection that after chemotherapy, surgery, or transplantation, leads to bacterial translocation and results in a severe sepsis.

The next step to a clinical application and validation in cancer patients was to study the efficacy as well as safety of the specific nutritional combination in a medical food in healthy volunteers. In this proof of concept study, twelve healthy men and women consumed the medical food for a period of one week as described in Chapter 5. Immune function was measured by the *ex vivo* LPS-stimulated cytokine production in whole blood cultures. An additional objective of this study was to investigate the incorporation kinetics of EPA and DHA (from fish-oil) into white blood cell phospholipids within one week of intervention, since a rapid and effective incorporation of these n-3 PUFA might be very important for cancer patients starting a treatment regimen soon after diagnosis.

After one day of nutritional intervention, the percentage of EPA in white blood cell (WBC) phospholipids increased significantly and rose even further towards the end of the week. No effect was observed on the percentage of DHA in WBC phospholipids after one week of nutritional supplementation, whereas the percentage of DPA (n-3) increased significantly and the percentage of AA was reduced from day two and onwards. Comparable results were obtained for the incorporation into plasma and red blood cell (RBC) phospholipids. Moreover, intervention with the medical food significantly enhanced the production of pro-inflammatory cytokines in LPS-stimulated whole blood cultures, indicating immune modulatory effects within one week of nutritional intervention, which might be beneficial for an

individual's ability to react to acute infectious triggers. These results confirm the potential for achieving rapid immune modulatory effects with the medical food in a clinical human setting.

In Chapter 6, the effect of a 4-week nutritional intervention with the medical food on immune function was investigated in an early phase in a group of sixty-four newly diagnosed esophageal cancer patients before the start of anti-cancer therapy. In this exploratory, randomized, double-blind study, the group receiving the specific medical food was compared with a Control group that received routine nutritional support. Immune function was measured by the *ex vivo* ConA- and LPS-stimulated proliferation and cytokine production in PBMC cultures. Secondary objectives were to assess the effects of the medical food on parameters of inflammation, nutritional status, product palatability and safety. Data on immune function, inflammation and nutritional status of healthy volunteers were obtained to compare baseline values and allow adequate interpretation of the data.

At baseline, no differences between healthy volunteers and the patient population were observed on the *ex vivo* stimulations of blood mononuclear cells and subsequently, no effect of the nutritional intervention could be detected. By contrast, after the four week intervention period, the incorporation of total n-3 PUFAs, EPA, DPA and DHA in plasma phospholipids was significantly increased in the medical food group compared to the Control group, whereas the incorporation of total n-6 PUFAs, AA and the ratio n-6/n-3 PUFAs was significantly decreased in the medical food group. Several inflammatory serum markers were significantly higher in patients compared to volunteers at baseline and after the nutritional intervention, serum PGE_2 levels were significantly decreased in the medical food group and increased in the Control group. In addition, body weight increased significantly and ECOG performance status was significantly improved after intervention with the medical food. Moreover, the medical food is well-appreciated with a high compliance rate of study product intake. No clinically relevant safety concerns were reported and no changes in blood safety parameters were measured.

The risk of immune suppression is high after anti-cancer treatment, leading to a reduced treatment efficacy and a higher frequency and severity of infectious and other complications. Therefore, the rapid-acting effect of the medical food was investigated in a group of thirty-eight cancer patients receiving radiotherapy within one week of nutritional intervention as described in Chapter 7. In this randomized, double-blind study, the group receiving the specific medical food was compared with a Control group receiving an iso-caloric and iso-nitrogenous product. EPA and DHA incorporation into white blood cell phospholipids was measured at baseline and after one week of intervention. The subsequent changes on immune function were measured by the *ex vivo* LPS-stimulated cytokine production in whole blood cultures. Secondary objectives of the study were to assess the effects of the medical food on inflammatory status, nutritional status, safety and compliance.

After one week of intervention, the incorporation of total n-3 PUFAs, EPA, DPA and DHA in WBC phospholipids was significantly increased in the medical food group compared to the Control group, whereas the incorporation of total n-6 PUFAs, AA and the ratio n-6/n-3 PUFAs was significantly decreased in the medical food group. Comparable results were ob-

tained for the incorporation into plasma and red blood cell (RBC) phospholipids. No effects were observed on the production of pro-inflammatory cytokines in *ex vivo* LPS-stimulated whole blood cultures after one week intervention with the medical food and moreover, no effects on serum levels of inflammatory cytokines could be detected, but these levels were relatively low. By contrast, serum levels of PGE_2 were significantly decreased in cancer patients receiving radiotherapy after a one week of nutritional intervention with a medical food and no clinically relevant safety concerns were reported.

The results of the present thesis show that nutritional intervention with a unique specific medical food may represent a new opportunity for applications in cancer patients being an integral part of disease management and enabling an early supportive care strategy.

SAMENVATTING

Kanker is een verzamelnaam voor meer dan honderd verschillende ziekten (Hoofdstuk 1). Al deze verschillende soorten kanker hebben één gemeenschappelijk kenmerk: een ongeremde deling van lichaamscellen. In het menselijk lichaam zorgen vele regelmechanismen voor een gecontroleerde groei en ontwikkeling van nieuwe cellen en voor de dood van beschadigde of verouderde cellen. Wanneer er echter veranderingen optreden in het stukje genetisch materiaal dat verantwoordelijk is voor deze regelmechanismen, kan een normale cel transformeren tot een kankercel, die uiteindelijk kan uitgroeien tot een tumor. Bij de ontwikkeling van kanker spelen verschillende factoren een rol. Erfelijke factoren en levensstijl factoren zijn betrokken bij dit proces, maar ook veroudering speelt een belangrijke rol bij het ontstaan van kanker. Kanker heeft invloed op veel processen in je lichaam waarbij verschillende aspecten, zoals het type en stadium van de tumor, individuele persoonskenmerken, anti-kanker behandelingen en de aanwezigheid van kanker cachexia, het klinische verloop en de prognose van de ziekte kunnen beïnvloeden.

Cachexia is een groot probleem in veel kankerpatiënten. Wereldwijd krijgt zo'n 30% tot 90% van de patiënten tijdens het verloop van hun ziekte te maken met een vorm van ondervoeding of cachexia. De belangrijkste kenmerken van deze chronische aandoening zijn progressief gewichtsverlies, anorexia, verlies van spier- en vetweefsel, spierzwakte en vermoeidheid. Er zijn verschillende factoren die een rol spelen bij het ontstaan van cachexia, waaronder tumor factoren, anti-kanker behandelingen, voedingsstatus, maar ook leeftijd en zelfs stress en depressie zijn betrokken bij dit proces. Dit kan leiden tot een chronische staat van ontsteking en tegenstrijdig genoeg, in een verlaagde reactiviteit van het immuunsysteem. Samen kunnen deze factoren leiden tot progressie van de ziekte, een verhoogde kans op infecties en andere complicaties, en een minder optimale behandeling. Voor een patiënt kan dit resulteren in een verminderde kwaliteit van leven en een slechte prognose.

Daarom is het essentieel dat gebruik wordt gemaakt van een multidisciplinaire aanpak, waarin voedingsondersteuning een belangrijke rol speelt. Dit begint op het moment van diagnose en loopt parallel aan de verschillende behandelingen die de patiënt ondergaat. Bovendien zijn er richtlijnen gepubliceerd door de European Society for Clinical Nutrition and Metabolism (ESPEN) die wetenschappelijk onderbouwde informatie verschaffen over voedingsondersteuning in relatie tot specifieke problemen zoals timing, dosering, de samenstelling en de route van toediening. Een eerste belangrijke stap is echter het identificeren van patiënten die er mogelijk baat bij zouden hebben. Dit kan doormiddel van een routine screening en de beoordeling van de voedingsstatus van de patiënt door een diëtist. Vervolgens kan voedingsondersteuning worden gegeven, afgestemd op de individuele behoeften van de patiënt, de medische conditie en de huidige en te verwachten bijwerkingen van de behandeling, met als doel gewichtsverlies tegen te gaan en een suboptimale behandeling te voorkomen om uiteindelijk de klinische resultaten en kwaliteit van leven voor de patiënt te verbeteren.

Om die reden is een specifieke voedingscombinatie ontwikkeld, gebaseerd op eerder uitgevoerd onderzoek, die toegepast kan worden in kankerpatiënten. Dit concept bevat hoge concentraties eiwit en leucine en is bovendien verrijkt met een visolie emulsie (die eicosapentaenoic acid (EPA) en docosahexaenoic acid (DHA)) bevat) en een specifieke mix van prebiotische oligosacchariden. Dit concept is ontwikkeld om complicaties te verminderen

en een optimale behandeling te ondersteunen door het remmen van de ontstekingsstaat, het ondersteunen van het immuunsysteem en het behouden van spiermassa en -functie. Om inzicht te krijgen in de werking en de effectiviteit van verschillende voedingsingrediënten, is een recent aangepast en gevalideerd diermodel voor kanker cachexia gebruikt. Dit model is gebaseerd op het dikke darm (C26) tumormodel, waarin onder andere parameters voor immuunfunctie worden gemeten.

In Hoofdstuk 2 is het optreden van immuunsuppressie gedurende het verloop van het cachexia proces bestudeerd door het meten van contact overgevoeligheid (CHS) tegen de stof oxazolone als een gevalideerde parameter voor Th1-gemedieerde immuunfunctie. CHS is gemeten op twee tijdspunten tijdens de studie om veranderingen in immuun status te bekijken tussen de pre-cachectische en de cachectische staat van de muizen. De resultaten laten zien dat immuunfunctie al verlaagd is voordat daadwerkelijk gewichtsverlies is opgetreden. Om die reden is immuunsuppressie geïdentificeerd als een mogelijke vroege risico factor voor cachexia. Dit zou pleiten voor een vroege ondersteuningsstrategie voor kankerpatiënten waarin specifieke voedingsondersteuning een belangrijke rol speelt. De overweging om voedingsondersteuning zo vroeg mogelijk aan te bieden, bij voorkeur te beginnen op het moment van diagnose en vervolgens parallel te laten lopen aan de verschillende behandelingen van de patiënt, zou dan ook van groot belang zijn om het immuunsysteem te ondersteunen en de ontsteking en andere cachexia kenmerken te remmen.

Om de mogelijke voordelen van specifieke voedingsondersteuning voor het immuunsysteem te bekijken, zijn de effecten van zowel de individuele ingrediënten als van de totale specifieke nutritionele combinatie (SNC) bestudeerd. Hierbij is gekeken naar het effect op de ontstekingsstaat, op immuunfunctie en op verschillende biomarkers voor cachexia in het dikke darm (C26) tumormodel (Hoofdstuk 3). Er werd geen effect gevonden van de afzonderlijke ingrediënten van de SNC op het gewicht van de dieren in vergelijking met een groep op een controle dieet. Echter, het dieet waaraan de complete combinatie (SNC) is toegevoegd verbeterde het gewicht van de dieren aanzienlijk, wat wijst op een minder cachectische staat van de dieren. Dit werd benadrukt door een positief effect op andere cachexia parameters, zoals een significante remming van gewichtsverlies van epididymus vet en skeletspieren. Bovendien werd geen effect van de afzonderlijke ingrediënten op CHS waargenomen in tumor-dragende muizen in een pre-cachectische staat. Na toevoeging van de complete combinatie (SNC) was de reactie van het immuunsysteem significant toegenomen wat wijst op een verbetering van de Th1-gemedieerde immuunfunctie voordat gewichtsverlies optreedt. Ook tijdens de cachectische staat heeft de toevoeging van de SNC bijgedragen aan een verlaging van ontstekings-gemedieerde cytokines (signaalstoffen) in plasma en een verbetering van de immuunfunctie *ex vivo*, wat betekent dat de ontstekingsstaat verlaagd is en de reactie van het immuunsysteem is verbeterd vergeleken met een iso-calorisch controle dieet.

Om deze positieve immuun modulerende effecten te vertalen naar een situatie waarin daadwerkelijk wordt gekeken naar de weerstand tegen een infectie met een relevante levende ziekteverwekker, is het effect van de SNC op *Pseudomonas aeruginosa* infecties bekeken in een muismodel voor chemotherapie-geïnduceerde neutropenie (tekort aan witte bloedcellen), waarin het immuunsysteem onderdrukt is (Hoofdstuk 4). Dit lijkt op de situatie in kankerpatiënten die lijden aan complicaties veroorzaakt door een behandeling

met chemotherapie, welke vaak in verband worden gebracht met een verhoogde morbiditeit en mortaliteit tijdens kanker. In dit model zijn infecties gemeten door middel van de incidentie en de ernst van de bacteriële translocatie naar de lever en de longen en door het meten van ontstekings-gemedieerde cytokines in plasma.

Na de chemotherapie behandeling werden de muizen sterk neutropeen. De SNC had echter geen effect op de waardes van de verschillende types witte bloedcellen. Desalniettemin was de groei in lichaamsgewicht van de muizen op het SNC dieet al hoger voor de chemotherapie behandeling vergeleken met muizen op een controle dieet, terwijl de voedselinname van de muizen gelijk was tussen de groepen. Na de chemotherapie behandeling ging het lichaamsgewicht in beide groepen omlaag, maar bleef significant hoger in de SNC groep vergeleken met de controle groep. Bovendien, verlaagde de interventie met de SNC de incidentie en de ernst van de *P. aeruginosa* infectie door de translocatie naar de lever te verminderen. Een vergelijkbaar beeld was ook in de longen te zien. Daarnaast verlaagde de SNC de fecale pH, wat een verklaring zou kunnen zijn (of op z'n minst gedeeltelijk) voor de lagere *P. aeruginosa* levels in fecale monsters gedurende de interventie fase van het experiment. Een verlaagde pH kan het gevolg zijn van de productie van korte keten vetzuren (SCFA) die gevormd worden door de omzetting van de prebiotische oligosacchariden. Dit kan bijdragen aan de remming van de groei en hechting van pathogene bacteriën en de ontwikkeling van nuttige bacteriën stimuleren. Plasmalevels van ontstekings-gemedieerde cytokines waren ook enigszins verlaagd en er was een sterke correlatie met bacteriële translocatie naar de lever. Er zijn hier waarschijnlijk verschillende mechanismen bij betrokken geweest, zoals de modulatie van de microbiota in de darmen, een verbeterde barrièrefunctie van de darmen, een verbeterde immuunfunctie en een verlaging van de ontstekingsstaat. Als deze resultaten worden vertaald naar de menselijke situatie, zouden ze wellicht zeer gunstig kunnen zijn voor patiënten die lijden aan een latente darminfectie en die na chemotherapie, chirurgie of transplantatie, kunnen leiden tot bacteriële translocatie en uiteindelijk in een ernstige sepsis.

De volgende stap naar een klinische toepassing en validatie van de resultaten in kankerpatiënten was om zowel de effectiviteit als de veiligheid van de specifieke voedingscombinatie, als onderdeel van een medische voeding, te bestuderen in gezonde vrijwilligers. In deze proof-of-concept studie hebben twaalf gezonde mannen en vrouwen de medische voeding genomen voor een periode van een week, zoals beschreven in Hoofdstuk 5. Immuunfunctie werd bepaald door het meten van de *ex vivo* LPS-gestimuleerde productie van cytokines in volbloed kweken. LPS is een component van een bacterie celwand en met deze toepassing bekijk je de capaciteit van bloedcellen om te reageren tegen een infectieuze trigger. Ook werd in deze studie gekeken naar de inbouwsnelheid van de vetzuren EPA en DHA (uit visolie) in witte bloedcellen binnen een interventieperiode van een week, omdat een snelle en effectieve inbouw van deze n-3 vetzuren belangrijk zou kunnen zijn voor kankerpatiënten die een behandeling starten kort na het moment van diagnose. Al na een dag van de voedingsinterventie is het percentage EPA in witte bloedcellen (WBC) aanzienlijk verhoogd, waarna het verder doorsteeg tot aan het eind van de interventie. Er was geen verandering te zien in het percentage DHA in de WBC, hoewel het percentage docosapentaenoic acid (DPA, n-3) significant verhoogd was en het percentage arachidonic acid (AA)

verlaagd was vanaf dag 2 tot aan het eind van de week. Voor de inbouw in rode bloedcellen (RBC) en plasma werden vergelijkbare resultaten gevonden.

Daarnaast verhoogde de interventie met de medische voeding de productie van ontstekings-gemedieerde cytokines in LPS-gestimuleerde volbloed kweken aanzienlijk, wat wijst op immuunmodulerende effecten reeds binnen een week van interventie. Dit zou gunstig kunnen zijn voor het vermogen om te reageren op acute infectieuze triggers. Deze resultaten bevestigen bovendien de mogelijkheid om snelle immuunmodulerende effecten te induceren met een medische voeding in de menselijke situatie.

In Hoofdstuk 6 is het effect bekeken van een 4-weekse interventie met de medische voeding op immuunfunctie in een vroeg fase in een groep van 64 nieuw gediagnosticeerde slokdarmkanker patiënten voor de start van een anti-kankerbehandeling. In deze exploratieve, gerandomiseerde, dubbel blinde studie, werd de groep die de specifieke medische voeding (actieve groep) kreeg vergeleken met een controle groep die een normale (routine) voedingsondersteuning kreeg. Immuunfunctie werd gemeten doormiddel van de *ex vivo* ConA- en LPS- gestimuleerde proliferatie en cytokine productie in witte bloedcel kweken. ConA is een stof die T-cellen kan stimuleren en aan zetten tot proliferatie. Verder werd het effect van de medische voeding onderzocht op ontstekingsparameters en voedingsstatus, en werd de veiligheid en smakelijkheid van het product bekeken. Gegevens over het immuunsysteem, ontsteking en voedingsstatus van gezonde vrijwilligers werden verkregen om de uitgangswaarden te kunnen vergelijken en een adequate interpretatie van de gegevens mogelijk te maken.

Bij aanvang van de studie werden geen verschillen waargenomen tussen de gezonde vrijwilligers en de patiënten populatie op de *ex vivo* stimulaties van witte bloedcellen. Ook na de voedingsinterventie werden geen verschillen gemeten. Daarentegen, na de 4-weeks interventie periode was de inbouw van de totale n-3 vetzuren, EPA, DPA en DHA in plasma significant verhoogd in de actieve groep vergeleken met de controle groep. De inbouw van de totale n-6 vetzuren, AA en de ratio n-6/n-3 vetzuren was aanzienlijk afgenomen in de actieve groep. Bij aanvang van de studie waren verschillende ontstekingsmarkers in serum hoger in patiënten vergeleken met de gezonde vrijwilligers. Echter, na de voedingsinterventie waren serum waardes van prostaglandine E_2 (PGE_2, een belangrijke ontstekingsmarker) significant verlaagd in de actieve groep vergeleken met de controle groep. Ook het lichaamsgewicht was toegenomen en de ECOG performance status was significant verbeterd na de interventie met de specifieke medische voeding. Bovendien werd de specifieke medische voeding goed gewaardeerd met een hoge inname van de producten tijdens de studie. Er zijn geen klinisch relevante problemen betreffende de veiligheid van het product opgetreden en er konden geen veranderingen waargenomen worden in veiligheidsparameters in het bloed.

Het risico op het optreden van immuun suppressie is hoger na een anti-kanker behandeling. Dit kan leiden tot een verminderde effectiviteit van de behandeling en een toename in het aantal en de ernst van infecties en andere complicaties. Om die reden is het snelwerkende effect van de specifieke medische voeding onderzocht in een groep van 38 kankerpatiënten die onder behandeling zijn met radiotherapie zoals beschreven in Hoofdstuk 7.

In deze gerandomiseerde, dubbel blinde studie, werd de groep die de specifieke medische voeding (actieve groep) kreeg vergeleken met een controle groep die een iso-calorisch en iso-nitrogeen product kreeg. De inbouw van de vetzuren EPA en DHA werd gemeten bij aanvang en na een week van interventie. De veranderingen in immuunfunctie werden gemeten door middel van de *ex vivo* LPS-gestimuleerde cytokine productie in volbloed kweken. Verder werd er gekeken naar het effect van de medische voeding op ontstekingsstatus, voedingsstatus, veiligheid en product inname.

Na de interventie periode van een week was de inbouw van de totale n-3 vetzuren, EPA, DPA en DHA in witte bloedcellen significant verhoogd in de medische voeding groep, ten opzichte van de controle groep. De inbouw van de totale n-6 vetzuren, AA en de ratio n-6/n-3 vetzuren was aanzienlijk afgenomen in de actieve groep. Voor de inbouw in RBC en plasma werden vergelijkbare resultaten gevonden. Na een week interventie met de specifieke medische voeding werden echter geen effecten gevonden op de productie van cytokines in LPS-gestimuleerd volbloed, of op de waardes van ontstekings-gemedieerde cytokines in serum, mogelijk verband houdend met het feit dat deze waardes erg laag waren. Daarentegen waren de serumwaardes van PGE_2 significant verlaagd na een week interventie met de specifieke medische voeding in kankerpatiënten onder behandeling van radiotherapie. Er zijn geen klinisch relevante problemen waargenomen betreffende de veiligheid van het product.

De resultaten die beschreven zijn in dit proefschrift laten zien dat de interventie met een specifieke medische voeding een nieuwe mogelijkheid zou kunnen zijn voor de toepassing in kankerpatiënten als geïntegreerd onderdeel van het ziekte management en die een strategie met vroege voedingsondersteuning mogelijk maken. Verder onderzoek is echter erg belangrijk om de mogelijke immunologische effecten van het voedingsconcept te onderzoeken en te valideren in verschillende kankersoorten en stadia van de ziekte. Hierbij zal gekeken moeten worden naar klinisch relevante parameters om zo een krachtige strategie te ontwikkelen die leidt tot voordelen voor de patiënt.

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CURRICULUM VITAE

Joyce Faber was born in Hengelo on 17 December 1979. In 1998 she graduated from the Twickelcollege in Hengelo and started the study Biology and Medical Laboratory Sciences at the Saxion Hogeschool in Enschede, specializing in Biochemistry/ Biotechnology. During her first internship she studied the expression of TGF-β in *Lactococcus lactis* under supervision of Jan Grijpstra and Dr. Jan Knol at Nutricia Advanced Medical Nutrition, Danone Research - Centre for Specialised Nutrition* in Wageningen. During her second internship, she studied the role of the enzymes COX and LOX in colon cancer under supervision of Simone Dusseljee and Dr. Mirjam Govers also at Danone Research. In January 2002 the author graduated and started working at Danone Research.

Working on the immunological aspects of arthritis, the author studied the effect of plant derived modulators on inflammation and cartilage metabolism under supervision of Dr. Sander Hougee and Dr. Yvo Graus. Afterwards, from 2004-2006, the author was involved in several projects on allergy and immune modulation under supervision of Prof. Dr. Johan Garssen. To build a bridge between the immunological science and disease targeted nutrition, the author participated in the oncology project where she studied the immune modulatory effects of a specific nutritional combination during cancer in a pre-clinical as well as in a clinical setting under supervision of Dr. Marchel Gorsselink (pre-clinical), Dr. Paul Vos and Dr. Ardy van Helvoort (pre-clinical and clinical). The research on this project generated the basis of the publications that appeared in this thesis.

Currently, the author continues to work as a scientist on the oncology project under supervision of Dr. Paul Vos and Dr. Niki Georgiou with the aim to develop the optimal nutritional support for cancer patients. In addition, she is involved in several other medical projects

within the immunology team under supervision of Prof. Dr. Johan Garssen. The author lives together with Arjan Stokreef on a farm with dairy cows.

* Nutricia Advanced Medical Nutrition, Danone Research - Centre for Specialised Nutrition was formerly called Numico-Research (before April 2008).

LIST OF PUBLICATIONS

ARTICLES

1 Faber J, Berkhout M, Fiedler U, Avlar M, Witteman BJ, Vos AP, Henke M, Garssen J, van Helvoort A, Otten MH, Arends J. Rapid incorporation of EPA and DHA into white blood cells and reduced serum PGE_2 levels after one week of nutritional intervention with a medical food in cancer patients receiving radiotherapy. Submitted for publication.

2 Faber J, Uitdehaag MJ, Spaander MCW, van Steenbergen SCL, Vos AP, Berkhout M, Lamers CHJ, Rümke HC, Tilanus HW, Siersema PD, van Helvoort A, van der Gaast A. Reduced serum PGE_2 levels and improved body weight and performance status after nutritional intervention with a specific medical food in newly diagnosed esophageal cancer patients. Submitted for publication.

3 Faber J, van Limpt K, Kegler D, Luiking Y, Garssen J, van Helvoort A, Vos AP, Knol J. Bacterial translocation is reduced by a specific nutritional combination in mice with chemotherapy-Induced neutropenia. J Nutr. 2011 Jul;141(7):1292-8.

4 Faber J, Berkhout M, Vos AP, Sijben JW, Calder PC, Garssen J, van Helvoort A. Supplementation with a fish oil-enriched, high-protein medical food leads to rapid incorporation of EPA into white blood cells and modulates immune responses within one week in healthy men and women. J Nutr. 2011 May;141(5):964-70.

5 Faber J, Vos AP, Kegler D, Argiles J, Laviano A, Garssen J, Van Helvoort A. Impaired immune function: an early marker for cancer cachexia. Oncol Rep. 2009;22:1403-6.

6 van Norren K, Kegler D, Argiles JM, Luiking Y, Gorselink M, Laviano A, Arts K, Faber J, Jansen H, et al. Dietary supplementation with a specific combination of high protein,

leucine, and fish oil improves muscle function and daily activity in tumour-bearing cachectic mice. Br J Cancer. 2009;100:713-22.

7 van Hoffen E, Ruiter B, Faber J, M'Rabet L, Knol EF, Stahl B, Arslanoglu S, Moro G, Boehm G, Garssen J. A specific mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides induces a beneficial immunoglobulin profile in infants at high risk for allergy. Allergy. 2009;64:484-7.

8 Faber J, Vos P, Kegler D, van Norren K, Argiles JM, Laviano A, Garssen J, van Helvoort A. Beneficial immune modulatory effects of a specific nutritional combination in a murine model for cancer cachexia. Br J Cancer. 2008;99:2029-36.

9 Hartog A, Hougee S, Faber J, Sanders A, Zuurman C, Smit HF, van der Kraan PM, Hoijer MA, Garssen J. The multicomponent phytopharmaceutical SKI306X inhibits *in vitro* cartilage degradation and the production of inflammatory mediators. Phytomedicine. 2008;15:313-20.

10 Hougee S, Faber J, Sanders A, Berg WB, Garssen J, Smit HF, Hoijer MA. Selective inhibition of COX-2 by a standardized CO_2 extract of Humulus lupulus *in vitro* and its activity in a mouse model of zymosan-induced arthritis. Planta Med. 2006;72:228-33.

11 Hougee S, Faber J, Sanders A, de Jong RB, van den Berg WB, Garssen J, Hoijer MA, Smit HF. Selective COX-2 inhibition by a Pterocarpus marsupium extract characterized by pterostilbene, and its activity in healthy human volunteers. Planta Med. 2005;71:387-92.

12 Hougee S, Sanders A, Faber J, Graus YM, van den Berg WB, Garssen J, Smit HF, Hoijer MA. Decreased pro-inflammatory cytokine production by LPS-stimulated PBMC upon *in vitro* incubation with the flavonoids apigenin, luteolin or chrysin, due to selective elimination of monocytes/macrophages. Biochem Pharmacol. 2005;69:241-8.

ORAL PRESENTATIONS

1 Faber J, van Limpt K, Kegler D, Luiking Y, Garssen J, van Helvoort A, Vos AP, Knol J. Reduced bacterial translocation after intervention with a specific nutritional combination in a murine model for chemotherapy-induced neutropenia. Pharma-Nutrition 2011, Amsterdam, the Netherlands.

2 Faber J, Berkhout M, Vos AP, Sijben JWC, Calder PC, Garssen J, van Helvoort A. Supplementation with a fish-oil enriched sip-feed leads to EPA incorporation into white blood cells and enhanced immune responses within one week. ISSFALL 2010, Maastricht, the Netherlands.

3 Faber J, Berkhout M, Vos AP, Calder PC, Garssen J, van Helvoort A. Supplementation of healthy elderly with a fish-oil enriched sip feed leads to fast incorporation of EPA into white and red blood cells and results in improved immune responses within one week. ESPEN 2009, Vienna, Austria.

POSTER PRESENTATIONS

1 Faber J, Berkhout M, Fiedler U, Witteman BJ, Vos AP, Henke M, van Helvoort A, Otten MH, Arends J. Rapid incorporation of EPA and DHA into white blood cells and reduced serum PGE_2 after one week of intervention with a medical food in cancer patients receiving radiotherapy. ESPEN 2011, Göthenborg, Sweden.

2 Faber J, Uitdehaag MJ, Spaander MCW, van Steenbergen SCL, Vos AP, Berkhout M, Lamers CHJ, Rümke HC, Tilanus HW, Siersema PD, van Helvoort A, van der Gaast A. newly diagnosed esophageal cancer patients serum PGE_2 levels are reduced after nutritional intervention with a medical food. ESPEN 2010, Florence, France.

3 Faber J, Vos AP, Kegler D, van Norren K, Argilés JM, Laviano A, Garssen J, van Helvoort A. Beneficial immune modulatory effects of a specific nutritional combination in a murine model for cancer cachexia. TIP spring meeting 2009, Utrecht, the Netherlands.

4 Faber J, Vos AP, Kegler D, van Norren K, Garssen J, van Helvoort A. Beneficial immune modulatory effects of a specific nutritional combination in a murine model for cancer cachexia. NVVI 2008, Lunteren, the Netherlands.

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