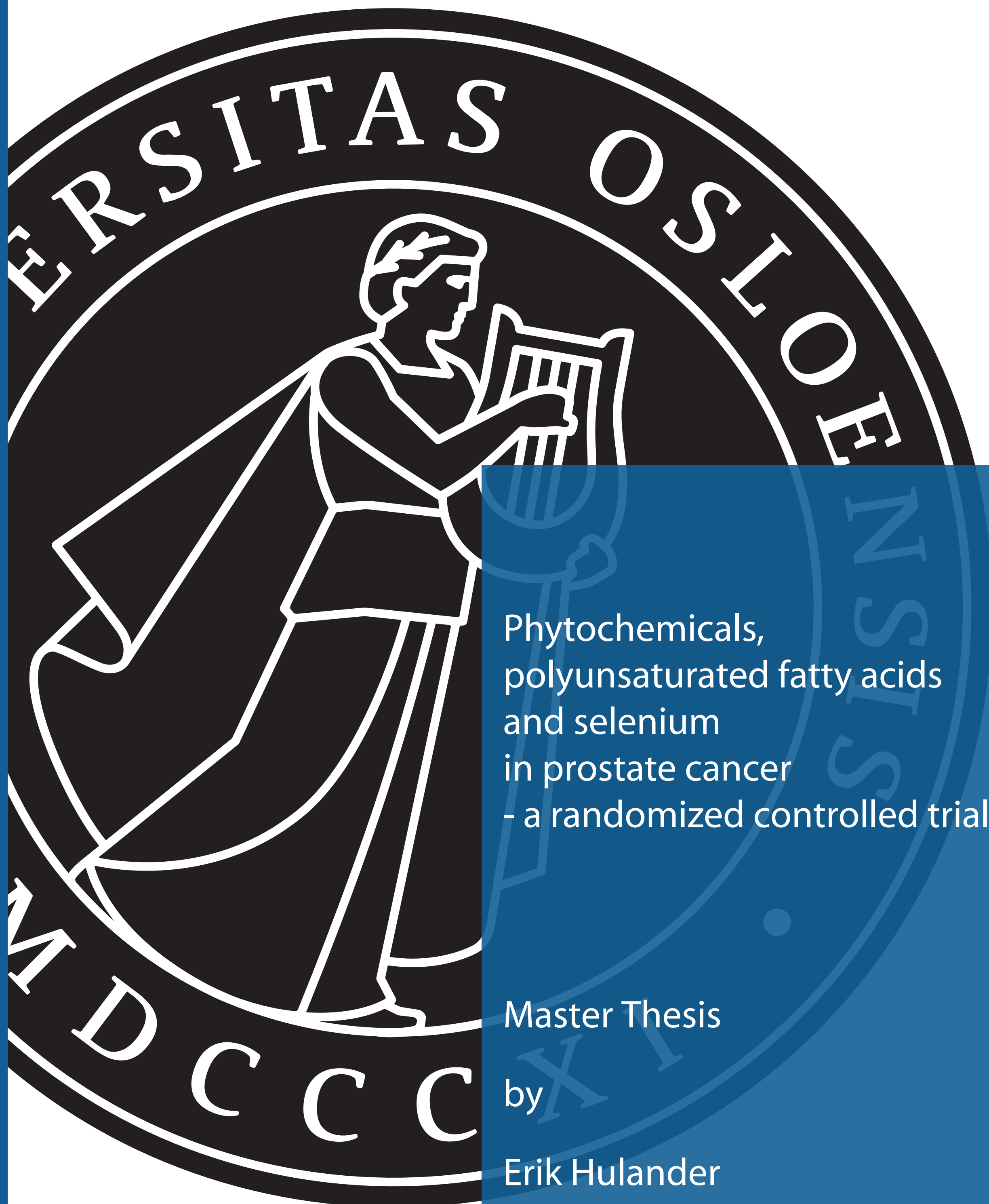

Phytochemicals, polyunsaturated fatty acids and selenium in prostate cancer - a randomized controlled trial

Master Thesis

by

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May 2013

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A randomized controlled trial

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UNIVERSITY OF OSLO

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Abstract

Background: Prostate cancer (PC) is the most commonly diagnosed type of cancer in the Norwegian population. Several dietary factors are proposed to affect PC development. The aim of this study was to evaluate whether a dietary intervention have effects on prostate specific antigen (PSA)-development, biomarkers of inflammation and insulin-like growth factor-1 (IGF-1) in the period between diagnosis and elective therapy in PC patients.

Methods: A randomized controlled trial was conducted, patients (n = 78) were allocated to control or one of two interventions; 1) tomato product containing 30 mg/day of lycopene, or 2) a "multi-diet" approach with the same tomato products plus daily supplementation of 200 μg selenium, 200 mg soy isoflavones, 5 g fish oil, 1 cup black and green tea as well as 0.33 L of pomegranate- and grape juice. The trial lasted a median of 21 days. Statistical analysis was stratified by baseline values as well as developments in biochemical markers of compliance. Primary endpoints were changes in PSA levels, secondary endpoints were IGF-1 and biomarkers of inflammation.

Results: Increase of total PSA values was lower in patients with intermediate tumor risk in both tomato and multi-diet intervention (p=0.015 and p=0.037 respectively). Patients with high increases in plasma lycopene, selenium and eicosapentaenoic acid combined during the intervention had lowered total and free PSA values (p=0.003 and p=0.004 respectively) compared to those with low increases. Similarly, when stratifying by plasma lycopene changes, different changes in both total PSA and free PSA levels were found (p=0.009 and p=0.039 respectively). No significant differences were seen in biomarkers of inflammation.

Conclusion: Tomato and multi-diet supplementation decreased PSA development in patients with intermediate tumor risk. Tomato supplementation decreased PSA development most efficiently in patients with high increase in plasma lycopene. There was an added effect on PSA in patients with corresponding increases also in selenium and omega-3 fatty acids. The results add substantially to the evidence from clinical human trials of diet as a modulating component of PSA-development on established PC, and warrant further studies.

Acknowledgements

First and foremost, I would like to thank my co-supervisor Rune Blomhoff for welcoming me to his research group and introducing me to this interesting, and rather groundbreaking, clinical research project.

Secondly, I would like to thank my main supervisor Ingvild Paur for her patient, optimistic and genuinely enthusiastic guidance throughout this thesis work. I feel blessed to have had her as a supervisor.

I am also grateful to everyone previously or currently involved in this project, who I have not gotten to know in person, but whose work has built the foundation of the study and allowed me to finish this master thesis. Including, but not limited to, Wolfgang Lilleby, Anette Karlsen and Sigbjørn Smeland.

Moreover, I would like to thank Siv Kjølsrud Bøhn, for her help with PCA analysis as well as valuable input in the work with my thesis. I would also like to thank Nasser Bastani, for helping me understand externally performed analyses. I am also grateful to Kari Holte who assisted in all practical procedures. Special thanks go also to Siv Åshild Wiik who helped me get started in the lab. Overall, I have felt privileged being a master student in the Blomhoff research group. There have been many invaluable group discussions which has allowed me to get an insight into the workings of professional researchers. It has been a time of great learning for me.

I would also like to thank all my friends and my family who have supported me throughout this period. Including (but not limited to) PhD Zhihui Wang who helped proof reading, clinical dietitian Patrik Hansson for enthusiastic discussions, and last but not least my father for his continued support during the course of my studies.

List of abbreviations

Aker	Aker Hospital
AR	Androgen receptor
BMI	Body mass index
BPH	Benign prostate hyperplasia
CRP	C-reactive protein
COX-2	Prostaglandin-endoperoxide synthase 2
DHA	Docosahexaenoic acid
DHT	Dihydrotestosteron
DNA	Deoxyribonucleic acid
DNR	The Norwegian Radium Hospital
DM	Diabetes mellitus
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
ELISA	Enzyme-linked immuno sorbent assay
EPA	Eicosapentaenoic acid
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FAME	Fatty acid methyl ester
fPSA	Free prostate specific antigen
HGPIN	High grade prostatic intraepithelial neoplasia
IGF-1	Insulin-like growth factor 1
IGFBP-3	Insulin-like growth factor binding protein 3
IL-6	Interleukin 6
n-3	Omega 3
n-6	Omega 6
p2PSA	2-pro prostate specific antigen
PC	Prostate cancer
PCA	Principal component analysis
PC(1-5)	Principal component 1-5
PFPI	Prostate Phytochemical & PUFA Intervention
Phi	Prostate health index
PSA	Prostate specific antigen
RCT	Randomized controlled trial
SNP	Single nucleotid polymorphism
suPAR	Soluble urokinase plasminogen activator receptor
tPSA	Total prostate specific antigen
tPSAV	Total prostate specific antigen velocity
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
WCRF	World cancer research fund

Table of contents

1 Introduction ... 1
1.1 The prostate and prostate cancer ... 1
1.2 Inflammation and prostate cancer ... 9
1.3 Nutritional factors and the risk of PC ... 13
2 Aims ... 18
2.1 Aims of the PFPI study ... 18
2.2 Specific aims of this master thesis ... 18
3 Methods ... 19
3.1 The Prostate Phytochemical & PUFA Intervention ... 19
3.2 Statistical analysis ... 24
3.3 Laboratory analyses ... 30
3.1 Contribution of the master candidate ... 38
4 Results ... 39
4.1 Descriptive data for the PFPI-study ... 39
4.2 Compliance to the dietary interventions ... 40
4.3 Effects of the intervention ... 44
5 Discussion ... 54
5.1 Methodological discussion ... 54
5.2 Discussion of results ... 59
5.3 Implications for the patient population ... 76
5.4 Future implications ... 77
6 Conclusion ... 78
7 References ... 79
8 Supplementary tables and figures ... 90
9 Appendix ... 98
9.1 Externally performed laboratory analyses ... 98

1 Introduction

This master thesis in clinical nutrition examines the effects of a nutrition intervention with tomato products, pomegranate- and grape juice, green- and black tea, isoflavone extract from soy, selenium and omega-3 fatty acids on patients with established prostate cancer (PC). The clinical trial was conducted between 2006 and 2011 in collaboration between the research groups of Professor Rune Blomhoff at the Department of Nutrition, University of Oslo, and clinicians at The Norwegian Radium Hospital (DNR) and Aker Hospital (Aker), both in Oslo. This master thesis is thus based on material obtained in the study, planning and conduction of the trial is not a part of this thesis.

1.1 The prostate and prostate cancer

1.1.1 Incidence of prostate cancer

Cancer is the second leading cause of death in the Norwegian population. During the years 2001 – 2011, about one of four deaths in Norway were due to malignant neoplasms (1). Among malignant diseases, PC was the most common type of cancer in the Norway, and 12.8% of Norwegian men develop PC by the age of 75 years (2), resulting in a 4% prevalence of prostate cancers in Norwegian men above 50 years of age (2, 3).

The incidence rate for PC increased by 21 % for 2006-2010 compared to 2001-2005 (2). The relative survival, measured as survival in a patient group divided by the expected survival of a comparable group in the general population, has at the same time increased from 80 to 89 % (2). Still, second to lung cancer, PC is accountable for the highest number of cancer attributed deaths. In the year of 2011 alone, 1052 Norwegian men died from PC (4).

In North America, statistics reveals a 16 % lifetime risk of being diagnosed with PC, but only about three percent lifetime risk of dying from the disease (5). PC can in some cases be a silent disease that may develop asymptomatic and go unnoticed. In 2008 Yin *et al.* (6) published a report where presumably healthy organ donors where examined post mortem for PC. Out of the 340 men examined, twelve percent were found to have undetected PC. The prevalence of incidental PC was even higher in older men, in the age groups 50-59, 60-69 and 70-81 years of age the prevalence was 23, 35 and 46% respectively.

1.1.2 The prostate organ

The prostate is a gland with functions in the male reproductive system and is located adjacent to the bladder in front of the rectum (figure 1.1). The prostate begins to form in the prenatal period and typically grows to its mature size at puberty. The development of the gland is dependent on androgens. Testosterone is taken up in the prostate and is through 5-α-reductase converted to dihydrotestosterone (DHT), which is considered to be the main intraprostatic androgen (7). The prostate is dependent on androgens to maintain its cellular content and functional activity, and in animal experiments castration led to a considerable decrease of the number of prostatic cells (8).

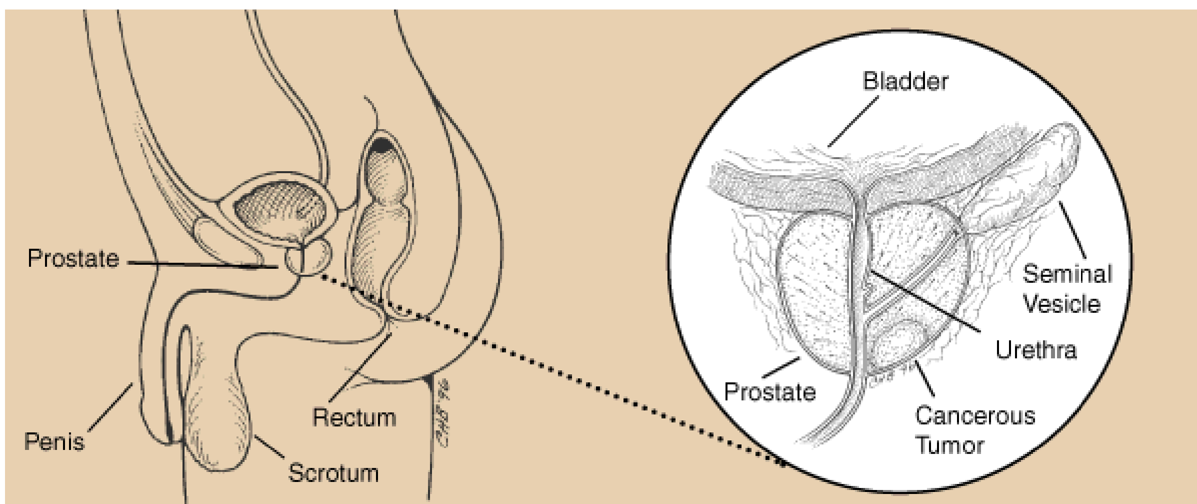

Figure 1.1 The prostate gland. Reprinted by the permission of the American Cancer Society, Inc from www.cancer.org. All rights reserved.

The prostate produces fluids containing calcium, citrate, phosphate ions, a clotting enzyme and the protein hydrolyzing enzyme Prostate Specific Antigen (PSA) (9). The urethra and seminal ducts join in the prostate and during ejaculation smooth muscle in the gland contract to aid in the process of expelling semen.

Enlargement of the prostate typically leads to a narrowing of the urethra, which may lead to obstructive problems when urinating. Benign enlargement of the inner prostate around the urethra is relatively common in aging men. This is a separate condition from PC and is referred to as benign prostatic hyperplasia (BPH). Coexistence of BPH and PC has been described in the literature, but it is currently unknown whether BPH can be seen as a causal link in developing PC or if the two conditions are simply driven by common risk factors (10).

1.1.3 **Prostate cancer development**

Unlike free-living cells like bacteria that compete to survive, the cells of multicellular organisms such as humans are dedicated to a complex collaboration. Cancer is characterized by an uncontrolled division of cells that disobey normal intra- and inter-cellular communication. The development of cancer can be generalized into three stages; initiation, promotion and metastasis.

The first step in cancer development is the initiation phase, where a significant change in a cell's deoxyribonucleic acid (DNA) occurs. Random mutations appear constantly and it is estimated that in a life time, every single gene will have undergone mutations about 10^{10} times (11). The body has several systems that repair damaged DNA and makes nonfunctional cells undergo apoptosis. If random mutations accumulate and lead to modulation of proteins that control essential behavior of the cell (e.g. cell growth, division and apoptosis) the cell may become a renegade cancer cell. Environmental factors can increase the number of mutations; these factors include ionizing radiation, viruses and chemical carcinogens. Chemical carcinogens can be components found in the diet or external exposure to toxic chemicals (11).

After the initiation, the cells no longer play by the rules of apoptosis and cell division, and the next phase is promotion. Promoters are typically compounds that promote proliferation and cell division, and can act through either interaction with cell receptors or by directly altering gene expressions in the cell (11). It is suggested that free radicals (12), hormones and growth factors play a role in this phase by stimulating further mutation and cell growth (11). Many effects from promoters are organ-specific, since density of receptors and accessibility to certain genes varies among different types of tissue. In this phase tumor growth is established.

Finally, metastasis may appear. The mechanisms behind the start of the metastasis process are not well understood. The cancerous tumor now grows uncontrollable without external stimulation and invades other tissues. In lack of nutrition and oxygen, the tumor cells sends out signals to nearby endothelial cells which respond by generating new capillaries to support the tumor. This angiogenesis supplies the tumor with oxygen and nutrients and helps heterogeneous cells to migrate to different parts in the body and develop distant metastases (reviewed in more detail by Gupta and Massagué (13)).

PC is often described as an androgen dependent disease, where development is thought to be dependent on the presence of testosterone and DHT. An optional treatment for PC is hence androgen therapy, deprivation of testosterone production or androgen signaling through chemical castration. This can halt progression and shrink tumor size. Tumor growth most often later resumes even in castrated men however, the disease is then classified as androgen independent PC and is generally more advanced (14).

1.1.4 Prostate Specific Antigen (PSA) in prostate cancer

PSA, which is produced in the prostate, has a physiological function of cleaving semenogelin in the seminal fluids after ejaculation, transforming the ejaculate to a more liquid form and enabling the spread of sperm cells (15, 16).

PSA is produced by all prostate tissue, whether normal, inflamed and cancerous. Increased serum or plasma PSA is however an indicator of prostate pathology, as PSA is normally not released into the circulation. Elevated PSA-levels are found not only in PC but also in prostatitis, BPH and after recent sexual activity (17, 18).

Most of the circulating PSA is bound to the protease inhibitor alpha-1-antichymotrypsin. A smaller proportion of PSA is not bound to proteins and is then referred to as free PSA (fPSA). The ratio of fPSA over total PSA levels (tPSA) has been described as an alternative predictor of PC for those with tPSA between 2 and 10 ng/mL, where a lower ratio of fPSA indicates increased probability of having PC (19).

Other proposed methods of predicting PC includes tPSA velocity (tPSAV), where one calculates the rate of increase in tPSA-values over time. The diagnostic value of tPSAV is controversial though. The European Association of Urology currently does not recommend tPSAV measurements over tPSA-values alone (20), and the American Urological Association has not refuted nor endorsed the use of tPSAV (21). A recent report by Wallner *et al.* (22) indicated that use of tPSAV improved prediction of PC in a retrospective cohort. Another way of measuring PSA values is tPSA doubling time, where the time required for a doubling of tPSA is calculated, this is primarily used in post-treatment settings.

Possibly upcoming methods to improve prediction of PC include measuring inactive pro-PSA. Specifically the ratio of isoform 2-pro-PSA (p2PSA) to tPSA has received attention, and has

been indicated to more accurately predict PC than tPSA and ratio of fPSA at diagnosis when tPSA is between 2 and 10 ng/mL (23, 24). Yet another proposed method to predict PC includes the Prostate Health Index (Phi), where all of these variables are included (see formula below). This index is, like the p2PSA-ratio, suggested to more accurately predict PC than tPSA or fPSA-ratio alone in tPSA values between 2 and 10 ng/mL (23, 24).

$$Phi = \left(\frac{p2PSA}{fPSA}\right) * \sqrt{tPSA}$$

Treatment of PC includes e.g. prostatectomy or radiotherapy, measures which may have debilitating side effects. Possible adverse effects of these treatments include erectile dysfunction, urinary incontinence and bowel dysfunction (25). Screening for PC involves quantification of PSA, however the value of PSA screening has been questioned due to uncertainty if harms of treatment and diagnostic procedures outweigh the benefits. The U.S. Preventive Services Task Force currently recommends against PSA-screening (26). A recent Cochrane review also failed to identify decreased mortality as an effect of PSA-screening (27).

While PSA is not cancer-specific but rather organ-specific, PSA quantification remains to date the most important blood based biomarker in detecting and monitoring PC. An increased risk of PC is indicated with higher tPSA also in lower ranges (< 4 µg/mL) (28).

In established PC, a decrease in tPSA-levels is an indicator of a beneficial treatment and potentially a decreased number of malignant prostatic cells.

1.1.5 Predisposition, age and race

Most types of cancer primarily affect the older segment of the population. Prostate cancer is no exception; over 99 % of all cases of PC in Norway are diagnosed at the age of 50 years or above (2).

Globally, the incidence of prostate cancer varies to a great extent with the highest recorded incidence in Australia and New Zealand, followed by Western and Northern Europe and North America (29). These countries have an incidence that is tenfold or more than the countries with the lowest incidence; South-Eastern, Eastern and South-Central Asia, as well as Northern Africa *(ibid)*.

The global variation in incidence is in part ascribed to differing PSA-screening practice, but ethnicity has also been proposed as an independent risk factor. In 2011, Gunderson *et al*. (30) published a study comparing epidemiologic data of PC incidence with migration patterns, where a link between historic European migration patterns and current incidence of PC is described. They propose a genetic susceptibility in the Northern European genome to be partially responsible for the uneven spread of PC.

When looking at migration patterns, populations moving from a low incidence areas to high incidence areas tend to increase the risk of PC. In ethnic Asian populations, such as Japanese and Korean men living in North America, there is a considerable higher incidence of PC compared to their home countries (31, 32). In the multiethnic society in North America, both incidence and mortality of PC is found to be highest in men of African-American descent, suggesting that environmental factors alongside with ethnicity may impact the pathogenesis (33). One example of such an environmental factor might be obesity, measured by BMI, which is indicated to be higher in men with Afro-American than European descent (34).

1.1.6 Metabolic risk factors

Prevalence of obesity, defined as BMI above 30, has increased globally during the last decade (35). Obesity and the metabolic syndrome is associated with a higher incidence of several types of cancer (36), and there is a correlation between BMI and risk of mortal PC (37-39).

The impact of the metabolic syndrome in development of PC, is however less clear. Observational studies have seen an inverse correlation with type-2 diabetes mellitus (DM) and risk of developing PC (40). This is in line with a recently published cohort, that found associations of high glucose and triglycerides with decreased risk of developing PC, but a significant association of increased risk of mortal PC with high blood pressure and BMI (39).

There are several theories to explain the seemingly disparate results (figure 1.2). there might be a detection bias in obese individuals; lower PSA values due to hemodilution, higher incidence of BPH leading to both difficulties taking biopsy samples and fewer positive samples, and a constant state of hyperinsulinemia leading to elevated levels of insulin-like growth factor 1 (IGF-1) (41, 42), which again is associated with increased risk for PC (43, 44).

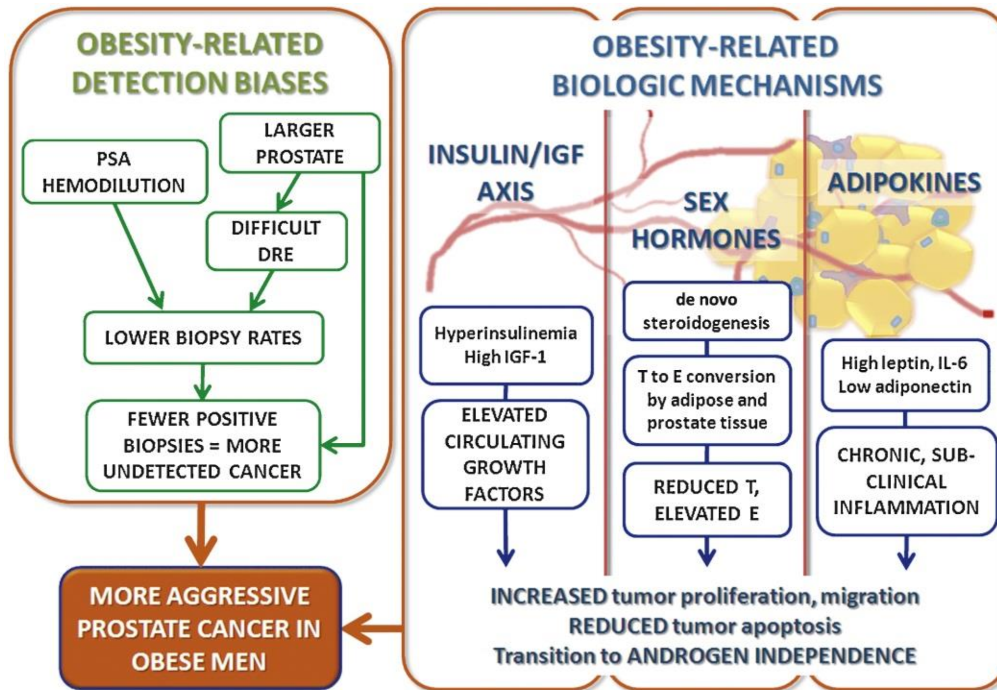

Figure 1.2 Suggested obesity-related mechanisms behind epidemiological links of obesity and mortal PC. It is suggested that obesity lead to more aggressive prostate cancer through detection biases as well as altered biologic signaling. DRE: Digital rectal examination, T: Testosterone, E: Estrogen. Reprinted from European Urology, Vol. 12, Emma H. Allott,Elizabeth M. Masko,Stephen J. Freedland, Obesity and Prostate Cancer: Weighing the evidence, 01344-9, Copyright 2012, with permission from Elsevier.

It is suggested that the endocrine activity from adipose tissue, secretion of leptin, interleukin-6 (IL-6) and lowered adiponectin levels favor subclinical chronic inflammation which in turn stimulates PC development (41, 42).

As described in figure 1.2, increased conversion of testosterone to estrogen may promote progression of PC. As reviewed by Bonkhoff & Berges (45), the human prostate has the estrogen receptors alpha (ERα) in stromal cells and beta (ERβ) in the luminal cells. The estrogen receptors are sometimes modulated during prostate carcinogenesis, where ERα expression extends also to the luminal cells while ERβ expression is partly lost. This has led to the suggestion that ERα have oncogenic and ERβ protective effects. This hypothesis is also supported by the upregulation of the progesterone receptor by ERα.

1.2 Inflammation and prostate cancer

The notion that inflammation could act as a modulator of cancer development was first proposed in the year of 1863 when Virchow published his hypothesis that cancer cells develops at sites of chronic inflammation (46). Data from both animal and human studies suggests a role of chronic inflammation in PC development (47).

Inflammation is a complex set of responses to tissue damage or pathogens that are perceived as harmful to the organism. An inflammatory response is essential to limit the spread of pathogens, eliminate infections, remove debris and resolve tissue injury. One of the cardinal physical signs of inflammation is swelling and locally increased blood flow, which is mediated by a number of cytokines and chemical agents that dilate blood vessels and increase capillary permeability (9). Neutralization of the cause of inflammation is normally followed by resolution and tissue repair. If the inflammation is not resolved however, a state of chronic inflammation may occur.

Prostatic inflammation can be caused by numerous factors (figure 1.3). Indications of inflammation in benign biopsy samples are associated with an increased risk of developing PC. Active inflammation in and around the prostate is associated with a worse outcome, suggesting that inflammation creates a procarcinogenic environment, and that inflammation is also driven by tumor progression or vice versa (48).

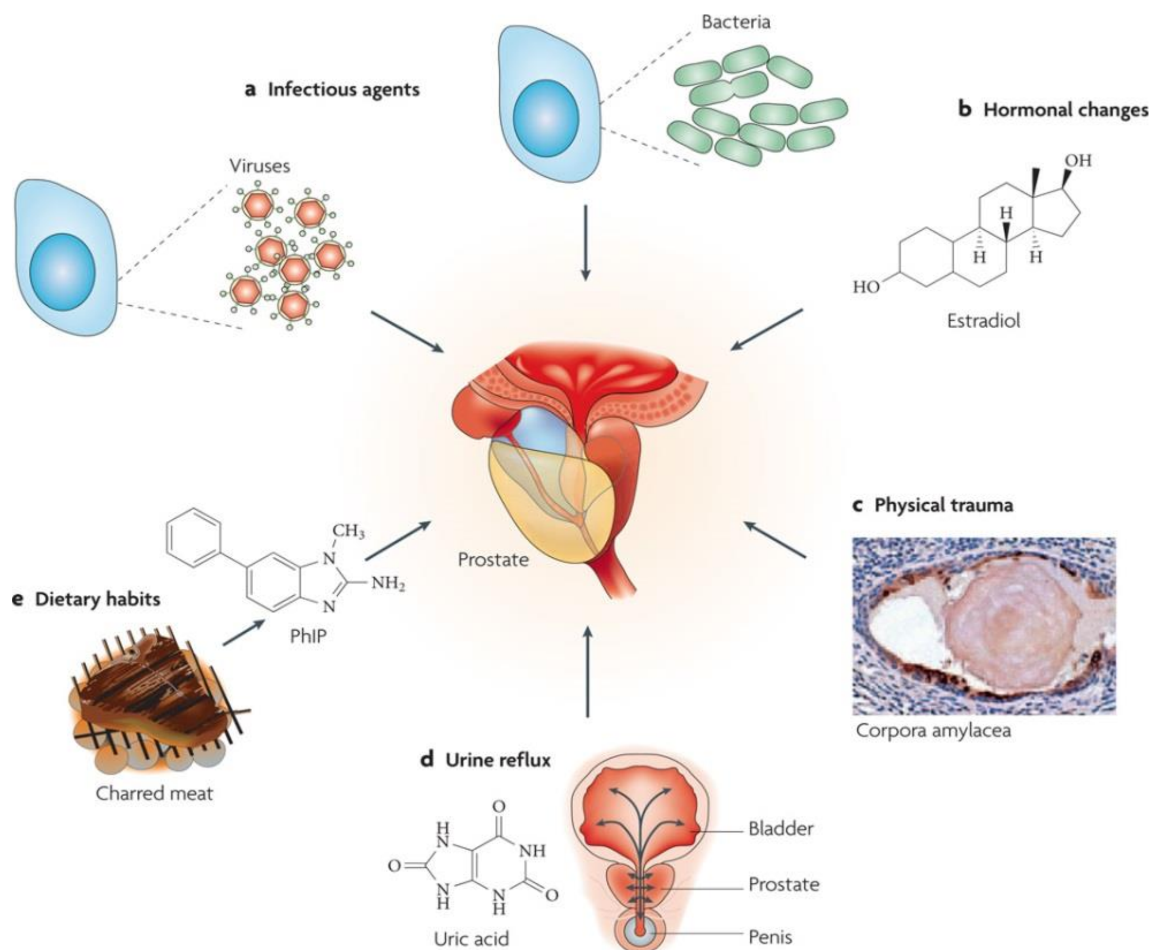

Figure 1.3. Possible causes of prostate inflammation. Inflammation in a healthy prostate can be initiated due to numerous causes such as a) infectious agents, b) hormonal alterations during early prostate development leading to anatomical alterations that induce inflammation, c) physical trauma, d) compounds in urine that during reflux can activate immune response, e) ingested dietary carcinogens such as the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhiP), commonly found in burned meat. Adapted by permission from Macmillan Publishers Ltd: Nat Rev Cancer. 2007 April; 7(4): 256–269, copyright 2007.

The immune system and its associated inflammatory response have a role in the immunosurveilance and continuous eradication of upcoming cancer cells (49). As recently reviewed by Candido & Hagemann (50), it has further become evident that an inflammatory pathway is activated, and taken advantage of, by the cancer cells themselves in the neoplastic process. Several proinflammatory cytokines have been linked to the development and stage of various types of cancers (50).

Several cytokines are potential modulators of inflammation and PC development, such as macrophage inhibitory cytokine 1 and IL-6 which both have been found to be upregulated in

PC (48). Activated nuclear factor kappa B(NF-κB) (a family of transcription factors important in immune- and inflammatory responses) has also been associated with progression of prostate cancers (51).

A differential expression of chemokines, which in turn attract leukocyte infiltration and modulate growth, angiogenesis and drive metastasis, have been seen in PC tumor and stromal cells along with progressing disease (52).

1.2.1 C-reactive protein

C-reactive protein (CRP) is a non-specific acute phase protein that is produced by hepatocytes in response to infection, trauma and inflammation (53). CRP plays a role in the clearance of apoptotic and necrotic cells, and some bacteria. An elevated level of CRP is described as an independent risk factor for decreased life expectancy and increased mortality among PC patients (54-56), and thus link systemic inflammation to poor outcome in PC patients. The expression of CRP is largely under control of the cytokine IL-6.

1.2.2 Interleukin-6

IL-6 is a multifaceted cytokine involved in a range of functions and is produced by immune cells as well as adipocytes and skeletal muscle. Elevated levels of IL-6 have been associated with several inflammatory, autoimmune and malignant disorders (57). IL-6 has a role in intensifying acute inflammation as well as in promoting chronic inflammation (58). In PC patients, levels of IL-6 are higher in metastatic and androgen independent conditions and has been shown to correlate with mortality (59), PC cell line experiments have further shown that IL-6 can induce conversion from benign to malignant prostate cells (60).

1.2.3 Soluble urokinase plasminogen activator receptor

Soluble urokinase plasminogen activator receptor (suPAR) is a relatively recently described biomarker of inflammation. The suPAR protein is formed form cleavage of the GPI-linked urokinase plasminogen activator receptor (uPAR) (figure 1.4), and is found in plasma, urine, serum and cerebrospinal fluid (61). The expression of uPAR is induced in situations of injury, inflammation and tissue remodeling (62). Elevated levels of suPAR have been found in patients with inflammatory diseases and it has been identified as an independent marker for a

negative prognosis in type-2 DM, cardiovascular diseases and cancer (63). Several types of tumor cells present more uPAR, and release more suPAR as compared to original healthy cells (64).

The level of suPAR is proposed to reflect a general activation of the immune system. In PC, levels of suPAR have been linked to aggressiveness of PC (65, 66). PC patients have shown to have elevated levels of suPAR compared to healthy men, and a markedly higher level is seen in patients with metastasis compared to men with localized PC (65). PC patients undergoing prostatectomy has displayed a subsequent decrease in circulating suPAR levels (65).

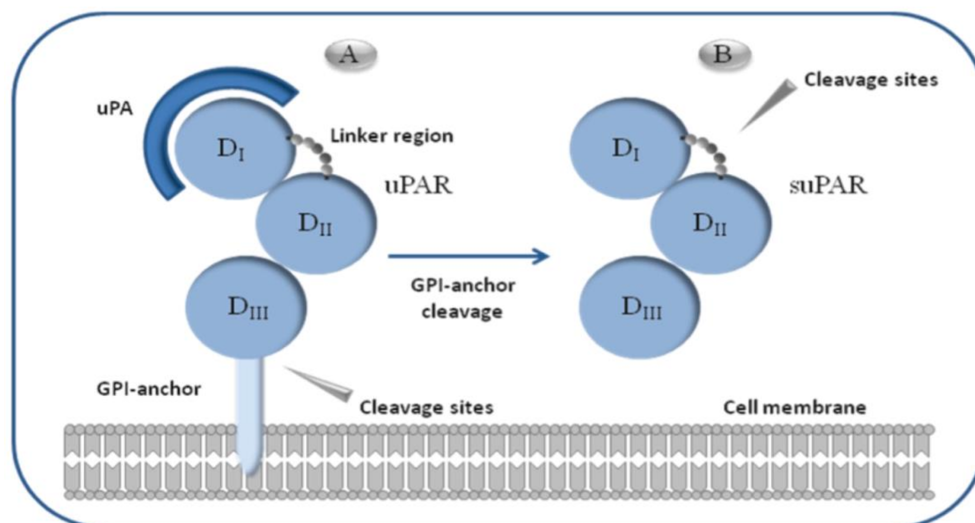

Figure 1.4 The figure displays uPAR interaction with its ligand uPA. Upon interaction with uPA uPAR cleaves between the D_{III} domain and the GPI anchor, forming free $suPAR_{I-III}$. Then $suPAR_{I-III}$ can later be cleaved between D_I and D_{II} by different proteases. In human serum, the molecule has been found in all the forms; $suPAR_{I-III}$, $suPAR_{II-III}$ and $suPAR_{I.}$ Image reprinted with permission from the author (61).

1.3 Nutritional factors and the risk of PC

The comprehensive Second Expert Report from the World Cancer Research Fund (WCRF) concluded about the evidence levels for foods and nutrients which has been indicated to modulate risk of PC (table 1.1) (67). The strongest evidence for decreased risk was found for foods containing lycopene and selenium.

Table 1.1. Statements from the Second Expert Report from the WCRF, 2007

Evidence level	Decreases risk	Increases risk
Probable	Foods containing lycopene Foods containing selenium Selenium supplement (200 µg/day)	Diets high in calcium (≥1.5 g calcium/day)
Limited-suggestive	Pulses / legumes Foods containing vitamin E Alpha-tocopherol supplement (50 mg/day)	Processed meat Milk and dairy products (≥1.5 g calcium/day & high dairy consumption)

1.3.1 Tomatoes and lycopene

The Second Expert Report from WCRF in 2007 concluded that foods containing lycopene probably decrease risk of developing PC (67). Lycopene is a carotenoid without vitamin A activity found in tomatoes, watermelon, papaya, grapefruit and in minute amounts in asparagus, red cabbage, mango and carrot (68). Upon consumption, this lipid-soluble compound is transported by lipoproteins and accumulates with varying density in different organs. The highest concentrations are found in the androgen producing adrenal glands and testes, followed by liver (69). Lycopene is also taken up in the human prostate and plasma levels have shown to correlate to the concentration within the prostate (70), and an increase in prostatic lycopene concentration occurs after supplementation (71).

Tomato is a frequently consumed food in the general Norwegian diet (72), and thus tomato and tomato products are assumed to be the major dietary source of lycopene. Circulating lycopene levels have been indicated to be inversely associated with advanced PC (73, 74), however, some studies have not seen this (75, 76). The effects of supplementation with foods rich in lycopene on PC have previously been examined in small study populations with varying results. A recent Cochrane review of Randomized Controlled Trials (RCT) concludes that there is currently insufficient evidence to neither support nor refute the use of lycopene

supplementation for prevention of prostate cancer (77), however this systematic review only found 3 studies of sufficient quality to be included.

The available literature on lycopene and PC indicate, with uncertainty, a potential effect of tomato products in PC development. Clinical intervention studies in established PC are not conclusive (78-84), there are few controlled trials (80, 82, 84) and more studies are needed to elucidate effects of tomatoes or lycopene in ongoing malignant tumors in the prostate.

1.3.2 Soy isoflavones

As mentioned, the Second Expert Report from the WCRF concluded that there is suggestive evidence that intake of pulses and legumes decrease risk of PC (67).

In a later meta-analysis, support for soy consumption and a risk reduction of developing PC is put forward. Yan & Spitznagel (85) found that, overall, a higher intake of soy foods was associated with a decreased risk of PC. This was particularly the case in Asian study populations while there was no significant association in western populations alone. Furthermore, there were no significant associations with fermented soy products and risk of PC.

Dieraty intake of soy products is considerably higher in Asian countries compared to North American and Europe (86), and the highest intake in the world is seen in Japan where the daily per capita consumption is about 9 grams of soy protein.

Another meta-analysis (87) found that total soy intake was associated with a decreased risk of PC. This study also examined individual soy foods, where high tofu intake had an association with decreased risk, whereas soy- bean, milk, or natto did not. The predominant isoflavones in soy products are daidzein and genistein (68). Among isoflavones, genistein and daidzein showed a significant association with decreased risk (87).

1.3.3 Selenium

Selenium is an essential trace element and serves as a cofactor for several antioxidant enzymes. In addition, selenium is essential for endocrine functions including thyroid and immune functions, and it has also been suggested that selenium may play a role in the expression of androgen receptor (AR) (88).

The Second Expert Report from the WCRF rated foods rich in selenium as well as supplements (200 µg/day) to "probably" decrease the risk of PC (67).

Since 2007, the potential beneficial effect from selenium has been further explored. The Selenium and Vitamin E Cancer Prevention Trial (SELECT) study (89) was a comprehensive RCT (n = 34888) aimed to examine effects from supplemental selenium, vitamin-E, and selenium and vitamin-E combined as compared to placebo. The SELECT study was prematurely aborted after a median of 5.5 years due to lack of evidence for a benefit, coupled with non-significant increased risks of PC and DM from vitamin-E and selenium respectively. A subsequent follow-up report (n = 34887) found no significant effect on PC risk nor DM from selenium after a three more years, but a significant increased risk of PC in the vitamin-E group (90). The group that had received vitamin-E and selenium combined, however, showed no increase in PC risk.

As a follow-up of the report by WCRF, a systematic review and meta-analysis was published in 2012 (91). The authors reported the findings of a gradual decreased risk of PC in subjects with serum or plasma selenium levels up to 170 ng/mL, as well as a decreased risk with toenail selenium between 0.85 and 0.94 µg/g. Total intake of selenium and PC risk was reported in only two studies, where one case control showed decreased risk in the intake range of 88-119 µg/day compared with both higher and lower intakes.

1.3.4 Omega-3 fatty acids

Long chained omega (n)-3 fatty acids are dietary components found in marine foods. These fatty acids are essential nutrients. Eicosapentaenoic acid (EPA) compete with the omega (n)-6 fatty acid arachidonic acid in the cyclooxygenase (COX) pathway and may thereby reduce the production of pro-inflammatory eicosanoids (92), and the n-3 fatty acid docosahexaenoic acid (DHA) may increase production of anti-inflammatory resolvins (93).

A systematic review and meta-analysis found significantly decreased risk of mortal PC with high fish intake, but only borderline significant associations with decreased incidence of PC (94). A more recent review concluded that the epidemiological data provides inconsistent results and no clear conclusions on n-3 fatty acids and risk of PC (95).

1.3.5 Green and black tea

Tea is one of the most widely consumed beverages in the world, and black and green teas are the most consumed. Black tea is processed by fermentation whereby components in the leaves are oxidized and/or polymerized. The black discoloration of the leaves is a result of this processing. Green tea on the other hand is quickly steamed before it is dried. As reviewed by Sharma and Lao (96), the concentration of catechines is several fold higher in green tea, whereas black tea contains markedly higher amounts of oxidized polyphenols.

A meta-analysis of 13 studies (97) examined observational studies of black and green tea and risk of PC. This study found borderline significant trends towards decreased risk of PC in high green tea consuming Asian populations. When differentiating between study designs, a significantly decreased risk of PC was seen in the case control studies but not in the cohorts. No associations were seen between black tea consumption and risk of PC.

An earlier Cochrane review examined green tea and risk of cancer incidence and mortality. This review only included one RCT, two cohort and two case control studies on PC (98). The RCT found a significant lowered risk of developing PC in patients with high grade prostate intraepithelial neoplasia compared to placebo after a one year green tea catechin supplementation. The included observational studies showed inconsistent results.

More recent individual studies examining effects on tea consumption on risk of PC have mixed results. A cohort on an ethnic Chinese population found increased risk of PC among weekly and daily black tea drinkers but no trend for green tea (99). A cohort on Scottish men found increased risk of PC among those who consumed ≥7 cups per day and increased risk of PC with increasing consumption (100).

1.3.1 Pomegranate and grapes

Both pomegranates and grapes have received research attention with regards to chemoprevention (101), however there is very limited from clinical human studies. Pomegranate contains high concentrations of the phytochemicals ellagitannins and anthocyanins, while grapes have received the most attention due to the contents of resveratrol.

A few animal studies have reported intriguing effects of pomegranate on PC. A study on male transgenic mice, prone to develop PC, tested pomegranate extract in two doses (equivalent to

an intake of 250 mL or 500mL in humans) compared to water (102). In this study, both intervention groups developed significantly less PC, and had a significantly lowered IGF-1/IGFBP3 ratio compared to controls. This study also assessed life-span, with the same intervention protocol but unlimited period of time. In this experiment, both supplemented groups lived significantly longer than the controls.

Data on effects of grapes in PC are predominantly from *in vitro* studies that suggest grape induce apoptosis in PC cells but not normal prostatic cells (103), and apoptosis as an effect of radiation is reportedly increased in cells treated with resveratrol (104). In prostate cancer calls treated with resveratrol, the expression of both PSA and androgen receptor were inhibited compared to control, and this effect was supported by changes in gene expression of other genes related to androgen receptor signaling (105).

2 Aims

2.1 Aims of the PFPI study

The main aim of the Prostate Phytochemical & PUFA Intervention (PFPI) study was to evaluate the effects of selected dietary components on developments in PSA in patients with established PC. Secondary endpoints included biomarkers of inflammation, oxidative stress, PC progression and compliance to the intervention, along with food frequency questionnaires, gene expression profiling and histological examinations of prostate tissue and general biochemical and anthropometric characteristics of the patients. The study was designed as a RCT with a control group and two parallel interventions; one group was set up to supplement their diet with tomato products, and the other group was given supplements of soy isoflavones, selenium, green and black tea as well as pomegranate- and grapejuice in addition to tomato products. The intervention lasted for 3 weeks in the period between diagnosis and scheduled treatment.

2.2 Specific aims of this master thesis

This master thesis is conducted within the PFPI study and with a special focus on the main endpoint PSA, as well as compliance and selected biomarkers. Thus, the specific aims of this master thesis are:

- Evaluate developments in PSA values as an effect of the tomato or multi-diet intervention in subjects with PC
- Study self-reported compliance and changes in selected biomarkers of compliance, to the two dietary interventions
- Evaluate changes in the biomarkers of inflammation (IL-6, suPAR and CRP) and the hormone IGF-1 as an effect of the tomato or multi-diet intervention in subjects with PC
- Evaluate if *a priori* defined subgroups might respond differently to the interventions based on baseline levels biomarkers for selected dietary components associated with PC
- Test whether changes in any of the endpoints or characteristics measured within the PFPI study, were correlated to changes in PSA-levels

3 Methods

In this section the clinical trial will be introduced followed by descriptions of the statistical analysis, the laboratory analysis and an overview of the contribution of the master student.

3.1 The Prostate Phytochemical & PUFA Intervention

The Prostate Phytochemical & PUFA Intervention study (short name: PFPI; ClinicalTrials.gov identifier: NCT00433797) is a phase I/II study in prostate cancer patients initiated in 2007 and with the last patient follow up completed in March 2012. The trial had consent from the regional ethics committee in Norway (REK Sør, nr. S-06187). The following sections describe the study in more detail.

3.1.1 Recruitment

Patients were recruited at DNR and Aker between the years 2007 and 2010. Inclusion criteria included above 5 years life expectancy and a histologically confirmed adenocarcinoma in the prostate gland. Complete inclusion and exclusion criteria are listed in table 2.1 below.

Table 2.1. Criteria for participation in the trial

Criteria are displayed as listed in the study protocol.

Inclusion Criteria:	Exclusion Criteria:
• Adenoarcinoma (as confirmed by histology) • N0/NXM0 and suitable for brachytherapy or radical prostatectomy • Serum tPSA< 20 ng/mL, and Gleason score =>6 or T1c- T3a, prostate volume < 60mL • Performance status 0-1, normal white blood cells and thromocytes, hemoglobin >11g/dl	• Previous endocrine treatment • Life expectancy < 5 år • Possible co-morbidity (cardiovascular disease, chronic obstructive pulmonary disease, diabetes type-I, vasculatory syndromes or inflammatory diseases that may affect quality of life and radiation therapy) • Urinary retention, incontinens or International Prostate Symptom Score (IPSS) score >12

A total of 160 patients were considered for the study, of which 86 agreed to participate, and 78 patients completed the trial (figure 2.1).

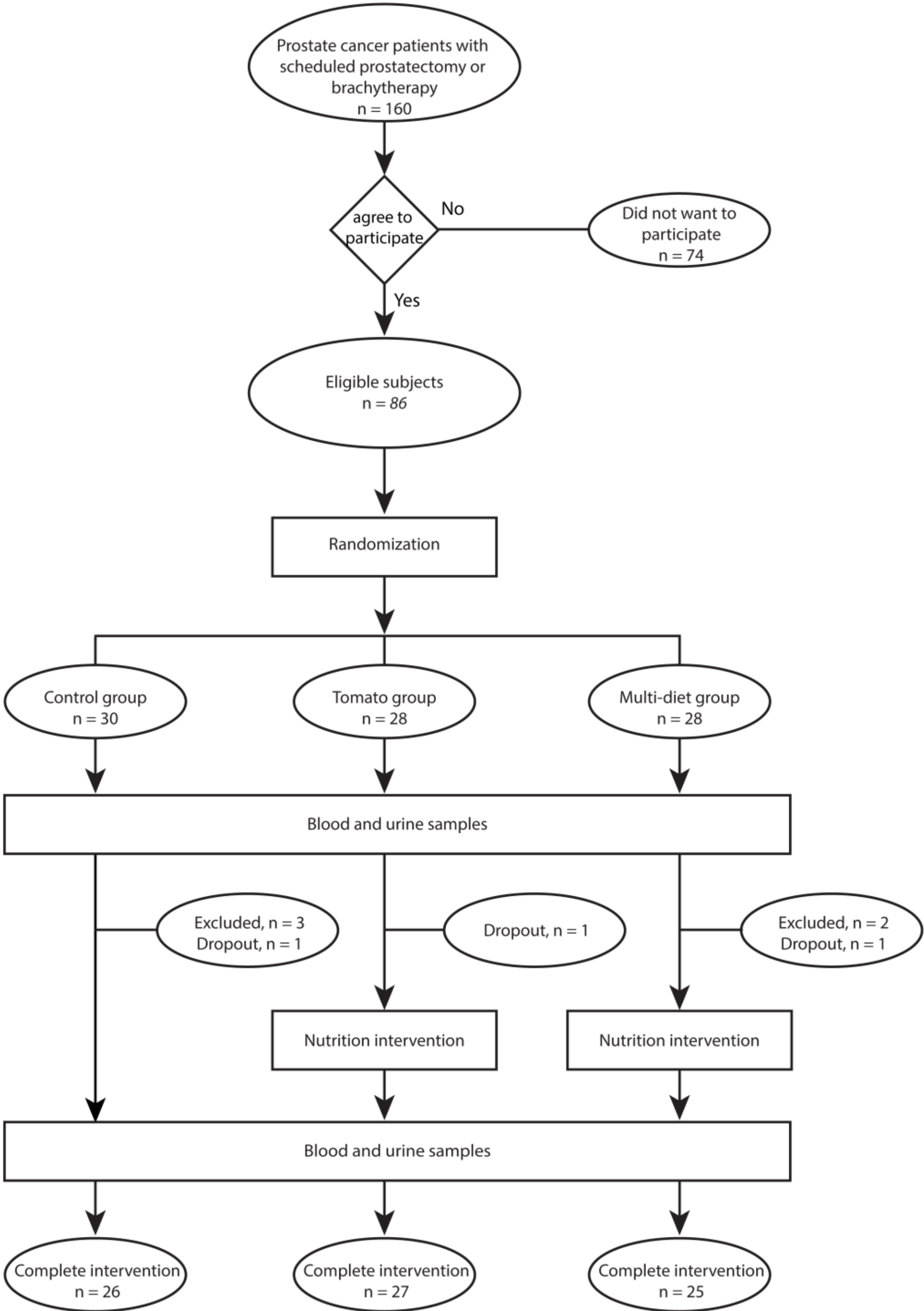

Figure 2.1. Subject recruitment. In total 78 subjects completed the trial. In the control group, three subjects were excluded due to comorbidities, and one dropped out for unknown reasons. One subject also dropped out of the tomato intervention for unknown reasons. In the multi-diet intervention, one subject was referred to a different hospital, one changed date of operation and one dropped out due to personal wishes.

3.1.2 **Randomization**

Subjects were randomly allocated to either a control group or one of two interventions; tomato intervention and multi-diet intervention. Subjects were block-randomized with varying block sizes by the research office of DNR.

3.1.3 **Power calculation**

Power calculation was performed based on an anticipated change in t PSA values. The relevant effect size was set to a 20 % decrease in tPSA compared to controls. Standard deviation was approximated to be 3.5 ng/mL, in order to reach a power of 0.8 and a significance level of 0.050 it was calculated that there would need to be 28 subjects in each group. A dropout rate of 20 % was anticipated and the total number of subjects to be recruited was set to 102.

3.1.4 **Intervention**

The intervention was carried out in the time window between diagnosis and elective treatment of either prostatectomy or brachytherapy. The timespan of the intervention period was around three weeks for both intervention groups (table 2.2). The control group was encouraged to continue their habitual diet, whereas the other two intervention groups received different supplemental dietary products described below.

Table 2.2. Length of the intervention

Data is displayed as median (range).

	Tomato Intervention	Multi-diet intervention
Time span [days]	21.5 (12-27)	21 (19-24)

Tomato intervention

Prior to the commencement of the study the concentration of lycopene in a wide range of tomato products had been examined (106). Subjects in the tomato group were provided with a selection of commercially available tomato products found to be high in lycopene content, the aim was to give an added daily intake of approximately 30 mg of lycopene per day (table 2.3).

Table 2.3. Food products used for the tomato intervention

Subjects were instructed to consume one daily serving from one of the tomato products. Serving size is displayed as a proportion of the prepackaged product size.

Brand	Product	Prepackaged amount	Lycopene [mg / 100 g]	Serving size	Lycopene per serving [mg]
Barilla	Pastasauce with basil	400 g	16.7	½	33.4
Dolmio	Pastasauce extra garlic	500 g	13.8	½	34.5
Dolmio	Pastasauce with sundried tomatoes	500 g	16.2	½	40.5
Knorr	Pastasauce with chili and onion	400 mL	14.1	½	28.2
Cadisco	Tomato juice	1 L	11.1	¼	27.8
ICA	Tomatoes, chopped	500 g	12.0	½	30.0

Multi-diet intervention

Participants in the multi-diet intervention group were provided with the same intervention as the tomato group, with the additional intake of grape and pomegranate juice, green and black tea, and supplements of selenium, isoflavones and fish oil (table 2.4).

Table 2.4. Products included in the multi-diet intervention

Producer	Product name	Product type	Amount/day
Tine	-	Grape juice	330 ml
Tine	-	Pomegranate juice	330 ml
Twinings of London	Jave green tea	Green tea	1 cup
Twinings of London	Earl Grey sort te	Black tea	1 cup
Nycoplus*	Omega-3 1000 mg	Fish oil	3 x 1000 mg 2 x 1000 mg
Solaray*	Selen	Selenium	1 x 100 µg 1 x 100 µg
Nature's sunshine*	Super soy extra	Isoflavones	2 x 40 mg 3 x 40 mg

*: The intake these of supplements were divided into a morning and evening dose.

The grape and pomegranate juices were provided from Tine specifically for the trial. The fish oil supplements were, according to the manufacturer, extracted from sardines and anchovies and contained 62.5 % n-3 fatty acids. The total intake of long-chain n-3 fatty acids in the multi-diet intervention was 2.8 grams (of which 1.6 of EPA, 1.1 grams of DHA and 0.1 gram of DPA) per day. The selenium supplement was in an organic form containing 1-selenomethionin. The isoflavone supplement came as an extract from soy beans, giving a daily intake of 200 mg isoflavones per day.

3.2 Statistical analysis

Normally distributed variables were first analyzed with an ANOVA test, if significance was found an individual student's t-test between groups was performed. Non-normally distributed variables were analyzed with Kruskal Wallis tests, if this displayed significance Mann Whitney tests between individual groups were performed. A majority of results were not appropriately distributed for parametric tests, thus the data in this thesis are displayed with the results from non-parametric analyses.

Relative changes in the multi-diet- and tomato group were compared to the control group in all end points. In order to decrease type-1 errors and limit the number of hypothesis tests, a predetermined scheme for statistical analysis was adopted. These predetermined analyses were subgroup analyses based on differing baseline characteristics (described in section 3.2.1), as well as possible group comparisons as subsequently indicated by a Principal Component Analysis (PCA) (described in section 3.2.2). Introduction of subgroup analyses introduce a multiplicity to the hypothesis testing, and p-values should be interpreted accordingly. Although 78 patients participated in the trial, the number of participants analyzed differs slightly between the endpoints due to missing samples from a few individuals.

3.2.1 Subgroup analysis

In order to elucidate possible divergent response to the intervention based on differing patient-characteristics, it was decided that subgroup based on baseline values should be compared (figure 3.1).

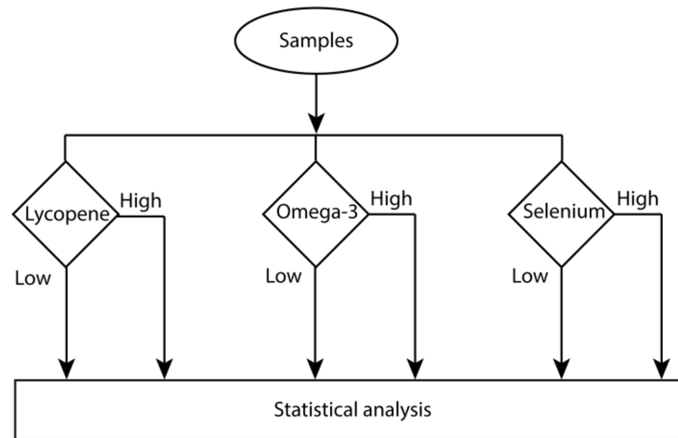

Figure 3.1. Samples were analyzed in subgroups derived from baseline characteristics. Median value of plasma lycopene, n-3 fatty acids in red blood cells and plasma selenium was used to differentiate subjects.

Tumor risk was classified based on prognostic risk factors, a criteria originally proposed by D'Amico *et al*. (107) in 1998 and has since been adapted by the European Association of Urology for use when considering treatment options for PC patients (20). These risk categories are applied in clinical evaluation of treatment options at DNR (table 3.1).

Table 3.1 Tumor risk classification

Risk group	Stratification group	T-classification	tPSA [ng/mL]	Gleason score
Low	1	T1c-T2a	tPSA ≤ 10	≤ 6
Intermediate	2	T2b-T2c	10 < tPSA < 20	7
High	3	T3x	20 ≤ tPSA	8-10

Stratification was based on factors indicating an unfavorable prognosis. Subjects were categorized based on gleason, T-score and tPSA values. If one or more factors were met the subject was placed in the respective group.

In brief, the criteria take into account tPSA levels, histological differentiation in cancer cells and size and invasiveness of the tumor. Cell differentiation pattern is classified by the Gleason grading system (figure 3.2), and the size and invasiveness of the tumor is classified by the Primary Tumor T-classification system (table 3.2).

Figure 3.2. The Gleason grading system. The gleason grading is based on the two most common patterns seen histologically in the tumor tissue, originally described by Gleason in 1966 (108). The pathologist assigns a score for these which are added together in a range between two and ten, with ten indicating the worst prognosis. The grade is further differentiated by a- and b-grading, where for example 7a and 7b indicates 3 respectively 4 as the most prevalent pattern. Image is reprinted with permission from the Oncolex online encyclopedia (109).

Table 3.2. The Primary Tumor (T) classification

T-value classification of prostate cancer as described in the European Association of Urology's guidelines on Prostate Cancer (20).

Primary Tumor (T) classification		Explanation
Tx		Primary tumor cannot be assessed
T0		No evidence of primary tumor
T1		Clinically inapparent tumour not palpable or visible by imaging
	T1a	Tumour incidental histological finding in 5% or less of tissue resected
	T1b	Tumor incidental histological finding in more than 5% of tissue resected
	T1c	Tumour identified by needle biopsy (e.g. because of elevated prostate-specific antigen [PSA] level)
T2		Tumour confined within the prostate
	T2a	Tumour involves one half of one lobe or less
	T2b	Tumour involves more than half of one lobe, but not both lobes
	T2c	Tumour involves both lobes
T3		Tumour extends through the prostatic capsule
	T3a	Extracapsular extension (unilateral or bilateral) including microscopic bladder neck involvement
	T3b	Tumour invades seminal vesicle(s)
T4		Tumour is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum,levator muscles, and/or pelvic wall

When surgery had been performed, Gleason and primary tumor classification (T-classification) were corrected based on the histological classifications performed by the pathologist post-surgery. In order to get the most accurate data for the statistical analysis, the post-surgical classifications for tumor risk categories were used.

3.2.2 **Principal Component Analysis**

A principal component analysis (PCA) was used to explore the variation between multiple parameters that otherwise might go unnoticed using conventional statistics.

PCA are mainly used to reduce the complexity of large datasets. For simplicity we can consider how PCA is used to reduce three dimensions (or parameters) into two dimensions. In three dimensions the whole data set can be seen as a swarm of points where, X_1, X_2 and X_3 represents three different variables. Subjects, as represented by blue dots, form a specific pattern in the three dimensional space (figure 3.3).

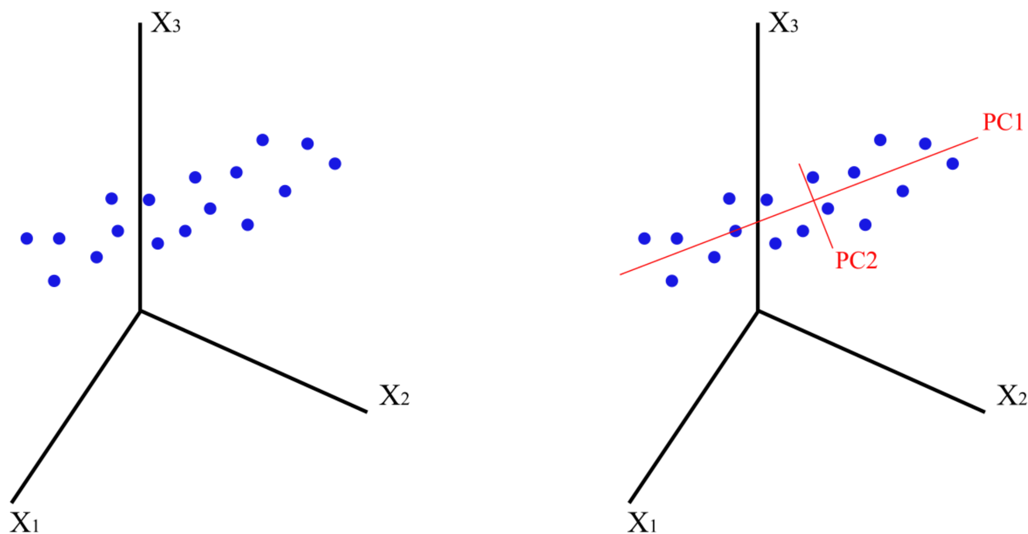

Figure 3.3. Dimension reduction. The data displayed in this scenario shows a clear pattern, which might not be detected by any individual test between X_1, X_2 or X_3. In the right figure the first and second principal component, is visualized in red, describing where the most variation in the data is found. Illustrations are adapted from *Multivariate Data Analysis in practice*, by Kim H. Ebsen (110).

In a PCA, the first principal component (PC) accounts for as much of the total variability as possible and is put as a straight line where most of the variation is explained (figure 3.4). The second principal component (PC2), is placed orthogonal to the first PC, and accounts for as much as possible of the remaining variation. If the number of total parameters are higher than 3, the number of PCs can be extended, where PC3 accounts for as much of the remaining variation as possible, and so on. The two dimensional plots, or score plots, visualizes how much the subjects are inter-related. The *correlation loading* which is interpreted together with

the score plot, shows the importance of the different variables for the PCs .Variables that are in the outer circle have largest influence on the PCs. Those that are in the same direction are correlated, whereas variables on opposite sides of the plot are indicated to be inversely correlated.

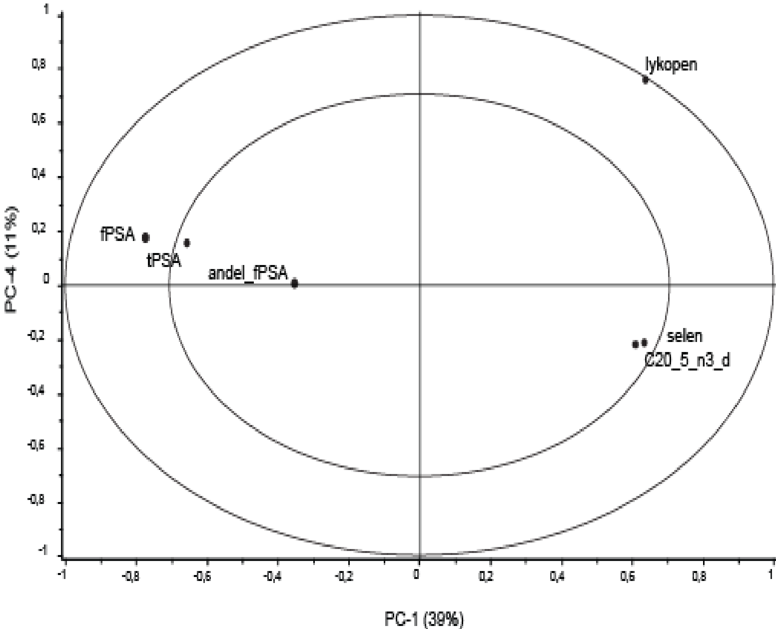

Figure 3.4. PCA correlation loading plot, PC1 and PC4. Variables that are close to our outside the inner circles are indicated to influence on the PCs. Those that are in the same direction are associated, whereas variables on opposite sides of the plot are indicated to be inversely correlated.

3.3 Laboratory analyses

Biochemical analysis on plasma samples and red blood cells were either performed in house by the master student, or by contracts with external laboratories. In the following section, all analyses are introduced. Internally performed methods are described in detail, whereas externally performed methods are briefly introduced with a more detailed description to be found in appendix 9.1.

3.3.1 Carotenoids in plasma

Carotenoids in plasma was quantified externally at Vitas (Oslo, Norway) using High Performance Liquid Chromatography.

3.3.2 Fatty acid composition in red blood cells

Fatty acids in red blood cells was quantified externally at Vitas using Gas chromatography with flame ionization detector (GC- FID).

3.3.3 C-reactive protein

CRP protein was quantified externally at the Oslo University Hospital by standard procedures. No information has been received regarding the method used.

3.3.4 Insulin-like growth factor-1

Insulin-like growth factor-1 was quantified externally at Oslo University Hospital using immunometric enzyme-labeled chemiluminescence.

3.3.5 Prostate specific antigen

Prostate specific antigen was quantified externally at DNR using the AutoDELFIA automatic immunoassay system.

3.3.6 **Selenium**

Selenium in plasma was quantified externally at Fürst Medical Laboratory using Inductively Coupled Plasma Mass Spectrometry.

3.3.7 **Interleukin-6**

IL-6 was quantified in-house using the *Human IL-6 UltraSensitive ELISA-Kit* from Invitrogen™ according to manufacturer's instructions.

Principle of the Assay

The kit comes with clear microtiter plates precoated with monoclonal IL-6 antibodies. Samples and standards are pipetted into these wells where IL-6 in samples bind to antibodies in the precoated wells (figure 3.5). After incubation, unbound material is washed away. Then a second monoclonal antibody-solution is added which binds to the already captured IL-6. After another wash step, the enzyme streptavidin-peroxidase is added. This enzyme binds to the second antibody by forming a non-covalent bond between biotin and streptavidin. After another incubation period the plate is washed again before a substrate solution is added. The streptavidin-peroxidase acts upon this substrate which produces a blue color tone proportional to amount of IL-6 in the wells. To halt the reaction, a stopping solution is added, inducing a shift in color to a yellow tone. A linear standard curve between absorbance and concentration based on standards supplied from the kit are used to calculate concentration of IL-6 in samples.

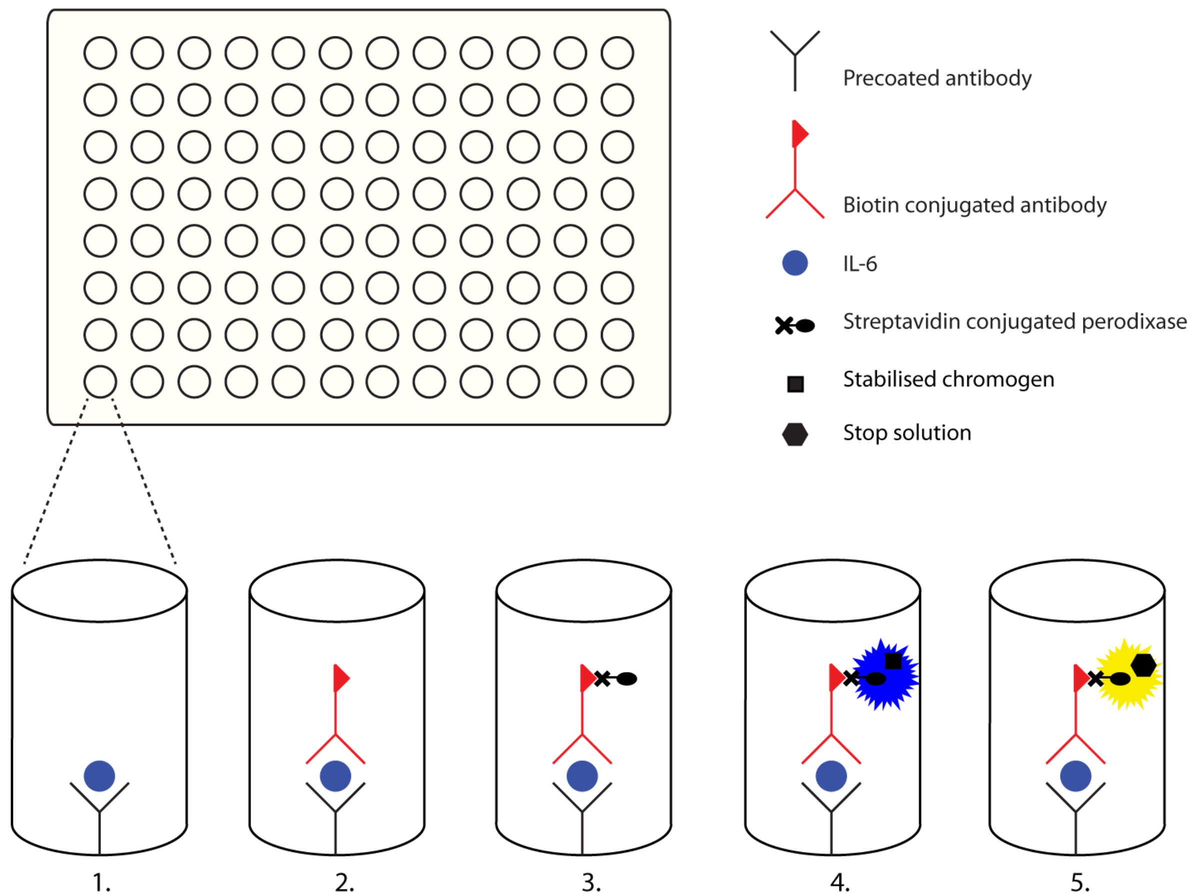

Figure 3.5. Overview of the IL-6 ELISA assay. 1) Wells precoated with antibodies bind to IL-6 in samples. 2) A solution containing biotin-conjugated IL-6-antibodies is added. 3) A streptavidin conjugated peroxidase is added, streptavidin and biotin forms a non-covalent bond. 4) Peroxidase acts on the stabilized chromogen solution and creates a blue color tone. 5) A stopping solution is added, creating a yellow color tone proportional to the amount of analyte.

Evaluation of the assay on plasma samples

This kit was designed for serum samples and is, according to the manufacturer, not properly tested on plasma samples. In preparation of analyses however, it became clear that serum samples were lacking from a majority of the subjects. An experimental approach to elucidate stability of the assay on plasma samples, as recommended by the manufacturer, was therefore performed. Briefly, plasma samples were spiked with a known amount of IL-6 and compared to wells with diluent buffer that had the same amount added. The recovery was then calculated in percentage, the predefined acceptable recovery range was described as between 80 and 120 percent, se formula below.

$$Recovery\ [\%] = \left(\frac{(Y + a) - Y}{(X + a) - X}\right) * 100$$

Recovery was calculated as percentage of IL-6 recovered in plasma. Where X= zero point standard, Y= sample, a = known amount of protein.

The experimental approach to evaluate analysis on plasma samples were done on lithium-heparin samples in triplicate. In this approach samples were spiked with an added concentration of 0.62 pg/mL. The experiment indicated a mean recovery of 55 %. It was decided, that quantity of IL-6 in plasma samples should still be explored, in spite of the indicated reliability.

Procedure

On the microtiter plate, 100 µl of samples and standards were pipetted in duplicate. The plate was then covered and incubated for three hours in 37ºC. All wells were washed six times, between each wash the contents were decanted over a sink and the plate was gently tapped on dry papertowels. After this, 100 µL biotinylated US Biotin Conjugate was added to each well and the plate was left for 45 minutes in room temperature. Then, the plates were again washed six times as previously described. 100 µL of Stabilized Chromogen was added to each well and the plate was left to incubate in complete darkness in room temperature for a maximum of 30 minutes. Color development was periodically evaluated during this time period and the reaction was stopped when colors appeared saturated. Finally, 100 µL stop solution was added to each well and absorbance read at 450 nm.

Reagents

Reagent
IL-6 Standard or samples
Standard diluent buffer
Hu IL-6 US Biotin Conjugate
Streptavidin-HRP Diluent
Wash buffer
Stabilized Chromogen, Tetramethylbenzidine
Stop solution

Materials used

Material	Producer	Country
Human IL-6 UltraSensitive ELISA-Kit	Invitrogen	USA
Titertek Multiskan PLUS	Eflab	Finland

3.3.8 Soluble urokinase plasminogen activator receptor

Soluble urokinase plasminogen activator receptor from patient specimens was quantified in-house using the *suPARnostic® Standard ELISA kit* from ViroGates® according to manufacturer's instructions.

Principle of the assay

The *suPARnostic® Standard ELISA kit* makes use of two monoclonal antibodies. Samples are first mixed with a monoclonal peroxidase conjugated suPAR-antibody solution. After mixing, samples are pipetted into wells on an optically clear microtiter plate that is precoated with a solid phase monoclonal suPAR-antibody. The aqueous and solid phase antiobodies bind to the D2-domain and D3-domain respectively, the kit thereby recognizes and quantifies $suPAR_{I\text{-}III}$ and $suPAR_{II\text{-}III}$.

After incubation the plate is washed to remove unbound material before a solution containing TMB-substrate and hydrogen peroxidase is added. The suPAR-bound peroxidase acts as a catalysing agent for the reaction between hydrogen peroxidase and the chromogenic TMB-substrate, creating a blue color tone.

After incubation with TMB-substrate, a stopping solution containing sulphuric acid is added, inducing a change in pH-value. The change in pH in turn induces a yellow color-tone and the strength of the color is measured (figure 3.6). A linear curve between absorbance and concentration based on standards supplied from the kit are then used to calculate concentration of suPAR in samples.

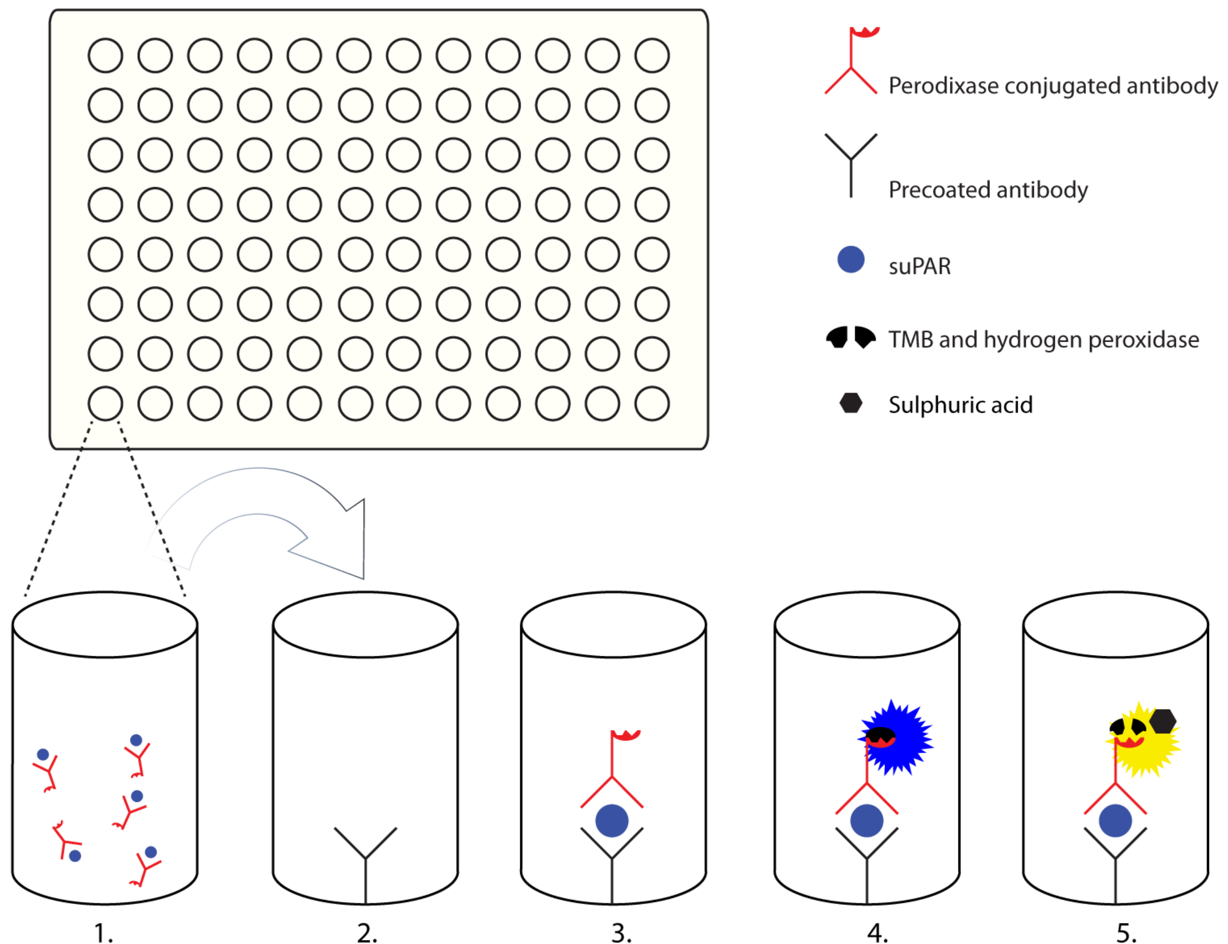

Figure 3.6. Overview of the suPARnostic ELISA assay. 1) Antibodies are mixed with sample, 2) Samples are pipetted into clear wells precoated with suPAR-antibody, 3) The solid phase suPAR-antibody binds and a sandwich is formed, 4) TMB substrate and hydrogen peroxidase is added. The suPAR-bound perodixase acts as a catalysator and a blue color tone is created, 5) Sulphur acid is added, halting the reaction and inducing a change in pH that creates a yellow colortone.

Procedure

Briefly, 25 μL of EDTA-plasma samples, standards, curve control and blanks were mixed, by pipetting with 225 μL peroxidase-conjugated suPAR-antibody solution in a white microtiter plate. 100 μL of the samples were then transferred in duplicate to the clear microtiter plate.

The plate was then sealed and after 60 minutes incubation in complete darkness, wells were then washed five times with 250 μL wash-buffer. After each wash wells were emptied by decanting contents in a sink and gently tapping the plate on dry paper towels.

After this, 100 μL TMB-substrate was added to each well and the plate was sealed and left for another 20 minutes incubation in darkness. Then 100 μL stopping solution was added, and absorbance read at 450 nm.

A linear standard curve between absorbance and concentration, calculated from blank wells and supplied standards from the kit, including a curve control, were used to calculate concentration of suPAR in samples. Standards came in pre-determined concentrations of 1.0, 5.1, 10.4, 15.5 and 20.7 ng/mL, curve control was at 2.6 ng/mL. The *suPARnostic-calculator* (software) supplied by the manufacturer was used to calculate concentrations.

Reagents

Reagent	Components
Peroxidase conjugate	Peroxidase conjugated suPAR-antibody solution
Standards	suPAR in phosphate buffered saline
Wash buffer	Phosphate buffered saline x1 MilliQ water x10
Dilution buffer*	Phosphate buffered saline x1 Peroxidase conjugate x9
Substrate	Tetramethylbenzidine and hydrogen peroxidase
Stopping solution	0.45 M sulphuric acid

*Only used for chromogen-blank wells

Materials used

Materials	Producer	Country
suPARnostic Standard ELISA kit	Virogates	Denmark
suPARnostic Calculator	Virogates	Denmark
Titertek Multiskan PLUS	Eflab	Finland

3.1 Contribution of the master candidate

External analyses described in this thesis were coordinated, samples prepared and delivered by the master candidate or Ingvild Paur. In-house laboratory analyses were planned, prepared and carried out by the master candidate under supervision from Ingvild Paur. PCA were done by PhD Siv Kjølsrud Bøhn, whereas other statistical analyses were performed by the master candidate.

The PFPI-trial as a whole involved a large number of researchers and clinicians at the DNR, Aker and the Department of Nutrition, Faculty of Medicine, University of Oslo. The project group was led by Prof. Rune Blomhoff, Dr. Wolfgang Lilleby, Prof. Sigbjørn Smeland and PhD Anette Karlsen.

4 Results

The results from the PFPI study will be displayed in three main sections; descriptive data, compliance to the interventions and results based on effects of the interventions.

4.1 Descriptive data for the PFPI-study

I n the PFPI-study, a total of 78 prostate cancer patients were randomly allocated to receive one of three interventions; tomato products, a "multi-diet" or controls. Descriptive data of the study populations at baseline were compared, and no significant differences between groups were detected for age, BMI, total cholesterol, HDL, LDL, treatment regime, center for recruitment or smoking status (table 4.1). The distribution of Gleason scores was somewhat skewed between the intervention groups, with the multi-diet group having a wider overall spread of Gleason scores (table 4.1).

Table 4.1. Descriptive data at baseline

Parameter	Control n = 26	Tomato n = 27	Multi-diet n = 25	P-value of between group differences
Age [a]	64 (51 – 74)	62.5 (48 – 72)	64 (54 – 75)	0.277
BMI [a]	26.4 (22.4 – 31.7)	25.5 18.4 – 33.5)	26.4 (20.4 – 48.2)	0.197
Total cholesterol [a]	5.6 (3.3 – 7.1)	5.7 (3.4 – 7.0)	5.6 (3.1 – 8.0)	0.805
HDL [a]	1.4 (0.8 – 2.6)	1.5 (0.9 – 2.7)	1.3 (0.5 – 1.9)	0.528
LDL [a]	3.5 (1.9 – 4.9)	3.5 (1.5 – 4.9)	3.3 (2.0 – 6.0)	0.911
Treatment regime (Surgery/Brachy therapy) [b]	21/5	25/2	23/2	0.447
Center (DNR/Aker) [b]	21/5	16/11	14/11	0.124
Current-/previous-/ never-smokers [b]	4/10/12	3/10/9 (data missing for 5 subjects)	3/8/11 (data missing for 3 subjects)	0.987
Gleason score [a]	7 (5 – 8)	6 (6 – 9)	6 (6 – 8)	0.106

a: Non-normally distributed parameter presented as median (range), Kruskal-Wallis tests was used to detect differences between groups.

b: Counts are compared statistically using the Fisher's Exact test.

4.2 Compliance to the dietary interventions

4.2.1 Self-reported compliance was high across all intervention items

Compliance to the intervention is a prerequisite in dietary intervention studies. The self-reported compliance is calculated as percentage intake of each supplement, where a 100% value means that a supplement was taken in full dosage every day during the trial. According to the self-reported compliance scheme, mean compliance was 96% or above in every single intervention item (table 4.2). One subject in the multi-diet group discontinued the fish oil supplement, whereas all other were taken continuously, and the period of missed intake was maximum two days in a row.

Table 4.2. Self-reported compliance in the tomato and multi-diet intervention groups
Parameter presented as mean (range) in percentage of total compliance to each product used in the intervention.

Intervention item	Tomato group n = 24	Multi-diet group n = 25
Tomato product	99 (89 – 100)	99 (95 – 100)
Selenium	-	99 (94 – 100)
Soy	-	99 (93 – 100)
Fish oil	-	96 (19 – 100)*
Green tea	-	97 (73 – 100)
Black tea	-	96 (77 – 100)
Grape juice	-	98 (91 – 100)
Pomegranate juice	-	98 (91 – 100)

*: One patient discontinued the fish oil supplements after 4 days; all other subjects reported a continuous intake throughout the trial.

4.2.2 Biomarkers of compliance reveal individual variations to interventions

As biomarkers of compliance, carotenoids in plasma, fatty acid composition in RBCs and selenium in plasma were measured. These biomarkers do not only reflect actual intake, but are also influenced by the absorption and metabolism of the compounds.

Carotenoids in plasma

Lycopene is the main carotenoid in tomatoes. It is thus important to measure change in plasma lycopene in order to evaluate the response to the intervention. Overall, there was a clear significant difference compared to the control group, with more than a doubling of median plasma lycopene in the two intervention groups (table 4.3). The individual response in plasma lycopene concentration based on the lycopene interventions varied. Most subjects in the intervention groups displayed strong increases, whereas a few subjects actually exhibited a decrease in plasma lycopene during the intervention period (figure 4.1). No significant differences between the groups were found in any other carotenoids (table 4.3).

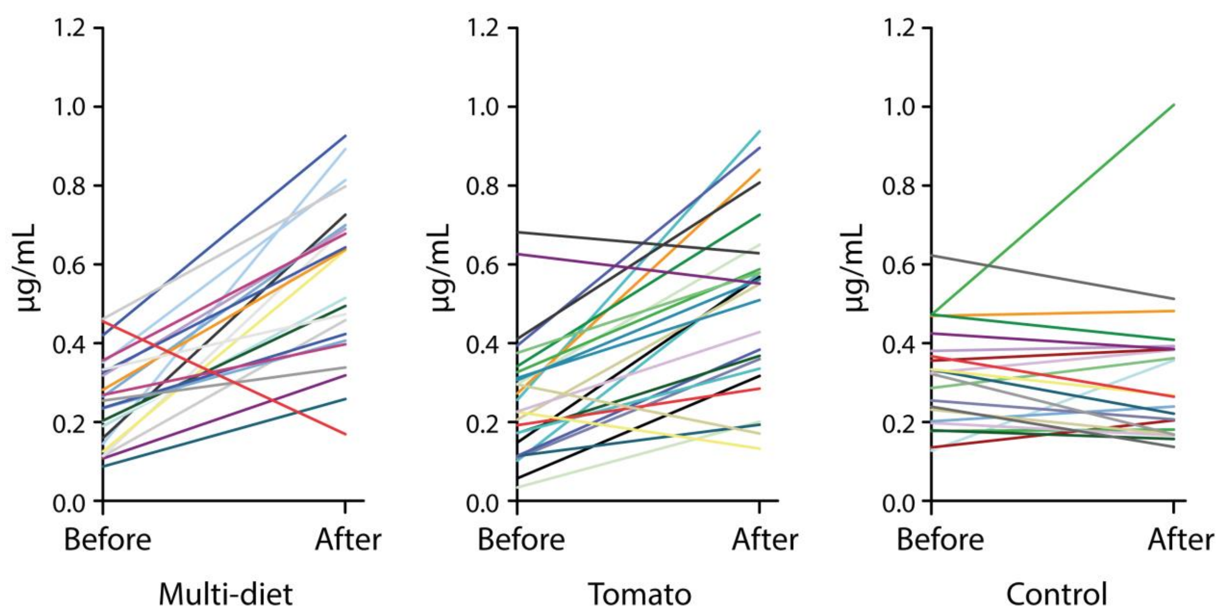

Figure 4.1. Individual changes in plasma lycopene within intervention groups and control.

Table 4.3. Plasma carotenoids and selenium, fatty acids in red blood cells, an overview and comparison between groups

Non-normally distributed parameters presented as median (range). Kruskal-Wallis tests were used to detect differences between groups, when significant Mann Whitney tests between groups were performed. A complete overview of all fatty acids analyzed is presented in supplementary table 8.1.

Parameter	Control group		Tomato intervention		Multi-diet intervention		P-value of between group differences	
	Baseline	Difference	Baseline	Difference	Baseline	Difference	Baseline	Difference
Carotenoids [µg/mL] (Control n = 23, Tomato n = 26, Multi-diet n = 24)								
Lutein	0.118 (0.04 – 0.39)	0.008 (-0.06 – 0.14)	0.120 (0.04 – 0.29)	0.027 (-0.12 – 0.09)	0.136 (0.04 – 0.24)	0.010 (-0.06 - 0.13)	0.498	0.272
Zeaxhantin	0.027 (0.01 – 0.14)	0.002 (-0.05 - 0.03)	0.29 (0.01 – 0.05)	0.001 (-0.02 – 0.02)	0.029 (0.02 – 0.06)	-0.007 (-0.02 – 0.02)	0.895	0.117
Betakrypto xantin	0.055 (0.01 – 0.18)	0.005 (-0.04 – 0.10)	0.052 (0.01 – 0.21)	0.023 (-0.17 – 0.10)	0.062 (0.03 – 0.21)	0.008 (-0.04 – 0.09)	0.345	0.088
Alfa-carotene	0.046 (0.01 – 0.24)	0.000 (-0.09 – 0.09)	0.032 (0.01 – 0.10)	-0.002 (-0.05 – 0.09)	0.037 (0.01 – 0.16)	-0.003 (-0.08 – 0.04)	0.358	0.399
Beta-carotene	0.177 (0.03 – 0.50)	0.017 (-0.18 - 0.18)	0.14 (0.03 – 0.30)	0.056 (-0.16 – 0.26)	0.131 (0.06 – 0.59)	0.058 (-0.18 – 0.18)	0.762	0.157
Lycopene	0.322 (0.13 – 0.62)	-0.022 (-0.15 – 0.53)	0.241 (0.03 – 0.68)	0.253 (-0.12 – 0.68)	0.245 (0.09 – 0.46)	0.323 (-0.29 – 0.75)	0.227	<0.001[a]
Fatty acid [% of total FAME weight] (Control n = 21, Tomato n = 24, Multi-diet n = 25)								
18:2 n-6	7.083 (5.309 – 10.197)	0.259 (-2.323 – 9.780)	7.240 (4.627 – 12.348)	-0.107 (-4.176 – 1.427)	7.087 (5.048 – 13.707)	-0.797 (-7.822 – 1.222)	0.605	0.023[b]
20:1 n-9	0.179 (0.119 – 0.238)	0.002 (-0.058 – 0.025)	0.205 (0.159 – 0.365)	-0.002 (-0.044 – 0.030)	0.212 (0.135 – 0.293)	-0.008 (-0.066 – 0.018)	0.034[c]	0.009[b]
20:2 n-6	0.167 (0.123 – 0.234)	0.002 (-0.028 – 0.032)	0.175 (0.125 – 0.212)	0.002 (-0.017 – 0.018)	0.174 (0.121 - 0.229)	-0.004 (-0.055 – 0.021)	0.325	0.024[b]
20:3 n-6	0.794 (0.606 – 1.291)	0.007 (-0.207 – 0.359)	0.881 (0.579 – 2.019)	0.001 (-0.111 – 0.211)	0.857 (0.540 – 1.608)	-0.127 (-0.567 – 0.146)	0.287	< 0.001[b]
20:4 n-6 & 22:1 n-9	9.907 (7.883 – 13.218)	0.070 (-3.138 – 2.212)	9.742 (7.427 – 12.688)	0.263 (-0.597 – 3.203)	10.147 (6.987 – 12.364)	-0.409 (-1.296 – 2.636)	0.909	< 0.001[b]
20:5 n-3	1.408 (0.685 – 2.154)	0.064 (-0.261 – 0.725)	1.489 (0.434 – 2.490)	0.023 (-0.293 – 0.401)	1.508 (0.698 – 3.742)	0.975 (0.100 – 1.718)	0.874	< 0.001[b]
22:5 n-3	2.484 (1.832 – 3.002)	0.025 (-1.015 – 1.062)	2.464 (1.211 – 3.270)	0.032 (-0.139 – 0.897)	2.580 (1.359 – 3.147)	0.343 (-0.203 – 1.040)	0.720	< 0.001[b]
22:6 n-3	6.097 (4.261 – 7.837)	0.024 (-2.101 – 2.982)	6.021 (2.906 – 8.084)	0.142 (-0.633 – 1.912)	6.049 (3.505 – 8.200)	0.487 (-0.394 – 2.151)	0.802	0.006[b]
Selenium [µmol/L] (Control n = 20, Tomato n = 24, Multi-diet n = 23)								
Selenium	1.1 (0.8 – 1.5)	0.0 (-0.3 – 0.2)	1.2 (0.8 – 1.8)	0.0 (-0.1 – 0.2)	1.1 (0.9 – 1.5)	0.9 (0.6 – 1.3)	0.449	< 0.001[d]

a: $p < 0.001$ between control and the tomato and multi-diet interventions respectively.
b: Non-significant difference between the control group and the tomato intervention, $p < 0.05$ between control and multi-diet intervention.
c: $p = 0.034$ between control and the tomato intervention, $p = 0.015$ between control and multi-diet intervention.
d: $p = 0.456$ between control and the tomato intervention, $p < 0.001$ between control and multi-diet intervention.

Fatty acid composition in red blood cells

Compared to the control group, the multi-diet group displayed an increase in the n-3 fatty acids, and a decrease in the n-6 fatty acids. The n-3 fatty acids 20:5 n-3 (Eicosapentaenoic acid (EPA)), 22:5 n-3 (Docosapentaenoic acid (DPA)) and 22:6 n-3 (Docosahexaenoic acid (DHA)) increased significantly ($p < 0.001$), $p < 0.001$ and $p = 0.002$ respectively). The n-6 fatty acids 18:2 n-6 (linoleic), 20:2 n-6 (eicosadienoic) and 20:3 n-6 (dihomo-gamma-linoleic) acid all decreased significantly ($p = 0.008$, $p = 0.013$ and $p = 0.002$ respectively). The jointly quantified 20:4 n-6/22:1 n-9 (erucic/arachidonic) acids also comparatively decreased ($p = 0.002$). Between the control and multi-diet groups, 20:1 n-9 (gondoic acid) differed at baseline ($p = 0.015$), and the changes were also significantly different ($p = 0.002$). An overview of fatty acids for which significant differences were detected is presented below (table 4.3), and a complete overview of all fatty acids analyzed is presented in supplementary table 8.1.

There were no significant differences in changes between the control and tomato intervention group in any fatty acid analyzed, although the control group had a significantly lower baseline value of the 20:1 n-9 (gondoic acid) ($p = 0.034$).

Selenium in plasma

The plasma selenium increased significantly in the multi-diet group and remained relatively stable in both the control and tomato groups (table 4.3). There were no significant differences in selenium levels at baseline.

4.3 Effects of the intervention

Effects of the intervention will be presented for PSA-measures (primary endpoints), the intervention groups and predefined subgroups, followed by hormones and biomarkers of inflammation in the intervention groups and subgroups. Finally, PCA and relevant subgroup analyses based on the results from the PCA will be presented.

4.3.1 Effect of the interventions on prostate specific antigen

Originally, the PFPI-study protocol was designed to include patients with intermediate risk prostate cancers. The post-surgery tumor classification is however the most accurate, and a relatively large proportion of the patients included in the PFPI study were reclassified from intermediate to high risk. The post-surgery classifications are displayed below (table 4.4), and this classification is used in the analysis.

Table 4.4 Tumor classification in the intervention groups

Post-surgery tumor risk classification, proportions between groups.

Tumor risk classification	**Control**	**Tomato**	**Multi-diet**	**Total**
Low	0	2	0	2
Intermediate	14	16	11	41
High	12	9	14	35
Total	26	27	25	78

In the comparison of PSA values without differentiating by tumor risk groups, there were no significant differences in changes of tPSA, fPSA or the ratio of fPSA/tPSA between the control and intervention groups. Nor were there any significant differences between the groups at baseline (table 4.5).

Table 4.5 PSA values in all subjects, an overview and comparison between groups

Non-normally distributed parameter presented as median (range), Kruskal-Wallis tests was used to detect difference between groups.

Parameter All subjects	Control group n = 22		Tomato intervention n = 25		Multi-diet intervention n = 25		P-value of between group differences	
	Baseline	Difference	Baseline	Difference	Baseline	Difference	Baseline	Difference
tPSA [ng/mL]	9.34 (4.42– 55.00)	0.41 (-1.63 – 4.00)	8.120 (1.52 – 25.90)	0.00 (-3.30 – 2.40)	10.60 (5.10 – 31.50)	0.14 (-12.40 – 4.80)	0.261	0.305
fPSA [ng/mL]	0.97 (0.55 – 2.18)	0.10 (-0.21 – 1.14)	0.82 (0.32 – 2.14)	0.04 (-0.48 – 0.42)	1.09 (0.43 – 4.96)	0.00 (-1.85 – 0.80)	0.207	0.111
Ratio fPSA [% free PSA]	9.59 (2.91 – 28.40)	0.37 (-3.77 – 7.96)	9.49 (4.10 – 21.05)	-0.05 (-1.47 – 5.07)	10.48 (4.46 – 37.69)	0.31 (-2.77 – 5.72)	0.976	0.823

Since the study was originally designed to primarily look at effects in intermediate risk patients, analysis were performed stratified by tumor risk classification. Intriguingly, in this original target population with intermediate tumor risk classification, increases in tPSA levels were significantly lower in the tomato and multi-diet groups, as compared to the controls (table 4.6). No significant differences in PSA-value development were detected in patients with high risk tumors.

Table 4.6 PSA values stratified by tumor classification, an overview and comparison between groups

Stratification based on tumor risk classification. Non-normally distributed parameter presented as median (range). Kruskal-Wallis tests were used to detect differences between groups, when significant Mann Whitney tests between individual groups were performed.

Parameter Intermediate risk	Control group n = 12		Tomato intervention n = 14		Multi-diet intervention n = 11		P-value of between group differences	
	Baseline	Difference	Baseline	Difference	Baseline	Difference	Baseline	Difference
tPSA [µg/mL]	6.68 (4.42 – 17.70)	0.55 (-0.26 – 2.24)	7.81 (1.52 – 18.00)	-0.34 (-1.12 – 1.90)	7.41 (5.10 – 23.50)	0.26 (-12.40 – 1.20)	0.783	0.029*
fPSA [ng/mL]	0.91 (0.59 – 1.65)	0.13 (-0.21 – 1.04)	0.57 (0.32 – 1.96)	0.00 (-0.18 – 0.28)	1.01 (0.43 – 4.26)	0.00 (-1.85 – 0.62)	0.143	0.160
Ratio fPSA [% free PSA]	12.59 (4.69 – 28.40)	0.58 (-3.77 – 6.35)	8.54 (4.10 – 21.05)	-0.31 (-1.47 – 5.07)	11.34 (6.28 – 37.69)	0.48 (-2.60 – 5.72)	0.063	0.931
High risk	**Control group n = 10**		**Tomato intervention n = 9**		**Multi-diet intervention n =14**		**P-value of between group differences**	
	Baseline	Difference	Baseline	Difference	Baseline	Difference	Baseline	Difference
tPSA [µg/mL]	12.75 (5.80 – 55.00)	0.10 (-1.63 – 4.00)	9.04 (5.06 – 25.90)	0.30 (-3.30 – 2.40)	13.05 (7.25 – 31.50)	0.06 (-3.80 – 4.80)	0.402	0.838
fPSA [µg/mL]	1.00 (0.55 – 2.18)	0.09 (-0.13 – 1.14)	1.15 (0.46 – 2.14)	0.10 (-0.48 – 0.42)	1.18 (0.48 – 4.96)	0.00 (-0.45 – 0.80)	0.769	0.431
Ratio fPSA [% free PSA]	7.61 (2.91 – 17.59)	0.37 (-1.23 – 7.96)	9.49 (7.88 – 17.61)	0.66 (-1.15 – 3.12)	8.56 (4.46 – 16.42)	0.16 (-2.77 – 2.44)	0.218	0.544

*: p = 0.015 between control and the tomato intervention, p = 0.037 between control and multi-diet intervention.

Effect on PSA based on differing baseline levels of compliance markers

Since PC development has been suggested to be affected by habitual intake and/or nutritional status of lycopene, n-3 fatty acids and selenium, *a priori* defined subgroup analysis was performed to see whether the intervention would have different effects based on the baseline levels of these markers. The subgroups were defined based on the median values for each biomarker. These stratified analyses did not detect any significantly different changes in PSA values based on baseline values for neither lycopene, n-3 fatty acids or selenium (supplementary table 8.2).

4.3.2 Hormones and biomarkers of inflammation

Both inflammation and the level of growth hormones have been implicated in prostate cancer etiology. In this thesis, the hormone IGF-1 and the inflammatory biomarkers CRP, suPAR and IL-6 were quantified in plasma from the PC patients in the PFPI study.

There were no significantly different changes in IGF-1, CRP, IL-6 or suPAR between intervention groups, nor were there any effect when stratifying by tumor risk classification (table 4.7). IGF-1 levels were however significantly higher at baseline in the tomato intervention compared to the control group in the total comparison (table 4.7).

In subgroups formed on differing baseline biomarkers, baseline IGF-1 levels were significantly higher in the tomato intervention compared to control in subgroups with either low n-3 fatty acid profile in RBC or high lycopene levels. Furthermore, there was a significant increase in changes of IGF-1 in the multi-diet intervention group compared to control in those with low baseline selenium levels (supplementary table 8.3). There were no differences in any biomarker of inflammation in the groups formed by different baseline values in biomarkers of compliance (supplementary table 8.3).

Table 4.7. CRP, IL-6, suPAR and IGF-1 values, an overview and comparison between groups and stratified by tumor risk classification

Non-normally distributed parameter presented as median (range), Kruskal-Wallis tests was used to detect difference between groups, when significant Mann Whitney tests between individual groups were performed.

	Parameter	Control group		Tomato intervention		Multi-diet intervention		P-value of between group differences	
		n Baseline	n Difference	n Baseline	n Difference	n Baseline	n Difference	Baseline	Difference
All subjects	CRP [mg/L]	n = 21 1.00 (0.60 – 7.10)	n = 21 -0.08 (-1.40 – 5.70)	n = 25 1.50 (0.60 – 6.00)	n = 25 0.00 (-2.90 – 5.20)	n = 25 1.40 (0.60 – 7.00)	n = 25 0.20 (-2.30 – 8.20)	0.833	0.173
	IGF-1 [nmol/L]	n = 20 18.5 (9.2 – 37.0)	n = 20 1.0 (-4.0 – 5.0)	n = 25 22.0 (14.0 – 37.0)	n = 25 -1.0 (-6.0 – 6.0)	n = 25 21.0 (9.5 – 32.0)	n = 25 1.0 (-5.0 – 6.0)	0.042*	0.189
	IL-6 [pg/mL]	n = 21 0.62 (0.00 – 4.22)	n = 21 0.00 (-1.04 – 1.77)	n = 25 0.81 (0.00 – 7.50)	n = 25 0.00 (-5.55 – 2.77)	n = 25 0.77 (0.00 – 11.19)	n = 25 0.00 (-1.25 – 3.19)	0.715	0.761
	suPAR [ng/mL]	n = 22 2.19 (1.01 – 6.66)	n = 22 0.23 (-0.69 – 1.23)	n = 26 2.19 (0.86 – 16.52)	n = 26 0.17 (-2.02 – 5.36)	n = 25 2.24 (1.04 – 3.15)	n = 25 0.25 (-1.27 – 1.15)	0.909	0.972
Intermediate risk	CRP [mg/L]	n = 11 1.00 (0.60 – 3.60)	n = 11 -0.04 (-1.10 – 5.70)	n = 14 1.15 (0.60 – 6.00)	n = 14 -0.15 (-2.90 – 5.20)	n = 11 1.70 (0.67 – 3.30)	n = 11 0.20 (-2.30 – 4.30)	0.380	0.581
	IGF-1 [nmol/L]	n = 10 14.5 (9.2 – 24.0)	n = 10 0.5 (-2.0 – 4.0)	n = 14 22.0 (14.0 – 37.0)	n = 14 -1.0 (-6.0 – 6.0)	n = 11 21.0 (12.0 – 30.0)	n = 11 2.0 (-5.0 – 6.0)	0.065	0.516
	IL-6 [pg/mL]	n = 12 1.10 0.00 – 4.22)	n = 12 0.00 -0.66 – 1.37)	n = 14 1.02 (0.00 – 7.50)	n = 14 0.00 (-5.55 – 2.77)	n = 11 0.28 (0.00 – 3.53)	n = 11 -0.021 (-1.25 – 3.19)	0.527	0.656
	suPAR [ng/mL]	n = 13 2.43 (1.57 – 4.36)	n = 13 0.18 (-0.69 – 0.90)	n = 15 2.23 (0.86 – 5.45)	n = 15 0.08 (- 2.02 – 1.07)	n = 11 2.24 (1.04 – 2.91)	n = 11 0.26 (-1.27 – 1.15)	0.598	0.407
High risk	CRP [mg/L]	n = 10 1.20 (0.60 – 7.10)	n = 10 -0.09 (-1.40 – 4.00)	n = 9 1.50 (0.60 – 5.40)	n = 9 0.00 (-0.81 – 1.00)	n = 14 0.99 (0.60 – 7.00)	n = 14 0.32 (-2.20 – 8.20)	0.712	0.207
	IGF-1 [nmol/L]	n = 10 19.0 (12.0 – 37.0)	n = 10 1.0 (-4.0 – 5.0)	n = 9 19.0 (15.0 – 31.0)	n = 9 0.0 (-2.0 – 4.0)	n = 14 20.5 (9.5 – 32.0)	n = 14 1.0 (-1.0 – 4.0)	0.530	0.802
	IL-6 [pg/mL]	n = 9 0.09 (0.00 – 2.01)	n = 9 0.00 (-1.04 – 1.77)	n = 9 0.46 (0.00 – 3.38)	n = 9 0.19 (-0.69 – 2.39)	n = 14 1.26 (0.00 – 11.19)	n = 14 0.06 (-0.73 – 0.98)	0.232	0.733
	suPAR [ng/mL]	n = 9 1.74 (1.01 – 6.66)	n = 9 0.56 (-0.24 – 1.23)	n = 9 2.21 (1.42 – 16.52)	n = 9 0.32 (-0.19 – 5.36)	n = 14 2.22 (1.22 – 3.15)	n = 14 0.24 (-0.59 – 1.12)	0.677	0.418

*: p = 0.016 between control and the tomato intervention, p = 0.080 between control and multi-diet intervention.

4.3.3 **Principal component analysis separate intervention groups based on changes in lycopene, selenium and EPA**

PCA was performed in order to identify possible patterns in the multidimensional data set, including all parameters measured in the study. These parameters include plasma biomarkers, patient- and tumor characteristics. No clear pattern could be seen for the baseline parameters, indicating that the multi-diet intervention, tomato intervention and control groups were similar with regards to these parameters at baseline (Figure 4.2). PCA on the changes during the intervention indicate that lycopene is important to separate the intervention groups from controls (Figure 4.3) while selenium and EPA (C20:5 n-3) is important to separate the multi-diet group from the two other groups.

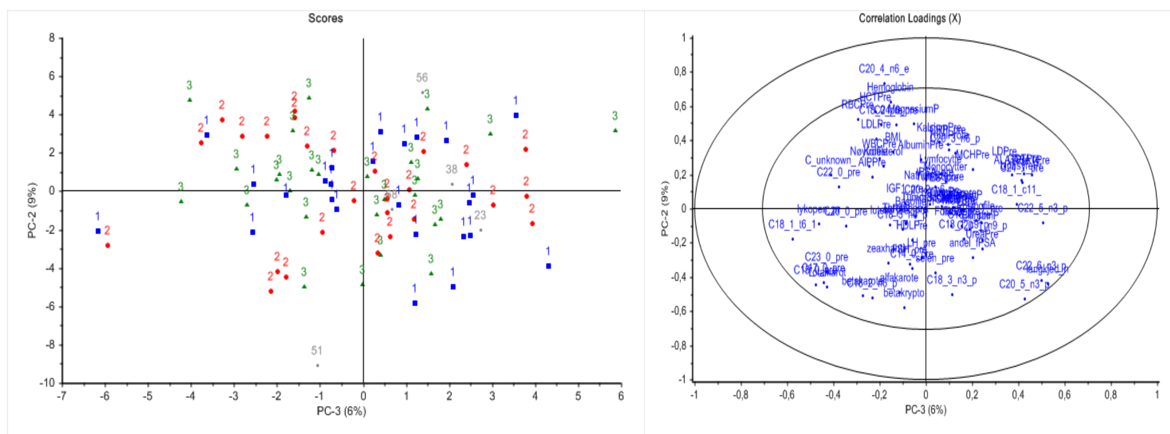

Figure 4.2. PCA analysis on baseline values, PC2 and PC3. 1) Multi-diet intervention, 2) Tomato intervention, 3) Control group. On the correlation loadings plot one can see that there are few markers that appear to be important. When interpreting this together with the scores plot, there is no pattern between the individual groups, confirming that subjects were not distinguished at baseline.

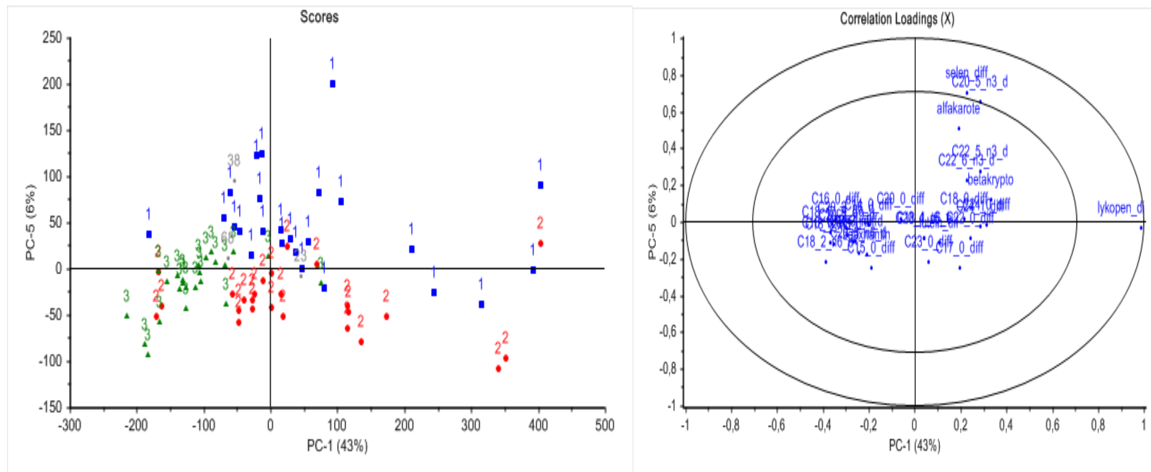

Figure 4.3. PCA analysis on baseline values, PC1 and PC5. 1) Multi-diet intervention, 2) Tomato intervention, 3) Control group. When analyzing changes in biomarkers, lycopene, selenium and EPA appear to be the most important variables and the groups are separated based on these biomarkers of compliance.

A more distinct effect on changes in PSA-values was found when performing a new analysis taking only these three biomarkers and PSA-values into account (figure 4.4). The control group seems to exhibit increases in PSA-values, indicated by the separation in the direction of PC-4. Subjects in the tomato group interventions seem to separate from the controls based on increases in lycopene which is equally influenced by PC-1 and PC-4. The multi-diet group further separates from both groups, seemingly dependent on increases in lycopene as well as EPA and selenium, with the two latter being inversely correlated with fPSA, tPSA and to some extent fPSA-ratio.

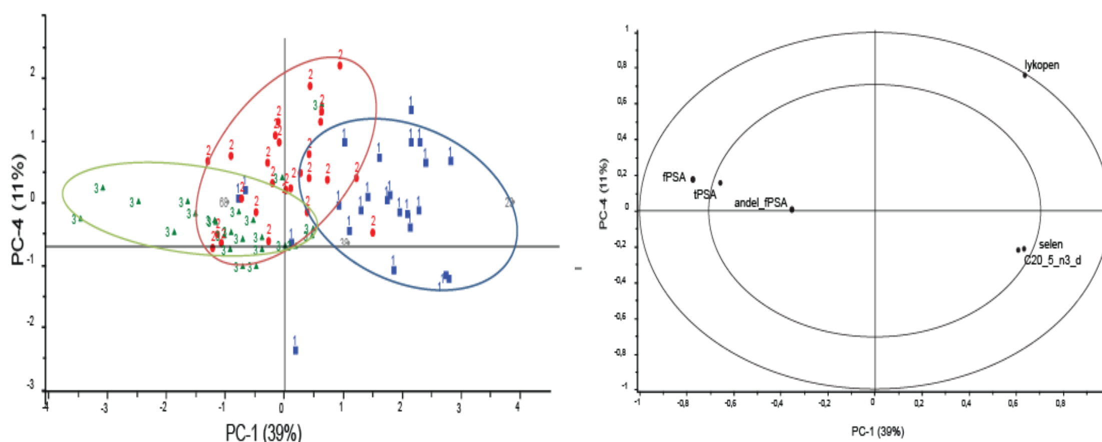

Figure 4.4. PCA analysis of PSA values and relevant biomarkers. PC1 and PC4 showing the 1) Multi-diet intervention (blue), 2) Tomato intervention (red), 3) Control group (green). In this analysis a clear pattern among the different groups can be seen. Lycopene differences pull the intervention and multi-diet group upwards, while selenium and EPA pulls the multi-diet group rightwards and downwards. The control group seem to only be influenced by an increase in fPSA, tPSA and slightly from fPSA-ratio.

Changes in biomarkers define effects on PSA

The results from the PCA indicate that changes on PSA-values are related to changes in EPA, lycopene and selenium. Based on the findings, subgroup analyses based on changes in these biomarkers of compliance were performed.

Subgroups analyzed are presented as above and below median changes in EPA, lycopene and selenium. Each subgroup analysis was performed on the three individually, as well as all the three biomarkers combined.

No significant effects on PSA levels were detected in subgroup analysis based on subjects with high or low changes in EPA or selenium alone (supplementary table 8.4 and 8.5 respectively).

Effect of changes in EPA, lycopene and selenium combined

There were significant differences in development of tPSA- and fPSA-values between subjects with an above or below median increase in all the three biomarkers simultaneously (table 4.8). This indicates a major impact of individual absorption and metabolism on the effects on PSA.

Table 4.8. PSA values compared between differing changes in EPA, lycopene and selenium, baseline values and changes during the intervention period

Non-normally distributed parameters presented as median (range), the Mann-Whitney test was used to compare groups. Groups are divided based on median change in plasma values.

Parameter	Low increase n = 17		High increase n = 15		P-value of difference	
	Baseline	Difference	Baseline	Difference	Baseline	Difference
tPSA [ng/mL]	8.44 (5.48 – 18.90)	0.72 (-1.63 – 2.40)	9.03 (5.38 – 30.20)	-0.10 (-12.40 – 1.47)	0.433	0.003
fPSA [ng/mL]	1.09 (0.56 – 2.14)	0.10 (-0.21 – 1.14)	0.86 (0.43 – 4.96)	0.00 (-1.85 – 0.24)	0.192	0.004
fPSA ratio [% fPSA]	12.41 (6.29 – 22.48)	0.19 (-3.77 – 7.96)	9.02 (5.89 – 18.13)	0.31 (-1.41 – 3.58)	0.082	0.852

Out of the subjects analyzed, 23% exhibited a low and 27% a high increase. When examining allocation within these subgroups, there were no apparent differences between the low versus high increase groups with respect to tumor classification (table 4.9). None from the multi-diet intervention appeared in the low increases group. In the group with high increases there were mainly subjects from the multi-diet intervention, however also one subject from the control and one from the tomato intervention group exhibited high increases in all combined biomarkers (table 4.9).

Table 4.9. Group characteristics of tumor risk classifications and intervention group allocations within high and low increases in all combined biomarkers of compliance

Cross tabulation of intervention group allocation and tumor risk classification between groups allocated by above and below median changes in EPA, lycopene and selenium.

	Low increase	High increase	Total
Tumor risk classification			
Low	0	0	0
Intermediate	9	6	15
High	8	9	17
Intervention groups			
Control	12	1	13
Tomato	5	1	6
Multi-diet	0	13	13
Total	**17**	**15**	**32**

Changes in plasma lycopene affect PSA development

There were significant differences in development of tPSA- and fPSA-levels between subjects with an above or below median increase in plasma lycopene (table 4.10). There was also a significant difference in baseline fPSA values between the two groups, where subjects that showed higher increase in plasma lycopene had lower baseline fPSA-values.

Furthermore, significant differences in tPSA-levels based on lycopene increases were detected in both intermediate and high tumor risk classification groups, with those with a high increase in lycopene having a decrease in tPSA (table 4.10). Also, the group with high increases in lycopene had significantly lower baseline fPSA in the intermediate risk group.

Table 4.10. PSA values compared between differing changes in plasma lycopene, baseline values and changes during the intervention period

Non-normally distributed parameters presented as median (range), the Mann-Whitney test was used to compare groups. Groups are formed based on median value of lycopene changes for the whole study population.

Parameter	Low increases		High increases		P-value of between group differences	
	Baseline	Difference	Baseline	Difference	Baseline	Difference
All subjects (low increases n = 35, high increases n = 35)						
tPSA [ng/mL]	9.74 (4.42 – 31.50)	0.45 (-3.30 – 4.80)	8.12 (1.52 – 30.20)	-0.02 (-12.40 – 1.70)	0.425	0.009
fPSA [ng/mL]	1.10 (0.51 – 3.30)	0.10 (-0.48 – 1.14)	0.84 (0.32 – 4.96)	0.02 (-1.85 – 0.62)	0.011	0.039
fPSA ratio [% fPSA]	11.26 (3.93 – 28.40)	0.19 (-3.77 – 7.96)	8.93 (4.10 – 37.69)	0.31 (-2.34 – 5.72)	0.127	0.907
Intermediate tumor risk (low increases n = 17, high increases n = 20)						
tPSA [ng/mL]	8.44 (4.42 – 17.70)	0.45 (-0.80 – 2.24)	7.39 (1.52 – 23.50)	-0.05 (-12.40 – 1.70)	0.828	0.030
fPSA [ng/mL]	1.08 (0.51 – 1.96)	0.10 (-0.21 – 1.04)	0.69 (0.32 – 4.26)	0.01 (-1.85 – 0.62)	0.041	0.061
Ratio fPSA [% free PSA]	12.41 (4.69 – 28.40)	0.48 (-3.77 – 6.35)	9.62 (4.10 – 37.69)	0.14 (-2.34 – 5.72)	0.104	0.869
High tumor risk (low increases n = 17, high increases n = 14)						
tPSA [ng/mL]	12.90 (5.06 – 31.50)	0.50 (-3.30 – 4.80)	9.67 (5.38 – 30.20)	-0.08 (-3.80 – 1.59)	0.316	0.048
fPSA [ng/mL]	1.20 (0.55 – 3.30)	0.10 (-0.48 – 1.14)	0.87 (0.46 – 4.96)	0.04 (-0.23 – 0.42)	0.072	0.203
Ratio fPSA [% free PSA]	9.98 (3.93 – 17.61)	0.19 (-2.77 – 7.96)	8.44 (5.89 – 16.42)	0.33 (-0.96 – 3.12)	0.493	0.493

There was no apparent disproportion in tumor classification between the differing response in plasma lycopene changes and thus lycopene changes seemed independent of tumor stage (table 4.11). Those with high increases in lycopene were expected to come mainly from the multi-diet and tomato intervention. Indeed a majority, but not all subjects, belonged to these groups. Two subjects from the control group also showed high increases in plasma lycopene (table 4.11).

Table 4.11. Group characteristics of tumor risk classifications and intervention group allocations within high and low increases in lycopene

Cross tabulation of intervention group allocation and tumor risk classification between groups allocated by above and below median changes in lycopene.

	Low increase	**High increase**	**Total**
Tumor risk classification			
Low	1	1	2
Intermediate	17	20	37
High	17	14	31
Intervention groups			
Control	19	2	21
Tomato	9	16	25
Multi-diet	7	17	24
Total	**35**	**35**	**70**

5 Discussion

In this section, a methodological discussion will first be presented, followed by discussion of the results.

5.1 Methodological discussion

5.1.1 Study design

A weakness of several prior intervention studies on PC and dietary factors has been the lack of control groups, and generally the study populations have been very small. This study was calculated to have a power of 80% for detecting changes in tPSA and was designed as a RCT with two parallel interventions, thus giving an overall solid study design.

Another factor to be taken into account in the current literature on PC and dietary factors lies within the selection of the study populations. Often either subject under “active surveillance” (subjects with asymptomatic/latent PC) or patients who have already undergone treatment and are in relapse are recruited, or sometimes both simultaneously.

The PFPI trial took advantage of the time window between diagnosis and treatment, which allowed exploring the effects from a nutrition intervention on untreated patients with established PC, and study the etiology of PC in a well-defined study population. Although significant effects on developing PSA-values were seen, functional relevance for the effects on PSA is still not clear.

There are few well designed studies on the supplements used in the PFPI-intervention in similar study populations and time frame. There has previously been one RCT on tomato-supplementation (80), one on soy grits (111), and one on soy isoflavone (112) supplementation on patients scheduled for prostatectomy, which could be comparable to our study population. However, none of these studies have stratified by composite tumor risk classification such as in the PFPI-trial. The RCT examining tomato-supplementation was furthermore considerably smaller than the PFPI study (n = 26), had a significant disproportion of tumor confined to the prostate between groups and did not compare compliance to changes in PSA (80).

5.1.2 IL-6 quantification

Prior to analysis, different ELISA-kits were compared, finally the Human IL-6 US UltraSensitive kit from Invitrogen was chosen due to the superior sensitivity with a valid detectable range within 0.16 - 10 pg/mL. The analysis was at this time intended to be done on serum samples. The reliability of the assay on plasma samples was not guaranteed by the manufacturer, and results from the previously described recovery-experiment indicated a relatively low recovery. As reported from the manufacturer (personal communication, L. Gao, Invitrogen, USA), a variable recovery on plasma samples had been indicated also in their own lab experiments.

Degradation

The time from patient recruitment to analysis of IL-6 was between 13 and 54 months, thus the stability of IL-6 over time is a factor to be taken into account. Data on the stability of frozen IL-6 over time is however somewhat scarce. A study on IL-6 in whole blood (n = 3) found that about 50% was recovered after four years storage in -80 ºC (113). Another study found IL-6 to be stable for 12 months in human colostrum (n = 10), when stored at -70 ºC (114). Yet another study examined stability in amniotic fluid (n = 28), and found below 80% of original levels after 12 months storage in -70 ºC. These reports are on small study populations and in different mediums than used in this study, nevertheless it suggests that IL-6 might have degraded over time. Results from the IL-6 analysis indicated that a large proportion of specimens collected in the very beginning of the trial were indeed below the detection range (figure 5.1).

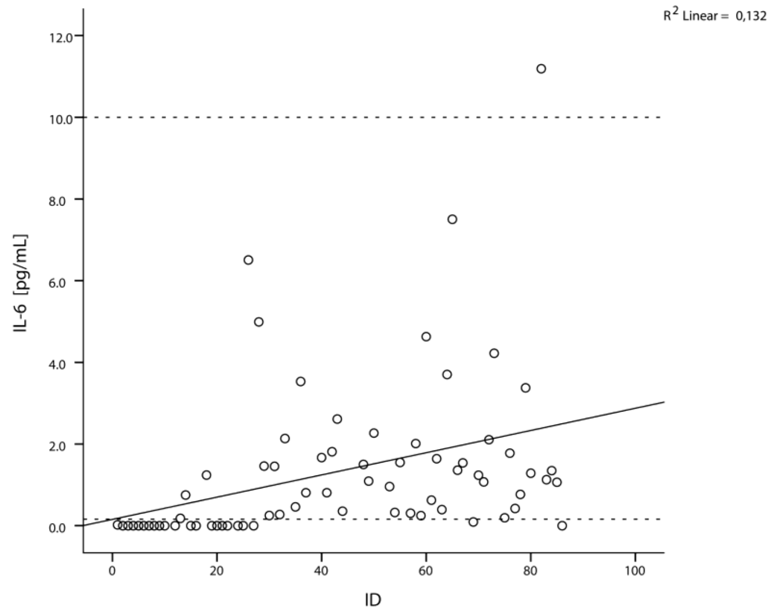

Figure 5.1. Pre-intervention plasma levels of IL-6. A low ID indicates that the patient was recruited early in the study and a high ID that it was recruited in the end. Dotted lines mark the valid detection range of the kit.

Other methods

The specific epitopes that antibodies in this assay bind to are unknown (personal communication, L. Gao, Invitrogen, USA) hence, perhaps a different kit utilizing other antibodies could have shown a different recovery rate. Other ways of measuring IL-6 could be by western blotting, but this is a more demanding method. One could also quantify mRNA expression by polymerase chain reaction (PCR) analysis, though, in this trial we had plasma and serum samples, which makes analysis on a protein level more feasible. In this trial, the volume of specimens to be tested was limited and we were not able to try different approaches in parallel, and the ELISA method was found to be most suitable.

As reviewed by Vgontzas *et al*. (115), there are circardian variations in the secretion and plasma levels of IL-6. The levels of IL-6 are furthermore increased following tough physical exercise which we cannot control for in our samples (116).

The indication of degradation together with the uncertain reliability of the assay on plasma samples, make it difficult to draw sound conclusions from the IL-6 quantification. Collection of morning samples over a shorter total time span, and with no strenuous exercise within the past 24 hours, would have been better suited in order to reveal possible effects of these interventions on IL-6.

5.1.3 suPAR quantification

suPAR is a relatively new biomarker and the number of available assays are limited. To the best of our knowledge, the only commercially available assays are the suPARnostic tools, marketed by ViroGates. These include ELISA kits as used in this thesis, as well as the "Quick Triage" kit which is used together with the "Quick Test Reader". This latter set is a faster and less precise measurement where a binary result is given, determining if suPAR-levels are above or below 5.5 ng/mL. For research purposes, the ELISA kit is therefore the best choice due to higher accuracy.

There are to date no published reports on the stability of suPAR over prolonged storage times, though the producers of suPARnostic have some data. When comparing values from the samples over time since 2006, a correlation of 0.9 has been estimated for repeat measurements (personal communication, T. Pielak, ViroGates, Denmark). There is, however, a batch to batch variation (personal communication, T. Pielak, ViroGates, Denmark), and in light of this, we made sure that we used the same batch lot for all the suPAR analyses in this thesis. In contrast to the IL-6 quantification, the data of suPAR levels prior to intervention (figure 5.2) did not indicate degradation.

Other methods of quantifying suPAR could have been mRNA expression through PCR-analysis. However, in this trial only plasma was available for these analyses.

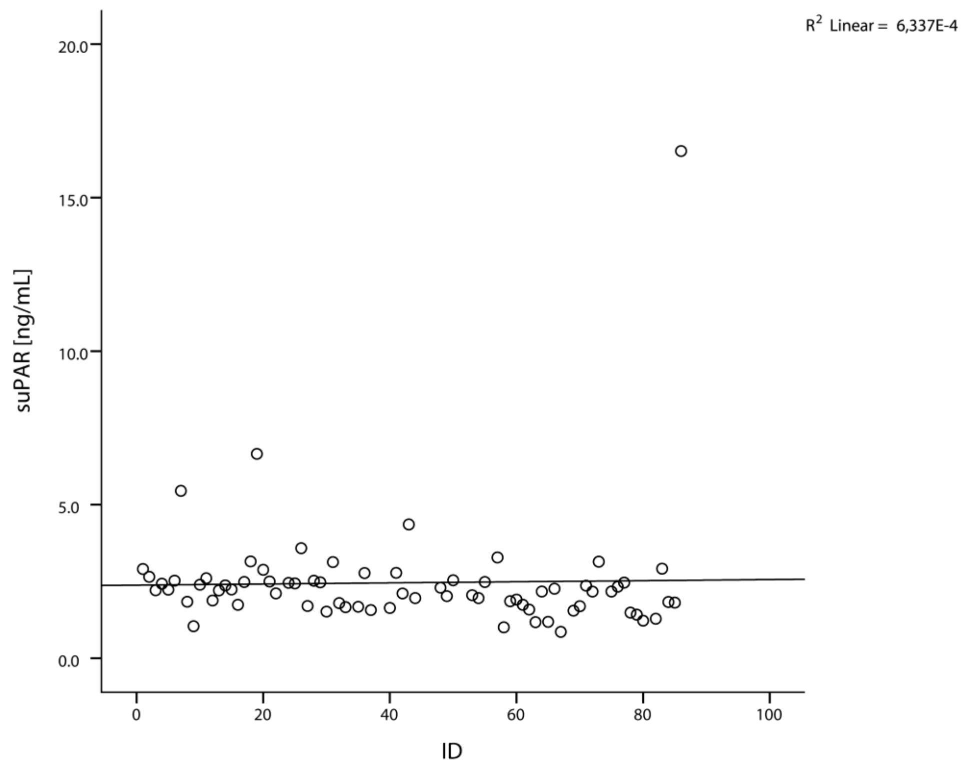

Figure 5.2. Pre-intervention plasma levels of suPAR. A low ID indicates that the patient was recruited early in the study and a high ID that it was recruited in the end.

5.2 Discussion of results

The aim of this thesis was to examine biomarkers of PC and compliance in a RCT on PC patients who supplemented their habitual diet with one of two interventions; tomato products; or tomato products and pomegranate- and grape juice, green- and black tea, soy extract, selenium and n-3 fatty acids as compared to a control group. The results from the PFPI study will be compared to the current evidence from clinical human studies. In this thesis, only statistically significant differences are described in the results section. In the current literature, a large proportions of the clinical trials report non-significant changes in biomarkers. Therefore, the following discussion will also include those papers to reflect the literature as a whole.

The main endpoint in this trial was development of PSA values. Significant differences in tPSA were found in patients in intermediate tumor risk classification, as well as responders to the intervention as measured by increase in biomarkers of compliance. A high increase in lycopene resulted in lowered tPSA-levels regardless of tumor risk classification. Furthermore, high increases in lycopene, selenium and n-3 fatty acids combined also gave significantly lowered tPSA and fPSA development compared to low increases.

5.2.1 Compliance to the intervention and corresponding effects on biomarkers

The self-reported compliance among participants was high; only one subject discontinued n-3 fish oil supplements while all other supplements were consumed with high compliance throughout the trial; the mean compliance rate was 96% or above for every supplement.

In regards to biomarkers of compliance, several studies have examined lycopene as a marker of tomato- or lycopene supplementation (78-80, 117, 118). To our best knowledge though, there are no clinical intervention studies utilizing this data to link this biochemical marker of compliance directly to the response in PSA or other biomarkers of PC. This is an important strength of our trial as one hypothesis, and a main reason why we measure biomarkers of compliance, is that our endpoints would be altered in responding patients only.

Another strength of the PFPI-study design is the linkage between self-reported compliance and the use of biochemical markers of compliance to evaluate compliance and bioavailability

of three supplements. These biomarkers showed clear, statistically significant increases when comparing intervention groups to the control, further underscoring a high overall compliance to the interventions. On an individual level, participants displayed close to uniform increases in the biochemical markers of compliance in response to the individual interventions, with the outstanding exception of lycopene. Lycopene quantification in plasma revealed a more pronounced differential response, or a possible false self-reported compliance, to the tomato supplementation for at least four subjects in the tomato group and one subject in the multi-diet group as illustrated in figure 4.1 in results section 4.2.2. Increases in selenium were uniform in the multi-diet group (supplemental figure 8.1). There was one individual in the multi-diet intervention that did not increase in n-3 fatty acids (measured as DHA, DPA and EPA combined) (supplemental figure 8.2), this individual was however at the highest level of all participants at baseline, and had only a minor decrease of 0.9%.

The PCA analysis further indicated that changes in biomarkers of compliance during the trial had a relation to changes in PSA. When stratifying the analysis based on changes in biomarkers of compliance, a clear statistically significant difference was seen in responders (i.e. those with large increases in lycopene or lycopene, selenium and EPA combined), with comparative decreases in tPSA and fPSA levels. This highlights the importance of possible differential uptake, bioavailability of supplements and biological response between individual subjects.

Our results indicate that a response to the tomato supplementation, measured as changes in plasma lycopene, was the biggest identifiable individual factor affecting tPSA-development. Although lycopene uptake and bioavailability varies among individuals, it is commonly used as a marker of compliance and habitual intake of tomato products in clinical trials.

The bioavailability of lycopene is influenced by concurrent lipid and fiber intake, which potentially increases respectively decreases uptake (119). The function of fat absorption will affect the uptake of fat soluble compounds, such as lycopene, from the gastrointestinal tract. Intake of phytosterols is indicated to decrease circulatory lycopene levels (120, 121). There are also indications that genetic differences affect lycopene uptake, as reported by Borel *et al.* (122), who found SNPs in genes regulating lipid metabolism to be significantly associated with plasma lycopene levels.

Epidemiologic studies have linked foods containing lycopene to decreased risk of PC (67). This association has however not been as evident in all epidemiologic studies (123) . Though, as previous systematic analyses by the Blomhoff research group underlined, lycopene contents differs widely between different tomato products (106), a variation that can be more precise controlled for in clinical trials.

Previous clinical trials have evaluated different dosage of the lycopene supplement *Lyc-O-Mato* (LycoRed Natural Products Industries, Ltd. (Beer-Sheva, Israel)). One study examined ranges from 15 to 120 mg/day over 9 months (118), and saw a significant trend towards increased plasma lycopene with increased dosage, as well as a tendency towards a plateau after three months of supplementation. Another study using the same supplement in dosages ranging from 15 to 45 mg/day indicated that the highest plasma concentration was found at 45 mg/day (79). A trial examining uptake of a single-dose of incremental lycopene amounts from tomato paste (10, 30, 60, 90 and 120 mg) in healthy men however, saw that the lower dosages (10 and 30 mg) absorbed significantly higher proportion of the consumed lycopene compared to those consuming 120 mg (124).

The previous clinical trials that have indicated effects on PSA in PC patients have used doses up to 30 mg (78, 80, 81). In terms of clinical application, the dosage used in the PFPI study (~30 mg lycopene/day) is an amount that could be covered in a tomato-rich habitual diet. Thus, the supplementation dose in our trial seems to be at a clinical and biological relevant level.

5.2.2 PSA development

Prior habitual diet did not affect PSA development

Since dietary factors have been linked to the development of PC in epidemiological studies (as summarized by WCRF(67)), Food Frequency Questionnaires were used to evaluate the habitual diet prior to the intervention. It would be interesting to analyze baseline dietary patterns and evaluate possible differential effects from these on primary endpoints, but it is beyond the scope of this thesis. Also, differences such as those found in the epidemiological studies, might not be expected since these subjects already have established PC. Stratifying

the statistical analysis based on baseline values of available compliance biomarkers had no effect on relative changes in PSA-values. This indicates that habitual intake and uptake was not an important factor for the PSA-response to the intervention in this study population.

The effects of the intervention was most pronounced in patients with intermediate risk tumors

When stratifying for tumor risk classification significantly different tPSA-developments were seen as a result of the interventions. This stratification is a general clinical criteria in use when evaluating treatment options at DNR and is recommended in guidelines by the European Association of Urology (20). The data from this analysis indicate that the intervention had an effect on those classified as intermediate tumor risk, who displayed significantly lowered tPSA-levels compared to controls. No significant difference was seen in high risk patients. For low risk patients (n = 2), there was not enough data for a statistical comparison.

In the latest available statistics on diagnosed PC in Norway (125), there was no report on composite tumor risk categories, but each individual marker was presented; T-score, Gleason score and pre-operative tPSA values. The proportion falling into intermediate risk group or below on each individual marker was 60, 70 and 65% respectively. An earlier report on diagnosed PC did categorize by tumor risk stage (126). The grouping criteria in this report were similar to that in use at DNR. In this report, 27% of patients were in the intermediate risk group. Hence, a substantial part of diagnosed cases of PC in Norway seems to be in the range of the intermediate risk, in which effects on tPSA was seen in our trial.

If the data from our trial reflect a general response in PC patients and considering a possibility of high prevalence of undetected PC as indicated by Yin *et al*. (6), it could have implications for future adjuvant health care approaches. As such, the results from our trial warrant further studies.

Significance of PSA as a biomarker

The data in our trial does not permit us to determine whether a comparative decrease in PSA levels, as an effect from the dietary intervention, is a direct measure of PC regression or stagnation of PC progression. Follow-ups of the PFPI-study might link these changes in PSA to endpoints such as PC recurrence or PC related death. There are not sufficient cases at this point, to perform those comparisons (personal communication, Dr. W. Lilleby).

High preoperative tPSA-values have been significantly associated with increased risk of post-operative biochemical recurrence (127), and the European association of Urology currently recommends tPSA-testing and digital rectal examination for evaluation of the effect of intended curative treatments (20).

Changes in PSA-values over time have previously been evaluated in PC patients under active surveillance (128-130). Ross *et al*. (128) examined post-diagnostic tPSA-developments in a well-defined population of low grade disease compared to results from annual biopsy and later prostatectomy. This study found significantly higher tPSA values prior to adverse biopsy results, and a trend (p = 0.06) towards higher tPSAV. Among those with a tPSA below 4 ng/mL at diagnosis, tPSAV was significantly associated with adverse biopsy results. The predictive value of tPSAV alone was however not accurate enough to be clinically useful for evaluating curative therapy. No association with PSA and adverse pathologic findings was later seen among those who underwent prostatectomy. This study concluded that tPSAV-measurements alone were not sufficient to distinguish progression. A recent study by Iremashvili *et al.* (129) studied the ability of tPSAV to predict progression in biopsy results in patients undergoing active surveillance. This study found a significant prediction only when 4 or more biopsies had been made, at a median duration at 3.6 years or more. A previous study by Ng *et al.* (130) examined PSA-developments in a two year period prior to repeated biopsy, and found significant association with baseline tPSA, tPSAV and tPSA doubling time with adverse biopsy results.

The rate of increase in tPSA may not alone be specific enough to accurately predict disease progression in a clinical setting where curative therapy is considered. However, there are statistical associations between PSA-values and PC-development; previous studies have linked increases in pre-treatment tPSA-vales to PC-related mortality (131-134).

The data from our trial is not within a sufficient time-frame to permit the calculation of tPSAV. Still, patients identified as responders by changes in biomarkers of compliance, actually exhibited a decrease in tPSA values. The clinical relevance of the observed changes in PSA as presented in this thesis is currently uncertain. Nevertheless, PSA-values remain an important prognostic biomarker in PC development, a marker in which developments were significantly altered during our 3 week nutritional intervention.

In this trial, we combined PSA-measurements with biomarkers of inflammation and the growth factor IGF-1. Additional potential biomarkers of PC include the human glandular kallikrein-2, (a protease involved in producing PSA from the inactive form pro-PSA) which has been linked to stage and progression of PC (135). The transforming growth factor-beta1 is another potential biomarker, and has been associated with metastatic disease and biochemical progression after prostatectomy (136). Other proposed ways of differentiating aggressive disease include genomic analysis and identification of mutations specific for aggressive PC (137). It would be interesting to explore if genetic profiles could affect the response to our intervention, but it is beyond the scope of this thesis.

5.2.3 Biomarkers of inflammation

Inflammation is an established risk factor in PC-development, there are also indications that the dietary components influence inflammatory activity. Reports of effects on inflammatory biomarkers as response of dietary interventions in PC in humans are very limited. Heymach *et al.* (138) found effects of interventions based on flaxseed and low-fat on plasma levels of selected cytokines in prostate cancer patients.

A study on male non-PC patients with low fruit and vegetable intake found significantly decreased CRP levels after an 8 week trial with daily supplements of lycopene, but no significance compared to the control group (139). In our trial, there were no significant differences in CRP between the interventions and the control group.

A controlled study examining effects of tomato paste enrichment in a single high-fat meal on healthy individuals, found decreased post-prandial IL-6 (at 6 hours) compared to the control meal (140). In our trial, there were no significantly different changes in IL-6 in any subgroup analyzed, however IL-6 was increased in the whole study population during the intervention period ($p = 0.043$). IL-6 was increased significantly in the control group ($p = 0.049$) but the changes were not statistically significant in any of the intervention groups. This might suggest higher inflammatory responses in the control group, but changes *within* intervention groups has to be interpreted with caution, or not at all, considering the imminent risk of type-1 errors (141).

A previous study has shown that increased suPAR is associated with increased risk of cancers and increased mortality (63). This is however, the first study to investigate the effect of a dietary intervention on suPAR levels. There were no significant differences between the intervention groups in development of suPAR values. Throughout the trial, there was an increase in the whole study population ($p < 0.001$), and significant increases in the control- and multi-diet group, but not in the tomato group.

5.2.4 **Tomato interventions**

The results in this trial indicate that the main single identifiable component affecting PSA-development was high increase in lycopene. Previous trials with tomato-interventions have given varied results in prostate cancer patients.

Chen *et al*. (78) found a significant 18% decrease in serum tPSA after a three week tomato product intervention containing 30 mg of lycopene per day, but the results are difficult to interpreted since it was an uncontrolled trial. Participants in this study had a total mean Gleason score of 6.5, which is the same as in our trial. A follow-up study from this trial on the same intervention subjects with an added reference group assessed apoptotic cell death (84). This trial found an increased cell death in malignant prostatic tissue in the intervention group, but no statistically significant chances compared to the reference group.

A randomized controlled trial by Kucuk *et al*. (80) used a tomato extract containing 30 mg of lycopene for a 3 week intervention prior to prostatectomy. This is the study with the most similar design to ours. A non-significant 18 % decrease of tPSA was seen in the intervention group. This study, however, was small and seems to have had a disproportion of tumors confined to the prostate, as well as a non-significantly larger proportion of small tumors in the intervention group.

Kumar *et al*. (79) explored the effect of 15, 30, and 45 mg of daily lycopene supplementation (no control group) during 30 days prior to prostatectomy, and stratified the analysis on Gleason grade, but found no significant differences in tPSA-development within or between groups.

A randomized controlled study by Mohanty *et al.* (142) in men with high grade intraepithelial neoplasia (HGPIN, a premalignant condition associated with increased risk of PC) tested 8 mg/day of lycopene supplementation from *Lyc-O-Mato*. The results indicated a decrease of tPSA in the intervention group and an increase in the control after one year, but no statistical analysis was presented. Another randomized controlled study on men with HGPIN or atypical foci (a biopsy result indicating suspicion of PC) explored effects from daily supplementation of 30 mg/day of lycopene from *Lyc-O-Mato* and multivitamins compared to multivitamins alone for 4 months (117). There were no differences between groups. Total PSA-levels decreased from baseline in both groups the first month, but at 4 months however, there were increases in tPSA-levels in both groups and no difference compared to baseline, and the authors proposed a transient effect.

In a randomized controlled study over 24 months, patients with metastatic PC undergoing orchidectomy (surgical castration) (82), consumption of four mg of daily lycopene supplementation after surgery yielded significantly lower tPSA values compared to surgical treatment alone.

In androgen independent metastatic PC, an uncontrolled trial testing a tomato based daily supplement containing 10 mg lycopene for three months resulted in decreased tPSA levels in 30% of the participants, and stable values in 50% (81). Another uncontrolled trial on androgen independent PC, with daily tomato based supplement containing 30 mg of lycopene for a median intervention period of three months, showed no effect on tPSA values (83). The results from these two trials are again difficult to interpret since no control groups were present.

Considering the methodological shortcomings of many clinical trials on PC and tomato supplementation, the results from our trial adds substantially to the available data. The WCRF report in 2007 rated foods containing lycopene as probable to decrease risk of PC (67), whether this effect is directly mediated by lycopene is however less clear.

Lycopene versus tomato

Earlier animal studies have examined the effects of lycopene and tomato extracts in PC models. A study on mice implanted with prostate tumor cells found a significant increase in tumor necrosis after a lycopene-rich diet (143). Another study by Boileau *et al.* (144) on male rats with hormonally- and chemically induced PC examined synthetic lycopene, tomato extracts or a control diet in relation to survival. In this report rats fed tomato extract lived significantly longer, as compared to both the group consuming synthetic lycopene and the control rats. The lycopene dosage in the synthetic lycopene group was about 10 times higher than in the tomato group though, which complicates a comparison.

A study on transgenic mice, prone to develop PC, examined supplementation of tomato paste and synthetic lycopene, with equal amounts of lycopene in each diet until 20 weeks of age (145). The number of benign samples was significantly higher in the lycopene group compared to both control and the tomato paste intervention. The tomato paste in use in this study was, contrary to the trial by Boileau *et al*. (144), produced from peeled tomatoes and without seeds. Another similar study on transgenic mice examined supplementation of a tomato paste produced on the whole tomato, including skin and seeds (146). This trial had two separate protocols examining survival and tumor development. The tomato supplemented mice lived significantly longer than the controls, and in the second protocol tumor development and serum IL-6 was significantly lower in the tomato supplemented group at 20 weeks into the intervention compared to control.

Yet another study on rats, examining a 4-day intervention with either lycopene, tomato extract or phytofluene noticed decreased levels of testosterone, but not DHT, in the intervention groups compared to control (147). Interestingly, the phytofluene group showed significantly lower testosterone also compared to the other intervention groups. In the PFPI-intervention, there were no significant differences in changes in testosterone, and DHT was not quantified.

Furthermore, a controlled study on male rats with implanted PC evaluated the supplementation of syntethic lycopene in differing concentration as well as tomato and broccoli, both alone and combined and in two different concentrations (148). Effects of the 5-α-reductase inhibiting drug finasteride as well as castration were also evaluated. Tumor weight was found to be significantly reduced in castrated rats, but also in those eating either broccoli or high dose broccoli and tomato combined, while there was a borderline significant

decrease in tomato and low dose tomato and broccoli combined ($p = 0.05$ and $p = 0.055$ respectively). When evaluating apoptosis rate in prostate tissue, the castration, tomato, broccoli, as well as broccoli and tomato combined increased apoptosis compared to controls. Synthethic lycopene however, which at the low dose was twice that consumed in the tomato group, had no effect on apoptotic rate.

While lycopene is a reliable marker for tomato product intake and uptake, these animal studies suggest lycopene alone might not be the only mediator of effects found in tomatoes, and that dosage may be integral to the response. There seems to be a need to examine the whole spectra of phytochemicals in tomatoes and other cruciferous plant based whole foods to gain more understanding of the complex interplay.

A study on sera from healthy adult males assigned to consume either red tomato paste, yellow tomato paste, lycopene or placebo, quantified mRNA expression of lymph node PC cells *in vitro* (149). This study found differential effects from red tomato and lycopene. Gene expression profiling of PC tissue in the PFPI intervention is planned, however it is beyond the scope of this master thesis.

5.2.5 **Individual components in the multi-diet intervention**

With the exception of tomatoes, it is not possible to evaluate individual effects from each supplement in the multi-diet intervention. Still, a brief introduction and discussion of previous trials for each supplement besides tomatoes will be presented.

Selenium

The compliance to selenium supplementation, as measured in plasma levels, showed a uniform increase in the multi diet intervention group (supplementary figure 8.1). In the most recent systematic review and meta-analysis (91), a decreased risk of PC was identified within the plasma/serum selenium range of ~135 – 170 ng/mL. This review did not have data on higher concentration ranges.

In the SELECT study (90), which did not see any decreased incidence of PC, median plasma selenium values were at 135 and 136 ng/mL at baseline for selenium and selenium plus vitamin-E interventions respectively. Hence, it could be that those participants were already selenium replete and had no further benefits of additional supplementation. In comparison, the median baseline plasma selenium level of participants in our trial was about 87 ng/mL. The multi-diet group exhibited an increase from a median value of 87 to 158 ng/mL after the trial, well within the proposed beneficial range.

The safety of selenium supplementation is a topic of debate. Selenium has previously been described to have insulin-mimetic effects (88). A randomized placebo controlled intervention evaluated 200 µg/day of selenium supplementation to the incidence of type-2 diabetes with a mean follow up of 7.7 years (150). This trial did not see decreased risk, instead there was a trend towards increased risk of type-2 diabetes ($p=0.050$). A cross sectional study on North American adults ($n = 8876$) found associations with serum selenium above 138 ng/mL and diabetes as compared to serum levels below 112 ng/mL (151), this association was however not valid for normoweight subjects ($BMI < 25$).

To date, there are few clinical trials on humans evaluating only selenium supplementation in established PC. A RCT that compared selenium supplementation (200 µg/day) to placebo in patients with HGPIN, found no differences in PC development between the groups (152). In a subgroup analysis there were indications of a decreased risk of PC in the most selenium depleted subjects (<106 ng/mL), but no statistical significance was detected. Another recent

double blinded placebo trial in men with raised tPSA-levels assessed effects of supplementation with selenium-yeast with a dosage of 200 and 400 µg/day compared to placebo (153). This trial went on for 5 years and found no significant effect on the risk of developing PC.

A randomized double-blinded placebo controlled trial on patients with established local PC under active surveillance examined effects of daily supplements of 200 and 800 µg selenium on tPSA-velocity (154). The median follow-up was between 33-39 months. This study did not see any significant beneficial effects between groups. Instead, those with the highest quartile of baseline selenium who received the 800 µg supplement significantly increased the tPSA-velocity compared to placebo.

The optimal selenium status and the mechanisms behind the observed associations need to be further explored. Determination of who might benefit from dietary selenium supplementation in PC trials may need to be assessed on an individual basis.

In the trials evaluating selenium supplementation on risk of PC in high-risk patients (152, 153), or on established local PC (154), IGF-1 levels were not quantified. However, in older adults, plasma selenium has previously been reported to be associated with circulating IGF-1 (155). In our trial, there were significant increases of IGF-1 as an effect from the intervention among participants in the multi-diet intervention compared to the control group, within the subgroups with low baseline plasma selenium.

Soy

As previously discussed, several studies have found inverse associations of high soy intake and risk of PC, an association most prominent in Asian populations. Soy products are generally more consumed in Asia and a possible explanation might be a dose dependent effect.

Studies on non-PC patients have not shown any clear effects on tPSA-levels (156-159). A RCT on patients with either HGPIN, preneoplastic lesions or low grade PC tested daily supplementation of 40 g of soy protein, alcohol-washed soy protein or milk protein (160). This trial did not see any effects on tPSA after 6 months, but a significant reduction in further PC incidence in the soy groups combined as compared to the milk protein group.

In established PC, there are some indications for an effect from soy supplementation. A randomized double blinded placebo trial tested the daily addition of 50 grams of soy grits baked in bread compared to wheat based bread in PC patients prior to prostatectomy (111). This trial found significantly lowered tPSA levels and increased ratio of fPSA after the soy intervention. The intervention time was about 3 weeks and mean Gleason score 6.5 and 5.7 for soy and wheat respectively, a similar respectively somewhat lower range than in PFPI. Two other RCTs with genistein or soy protein interventions in early stage PC, did not find significant effects on PSA-levels (161, 162).

A recent randomized double blind placebo trial tested the effects of 30 mg of genistein on Norwegian PC patients during a mean of 33 days (112). This study found a decrease in tPSA in the genistein group and an increase in the control group with borderline significant difference ($p = 0.051$). This trial had similar Gleason grade, but less extended primary tumors than the PFPI-study.

In 2002 the equol hypothesis was presented. In brief, equol which is a microbiotic metabolite of daidzein, was identified as the most potent phytoestrogenic compound in soy isoflavones (163). Equol is produced to a different extent in different populations, and a need to separate equol-producers from non-equol producers in order to efficiently elucidate the effect of consumption of soy isoflavonoids was proposed. The isomer s-equol, a compound with high affinity ERβ receptor, has since been identified as the compound produced by the intestinal microbiota (164). In a case control study, Akaza *et al*. (165) found a significantly lower proportion of equol producers in PC cases than controls in Japanese and Korean men. While the equol-hypothesis is intriguing, the relevance of equol producing ability could not be controlled for in our trial.

Fish oil

The main proposed mechanism of action for n-3 fatty acids in PC is the participation in COX-2 regulated pathways where it competes with n-6 fish oils and decreases the production of proinflammatory eicosanoids and thus could reduce inflammation. Plasma n-3 fatty acids have previously been inversely associated with circulating biomarkers of inflammation, including IL-6 and CRP, in the general population (166). In our trial there were no effects on biomarkers of inflammation in any analysis, irrespective of stratification by changes or baseline values of n-3 fatty acid composition in RBC.

A case-control study examined fatty acid intake as well as single nucleotide polymorphism (SNP) in COX-2 in relation to PC risk (167). Overall, this study found significantly lower risk of PC in those with high n-3 fatty acid intake. When stratifying by SNPs an even stronger effect was seen in one of the genotypes, suggesting that this SNP modulated the response to n-3 fatty acid intake. An earlier small study (n = 9) examined COX-2 expression in PC patients after a three months n-3 supplemented low fat nutrition intervention (168). Compared to baseline, four patients showed significant decrease in COX-2 expression.

The daily dosage consumed in our trial was about 2.8 grams of long-chain n-3 fatty acids per day. Although controversial, long chain n-3 fatty acids are at times used as adjuvant therapy in clinical management of certain autoimmune disorders such as rheumatoid arthritis, where doses ≥ 2.7 grams per day is suggested to have clinical effects when used over time (169). These trials on inflammatory diseases are over a longer period of time than our study, and it could be that a 3 week intervention is not sufficient to see an effect on inflammatory biomarkers. We could also not control for individual response based on genetic differences.

Tea

To date, there are few studies that have examined effects of tea on established PC. An uncontrolled trial by McLarty *et al*. (170) examined effects of a green tea extract containing 0.8 mg epigallocatechin-3-gallate (EGCG) for a median of 35 days on PC patients, with a majority (62%) of Afro-American subjects. This study found significant decreases in circulatory tPSA, IGF-1 and the ratio of IGF1 to Insulin-like growth factor binding protein 3 (IGFBP-3). Data on tumor stage was not reported and overall the results are difficult to interpret due to the lack of a control group.

A better designed, double-blinded placebo controlled trial also evaluated green tea catechin extract giving 0.8 grams of EGCG per day in PC patients in the time window between diagnosis and prostatectomy (171). In this study the median intervention time was 28 days. This study found no significant differences in tPSA, IGF-1 or IGFBP-3. The majority of patients in this trial had a Gleason score of 6 (80.8%) or 7 (16.7%), which is slightly lower and in a more confined range as compared to the PFPI study population

Pomegranate and grapes

A uncontrolled clinical trial examined the effect of 240 ml of pomegranate juice in relation to tPSA doubling time in patients that had previously undergone surgery, radiation- or cryotherapy for PC (172). The time of doubling tPSA values was compared to that prior to the intervention. A majority of the participants, 83%, showed improvements in the tPSA doubling time. Another uncontrolled double blinded trial explored two different doses of pomegranate extract, giving the equivalent amounts of polyphenols to about 240 and 720 ml of juice, on PC patients that previously had undergone operation, radiation- or cryotherapy (173). This trial found significantly prolonged tPSA doubling time after the intervention in both groups, with no significant differences between the groups. The results from both these trials should again be interpreted with caution, as both are lacking proper control groups.

To date, there are no intervention studies on grapes in PC patients. Though, one cohort study evaluated dietary supplements and risk of PC including grape seed extracts (174). This study found the use of grape seed supplements to be significantly associated with a decreased risk of total PC. When stratifying by Gleason score after histological examination, there was a significantly decreased risk in low (total Gleason ≤ 7 with 3 as the most prevalent histologic

pattern) but not high-grade PC, though the number cases was very low (n = 3 and n = 4 respectively).

Dietary interventions with multiple components

To date, there are few clinical trials examining a combined intervention of several nutritional supplements such as the multi-diet intervention in the PFPI-trial.

One RCT examined effects of daily supplementation of 200 µg selenium, 40 grams of soy protein and 800 international units of vitamin-E for a duration of 3 years on patients with HGPIN (175). This trial saw no significant difference in the progression to PC compared to the control group.

Another RCT examined a multiple intervention with soy isoflavones (62.5 mg/day), lycopene (15 mg/day from *Lyc-O-Mato*), selenium (128 µg/day), silymarin and several vitamins and antioxidative compounds in a placebo controlled double blinded cross-over design on patients previously undergone radiotherapy or prostatectomy (176). The intervention lasted 10 weeks with 4 weeks washout periods. This study found significantly decreased tPSA-slope in the intervention group, indicating a longer tPSA-doubling time. No significance between groups was seen in direct tPSA-measurements.

A similarly designed double-blind placebo cross-over trial examined effects from a 6 week intervention with a two week washout period on a population of patients under either active surveillance or after radiotherapy or prostatectomy with rising tPSA-values (177). The intervention included vitamin-E, selenium (200 µg/day), soy isoflavones (100 mg/day), green tea (3 cups/day) and lycopene (10 mg/day) as well as phytosterols and other carotenoids. This trial did not see any differences in tPSA, but fPSA, DHT and testosterone decreased significantly in the intervention group.

A RCT on patients with either local untreated PC or patients with rising tPSA-values after prostatectomy respectively curative radiotherapy , tested supplementation vitamin-E, selenium (200 µg/day), vitamin-C and Coenzyme Q10 (178). This study found no significant effect in any endpoints analyzed.

Another double blinded randomized study examined effects of isoflavones (40 mg) and curcumin (100 mg) combined on patients with increased tPSA-levels but negative biopsies

(179). This trial lasted 6 months and found a significantly lowered tPSA in subjects with high baseline levels (tPSA ≥ 10 µg/ml).

An uncontrolled randomized trial tested supplementation of either lycopene (30 mg from *Lyc-O-Mato*) alone (median of 6 months) or combined with soy isoflavones (40 mg/day) (median 5.5 months) on a diverse population of local or metastatic, androgen dependent or non-androgen dependent PC (180). Patients with hormone refractory PC receiving lycopene alone had a significantly lower rate of tPSA rise as compared to the combined intervention, but no differences was seen in hormone sensitive PC. In contrast to these latter findings, a possible synergistic effect was found in the PFPI study when increases in selenium, EPA and lycopene were combined, as compared to increases in lycopene alone.

5.3 Implications for the patient population

With the large and increasing number of PC cases, the possible discovery of preventive agents or therapy for PC would not only be of public health interest, but also of great public interest. A North American prostate cancer risk screening program reported in 2004 that about 50% of participants took one or more un-prescribed supplements in the hope of decreasing PC risk (181).

Common treatment for PC includes radiotherapy and prostatectomy, which as previously discussed may have severe side-effects. There are to date no absolute cure for high grade PC, 5-α-reductase inhibitors have shown to decrease incidence of PC (182, 183). But in fact, high grade PC was significantly increased in the treatment group in one of these studies (183). These treatments also showed increased side-effects such as breast enlargement, decreased libido and erectile dysfunction compared to placebo. This is to be taken into account in light of the results from our, rather short, dietary intervention.

Given the large interest in what is perceived as “natural” compounds from the general public, together with the results from the present and previous trials that indicate that tomato products may in fact play a role in PC development, there is a dire need for replication and evaluation of long term effects of a dietary intervention containing these compounds.

5.4 Future implications

Undiagnosed PC could be a more common feature in aging men than diagnostic statistics reveal (6). Hence, identifying potentially beneficiary effects from diet on PC development could have major implications on preventive and adjuvant health-care strategies.

There are several theories behind the potential effects of lycopene-rich foods on PC development. One wide-spread theory is that exogenous antioxidants may have an effect of directly dampening oxidative stress *in vivo* and thereby reduce the risk of chronic diseases and cancer. However, it is unlikely that this is the sole mechanism and data on the biological function of lycopene has previously been reviewed (184, 185). It was proposed that metabolites of lycopene may inhibit cell proliferation and induce apoptosis and enhance gap junction communication, modulate inflammation, as well as activate phase-2 detoxifying/antioxidant enzymes by activation of Nrf2. It was furthermore proposed that lycopene might alter IGF-1 signaling by modulating IGFBP-3 expression, as well as reduce expression of 5-α-reductase in the prostate. The data presented in this thesis, however, does not permit an evaluation of the underlying molecular mechanisms.

The end results data from the PFPI-intervention as a whole is in no way completely reported in this thesis, gene expression profiling of both tumor and normal tissues, analysis of habitual food intake and immunohistochemistry of tumors are yet to be undertaken.

The effects identified in this trial could be transient. In order to illuminate long term effects, an option would be to study hard end points on early stage PC under active surveillance over an extended time period. Moreover, assuming high prevalence of undetected prostate cancer, a preventive trial on a healthy study population would be of great interest, even though such a trial would be costly.

As recently reviewed by Sutcliffe and Colditz (186), a majority of PC studies have been performed on the aging population, on a disease that takes decades to develop and manifest. Hence, these authors propose the studies of early life exposures on later PC incidence. It would seem plausible that preventive measures could have a more prominent effect on prostate development in very early life and on pre-malignant conditions in younger adults.

6 Conclusion

In the PFPI study there was significantly lowered tPSA-values in patients with intermediate tumor risk in both intervention groups compared to controls. No effects on PSA-values of either a tomato- or a multi-diet intervention were seen in the statistical analysis when the intervention groups (i.e. combined analysis of low, intermediate and high tumor risk) were compared to the control group.

Self-reported compliance to the interventions was high, and there were clear statistically significant differences in biomarkers of compliance between interventions and the control group.

Furthermore, the two dietary interventions did not significantly modify IL-6, suPAR, CRP nor IGF-1. Levels of IGF-1 were increased in subjects with low baseline plasma selenium that received multi-diet supplementation, but no other changes were observed in subgroups based on baseline levels of lycopene, selenium or omega-3.

Changes in biomarkers of compliance were linked to changes in PSA. Above median increases in lycopene, and EPA, selenium and lycopene combined, gave lowering of tPSA and fPSA increases regardless of tumor stage. Increase in plasma lycopene was identified as the single most important factor for development in tPSA-values, with an added possibly synergistic effect from increases in compliance markers of fish oil and selenium supplementation.

In conclusion, the results from this trial support a role of diet as a modulating component in PC development and add substantially to the current body of evidence. Well-designed studies over a longer period of time are needed to evaluate long term effects and clinical relevance from tomato, or a combined phytochemical rich dietary intervention combined, on PC development.

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8 Supplementary tables and figures

Table 8.1. Fatty acid composition in red blood cells, baseline values and changes during the intervention period

Non-normally distributed parameter presented as median (range), Kruskal-Wallis tests was used to detect difference between groups, when significant Mann Whitney tests between individual groups were performed.

Fatty acid [% of total FAME weight]	Control group (n = 21)		Tomato intervention (n = 24)		Multi-diet intervention (n = 25)		P-value of between group differences	
	Baseline	Difference	Baseline	Difference	Baseline	Difference	Baseline	Difference
14:0	0.293 (0.181 – 0.406)	-0.004 (-0.084 – 0.382)	0.297 (0.200 – 0.565)	0.008 (-0.357 – 0.151)	0.274 (0.190 – 0.892)	0.010 (-0.650 – 0.112)	0.988	0.802
15:0	0.093 (0.054 – 0.136)	0.002 (-0.020 – 0.041)	0.088 (0.064 – 0.147)	-0.001 (-0.048 – 0.014)	0.096 (0.058 – 0.148)	-0.003 (-0.025 – 0.020)	0.997	0.343
16:0	18.81 (18.019 – 21.240)	0.164 (-1.925 – 2.277)	18.780 (17.775 – 20.87)	-0.115 (-2.615 – 1.367)	18.856 (16.079 -21.734)	0.073 (-2.816 – 4.059)	0.948	0.191
16:1	0.334 (0.170 – 0.932)	-0.011 (-0.212 – 1.039)	0.338 (0.180 – 0.568)	0.004 (-0.283 – 0.424)	0.263 (0.161 – 1.984)	-0.012 (-1.535 – 0.345)	0.471	0.868
17:0	0.279 (0.152 – 0.336)	-0.008 (-0.065 – 0.032)	0.265 (0.192 – 0.380)	0.003 (-0.042 – 0.043)	0.277 (0.161 – 0.369)	-0.013 (-0.044 – 0.040)	0.604	0.423
18:0	13.998 (13.092 – 14.780)	-0.066 (-3.574 – 1.057)	14.106 (12.932 – 14.983)	-0.021 (-0.757 – 1.877)	13.901 (10.364 – 15.105)	0.103 (-1.003 – 3.367)	0.674	0.736
18:1 (carbon atom 6-11)	0.248 (0.222 – 0.361)	-0.037 (-0.103 – 0.048)	0.279 (0.190 – 0.429)	-0.024 (-0.163 – 0.020)	0.266 (0.170 – 0.608)	-0.036 (-0.239 – 0.009)	0.502	0.250
18:1 (carbon atom 9)	9.779 (9.104 – 10.977)	0.021 (-0.868 – 4.549)	9.904 (8.413 – 11.994)	0.243 (-2.559 – 1.828)	9.820 (7.676 – 17.855)	-0.254 (-7.229 – 1.837)	0.991	0.564
18:1 (carbon atom 11)	0.782 (0.631 – 1.085)	0.052 (-0.091 – 0.310)	0.825 (0.612 – 1.055)	0.011 (-0.132 – 0.143)	0.808 (0.591 -1.470)	-0.001 (-0.461 – 0.204)	0.444	0.085
18:2 n-6	7.083 (5.309 – 10.197)	0.259 (-2.323 – 9.780)	7.240 (4.627 – 12.348)	-0.107 (-4.176 – 1.427)	7.087 (5.048 – 13.707)	-0.797 (-7.822 – 1.222)	0.605	0.023[a]
20:0	0.351 (0.271 – 0.449)	-0.008 (-0.057 – 0.026)	0.360 (0.282 – 0.505)	-0.006 (-0.068 – 0.038)	0.378 (0.294 – 0.459)	-0.006 (-0.033 – 0.081)	0.432	0.730
18:3 n-6	0.068 (0.051 – 0.097)	-0.004 (-0.039 – 0.156)	0.069 (0.041 – 0.222)	0.001 (-0.134 – 0.109)	0.067 (0.046 – 0.250)	-0.007 (-0.176 – 0.032)	0.562	0.635
18:3 n-3	0.122 (0.092 – 0.191)	0.000 (-0.040 – 0.240)	0.126 (0.000 – 0.246)	0.002 (-0.141 – 0.138)	0.113 (0.086 – 0.617)	-0.005 (-0.492 – 0.035)	0.731	0.154
20:1 n-9	0.179 (0.119 – 0.238)	0.002 (-0.058 – 0.025)	0.205 (0.159 – 0.365)	-0.002 (-0.044 – 0.030)	0.212 (0.135 – 0.293)	-0.008 (-0.066 – 0.018)	0.034[b]	0.009[a]
20:2 n-6	0.167 (0.123 – 0.234)	0.002 (-0.028 – 0.032)	0.175 (0.125 – 0.212)	0.002 (-0.017 – 0.018)	0.174 (0.121 - 0.229)	-0.004 (-0.055 – 0.021)	0.325	0.024[a]
22:0	1.887 (1.436 – 2.333)	-0.024 (-0.733 – 0.230)	1.831 (1.478 – 2.319)	0.002 (-0.269 – 0.273)	1.777 (1.148 – 2.584)	-0.036 (-0.257 – 0.476)	0.292	0.599
20:3 n-6	0.794 (0.606 – 1.291)	0.007 (-0.207 – 0.359)	0.881 (0.579 – 2.019)	0.001 (-0.111 – 0.211)	0.857 (0.540 – 1.608)	-0.127 (-0.567 – 0.146)	0.287	< 0.001[a]
20:4 n-6 & 22:1 n-9	9.907 (7.883 – 13.218)	0.070 (-3.138 – 2.212)	9.742 (7.427 – 12.688)	0.263 (-0.597 – 3.203)	10.147 (6.987 – 12.364)	-0.409 (-1.296 – 2.636)	0.909	< 0.001[a]
23:0	0.222 (0.145 – 0.299)	-0.001 (-0.015 – 0.013)	0.218 (0.154 – 0.288)	-0.004 (-0.047 – 0.013)	0.227 (0.131 – 0.279)	-0.003 (-0.017 – 0.056)	0.969	0.655
20:5 n-3	1.408 (0.685 – 2.154)	0.064 (-0.261 – 0.725)	1.489 (0.434 – 2.490)	0.023 (-0.293 – 0.401)	1.508 (0.698 – 3.742)	0.975 (0.100 – 1.718)	0.874	< 0.001[a]
24:0	5.058 (4.389 – 5.755)	-0.064 (-2.128 – 0.467)	5.186 (3.840 – 5.642)	-0.048 (-0.692 – 1.242)	4.952 (2.500 – 5.936)	-0.002 (-0.705 – 2.877)	0.495	0.719
24:1	4.532 (3.938 – 5.520)	-0.035 (-2.165 – 1.067)	4.643 (3.629 – 5.374)	0.009 (-0.529 – 1.062)	4.668 (2.438 – 5.654)	-0.074 (-0.714 – 2.365)	0.598	0.575
22:5 n-3	2.484 (1.832 – 3.002)	0.025 (-1.015 – 1.062)	2.464 (1.211 – 3.270)	0.032 (-0.139 – 0.897)	2.580 (1.359 – 3.147)	0.343 (-0.203 – 1.040)	0.720	<0.001[a]
22:6 n-3	6.097 (4.261 – 7.837)	0.024 (-2.101 – 2.982)	6.021 (2.906 – 8.084)	0.142 (-0.633 – 1.912)	6.049 (3.505 – 8.200)	0.487 (-0.394 – 2.151)	0.802	0.006[a]
Unknown	13.634 (12.312 – 17.508)	-0.478 (-4.101 – 1.102)	13.765 (11.386 – 15.539)	-0.034 (-2.256 – 2.458)	13.747 (8.872 – 28.854)	-0.036 (-15.045 – 6.329)	0.978	0.346

a: Non-significant difference between the control group and the tomato intervention, $p < 0.05$ between control and multi-diet intervention.

b: $p = 0.015$ Between control and tomato, $p = 0.034$ between control and multi-diet group.

Table 8.2. PSA values in subjects with low and high baseline lycopene, n-3 fatty acids and selenium, an overview and comparison between groups

Subjects were divided into groups above and below baseline median value of plasma lycopene, plasma levels of DHA, DPA and EPA in RBC combined, and plasma selenium. Non-normally distributed parameters presented as median (range), Kruskal-Wallis tests was used to detect differences between groups.

	Parameter	Control group		Tomato intervention		Multi-diet intervention		P-value of between group differences	
		Baseline	Difference	Baseline	Difference	Baseline	Difference	Baseline	Difference
Lycopene	**Low baseline lycopene (control n = 9, tomato intervention n = 13, multi-diet intervention n = 13)**								
	tPSA [ng/mL]	9.36 (5.80 – 17.50)	0.20 (-0.90 – 2.24)	8.12 (4.58 – 25.90)	0.00 (-3.30 – 2.40)	12.30 (5.10 – 31.50)	0.40 (-3.80 – 4.80)	0.246	0.691
	fPSA [ng/mL]	1.02 (0.77 – 1.45)	0.21 (-0.21 – 1.04)	0.82 (0.52 – 2.14)	0.00 (-0.48 – 0.42)	1.09 (0.51 – 4.96)	0.02 (-0.16 – 0.80)	0.408	0.224
	Ratio fPSA [% free PSA]	11.26 (6.29 – 22.48)	1.58 (-3.77 – 6.35)	9.49 (7.73 – 17.61)	0.14 (-1.47 – 5.07)	9.00 (4.46 – 37.69)	0.27 (-2.60 – 5.72)	0.566	0.493
	High baseline lycopene (control n = 12, tomato intervention n = 12, multi-diet intervention n = 11)								
	tPSA [ng/mL]	8.11 (4.42 – 23.70)	0.57 (-1.63 – 1.64)	8.58 (1.52 – 19.60)	-0.15 (-1.00 – 1.90)	9.72 (5.92 – 23.50)	-0.10 (-12.40 – 1.20)	0.795	0.186
	fPSA [ng/mL]	0.84 (0.55 – 2.18)	0.07 (-0.13 – 1.14)	0.81 (0.32 – 1.96)	0.07 (-0.07 – 0.24)	1.02 (0.43 – 4.26)	-0.01 (-1.85 – 0.31)	0.528	0.123
	Ratio fPSA [% free PSA]	9.59 (3.93 –28.40)	-0.17 (-1.50 – 7.96)	9.38 (4.10 – 21.05)	-0.06 (-1.31 – 3.12)	11.34 (5.93 – 18.13)	0.33 (-2.77 – 3.58)	0.859	0.950
n-3 fatty acids	**Low baseline DHA, DPA and EPA (control n = 10, tomato intervention n = 12, multi-diet intervention n = 13)**								
	tPSA [ng/mL]	9.09 (4.42 – 17.70)	0.51 (-0.20 – 1.47)	8.51 (5.22 – 25.90)	-0.29 (-3.30 – 1.76)	11.20 (5.10 – 31.50)	-0.02 (-12.40 – 4.80)	0.288	0.248
	fPSA [ng/mL]	0.85 (0.59 – 1.66)	0.08 (-0.21 – 1.14)	0.74 (0.38 – 2.04)	0.04 (-0.48 – 0.24)	1.01 (0.48 – 4.26)	-0.04 (-1.85 – 0.80)	0.282	0.175
	Ratio fPSA [% free PSA]	10.05 (4.69 – 18.33)	0.38 (-3.77 – 7.96)	8.44 (4.10 – 18.92)	-0.31 (-1.15 – 3.12)	10.00 (4.46 – 18.13)	-0.23 (-2.60 – 3.58)	0.721	0.786
	High baseline DHA, DPA and EPA (control n = 11, tomato intervention n = 12, multi-diet intervention n = 12)								
	tPSA [ng/mL]	9.31 (5.80 – 23.70)	0.36 (-1.63 – 2.24)	8.54 (1.52 – 18.90)	0.03 (-1.12 – 2.40)	9.82 (5-92 – 30.20)	0.20 (-3.80 – 1.30)	0.766	0.671
	fPSA [ng/mL]	1.09 (0.55 – 2.18)	0.11 (-0.13 – 1.04)	0.97 (0.32 – 2.14)	0.05 (-0.18 – 0.28)	1.12 (0.43 – 4.96)	0.04 (-0.45 – 0.62)	0.708	0.651
	Ratio fPSA [% free PSA]	9.98 (3.93 – 28.40)	0.54 (-1.50 – 6.35)	10.77 (6.77 – 21.05)	0.04 (-1.47 – 5.07)	11.66 (5.89 – 37.69)	0.34 (-2.77 – 5.72)	0.974	0.685
Selenium	**Low baseline selenium (control n = 10, tomato intervention n = 7, multi-diet intervention n = 8)**								
	tPSA [ng/mL]	7.41 (4.42 – 17.70)	0.51 (-1.63 – 1.64)	6.99 (1.52 – 25.90)	0.07 (-3.30 – 1.70)	7.75 (5.10 – 29.40)	0.22 (-0.78 – 4.80)	0.721	0.615
	fPSA [ng/mL]	0.91 (0.63 – 1.66)	0.12 (-0.21 – 1.14)	0.54 (0.32 – 2.04)	0.04 (-0.48 – 0.28)	0.86 (0.48 – 1.85)	0.02 (-0.16 – 0.80)	0.474	0.486
	Ratio fPSA [% free PSA]	11.44 (4.69 – 18.33)	0.81 (-3.77 – 7.96)	8.52 (5.94 – 21.05)	-0.75 (-1.31 – 5.07)	10.21 (4.46 – 14.80)	0.46 (-2.60 – 1.71)	0.666	0.813
	High baseline selenium (control n = 7, tomato intervention n = 13, multi-diet intervention n = 10)								
	tPSA [ng/mL]	9.31 (5.80 – 23.70)	0.36 (-0.90 – 2.24)	8.12 (5.06 – 18.90)	0.30 (-1.12 – 2.40)	11.60 (6.61 – 31.50)	0.08 (-12.40 – 4.50)	0.213	0.596
	fPSA [ng/mL]	1.10 (0.59 – 2.18)	0.09 (-0.13 – 1.04)	0.95 (0.46 – 2.14)	0.10 (-0.18 – 0.42)	1.18 (0.43 – 4.26)	0.02 (-1.85 – 0.54)	0.465	0.531
	Ratio fPSA [% free PSA]	9.20 (6.29 – 28.40)	-0.25 (-1.37 – 6.35)	11.32 (6.77 – 17.61)	0.43 (-1.47 – 3.12)	10.65 (6.28 – 18.13)	0.23 (-2.77 – 3.58)	0.727	0.961

Table 8.3. Biomarkers of inflammation and IGF-1, an overview and comparison between groups, stratified by biomarkers of compliance at baseline

Non-normally distributed parameter presented as median (range), Kruskal-Wallis tests was used to detect difference between groups, when significant Mann Whitney tests between individual groups were performed.

	Parameter	Control group		Tomato intervention		Multi-diet intervention		P-value of between group differences	
		n Baseline	n Difference	n Baseline	n Difference	n Baseline	n Difference	Baseline	Difference
Lycopene	**Low baseline plasma lycopene**								
	CRP [mg/L]	n = 7 1.00 (0.60 – 7.10)	n = 7 -0.04 (-1.4 – 1.00)	n = 13 1.80 (0.60 – 5.40)	n = 13 0.06 (-1.30 – 1.00)	n = 13 1.70 (0.60 – 7.00)	n = 13 0.60 (-2.20 – 7.90)	0.780	0.438
	IGF-1 [nmol/L]	n = 7 18.0 (12.0 – 22.0)	n = 7 1.0 (-2.0 – 5.0)	n = 13 18.0 (14.0 – 31.0)	n = 13 3.0 (-6.0 – 6.0)	n = 13 19.0 (9.5 – 24.0)	n = 13 1.0 (-5.0 – 6.0)	0.565	0.929
	IL-6 [pg/mL]	n = 9 1.24 (0.00 – 4.22)	n = 9 0.00 (-1.04 – 1.37)	n = 13 0.46 (0.00 – 7.50)	n = 13 0.17 (-5.55 – 2.76)	n = 13 1.28 (0.00 – 11.19)	n = 13 0.00 (-0.73 – 1.56)	0.627	0.750
	suPAR [ng/mL]	n = 9 2.39 (1.22 – 3.15)	n = 9 0.38 (-0.29 – 0.90)	n = 14 2.30 (1.19 – 16.52)	n = 14 0.16 (-2.02 – 3.14)	n = 13 2.88 (1.01 – 6.66)	n = 13 0.23 (-1.27 – 0.79)	0.815	0.430
	High baseline plasma lycopene								
	CRP [mg/L]	n = 12 1.05 (0.60 – 3.60)	n = 12 -0.06 (-1.10 – 5.70)	n = 12 1.02 (0.60 – 6.00)	n = 12 -0.10 (-2.90 – 5.20)	n = 11 1.40 (0.66 – 3.30)	n = 11 0.20 (-2.30 – 8.20)	0.711	0.429
	IGF-1 [nmol/L]	n = 11 19.0 (12.0 – 37.0)	n = 11 0.0 (-4.0 – 4.0)	n = 12 25.5 (16.0 – 37.0)	n = 12 -2.0 (-5.0 – 5.0)	n = 11 21.0 (15.0 – 30.0)	n = 11 1.0 (-2.0 – 5.0)	0.044[a]	0.025[b]
	IL-6 [pg/mL]	n = 12 0.14 (0.00 – 1.67)	n = 12 0.00 (-0.07 – 1.77)	n = 12 1.21 (0.00 – 6.51)	n = 12 0.00 (-1.64 – 2.77)	n = 11 0.32 (0.00 – 2.11)	n = 11 0.00 (-1.25 – 3.19)	0.180	0.364
	suPAR [ng/mL]	n = 13 1.88 (1.52 – 3.13)	n = 13 0.18 (-0.69 – 1.23)	n = 12 2.11 (0.86 – 3.58)	n = 12 0.24 (-0.36 – 5.36)	n = 11 2.11 (1.04 – 2.53)	n = 11 0.25 (-0.28 – 1.15)	0.935	0.742
n-3 fatty acids	**Low baseline DHA, DPA and EPA in RBCs**								
	CRP [mg/L]	n = 10 1.20 (0.60 – 7.10)	n = 10 -0.39 (-1.30 – 1.00)	n = 12 1.65 (0.60 – 4.60)	n = 12 0.00 (-2.90 – 5.20)	n = 13 1.50 (0.60 – 7.00)	n = 13 0.20 (-2.30 – 8.20)	0.836	0.370
	IGF-1 [nmol/L]	n = 10 17.0 (12.0 – 24.0)	n = 10 0.5 (-3.0 – 5.0)	n = 12 27.5 (16.0 – 37.0)	n = 12 -1.5 (-6.0 – 4.0)	n = 13 21.0 (12.0 – 30.0)	n = 13 1.0 (-2.0 – 6.0)	0.017[c]	0.011[d]
	IL-6 [pg/mL]	n = 10 0.49 (0.00 – 4.22)	n = 10 0.00 (-1.04 – 1.37)	n = 12 1.01 (0.00 – 4.99)	n = 12 0.00 (-1.39 – 2.77)	n = 13 1.50 (0.00 – 11.19)	n = 13 0.08 (-1.25 – 3.19)	0.949	0.717
	suPAR [ng/mL]	n = 10 2.19 (1.01 – 6.66)	n = 10 0.26 (-0.69 – 0.89)	n = 12 1.95 (0.86 – 5.45)	n = 12 0.48 (-2.02 – 5.36)	n = 13 2.30 (1.29 – 2.91)	n = 13 0.23 (-1.27 – 0.73)	0.623	0.523
	High baseline DHA, DPA and EPA in RBCs								
	CRP [mg/L]	n = 9 0.98 (0.60 – 2.10)	n = 9 0.00 (-1.40 – 5.70)	n = 12 1.50 (0.60 – 6.00)	n = 12 -0.40 (-2.70 – 0.70)	n = 12 0.84 (0.60 – 2.50)	n = 12 0.32 (-1.90 – 4.30)	0.426	0.097
	IGF-1 [nmol/L]	n = 8 19.0 (12.0 – 37.0)	n = 8 1.0 (-4.0 – 4.0)	n = 12 19.5 (14.0 – 31.0)	n = 12 3.0 (-5.0 – 6.0)	n = 12 0.5 (-5.0 – 4.0)	n = 12 20.5 (9.5 – 32.0)	0.771	0.794
	IL-6 [pg/mL]	n = 11 0.62 (0.00 – 1.67)	n = 11 0.64 (-0.07 – 1.77)	n = 12 0.78 (0.00 – 7.50)	n = 12 0.09 (-5.5 – 2.76)	n = 12 0.58 (0.00 – 3.53)	n = 12 0.00 (-0.73 – 1.56)	0.745	0.258
	suPAR [ng/mL]	n = 11 1.81 (1.52 – 3.13)	n = 11 0.21 (-0.46 – 1.23)	n = 12 2.30 (1.19 – 16.52)	n = 12 0.13 (-0.25 – 3-14)	n = 12 2.05 (1.04 – 3.15)	n = 12 0.37 (-0.28 – 1.15)	0.443	0.660

Table 8.3 continued

	Parameter	Control group		Tomato intervention		Multi-diet intervention		P-value of between group differences	
		n Baseline	n Difference	n Baseline	n Difference	n Baseline	n Difference	Baseline	Difference
Selenium	**Low baseline plasma selenium**								
	CRP [mg/L]	n = 10 1.45 (0.87 – 7.10)	n = 10 -0.39 (-1.30 – 5.70)	n = 7 1.5 (0.60 – 2.40)	n = 7 0.00 (-1.10 – 5.20)	n = 8 1.3 (0.60 – 2.10)	n = 8 0.40 (-0.94 – 7.90)	0.736	0.267
	IGF-1 [nmol/L]	n = 9 18.0 (12.0 – 24.0)	n = 9 1.0 (-3.0 – 3.0)	n = 7 22.0 (14.0 – 29.0)	n = 7 -1.0 (-2.0 – 6.0)	n = 8 20.0 (12.0 – 30.0)	n = 8 4.0 (0.0 – 6.0)	0.404	0.049[e]
	IL-6 [pg/mL]	n = 10 0.05 (0.00 – 4.22)	n = 10 0.00 (-0.66 – 1.68)	n = 7 2.13 (0.31 – 4.99)	n = 7 -0.55 (-1.39 – 2.76)	n = 8 0.89 (0.00 – 2.27)	n = 8 0.09 (-0.35 – 3.19)	0.082	0.505
	suPAR [ng/mL]	n = 10 2.32 (1.55 – 6.66)	n = 10 0.26 (-0.69 – 1.23)	n = 8 1.79 (1.42 – 3.28)	n = 8 -0.20 (-0.57 – 0.70)	n = 8 2.46 (1.96 – 2.91)	n = 8 -0.04 (-1.27 – 0.60)	0.291	0.418
	High baseline plasma selenium								
	CRP [mg/L]	n = 6 0.74 (0.60 – 2.00)	n = 6 0.06 (-1.40 – 0.93)	n = 13 0.93 (0.60 – 7.00)	n = 13 0.00 (-1.30 – 1.50)	n = 10 1.70 (0.60 – 7.0)	n = 10 0.00 (-2.30 – 4.30)	0.371	0.897
	IGF-1 [nmol/L]	n = 6 19.0 (12.0 – 37.0)	n = 6 -0.5 (-4.0 – 4.0)	n = 13 19.0 (15.0 – 37.0)	n = 13 -1.0 (-5.0 – 4.0)	n = 10 21.0 (15.0 – 32.0)	n = 10 0.5 (-2.0 – 4.0)	0.977	0.451
	IL-6 [pg/mL]	n = 7 1.06 (0.00 – 1.67)	n = 7 0.44 (-0.07 – 1.32)	n = 13 0.35 (0.00 – 7.50)	n = 13 0.00 (-5.55 – 2.77)	n = 10 0.33 (0.00 – 1.55)	n = 10 0.00 (-1.25 – 0.98)	0.354	0.149
	suPAR [ng/mL]	n = 7 1.74 (1.57 – 3.13)	n = 7 0.18 (-0.32 – 0.90)	n = 13 2.21 (0.86 – 16.52)	n = 13 0.32 (-0.19 – 5.36)	n = 10 2.05 (1.18 – 3.15)	n = 10 0.32 (-0.28 – 1.12)	0.687	0.488

a: p = 0.019 between control and tomato, p = 0.222 between control and multi-diet
b: p = 0.143 between control and tomato, p = 0.063 between control and multi-diet
c: p = 0.006 between control and tomato, p = 0.139 between control and multi-diet
d: p = 0.139 between control and tomato, p = 0.148 between control and multi-diet
e: p = 0.541 between control and tomato, p = 0.008 between control and multi-diet

Table 8.4. PSA values compared between differing changes in EPA, baseline values and changes during the intervention period

Non-normally distributed parameters presented as median (range), the Mann-Whitney test was used to compare groups. Groups are divided based on into groups for below (low increase) and above (high increase) median change.

Parameter	Low EPA increase		High EPA increase		P-value of between group differences	
	Baseline	Difference	Baseline	Difference	Baseline	Difference
All subjects (low increases n = 35, high increases n = 35)						
tPSA [ng/mL]	8.12 (1.52 – 25.90)	0.29 (-3.30 – 2.40)	10.60 (4.42 – 31.50)	-0.10 (-12.40 – 4.80)	0.051	0.243
fPSA [ng/mL]	0.96 (0.32 – 2.14)	0.09 (-0.48 – 1.14)	1.01 (0.43 – 4.96)	0.02 (-1.85 – 0.80)	0.260	0.425
fPSA ratio [% free fPSA]	10.22 (4.10 – 22.48)	-0.07 (-3.77 – 7.96)	10.00 (3.93 – 37.69)	0.33 (-2.77 – 5.72)	0.640	0.282
Intermediate tumor risk (low increases n = 21, high increases n = 16)						
tPSA [ng/mL]	6.99 (1.52 – 18.00)	0.29 (-1.12 – 2.24)	8.55 (4.42 – 23.50)	-0.15 (-12.40 – 1.47)	0.284	0.335
fPSA [ng/mL]	0.64 (0.32 – 1.96)	0.09 (-0.21 – 1.04)	0.98 (0.43 – 4.26)	0.05 (-1.85 – 0.62)	0.090	0.922
Ratio fPSA [% free PSA]	10.22 (4.10 – 22.48)	-0.57 (-3.77 – 6.35)	11.48 (4.69 – 37.69)	0.97 (-2.60 – 5.72)	0.241	0.206
High tumor risk (low increases n = 13, high increases n = 19)						
tPSA [ng/mL]	11.65 (5.80 – 25.90)	0.25 (-3.30 – 2.40)	12.30 (5.38 – 31-50)	-0.02 (-3.80 – 4.80)	0.704	0.646
fPSA [ng/mL]	1.13 (0.63 – 2.14)	0.10 (-0.48 – 1.14)	1.09 (0.46 – 4.96)	0.00 (-0.45 – 0.80)	0.542	0.193
Ratio fPSA [% free PSA]	9.18 (6.29 – 17.61)	0.55 (-1.23 – 7.96)	8.55 (3.93 – 16.42)	0.14 (-2.77 – 3.12)	0.287	0.562

Table 8.5. PSA values compared between differing changes in selenium, baseline values and changes during the intervention period

Non-normally distributed parameters presented as median (range), the Mann-Whitney test was used to compare groups. Groups are divided based on into groups for below (low increase) and above (high increase) median change.

Parameter	Low selenium increase		High selenium increase		P-value of between group differences	
	Baseline	Difference	Baseline	Difference	Baseline	Difference
All subjects (low increases n = 34, high increases n = 32)						
tPSA [ng/mL]	8.70 (1.52 – 19.60)	0.17 (-1.63 – 2.40)	10.16 (4.58 – 31.50)	0.06 (-12.40 – 4.80)	0.119	0.187
fPSA [ng/mL]	0.95 (0.32 – 2.14)	0.08 (-0.21 – 1.14)	1.06 (0.43 – 4.96)	0.01 (-1.85 – 0.80)	0.284	0.057
fPSA ratio [% free fPSA]	10.10 (3.93 – 28.40)	0.17 (-3.77 – 7.96)	10.45 (4.46 – 18.92)	0.23 (-2.77 – 5.07)	0.633	0.669
Intermediate tumor risk (low increases n = 21, high increases n = 15)						
tPSA [ng/mL]	7.49 (1.52 – 18.00)	0.13 (-0.97 – 2.24)	7.36 (4.58 – 23.50)	0.26 (-12.40 – 1.47)	0.794	0.320
fPSA [ng/mL]	0.86 (0.32 – 1.96)	0.07 (-0.21 – 1.04)	0.84 (0.43 – 4.26)	0.00 (-1.85 – 0.31)	0.918	0.344
Ratio fPSA [% free PSA]	10.22 (4.10 – 28.40)	-0.27 (-3.77 – 6.35)	11.34 (4.69 – 18.13)	0.48 (-2.60 – 5.07)	0.751	0.975
High tumor risk (low increases n = 13, high increases n = 16)						
tPSA [ng/mL]	9.74 (5.06 – 19.60)	0.20 (-1.63 – 2.40)	15.60 (5.38 – 31.50)	-0.41 (-3.80 – 4.80)	0.132	0.308
fPSA [ng/mL]	0.99 (0.55 – 2.14)	0.10 (-0.09 – 1.14)	1.20 (0.46 – 4.96)	-0.03 (-0.48 – 0.80)	0.324	0.060
Ratio fPSA [% free PSA]	9.98 (3.93 – 17.61)	0.56 (-1.23 – 7.96)	8.77 (4.46 – 16.42)	0.09 (-2.77 – 3.12)	0.249	0.249

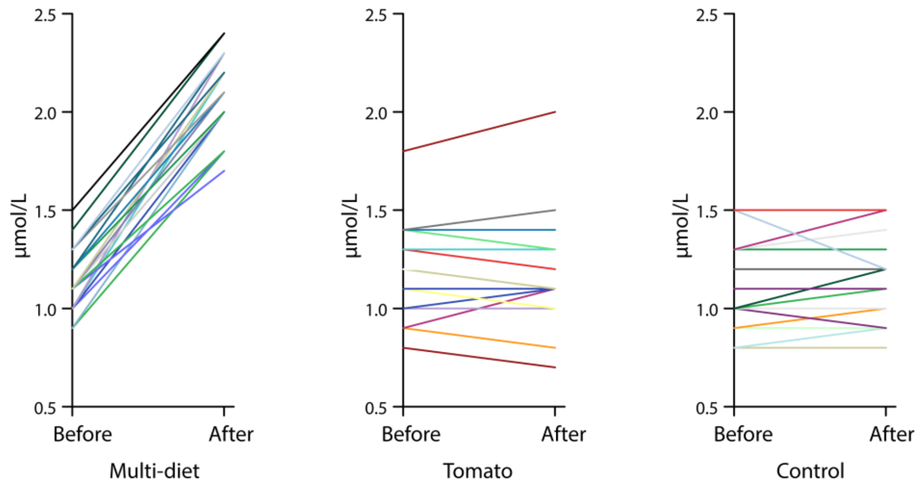

Figure 8.1. Individual development in plasma selenium levels.

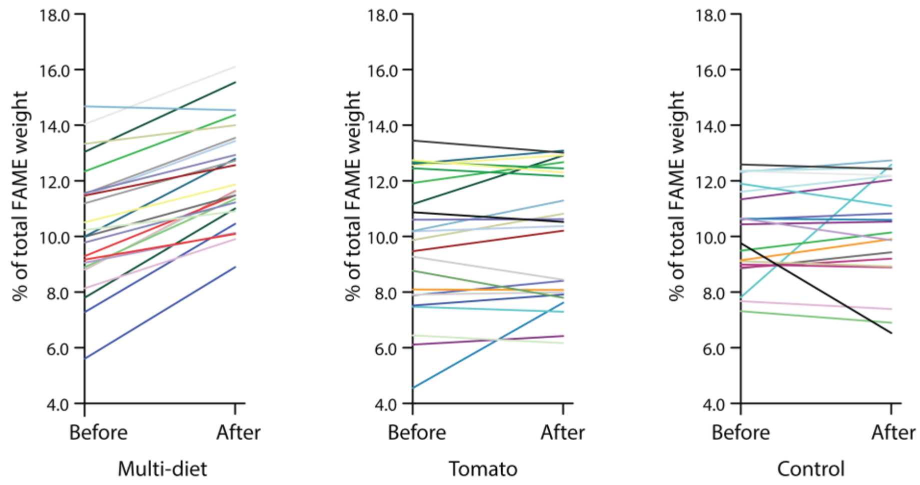

Figure 8.2. Individual development in RBC level of DHA, DPA and EPA combined.

9 Appendix

9.1 Externally performed laboratory analyses

Externally performed methods are briefly introduced in the methods section and described in more detailed in the following sections.

9.1.1 Carotenoids in plasma

Carotenoids in plasma was quantified externally at *Vitas* (Oslo, Norway) using High Performance Liquid Chromatography.

Principle of the assay

In brief, a High Performance Liquid Chromatography system is used to create high pressure on a liquid solvent to separate a mixture of compounds in an analytical column. Samples are injected into the mobile phase and, with the high pressure, the samples are forced through a column. The column contains packing materials that hold a stationary phase in place. The task of the stationary phase is to separate molecules in the samples. In this specific analysis, a lipophilic column is used which separates the molecules based on shape and size. After molecules in the samples are separated they can be read by a detector that records time and amount of each specific molecule. The detector is based on fixed UV-wavelength detection, which records the absorption of the analyte. The amount and type of analyte can then be quantified based on comparison to references.

Procedure

25 µL plasma are pipetted into vials and proteins are precipitated and carotenoids extracted with isopropanol added internal standard (β-Apo-8-carotenal). After thorough mixing and subsequent centrifugation, an aliquot of the isopropanol phase is injected into the HPLC-UV.

Analysis is performed on an 1100-series HPLC with a 1260 diode array detector (453nm) (Agilent Technologies, Palo Alto, CA). Separation is performed on a 3 µm, YMC C30 (150 mm × 4.6 mm i.d.) column from (YMC, Japan).

9.1.2 Fatty acids in red blood cells

Fatty acids in red blood cells was quantified externally at as Vitas using Gas chromatography (GC) with flame ionization detector (GC- FID). Table 8.1 lists all quantified fatty acids with the corresponding trivial names.

Principle of the assay

GC like other chromatographic techniques requires a mobile phase (carrier gas) and a stationary phase (liquid, immiscible polymer or inert solid). The GC column is a large length and coiled capillary column packed with fused silica fitted inside of an oven. The sample inside the column is turned into a gas, and then carried through a column by an inert or un-reactive gas (e.g. helium, argon, nitrogen). After molecules in the samples are separated they can be read by a detector that records time and amount of each specific molecule. The detector in this assay is a flame ionization detector, which burns the fatty acids and records the ionic composition. The amount and type of the specific analyte can then be quantified based on comparison to standards.

Procedure

Analysis was performed on a 7890A GC with a split/splitless injector, a 7683B automatic liquid sampler, and flame ionization detection (Agilent Technologies, Palo Alto, CA). Separations was performed on a SP-2380 (30 m × 0.25 mm i.d. × 0.25 µm film thickness) column from Supelco.

RBC samples, thawed in fridge overnight, were vortexed and pipetted into vials. Samples were methylated with 3N MeOH HCl. FAMEs were extracted with hexane, then samples were neutralized with 3N KOH in water. After mixing and centrifuging the hexane phase was injected into the GC-FID.

Table 8.1. Fatty acids quantified in RBC.

Fatty acids quantified	
Lipid	**Trivial name**
C14:0	Myristic
C15:0	Pentadecylic
C16:0	Palmitic
C16:1	
C17:0	Margaric
C18:0	Stearic
C18:1,t6-11	
C18:1,c9	Oleic
C18:1,c11	
C18:2,n-6	Linoleic
C20:0	Arachidic
C18:3,n-6	Gamma-linoleic
C18:3,n-3	Alpha-linolenic
C20:1,n-9	Gondoic
C20:2,n-6	Eicosadienoic
C22:0	Arachidic
C20:3,n-6	Dihomo-gamma-linoleic
C20:4,n-6/C22:1,n-9	Arachidonic / Erucic
C23:0	Tricosylic
C20:5,n-3	Eicosapentaenoic (EPA)
C24:0	Lignoceric
C24:1	
C22:5,n-3	Docosapentaenoic (DPA)
C22:6,n-3	Docosahexaenoic (DHA)
Unknown	

9.1.3 High sensitive C-reactive protein

High sensitive C-reactive protein was quantified externally at the Oslo University Hospital by standard procedures. No information has been disclosed regarding the method used.

9.1.4 Insulin-like growth factor-I

Insulin-like growth factor-I was quantified externally at Oslo University Hospital using immunometric enzyme-labeled chemiluminescence.

Principle of the assay

Briefly, samples and alkaline phosphatase-conjugated IGF-1 antibodies are simultaneously added to wells precoated with IGF-1-antibodies. Hence, a sandwich assay is formed with IGF-1 in between. After incubation unbound material is washed away and a substrate, phosphate ester of adamantyl dioxetane, is added. This substrate undergoes hydrolysis under the influence of alkaline phosphatase and creates an unstable intermediate that emits light.

The production of light is then measured and compared to cut off values and concentration of IGF-1 is then calculated.

9.1.5 Prostate specific antigen

Prostate specific antigen was quantified externally at DNR using the *AutoDELFIA automatic immunoassay system*.

Principle of the assay

Briefly, a kit containing 96-well mictrotiter plates precoated with a monoclonal PSA-antibody is used. Samples are automatically transferred to the plate wells, where the precoated antibody binds equally specific to both free and antichymotrypsin-bound PSA in the samples. After a washing step, two different monoclonal tracer antibodies are added. One is europium-labelled and binds to fPSA and one that is samarium-labelled and binds equally to both free– and antichymotrypsinbound PSA (figure 8.1). Then another wash to remove unbound material is performed before the amount of PSA is quantified by the use of time-resolved fluorometry.

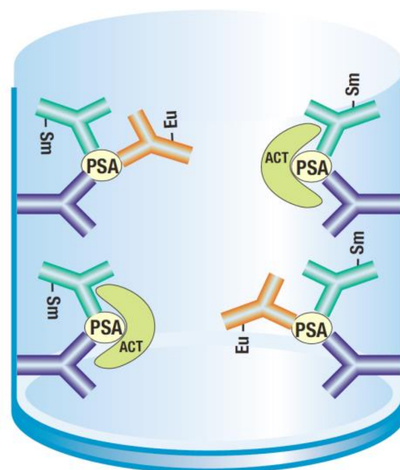

Figure 8.1. The basics of the separation of total and free PSA. The precoated antibody (depicted in purple) binds to both forms of PSA. The samarium labeled antibody (depicted in green) also binds to both free and total PSA. The europium labeled antibodies binds to only free PSA (depicted in orange). The illustration is reprinted with permission from Maija Ahti, PerkinElmer Life and Analytical Sciences, www.perkinelmer.com.

In brief, time resolved fluorometry takes advantage of the fluorescent properties of samarium and europium. Wells are exposed to flashes (x 1000/second) of light (wavelength 360 nm) and the emitted light in response to the illumination is then measured (x 1000/second). The different labels emitt light of specific wavelengths, and the amount of analyte can then be compared to standards (figure 8.2).

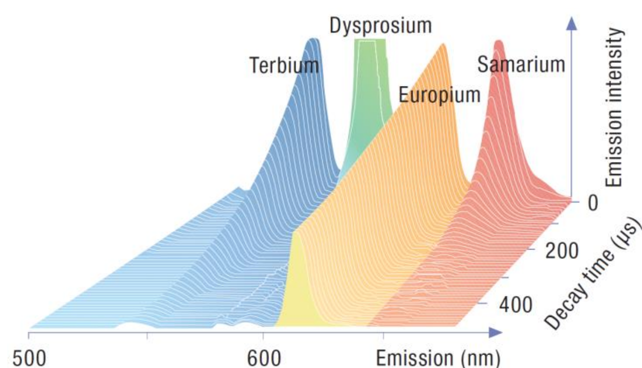

Figure 8.2. The fluorescent labels respond to flashes of light by emitting light in different wavelengths, allowing simultaneous quantification of different analytes. The illustration is reprinted with permission from Maija Ahti, PerkinElmer Life and Analytical Sciences, www.perkinelmer.com.

9.1.6 **Selenium**

Selenium in plasma was quantified externally at Fürst Medical Laboratory using Inductively Coupled Plasma Mass Spectrometry.

Principle of the assay

In brief, samples are first gone through a nebulizer which creates small aerosol droplets. These droplets are then gone through a spray chamber in order to eliminate the largest droplets. After this, samples are sent through a tube surrounded by an electrical coil together with argon gas.

A spark is used to create some ions from the argon. Energy to the electrical coil is supplied through magnetic induction from a radio frequency generator. This creates an electrical field that changes with high frequency, making electrons travel back and forth and generate heat as they collide with other argon atoms. The temperature in this torch reaches approximately 6000ºC. When a sample enters this heated cloud it is broken down into solids and then ionized. The samples are then sent out to a vacuum chamber where ions are separated from neutral matters. Based on the relation between the mass and electrical charge of the atoms, only one type of atom is going through to the detector at a time. The amount of analyte is then quantified by measuring the number of hits compared to results from known standards.

Procedure

Selenium was measured with PerkinElmer Sciex, Elan® DRC™ II (Shelton, USA) ICP-MS (Inductively Coupled Plasma – Mass Spectrometry) instrument. The 82Se isotope was measured in standard mode. External calibration was used and the standard was matched with sample-matrix by adding Selenium (Se) PerkinElmer Pure Atomic Spectroscopy Calibration Standard, Matrix 2% HNO3, 1000 µg/mL (Shelton, USA) to Autonorm™(Billingstad, Norway).

Samples, standard and quality controls were diluted 1:20 with Milli-Q™ de-ionized water (Millipore, Bedford, MA, USA) with 0,1% (v/v) Nitric acid (65% m/v, Suprapur®, Merck, Darmstadt, Germany) and 0,5% (v/v) 1-Butanol (74,12 g/mol pro analysi, Merck, Darmstadt, Germany). 10 µg/L Rhodium (Rh) PerkinElmer Pure Atomic Spectroscopy Calibration Standard, Matrix 10% HCl, 1000 µg/mL (Shelton, USA) was added directly to the diluent and was used as an internal standard.