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Short term fasting, IGF/insulin-axis and therapy outcome in patients with cancer

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S. de Groot

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Short term fasting, IGF/insulin-axis and therapy outcome in patients with cancer

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Chapter 1

General introduction and outline

Introduction

Effective approaches of prevention, diagnosis and treatment of cancer are necessary as cancer is the second leading contributor to mortality worldwide after cardiovascular diseases and its incidence is still increasing[1]. The obesity epidemic is one of the explanations of the increasing incidence and mortality due to cancer[2] and increasing our understanding of its relation with cancer may lead to more effective treatment of cancer.

Long-term caloric restriction without malnutrition decreases cancer incidence and mortality in non-human primates[3]. Moreover, short periods of fasting improve cancer treatment outcome in rodents[4] and may be feasible in humans as an addition to cancer treatment[5]. However, research in humans is in its infancy and the exact mechanism and effects are not established yet.

Short-term fasting as an adjunct to cancer treatment

Short-term fasting (STF) protects rodents from toxic effects of chemotherapy, radiotherapy and targeted therapy, while enhancing the efficacy of the cancer therapy in distinct malignancies[6,7]. This distinct response of healthy versus cancer cells to periods of nutrient deprivation is called differential stress resistance (DSR). Healthy cells are protected during starvation due to activation of nutrient sensing pathways. These pathways are highly preserved between species[8,9] and regulate that healthy cells invest energy in repair and maintenance rather than growth[10,11]. For example, autophagy (Greek for "self-eating") is a conserved catabolic process among eukaryotes to survive periods of nutrient deprivation[12].

In contrast, cancer cells are unable to slow down growth due to mutations in tumor suppressor genes and mitogenic pathways and may be more vulnerable during periods of starvation[7,12]. Moreover, declines of plasma levels of insulin like growth factor-1 (IGF-1), insulin and glucose are among the mediators of the effects of fasting on cancer cells[13,14].

Therefore, short-term fasting is a promising strategy in humans to increase tolerability and efficacy of cancer treatment[15], at least in non-cachectic patients. However, so far no randomized data on efficacy were available. Besides, it is unknown for which human tumors short-term fasting could be effective and what the optimal timing and duration of the fasting and refeeding should be. Additionally, some difficulties need to be addressed as how to increase compliance in patients: though as promising as STF seems, it puts additional burden on the patient. Perhaps, to ease the burden of fasting a fasting mimicking diet (FMD) may be an alternative to increase compliance, as it mimics the effects of short-term fasting on metabolism[16]. Another difficulty that needs to be addressed is how to introduce short-term fasting in treatment regimens with

corticosteroids. These agents are commonly used in chemotherapy schedules and may interfere with the benefits of short-term fasting due to hyperglycemia induction.

IGF-1 and insulin in cancer

Insulin-like growth factor 1 (IGF-1) and insulin are members of the IGF-1 pathway, which is involved in cell growth and proliferation by activation of the Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/AKT pathways[17]. Elevated levels of IGF-1 and insulin, due to obesity or diabetes mellitus for example[18], have been associated with development, progression, metastasis and worse survival of cancer[14]. IGF-1 and insulin activate the IGF-1 receptor (IGF-1R) and the insulin receptor isoform A (IR-A), which are both frequently upregulated in distinct types of cancer[19].

IGF-1R inhibitors, which are highly effective in preclinical studies, did not show convincing benefits in clinical studies[20]. One of the explanations of these disappointing results might be that IGF-1R inhibition causes upregulation of the ligands due to abrogation of negative feedback in the pituitary. To overcome this resistance to IGF-1R inhibitors in humans short-term fasting may be the key solution as insulin and IGF-1 decrease dramatically during fasting[13].

Outline

This thesis focusses on the effects of short-term fasting and the IGF-1 and insulin pathway on toxicity and efficacy of chemotherapy in patients with cancer.

Outline of this thesis

***Part I.* Short-term fasting and fasting mimicking diets as an adjunct to cancer treatment**

The subject of Part 1 of the thesis is further introduced in a review article (**Chapter 2**) on the effects of short-term fasting on cancer treatment. The current knowledge of the molecular mechanisms explaining differential stress resistance (DSR) of healthy versus cancer cells in response to fasting are described. Additionally, the available (ongoing) clinical data reflecting the impact of short-term fasting on the effects of chemotherapy in cancer patients are summarized and critically reviewed.

The subsequent chapters describe clinical trials of short-term fasting in patients with breast cancer. A randomized-controlled pilot trial (NCT01304251) was performed to identify the effects of 48-hours of short-term fasting on chemotherapy-induced side effects in patients with breast cancer, who received docetaxel, doxorubicin and cyclophosphamide (TAC) chemotherapy **(Chapter 3)**. A subsequent study, the

multicentre, randomized, phase II/III DIRECT study (NCT02126449) evaluated the impact of a fasting mimicking diet (FMD) on toxicity and efficacy of chemotherapy in patients with breast cancer **(Chapter 4)**. The aim of these two clinical studies was to evaluate the feasibility of short-term fasting/FMD in patients with cancer. Furthermore, these studies explored the effects of short-term fasting/FMD on growth factors, such as IGF-1, insulin and glucose, and the effects of fasting on DNA damage in healthy cells. The main goal was to evaluate the effects of fasting/FMD on toxicity and efficacy on standard chemotherapy.

***Part II*. IGF-1 and insulin pathway in cancer treatment**

In Part II of this thesis, the effects of the IGF-1 pathway on chemotherapy outcome and the pathway itself as target for cancer therapy are described. In **Chapter 5** the current knowledge of IGF-1R inhibitors for treatment of cancer and hypotheses to overcome the resistance mechanisms to these inhibitors are reviewed, supported with preclinical data. Ewing sarcoma, a rare cancer localized in the skeleton or soft-tissue, is used as a simple model, as the IGF-1 pathway may play a major part in its pathogenesis[21]. In the subsequent chapters tissue of the breast cancer patient cohort of the NEOZOTAC trial (NCT01099436) is used to evaluate the expression of the IGF-1R of the tumor before and after neoadjuvant chemotherapy and whether it predicts pathological response according to the Miller and Payne classification after neoadjuvant chemotherapy. Furthermore, the aim was to identify single nucleotide polymorphisms (SNPs), which have been described to influence the activity of the IGF-1 pathway, to predict chemotherapy efficacy and toxicity in this cohort (**Chapter 6**). In **Chapter 7** the same cohort is used for translational research of the IGF-1 pathway in patients with breast cancer, where associations between IGF-1R expression and serum levels of IGF-1 and insulin, and survival are examined. The last study of this thesis evaluates whether circulating levels of IGF-1 and IGF-binding protein 3 (IGF-BP3) can predict the (event-free) survival of patients with Ewing sarcoma treated with vincristine/ifosfamide/doxorubicin/etoposide (VIDE) chemotherapy **(Chapter 8)**. Finally, the thesis is concluded with a summary, general discussion and future recommendations.

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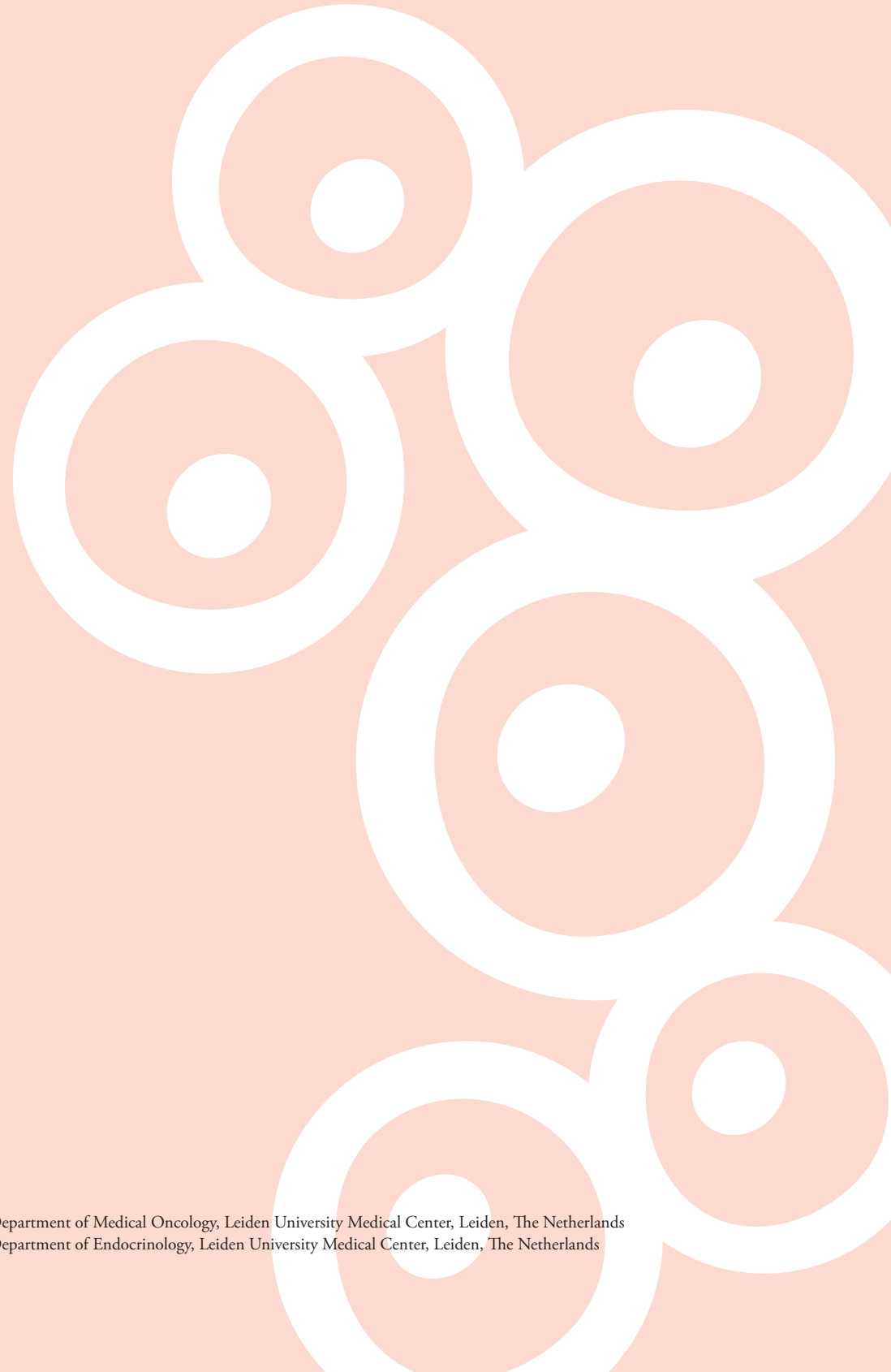
Part I

Short-term fasting and fasting mimicking diets as an adjunct to chemotherapy



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Chapter 2

Effects of short-term fasting on cancer treatment

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Abstract

Growing preclinical evidence shows that short-term fasting (STF) protects from toxicity while enhancing the efficacy of a variety of chemotherapeutic agents in the treatment of various tumour types. STF reinforces stress resistance of healthy cells, while tumor cells become even more sensitive to toxins, perhaps through shortage of nutrients to satisfy their needs in the context of high proliferation rates and/or loss of flexibility to respond to extreme circumstances. In humans, STF may be a feasible approach to enhance the efficacy and tolerability of chemotherapy. Clinical research evaluating the potential of STF is in its infancy. This review focuses on the molecular background, current knowledge and clinical trials evaluating the effects of STF in cancer treatment. Preliminary data show that STF is safe, but challenging in cancer patients receiving chemotherapy. Ongoing clinical trials need to unravel if STF can also diminish toxicity and increase efficacy of chemotherapeutic regimes in daily practice.

Background

Chronic caloric restriction reduces and delays cancer incidence, and inhibits tumor progression and metastasis in rodents[1-5]. Accordingly, cancer incidence and mortality are strongly reduced in chronic calorie restricted non-human primates[6]. Studies of long-term calorie restricted human subjects have shown a reduction of metabolic and hormonal factors associated with cancer risk[7-9]. However, chronic caloric restriction is not a feasible clinical intervention. Evident difficulties, such as the long period required to be effective, and unacceptable weight loss[10,11], hamper clinical application in cancer patients.

Preclinical studies suggest that short-term fasting (STF) protects rodents from toxic effects of chemotherapy, while simultaneously enhances the efficacy of a variety of chemotherapeutic agents in numerous distinct malignancies, e.g. breast cancer, melanoma, neuroblastoma, pancreatic cancer, and colorectal cancer[12]. In distinct strains of mice bearing xenograft malignancies, tumor growth clearly slows down in response to chemotherapy combined with a 24-60 hour fast as compared to treatment with chemotherapy alone[13-17]. STF simultaneously protects mice from chemotoxicity as well, because it reinforces stress resistance of healthy cells[16,18-24]. The distinct response of healthy *versus* tumor cells to STF is called differential stress resistance (DSR). During nutrient deprivation, healthy cells re-invest energy in maintenance and repair that contribute to resistance to chemotherapy, while tumor cells are unable to slow down growth due to mutations in tumor suppressor genes and mitogenic pathways[19,25]. Moreover, low serum levels of glucose during STF impose extra stress on tumor cells, as their energy needs under these circumstances are primarily met by means of glycolysis[14]. As a consequence of these differential responses of healthy *versus* cancer cells to STF, chemotherapy causes more DNA damage and apoptosis in tumor cells, while leaving healthy cells unharmed when it is combined with STF. Thus, STF protects healthy cells against the toxic properties of chemotherapy and renders tumor cells more sensitive, a phenomenon called differential stress sensitization (DSS).

In contrast to most cancer therapies, STF has only mild side effects, such as headaches, dizziness, nausea, weakness and short-term weight loss in humans[26]. Therefore, STF is a promising strategy to enhance the efficacy and tolerability of chemotherapy in cancer patients, especially as STF is an affordable and accessible approach and is potentially effective in a wide variety of tumors[12]. However, patients with severe weight loss, sarcopenia, cachexia or malnutrition are probably not good candidates for a STF intervention[27,28]. Recent guidelines recommend to increase protein and fat consumption in patients with cachexia[29,30]. Thus, STF may be particularly useful for relatively fit patients treated with (neo)adjuvant chemotherapy.

This narrative review will cover the current knowledge of the molecular mechanisms explaining "differential stress resistance" of healthy- and cancer cells in response to STF. Moreover, it summarizes the available clinical data reflecting the impact of STF on the effects of chemotherapy in cancer patients. Finally, ongoing clinical studies of the effects of STF in cancer treatment will be critically reviewed.

Differential stress resistance and sensitization in response to STF

In healthy cells, nutrient deprivation shuts down pathways promoting growth to re-invest energy in maintenance and repair pathways (Figure 1)[25,31,32]. This results in increased cellular protection, contributing to enhanced resistance to distinct stressors including chemotherapy and radiotherapy[19,33]. In contrast, tumor cells are unable to activate this protective response, due to: 1) uncontrolled activation of growth pathways and self-sufficiency in growth signals caused by oncogenic mutations or autocrine production of growth factors, and 2) loss of anti-proliferative signals due to mutations in tumor suppressor genes[34]. Thus, acquiring the ability to increase growth, tumor cells lose the ability to adapt to extreme environments, including nutrient deprivation. Additionally, the persistent increased growth rate of tumor cells requires abundant nutrients[35]. Therefore, STF increases DSS of tumor cells to several chemotherapeutic agents, radiotherapy and tyrosine kinase inhibitors (TKIs) (Table 1)[12-15,17-20,36-40]. Although the exact mechanism of DSR and DSS by STF is unknown, several growth factors and nutrient sensing pathways have been proposed to be key regulators, of which insulin-like growth factor-1 (IGF-1) is the most examined[41-43]. Nutrient sensing pathways are activated or inhibited in response to a low amount of available nutrients and are highly conserved among distinct organisms to overcome periods of famine[44]. During nutrient scarcity, these pathways guide cells to invest energy in repair and maintenance rather than reproduction and growth[45-47], presumably to enhance survival of periods of famine. Analogously, infection-induced anorexia is a common sign of sickness and may be an important strategy for host defence[48,49].

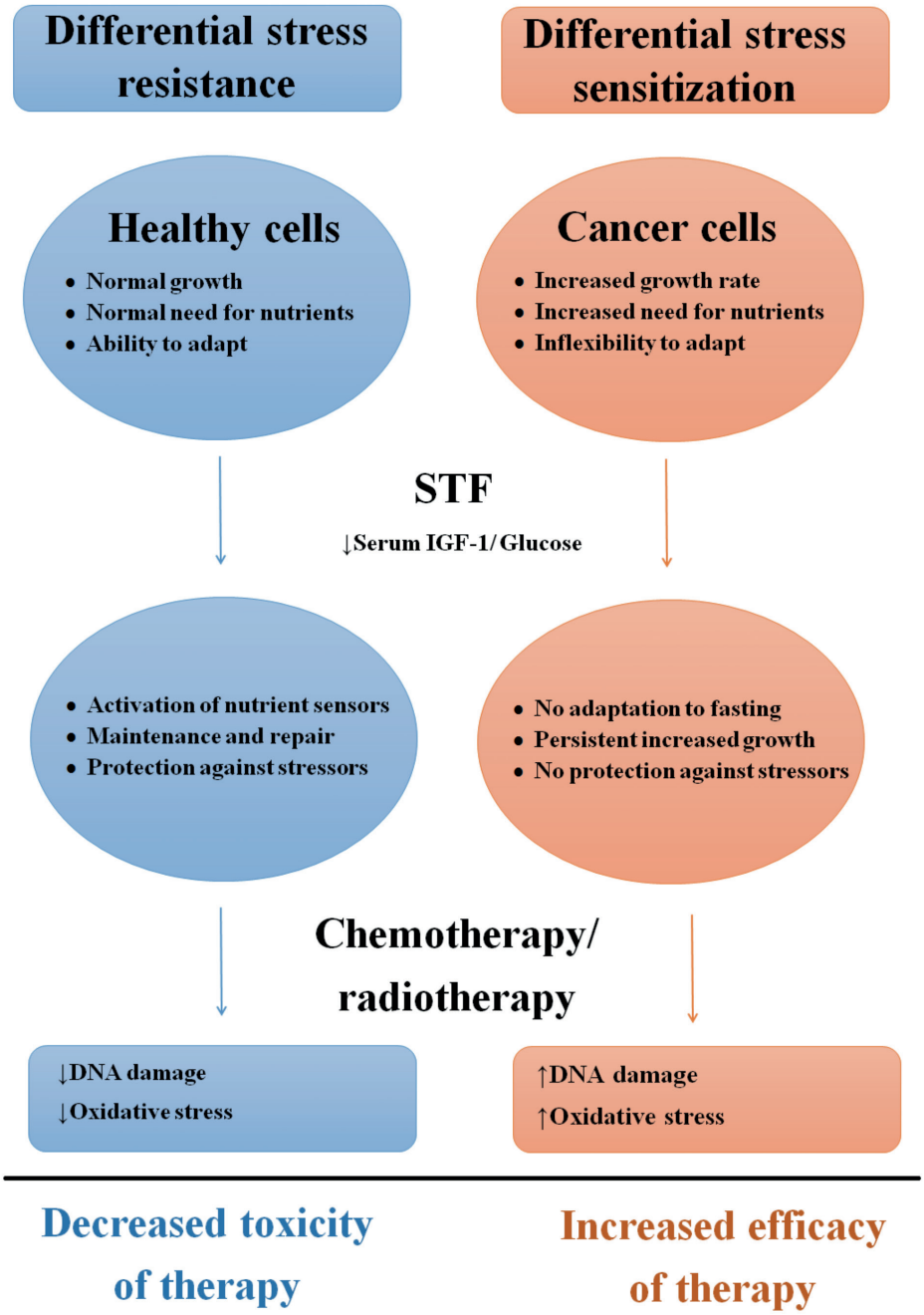

Figure 1: Schematic overview of differential effects of short-term fasting on healthy and cancer cells. This figure shows the global differential effect of STF between healthy and cancer cells. Abbreviations: STF; short term fasting, IGF-1: insulin growth factor-1.

Table 1: Overview of in vivo studies of the effect of STF on the toxicity and/or efficacy of chemotherapy, radiotherapy and tyrosine kinase inhibitors.

Author	Strain	Treatment	Outcomes of STF
Raffaghello *et al.* 2008[19]	A/J, CD-1, nude mice and A/J mice bearing subcutaneous NXS2 neuroblastoma	High dose etoposide ± 48-60 hours STF	Decreased mortality (toxicity) after high dose etoposide
Lee *et al.* 2012[12]	BALB/c, C57BL/6 and nude mice bearing subcutaneous:	±48-60 hours STF and:	
	4T1 breast cancer	Cyclophosphamide	Increased efficacy of CT, STF alone was as effective as CT alone, Increased survival
	B16 melanoma	Doxorubicin	Increased efficacy of CT, Increased survival, Decreased metastasis
	GL26 glioma	Doxorubicin	Increased efficacy of CT STF alone was as effective as CT alone
	ACN human neuroblastoma	Doxorubicin	Increased efficacy of CT
	MDAMB-231 breast cancer	Doxorubicin	Increased efficacy of CT
	OVCAR3 ovarian cancer	Doxorubicin	Increased efficacy of CT
	NXS2 neuroblastoma	Only STF	STF alone was effective, Increased survival
Safdie *et al.* 2012[13]	C57BL/6N mice bearing subcutaneous or intracranial GL26 glioma	Temozolomide ± 48 hours STF	Increased efficacy of CT, STF alone was as effective as CT alone (subcutaneous model only)
		Radiotherapy ± 48 hours STF	Increased efficacy of radiotherapy
Shi *et al.* 2012[37]	CD-1 Nude mice bearing subcutaneous ZL55 mesothelioma and A549 lung carcinoma	Cisplatin ± 48 hours STF	Increased efficacy of CT, STF alone was more effective as CT alone (mesothelioma only)
Kawaguchi *et al.* 2012[50]	GFP-LC3 mice	Doxorubicin ± 48 hours STF	Decreased cardiotoxicity after high dose doxorubicin.
Brandhorst *et al.* 2013[18]	AIN93G Mice	High dose doxorubicin ± 60 hours STF	Decreased mortality (toxicity) after high dose doxorubicin.
Saleh *et al.* 2013[38]	BALB/c mice bearing subcutaneous 67NR or NIH3 triple negative breast cancer	Radiotherapy ± 24 hours STF (alternate)	Increased efficacy of radiotherapy
Cheng *et al.* 2014[22]	C57BL/6J mice	manuscript (12178_ Proefschriftvoordrukker S.deGroot)	Decreased mortality (toxicity) after high dose cyclophosphamide.
Bianchi *et al.* 2015[14]	BALB/c mice bearing subcutaneous CT26 colon cancer	Oxaliplatin ± 48 hours STF	Increased efficacy of CT
Shim *et al.* 2015[15]	C57BL/6J mice bearing subcutaneous B16 melanoma	Doxorubicin or Cyclophosphamide ± 48 hours STF	Increased efficacy of CT STF alone was as effective as CT alone
D'aronzo *et al.* 2015[36]	Nu/Nu mice bearing subcutaneous BxPC-3-luc pancreatic cancer	Gemcitabine ± 24 hours STF	Increased efficacy of CT
Huisman *et al.* 2015[20]	FabplCre;Apc15lox/C mice bearing spontaneous intestinal malignancies	Irinotecan ± 48 hours STF	Decreased toxicity to CT No effect on efficacy of CT

Tinkum *et al.* 2015[21]	B6(Cg)-Tyrc-2J/J, Bmi1CreERT/+;Rosa26R/+ HopXCreERT/+;Rosa26R/+ Lgr5EGFP-IRES-CreERT2/+ ;Rosa26R/+, Lgr5EGFP-IRES-CreERT2/+ mice	High dose etoposide ± 24 hours STF	Decreased mortality (toxicity) after high dose etoposide
Caffa *et al.* 2015[39]	BALB/c mice	Crizotinib/regorafinib ± 48 hours STF	Increased efficacy of crizotinib/ regorafinib
Huisman *et al.* 2015[23]	BALB/c mice bearing subcutaneous C26 colon cancer	Irinotecan ± 72 hours STF	Decreased toxicity to CT No effect on efficacy of CT
Di Biase *et al.* 2016[17]	BALB/c, BALB/c-nude and C57BL/6 mice bearing subcutaneous MCF7 and 4T1 breast cancer, B16 melanoma	Doxorubicin and cyclophosphamide ± 48 hours STF or 96 hours FMD	Increased efficacy of CT
Pietrocola *et al.* 2016[40]	Wild-type C57BL/6 and athymic (nu/nu) mice	Mitoxantrone or oxaliplatin ± 48 hours STF	Increased efficacy of CT
Di Biase *et al.* 2017[16]	C57BL/6 mice	Doxorubicin ± 24-72 hours STF	Decreased cardiotoxicity after high dose doxorubicin.
Jongbloed *et al.* 2019[24]	BALB/c mice	Irinotecan ± 72 hours STF	Decreased toxicity to CT
Authors, site	**Subjects**	**Treatment**	**Outcome of STF**
Withers *et al.* 2014, UC Davis, USA[51]	20 dogs with lymphoma	Doxorubicin ± 24 hours STF	Safe and feasible Reduction in vomiting No reduction in IGF-1

STF: short-term fasting, CT: chemotherapy, FMD: fasting mimicking diet.

IGF-1 and insulin as key regulators of DSR

IGF-1 and insulin stimulate proliferation and growth and inhibit apoptosis in response to calorie and protein availability through signalling via the IGF-1 receptor (IGF-1R) and insulin receptor isoform A (IR-A), respectively[52-55]. Serum IGF-1 levels decrease during STF[56-58], because low insulin levels cause growth hormone (GH) resistance of the liver, which inhibits hepatic IGF-1 production[56,59,60]. Both insulin and IGF-1 activate the Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt pathways. In healthy cells, inhibition of proliferation and/or investment in maintenance may contribute to increased stress resistance. For example, mice with a liver *Igf1* gene deletion (LID), which have decreased IGF-1 levels similar to those during STF[61-63], exhibit increased resistance to high doses of various chemotherapeutic agents[42] and this benefit was nullified through IGF-1 administration[12,42]. Thus, the IGF-1R pathway seems to be a key mediator of stress resistance in response to STF in healthy cells.

During STF, the Ras/MAPK and PI3K/Akt pathways are down-regulated in cancer cells, whereby proliferation is inhibited[64]. Notably, resistance to the growth limiting effects of STF has been observed in cancer cells carrying mutations that cause a constitutive activation of the PI3K pathway, since these cells proliferate even in the absence of

insulin or IGF-1[65]. Therefore, the IGF-1R pathway is a key mediator of cancer cell growth and cancer resistance to commonly used therapeutics[42,66,67]. Thus, the reduction in circulating levels of IGF-1 and insulin during STF may contribute to the anticancer activity as well[68].

AMPK and autophagy

AMP-activated protein kinase (AMPK) may play a major part in DSR due to STF[69]. AMPK monitors cellular energy levels and becomes activated when ADP:ATP or AMP:ATP ratios in the cell increase[70]. AMPK inhibits energy consuming processes, such as cell proliferation and protein synthesis, and activates energy generating processes, such as glycolysis and fatty acid oxidation. It inhibits cell growth and stimulates autophagy[71]. Autophagy (Greek for "self-eating") is a highly conserved catabolic process among eukaryotes to survive periods of nutrient deprivation. This adaptive response of the cell involves damaged protein and organelle degradation to generate amino acids as an alternative energy source[72,73]. Activation of AMPK and autophagy seems to play a major part in de protective effects of STF in healthy cells[6].

However, the effects of AMPK activation in distinct tumor cells may vary, as some tumors harbour constitutively active AMPK[74,75] and others exhibit low AMPK activity[76-78]. Tumors with diminished AMPK activity or autophagy may be highly sensitive to STF, as AMPK activation enhances immune surveillance[40], whereas tumors with highly active AMPK or autophagy may be resistant[77,79-82].

Glucose metabolism and the "Warburg effect"

During STF, healthy cells, have the metabolic flexibility to cope with nutrient deprivation, since glucose can be replaced by ketone bodies and fatty acids as primary energy source.

In contrast, tumor cells depend on glucose to maintain the high rate of cellular proliferation[83,84]. Akt stimulates the so called "Warburg effect", characterized by an increased rate of glycolysis rather than oxidative phosphorylation even in the presence of oxygen[83-85]. STF down-regulates anaerobic glycolysis while up-regulating oxidative phosphorylation in tumor cells, and this "anti-Warburg effect" results in oxidative stress and apoptosis[14]. Also, a counterintuitive increase in protein translation during STF increases unmet energy needs, leading to cell death[12]. Moreover, the 20-40% reduction in circulating glucose during STF may be enough to kill anoxic tumor cells[85]. Thus, a decrease in nutrient availability during STF makes cancer cells more vulnerable to any challenge, including chemotherapy. However, overconsumption after a STF period might accelerate tumor growth, due to high glucose conditions and increased glycolysis[86].

Reactive oxygen species and DNA damage

Chemotherapeutic agents inflict oxidative stress and DNA damage upon healthy cells, which are underlying mechanisms of toxicity[44,87]. STF dampens oxidative stress in healthy cells by down-regulating metabolic rate and increasing scavenging of reactive oxygen species (ROS), which may contribute to DSR[33,44]. As serum glucose levels decrease during STF, fatty acids serve as the main energy source. Beta-oxidation of fatty acids produces ketone bodies, which can be used as an alternative/additional fuel. Ketone bodies can also activate pathways involved in protection against ROS[88]. Moreover, STF presumably activates DNA repair processes in healthy cells[22]. For example, in mice fasted for 24 hours before high dose infusion of etoposide, less DNA damage was seen in small intestinal stem cells 3 hours after the infusion compared to mice who ate *ad libitum*. As 1.5 hours post-treatment DNA damage was similar, DNA repair was likely more efficient in healthy cells due to STF[21].

In contrast, tumor cells exhibit increased ROS production if chemotherapy is combined with STF *in vitro*[12]. In breast cancer cells cultured in low glucose medium or serum of fasting mice, a 20-fold increase in DNA damage was seen in response to chemotherapy, as compared to cells cultured in regular medium or in serum of *ad libitum* fed mice[12].

Immune competence

Chemotherapy causes bone marrow toxicity and depletion of circulating immune cells, especially myeloid cell depletion[89,90]. Fasting protects hematopoietic stem cells and circulating immune cells from the detrimental effects of chemotherapy in mice[22,91]. Additionally, more efficient immunity as a result of STF presumably causes a lower rate of infections and febrile neutropenia as well[92].

On the other hand, fasting improves the therapeutic effect of chemotherapy on the tumor possibly through cellular immunity in mice, as this effect is nullified in nu/nu mice, which lack T cells[40,93]. Thus, STF may promote immunity and presentation of tumor-associated antigens (TAA), which promote efficient antitumor immunity contributing to increased efficacy of chemotherapy[94].

From animal models to the clinic

Preclinical data documenting the benefits of STF is abundant and promising. However, words of caution are appropriate regarding its application in patients with cancer. For instance, preclinical studies show severe, albeit transient, weight loss in animal models (20-40% of total bodyweight after 24-48 hours of fasting[12,19,23,39,95]. In contrast, the impact of a few days of fasting on bodyweight of humans appears far more modest (~1kg per day, largely water loss)[26], which is probably explained by metabolic differences

between humans and mice[95]. This is reassuring in the context of safety. However, it may also mean that humans need to fast for a (much) longer period of time than mice to obtain the same benefits (see discussion below). Therefore, carefully controlled clinical trials monitoring tumor growth as well as adverse effects of distinct dietary regimes are required before FMDs can be applied in clinical practice.

Metabolic risk factors for cancer

Obesity is associated with an increased risk of developing several cancers, such as breast cancer, colon cancer, ovarian cancer, endometrial cancer and thyroid cancer[96,97] and IGF-1 levels are positively associated with the risk of developing breast and prostate cancer[98,99]. Moreover, obesity and high levels of insulin and IGF-1, as well as having diabetes mellitus are associated with worse survival in cancer[100-103]. Obese subjects are often hyperglycemic and hyperinsulinemic, as a result of insulin resistance. Although circulating levels of total (free + bound) IGF-1 are normal or even low in obese subjects, levels of free (bioactive) IGF-1 are higher than in lean subjects [104]. Both insulin and free IGF-1 can bind the IGF-1R and IR-A[105] and activate the Ras/MAPK and PI3K/AKT pathway, through which cell proliferation is stimulated and apoptosis is inhibited, respectively[106]. Moreover, preclinically, obesity is associated with macrophage accumulation in adipose tissue resulting in an immune suppressive microenvironment[107]. These metabolic mechanisms may explain the increased risk of cancer as well as the worse prognosis of several cancers in obese subjects.

Clinical studies of fasting

Voluntary fasting has been performed for many centuries and purposes, such as religious, ethical and cosmetic[26,108]. Hippocrates was probably one of the first advocates of fasting for medical purposes (he recommended to fast during sickness). Since then, several doctors advised their patients to listen to their 'fasting instinct' (the natural loss of appetite during disease). Scientific research on the biomedical effects of fasting was performed from the late nineteenth century on, when several non-obese humans fasted for 20-40 days[26]. The first clinical study of medical fasting for the treatment of obesity was performed in 1915[109]. The authors reported that short periods of four to six days of fasting is a safe and effective method for reducing bodyweight in obese humans. Since that time several studies were performed in obese subjects, with the longest fasting period lasting 382 days (!)[110,111]. Fasting therapy was observed to be generally safe and well tolerated. Only mild side effects were reported, including headaches, dizziness, nausea, dyspepsia and fatigue[109-114]. However, in rare cases fasting for periods longer than 2 weeks was fatal in obese subjects with comorbidities as cardiac disease or diabetes mellitus[26,115-117], and in one rare case a 53-day fast caused Wernicke encephalopathy in

a patient with a lymphoma[118]. Additionally, fasting is not suitable for patients with rare metabolic illnesses such as glycogen storage disease or disorders of gluconeogenesis[119]. Benefits of fasting are improved cardiovascular risk factors, such as a decrease in blood pressure, improvement of lipid profile and insulin sensitivity, and weight loss in obese and non-obese subjects[114,120]. The weight loss during STF is approximately 0.9 kg per day and decreases during prolonged fasting to 0.3 kg per day by the third week[26,121]. Various studies examined the potential of fasting in the treatment of mood disorders, rheumatic diseases, asthma, chronic pain syndromes, hypertension, and metabolic syndrome[122,123]. For example, a large cohort study of more than 2000 subjects with chronic illness and pain syndromes, who used a very low-calorie diet of 350 kcal per day for 7 days, showed an increase in quality of life without any serious side effect[123]. In healthy subjects, STF by 3 cycles of a fasting mimicking diet (FMD) reduces common risk factors for cardiovascular diseases, diabetes and ageing, such as lowering blood pressure, body weight, glucose, triglycerides and cholesterol[124]. Additionally, STF may improve clinical outcome in patients undergoing a partial liver resection and may prevent acute kidney injury after cardiac surgery[125,126].

Metabolic changes during STF in humans

STF has profound metabolic effects in humans[127]. Serum glucose levels drop after a few hours and are maintained at a lower level by endogenous glucose production, stimulated by glucagon. Glycogen storage capacity is limited so that stores are virtually depleted after 24 hours. From then on, gluconeogenesis provides the brain with glucose as its major fuel source. Fatty acids are the primary fuel for the rest of the body. Beta-oxidation of fatty acids produces ketone bodies, which can serve as auxiliary energy source for the brain and the rest of the body. Insulin levels decrease rapidly and IGF-1 decreases dramatically after 36-72 hours[41]. Since the liver is resistant to GH during prolonged fasting, IGF-1 production is profoundly reduced[128]. Diminished negative feedback control through reduction of circulating insulin and IGF-1 causes plasma GH levels to increase[129,130]. IGF binding proteins, which regulate the bio-availability of IGF-1, change during fasting as well[41,131,132]. IGF-BP3 levels decrease, while IGF-BP1 levels increase 5-10-fold[133]. The decrease of IGF-I, downregulates the Ras/MAPK and PI3K/Akt pathways, through which cell proliferation is stimulated and apoptosis inhibited[12,19]. Moreover, fasting down-regulates the hypothalamus-pituitary-thyroid axis activity. It particularly lowers triiodothyronine (T3), while thyroid stimulating hormone (TSH) and free thyroxine (fT4) are slightly decreased or not affected[134]. Clinical research shows that fasting periods longer than 48 hours are required to facilitate a robust decrease in IGF-1 levels[41]. Therefore, it is likely that the positive effects of STF will be enhanced if the period of fasting is prolonged. A low sugar, low protein FMD may be an alternative to ease the burden of fasting, as it mimics the effects of STF on metabolism[91].

Clinical studies of STF during chemotherapy

To date, a few small clinical studies in humans exploring the effects of STF combined with chemotherapy have been published (Table 2)[22,131,135-138]. The design and results of these studies in humans are summarized below.

Table 2: Overview of clinical studies on the effect of STF on the toxicity of chemotherapy.

Authors, site	Human Subjects	Treatment	Outcome
Safdie *et al.* 2009, USC, USA[136]	10 human subjects with distinct malignancies	Distinct, + STF varying from 48-140 hours prior and 5-56 hours after CT	Safe and feasible. Reduction in CT-induced side effects.
Badar *et al.* 2014, KFMC, Saudi Arabia, NCT00757094[135]	11 human subjects with distinct malignancies	IF during Ramadan when receiving CT	Safe and feasible. Reduction in CT-induced side effects*.
Dorff et al. 2016, USC, USA, NCT00936364,[22,137]	20 human subjects with distinct malignancies	Platinum based CT + 24hours, 48 hours or 72 hours STF	Safe and feasible . Reduces DNA damage in leukocytes (dose response). Reduction of IGF-1 (dose response).
de Groot *et al.* 2015, LUMC, The Netherlands NCT01304251[131]	13 women with stage II and III HER2 negative breast cancer	TAC CT ± 48 hours STF	Safe and feasible. Reduction in IGF-1 Beneficial effect on erythrocytes and thrombocytes Possible reduction in DNA damage in healthy cells
Bauersfeld *et al.* 2018, Charite University, Germany, NCT01954836[138]	34 women with breast and ovarian cancer	CT ± 60 hours STF (cross-over)	Safe and feasible Beneficial effect on QOL, fatigue and well-being

USC: University of Southern California, KFMC: King Fahad Medical City, LUMC, Leiden University Medical Center, UC Davis, University of California, Davis School of Veterinary Medicine, STF: Short-term fasting, IF: intermittent fasting, CT: chemotherapy, * no statistics performed.

In a case series from the University of Southern California (USC), 10 patients with distinct malignancies fasted in combination with docetaxel, carboplatin, paclitaxel and/or gemcitabine[136,139]. Seven female and three male patients, with a median age of 61 years, diagnosed with breast (*N=4*), prostate (*N=2*), esophagus, non-small cell lung cancer, uterus and ovary cancer were described. Patients fasted for 48-140 hours prior to, and 5-56 after commencing chemotherapy. Six of the ten patients fasted alternately during the chemotherapy cycles (the other four fasted every cycle) and side effects were compared between cycles combined with STF and chemotherapy alone. Side effects were scored according to the Common Terminology Criteria for Adverse Events (CTCAE) 4.0. Besides hunger and dizziness, fasting had no significant side effects. The authors reported a decrease in chemotherapy-induced side effects, including fatigue, weakness, vomiting and diarrhea, when chemotherapy was combined with STF compared to chemotherapy alone. In five patients the tumor volume (evaluated with PET or

PET-CT) or tumor markers (PSA or CA-125) were evaluated. STF did not diminish chemotherapy-induced reduction of tumor volume and tumor markers, suggesting that STF did not interfere with the efficacy of chemotherapy.

In the King Fahad Medical City a clinical trial (NCT00757094) was conducted to evaluate the safety and feasibility of combining chemotherapy and intermittent fasting (including liquids) during the Ramadan[135]. Eleven patients, with distinct types of malignancies, received one gift of chemotherapy. Side effects and blood counts were compared with values measured in response to a similar dose of chemotherapy, given 2 weeks after the end of Ramadan. The authors concluded that combining fasting and chemotherapy during the month of Ramadan was well tolerated and safe. Side effects of chemotherapy tended to be less. However, because the study group was small, no statistics were performed. Moreover, due to the short fasting period (approximately 12 hours), major benefits may not be expected, as IGF-1 levels will evidently not be reduced[140].

We performed a randomized pilot study (NCT01304251) to evaluate the effects of short-term fasting on tolerance to (neo) adjuvant chemotherapy in HER2-negative breast cancer patients in the Leiden University Medical Center (LUMC)[131]. Eligible patients had stage II/III breast cancer and received (neo)-adjuvant TAC (docetaxel/doxorubicin/cyclophosphamide) chemotherapy. Patients were randomized to fast 24 hours before and 24 hours after chemotherapy, or to eat according to the guidelines for healthy nutrition. Metabolic parameters (glucose, insulin and IGF-1) at baseline and immediately before chemotherapy infusion –when patients in the STF group had fasted for 24 hours were compared. Toxicity in the two groups was compared as well. Additionally, chemotherapy-induced DNA damage was quantified in peripheral blood mononuclear cells (PBMCs) by the level of γ-H2AX, as determined by flowcytometry. Thirteen patients were included, of whom seven were randomized to the STF arm. STF was well tolerated in our study. Plasma glucose levels increased and insulin levels remained constant in response to STF. We inferred that this phenomenon was the result of the concomitant use of dexamethasone, which was administered as an anti-emetic, for reduction of fluid retention and dampening of hypersensitivity reactions in response to docetaxel. Circulating IGF-1 levels were only modestly reduced in the study, which could be due to the use of dexamethasone as well[141,142] or to the relatively short duration (24 hours) of fasting prior to chemotherapy. Non-hematological toxicity did not differ between the groups. However, mean erythrocyte- and thrombocyte counts 7 days post-chemotherapy were significantly higher in the STF group compared to the non-STF group. Levels of γ-H2AX were significantly increased 30 minutes post-chemotherapy in CD45 + CD3- cells in non-STF, but not in STF patients[131]. This study provides evidence that STF attenuates bone marrow toxicity in these patients and reduces chemotherapy-

induced DNA damage in PBMCs and/or accelerates its recovery.

Moreover, Dorff et al. reported results from a dose-escalating phase I study (NCT00936364), wherein 20 human subjects with distinct malignancies were treated with platinum-based chemotherapy combined with 24, 48 or 72 hours STF to identify the optimal fasting duration[22,137]. Eligible patients had distinct cancer types for which platinum-based combination chemotherapy was given with curative or palliative intent. Metabolic parameters (glucose, insulin, IGF-1 and IGF-BP1) at baseline and immediately before chemotherapy were compared. Moreover, toxicities and chemotherapy-induced DNA damage in PBMCs (determined by the COMET assay) between the three groups were compared. Twenty patients were included, 6 in the 24 hours group and 7 in the 48 and 72 hours group. The fasting was feasible and fasting-related toxicities were limited to grade 1 according CTCAE 4.0. The authors reported that 72 hours of STF was associated with normal lymphocyte counts and maintenance of a normal lineage balance in white blood counts (lymphoid/myeloid ratio) after 2 cycles of chemotherapy, while 24 hours STF was not[22]. IGF-1 levels decreased by 30, 33 and 8% in the 24, 48 and 72 hours fasting cohorts, respectively, after the first fasting period. Additionally, the COMET assay showed reduced DNA damage 24 hours after chemotherapy in leukocytes from subjects who fasted for more than 48 hours compared with subjects fasted for 24 hours *(P=0.08).*

Finally, Bauerfeld et al. published a randomized cross-over trial (NCT01954836) evaluating the effect of STF on quality of life in breast cancer and ovarian cancer patients treated with chemotherapy[138]. Patients were randomized to fast, using an FMD, 36 hours before and 24 hours after chemotherapy or to eat a normocaloric Mediterranean diet for the first three cycles of chemotherapy. After three cycles the patient crossed over to the other group of nutrition (Mediterranean diet or fasting). The design of the study allows intra-individual comparisons regarding side effects of treatment, but precludes conclusions as efficacy of chemotherapy. In total, 50 patients were included in the study, but only 34 were analyzed because of early study discontinuation. The fasting was safe and feasible and five patients (14.7%) continued fasting after three cycles and did not cross over to the normocaloric diet. The authors concluded that STF led to a better tolerance to chemotherapy with less compromised quality of life (QOL) and reduced fatigue within the 8 days after chemotherapy. Moreover, 31 patients declared that they would fast again during chemotherapy, while only 3 patients declared that they would not fast again during chemotherapy.

These first clinical studies lack enough power to draw definite conclusions. However, the first results suggest that STF is safe, while it reduces toxicity of chemotherapy. Large scale randomized studies are required to get more insight in the benefits of STF in cancer treatment in humans.

Ongoing studies

The first clinical studies have shown that STF combined with chemotherapy is safe and feasible in small patient groups[131,136,138]. Moreover, STF may reduce chemotherapy-induced toxicity. Additionally, chemotherapy-induced DNA damage in healthy cells may be decreased due to STF. However, large randomized clinical studies are required to generate (more) insight and validate the possible benefits of STF during chemotherapy. In table 3, an overview is shown of the ongoing trials with STF combined with cancer treatment.

Table 3: Overview of ongoing or unpublished clinical trials of STF combined with chemotherapy or radiotherapy.

Ongoing studies of STF during chemotherapy					
Trial, site	***N***	**Start**	**Tumor type and treatment**	**STF**	**Primary endpoint**
Non-randomized trial, NCT01175837, Mayo clinic, USA	12	2010	Distinct malignancies treated with CT	+24-48 hours prior to chemotherapy (distinct regimens)	Safety and feasibility
Phase II randomized trial, NCT01802346, USC, USA	120	2013	Breast cancer treated with AC and prostate cancer treated with docetaxel	±96 hours (using FMD) during CT	Toxicity of CT
Phase II/III Randomized study, NCT02126449, LUMC, the Netherlands	250	2014	Stage II and III Her2 negative breast cancer treated with AC-T or FEC-T.	±96 hours (using FMD), during AC-T or FEC-T, no corticosteroids in control arm during AC or FEC	Phase II: toxicity of CT Phase III: pCR
Phase Ib non- randomized trial, NCT02379585, Western Regional Medical Center, USA	10	2015	Breast cancer	CT ± 48 hours STF	pCR
Randomized trial, NCT02710721 Charite University, Berlin, Germany	60	2016	Advanced metastatic prostate cancer	±60 hours (using FMD) during CT vs. Mediterranean diet	QOL
Randomized crossover study, NTR5731, Erasmus medical center	18	2016	Metastatic colorectal cancer or other solid tumors receiving irinotecan	Dietary restriction including STF	25% reduction of the active irinotecan metabolite, SN38, in healthy liver tissue
Randomized trial, NCT03162289 Charite University, Berlin, Germany	150	2017	Ovarian or breast cancer	±60 hours (using FMD) during CT	QOL
Non-randomized trial, NCT03340935, University of Milan, Italy	85	2017	Distinct	5 days (using FMD), 700kcal a day during cancer treatment	Toxicity of CT
Non-randomized trial, NCT03595540, Genova, Italy	60	2017	Distinct	5 days (using FMD), 700kcal a day during cancer treatment	Feasibility

Table 3: Continued

Trial, site	*N*	Start	Tumor type and treatment	STF	Primary endpoint
Randomized trial, NCT03709147, Milan, Italy	88	2018	Lung adenocarcinoma	± 5 days (using FMD) during CT in combination with metformin	PFS
Randomized trial. NCT03700437, Indiana University, USA	40	2018	Non-small cell lung cancer	± 96 hours (using FMD) during carboplatin, pemetrexed and pembrolizumab	DNA damage in and count of circulating tumor cells
Studies of STF during radiotherapy					
Randomized trial, NCT01754350, Johann Wolfgang Goethe University Hospitals, Germany	50	2013	Glioblastoma Multiforme	±72 hours during reirradiation	PFS

USC: University of Southern California, CT: chemotherapy, LUMC: Leiden University Medical Center, FMD: fasting mimicking diet, pCR: pathological complete response, QOL: quality of life, PFS: progression-free survival.

One study to date investigates the effects of STF on the effects of radiotherapy. This randomized study (NCT01754350) conducted in Johann Wolfgang Goethe University Hospitals, includes patients with recurrent glioblastoma or gliosarcoma. The intervention entails 3 days of STF and 6 days of ketogenic diet during re-irradiation. The primary endpoint of the study is progression free survival.

A phase II study (NCT01802346), ongoing in the University of Southern California, examines the effects of an FMD on toxicity of chemotherapy in patients with breast and prostate cancer.

The phase II/III study (NCT02126449) from the LUMC, investigates the effects of STF using an FMD on toxicity (phase II part) and efficacy (phase III part) of neo-adjuvant AC-T or FEC-T chemotherapy. In this study prophylactic dexamethasone is omitted in the FMD arm during the AC and FEC chemotherapy cycles to reduce its potentially counteractive metabolic effects. Final results of the study are awaited (68). The same FMD will be used to investigate the effect on circulating tumor cells in non-small cell lung cancer during treatment with carboplatin, pemetrexed and pembrolizumab.

Another FMD, described by Bauerfeld (138), is tested in two studies (NCT02710721, NCT03162289) conducted in the Charité University in Berlin, one in advanced metastatic prostate cancer and another in ovarian or breast cancer. Primary endpoint of both studies is QOL.

Finally, three studies (NCT03340935, NCT03595540 and NCT03709147) investigate the feasibility and effect of a 5-day FMD (approximately 700 kcal a day) on chemotherapy in distinct tumors and distinct chemotherapy regimens.

Discussion and clinical implications

Clinical research evaluating the potential of STF is still in its infancy and more research is needed as the exact mechanism and effects are not established yet. Remaining questions are: is STF clinically effective in patients with solid tumors, in which tumors is STF effective, which markers are useful for prediction and monitoring of efficacy, what is the optimal length and timing of STF and refeeding, is STF safe in all patients, what is the optimal composition of an FMD, how can we increase patient's compliance?

STF may be an affordable and safe intervention - at least in patients without severe weight loss or malnutrition -, which potentially dampens the side effects of chemotherapy, radiotherapy and TKI's, while reinforcing their efficacy. Furthermore, it is potentially effective in a wide variety of tumors, although there is evidence that tumors with PI3K mutations or highly active AMPK are not sensitive (65, 82). Reduction of side effects would improve quality of life and potentially reduce costs of hospitalization and the use of drugs such as anti-emetics or antibiotics. Moreover, STF may broaden the therapeutic window of cancer treatments, allowing for an increase of the dosage of (chemo) therapeutic agents, thereby enhancing their efficacy. However, STF might be only feasible in chemotherapeutic regimens characterized by: 1) bolus infusions on one day, to keep the fasting period short, 2) a long interval between two cycles, to ensure sufficient recovery time between cycles and 3) low dose or no use of corticosteroids, to avoid hyperglycemia, which might interfere with the benefits of STF (131).

Patients at risk for malnutrition or cachexia may not be candidates for STF, as it may be unsafe to further limit nutrient intake in these patients for even a short time (27). However, notably, in preclinical setting caloric restriction showed even preservation of muscle strength in cancer cachexia (143). Therefore, robust clinical trials are needed to establish the safety and efficacy of FMD in patients at high risk of cachexia.

Close monitoring of patients by nutritionists with expertise in fasting may be needed to increase compliance in future studies and to prevent patients unacceptable weight loss. Moreover, in our opinion, STF or FMDs should only be applied in the context of clinical research in patients with cancer until there is robust evidence for their safety and benefits.

Conclusion

Abundant and convincing preclinical evidence shows that STF can decrease toxicity and simultaneously increase efficacy of a wide variety of chemotherapeutic agents. Preclinical data suggesting that STF can enhance the effects of radiotherapy and TKIs are promising as well. In clinical studies, STF emerges as a promising strategy to enhance the efficacy and tolerability of chemotherapy. It appears safe as an adjunct to chemotherapy in humans, and it may reduce side effects and DNA damage in healthy cells in response to chemotherapy. However, more research is needed to firmly establish clinical efficacy and safety.

List of abbreviations

AMPK:	AMP-activated protein kinase
CTCAE:	Common Terminology Criteria for Adverse Events
DSR:	differential stress resistance
DSS:	differential stress sensitization
FMD:	fasting mimicking diet
fT4:	free thyroxine
GH:	growth hormone
IGF-1:	insulin-like growth factor-1
IGF-1R:	IGF-1 receptor
IR-A:	Insulin receptor isoform A
LID:	liver Igf1 gene deletion
LUMC:	Leiden University Medical Center
MAPK:	mitogen-activated protein kinase
PBMCs:	peripheral blood mononuclear cells
PI3K:	phosphatidylinositol-3-kinase
ROS:	reactive oxygen species
STF:	short-term fasting
T3:	lowers triiodothyronine
TKIs:	tyrosine kinase inhibitors
TSH:	thyroid stimulating hormone
USC:	University of Southern California

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Chapter 3

The effects of short-term fasting on tolerance to (neo) adjuvant chemotherapy in HER2-negative breast cancer patients: a randomized pilot study

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Abstract

Background: Preclinical evidence shows that short-term fasting (STF) protects healthy cells against side effects of chemotherapy and makes cancer cells more vulnerable to it. This pilot study examines the feasibility of STF and its effects on tolerance of chemotherapy in a homogeneous patient group with early breast cancer (BC).

Methods: Eligible patients had HER2-negative, stage II/III BC. Women receiving (neo)-adjuvant TAC (docetaxel/doxorubicin/cyclophosphamide) were randomized to fast 24 hours before and after commencing chemotherapy, or to eat according to the guidelines for healthy nutrition. Toxicity in the two groups was compared. Chemotherapy-induced DNA damage in peripheral blood mononuclear cells (PBMCs) was quantified by the level of γ-H2AX analyzed by flow cytometry.

Results: Thirteen patients were included of whom seven were randomized to the STF arm. STF was well tolerated. Mean erythrocyte- and thrombocyte counts 7 days post-chemotherapy were significantly higher (*P=0.007,* 95% CI 0.106-0.638 and *P=0.00007,* 95% CI 38.7-104, respectively) in the STF group compared to the non-STF group. Non-hematological toxicity did not differ between the groups. Levels of γ-H2AX were significantly increased 30 minutes post-chemotherapy in CD45+CD3- cells in non-STF, but not in STF patients.

Conclusions: STF during chemotherapy was well tolerated and reduced hematological toxicity of TAC in HER2-negative BC patients. Moreover, STF may reduce a transient increase in, and/or induce a faster recovery of DNA damage in PBMCs after chemotherapy. Larger studies, investigating a longer fasting period, are required to generate more insight into the possible benefits of STF during chemotherapy.

Background

Chronic reduction of calorie intake without malnutrition reduces spontaneous cancer incidence and delays progression in a variety of tumors in rodents[1-4]. In long-term calorie restricted non-human primates, cancer incidence and mortality are reduced[5], and studies of long-term calorie restricted human subjects have shown a reduction of metabolic and hormonal factors associated with cancer risk[6-8]. Chronic calorie restriction is not practical for clinical use since it causes unacceptable weight loss in cancer patients[9]. However, brief periods of fasting may be feasible in patients and, in mice have been shown to slow cancer growth at least as effectively as chronic calorie restriction without compromising bodyweight[10-12]. Even more importantly, the effects of short-term fasting (STF) on susceptibility to chemotherapy differ between healthy somatic and cancer cells, a phenomenon called differential stress resistance (DSR)[10,11,13,14]. In healthy cells, nutrient deprivation shuts down pathways promoting growth to invest energy in maintenance and repair pathways that contribute to resistance to chemotherapy[15,16]. In contrast, tumor cells are unable to activate this protective response due to uncontrolled activation of growth pathways by oncogenic mutations. Indeed, the persistently increased growth rate of tumor cells requires abundant nutrients, and therefore, STF renders tumor cells more sensitive to chemotherapy[10-12]. Hence, STF is a promising strategy to enhance the efficacy and tolerability of chemotherapy.

In human subjects, STF is safe and well tolerated[17-19]. A case series of 10 patients with various types of cancer demonstrated that fasting in combination with chemotherapy is feasible and might reduce chemotherapy-induced side effects[20]. We conducted a randomized-controlled pilot trial to identify the effects of 48-hours of STF on chemotherapy-induced side effects and hematologic parameters in breast cancer (BC) patients, who received TAC (docetaxel, doxorubicin and cyclophosphamide) chemotherapy. Furthermore, we quantified chemotherapy-induced DNA damage in peripheral blood mononucleated cells (PBMCs) by measuring γ-H2AX accumulation[21]. Upon induction of DNA double strand breaks (DSBs), H2AX is rapidly phosphorylated at the site of DNA damage[22]. γ-H2AX has been widely used to quantify DNA damage after irradiation[23-26], where the expression has been shown to be associated with healthy tissue damage[22,27-30]. However, use of γ-H2AX as a marker for chemotherapy toxicity to healthy cells is relatively unexplored.

Methods

Patients

All women included in the study had a histologically confirmed diagnosis of HER2-negative stage II and III BC and were receiving (neo) adjuvant TAC-chemotherapy (see below). Eligibility criteria included age ≥ 18 years; BMI ≥19kg/m^2; WHO performance status 0-2; life expectancy of >3 months; adequate bone marrow function (i.e. white blood counts >3.0 x 10^9/L, absolute neutrophil count ≥1.5 x 10^9/l and platelet count ≥ 100 x 10^9/l); adequate liver function (i.e. bilirubin ≤1.5 x upper limit of normal (UNL) range, ALAT and/or ASAT ≤2.5 x UNL, Alkaline Phosphatase ≤5 x UNL); adequate renal function (i.e. calculated creatinine clearance ≥50 mL/min); adequate cardiac function; absence of diabetes mellitus; absence of pregnancy or current lactation; and written informed consent. TNM classification system was used to record stage of disease in accordance with Dutch guidelines of clinical practice (http://www.oncoline.nl).

Study design

Patients were randomized in a 1:1 ratio to fast beginning 24 hours before and lasting until 24 hours after start of chemotherapy ('STF' group) or to eat according to the guidelines for healthy nutrition with a minimum of two pieces of fruit per day ('non-STF' group). STF subjects were only allowed to drink water and coffee or tea without sugar. All patients kept a food diary of the consumption of food and drinks during the 24 hours pre- and post-chemotherapy. All patients gave informed consent in writing. The study (NCT01304251) was conducted in accordance with the Declaration of Helsinki (October 2008) and was approved by the Ethics Committee of the LUMC in agreement with the Dutch law for medical research involving human subjects.

Drugs

On the first day of each 3-weekly cycle (six in total), women received TAC (docetaxel 75mg/m^2 IV for 1 hour, adriamycin 50mg/m^2 IV for 15 minutes and cyclophosphamide 500mg/m^2 IV for 1 hour) with granulocyte-colony stimulating factor (G-CSF; pegfilgrastim 6mg) support the day after chemotherapy administration. Patients received prophylactic dexamethasone (8mg, BID the day before, the day of and the day after chemotherapy administration) in order to prevent fluid retention and hypersensitivity reactions. The anti-emetic agent granisetron (serotonin 5-HT$_3$ receptor antagonist; 1mg) was administered prior to chemotherapy infusion.

Blood sampling

Venous blood samples were drawn before randomization, at a maximum of 2 weeks prior to treatment (baseline) and directly before each chemotherapy administration (pre-chemotherapy, day 0). Non-fasting blood samples were drawn from subjects in

the non-STF group. The effect of fasting was determined by recording 1) metabolic parameters (insulin, glucose, insulin growth factor 1 (IGF-1), insulin growth factor binding protein 3 (IGFBP3)); 2) endocrine parameters (thyroid-stimulating hormone (TSH), triiodothyronine (T3) and free thyroxine (FT4)); 3) hematologic parameters (erythrocyte-, thrombocytes- and leukocyte count) and 4) inflammatory response (C-Reactive Protein (CRP)). For measurement of metabolic, endocrine and inflammatory parameters , blood was collected in a serum-separating tube and for hematologic parameters, blood was collected in an EDTA tube. In addition, hematologic parameters and CRP were measured on day 7 after each chemotherapy cycle. All samples were analyzed by the accredited clinical laboratory of the LUMC.

To investigate the effect of STF on chemotherapy-induced DNA damage in PBMCs, heparinized venous blood samples (9mL) were collected for both patient groups during each cycle just prior to chemotherapy, for some patients at 30 minutes after completion of chemotherapy, and on day 7 after administration. Samples were stored at room temperature until processing (in most cases directly after withdrawal or at least within 24 hours).

Toxicity

During each cycle, patients were instructed to report the experienced side effects, graded as mild, moderate or severe. Self-reported side effects, side effects documented by the physician and hematological toxicity were graded according to the Common Terminology Criteria for Adverse Events version 4.03 (CTCAE v.4.03)[31].

Isolation of PBMCs and γ-H2AX staining

PBMCs were isolated using Ficoll Paque Plus (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Isolated PBMCs were carefully resuspended in 1 ml of Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 40% fetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria) and 10% dimethyl sulfoxide (DMSO) and divided over two cryovials. Samples were directly transferred to an isopropanol chamber and incubated at -80°C for a minimum of 24 hours to cryopreserve before they were stored in the vapor phase of liquid nitrogen.

Samples were processed batch wise, so that samples from distinct time points within each cycle were processed simultaneously for each patient. After thawing in RPMI at room temperature, PBMCs were fixed in 1.5% formaldehyde and permealized in ice-cold methanol. Cells were washed 3 times in staining buffer (PBS with 5% bovine serum albumin (BSA, Sigma)) and stained for 30 minutes on ice with anti-CD45-PerCP-Cy5.5 (1:20, BD, clone 2D1), anti-CD3-PE (1:10, BD, clone SK7), anti-CD14-AF700 (1:80, BD, clone M5E2), anti-CD15-PE CF594 (1:100, BD, clone W6D3)

and anti-γ-H2AX-AF488 (1:100, Biolegend, clone 2F3), followed by another washing step. The cell acquisition was performed immediately after the staining procedure (BD LSR Fortessa Flow Cytometer analyzer, BD Bioscience, Breda, The Netherlands) and data was analyzed using BD FACS Diva Software version 6.2. Compensations were set using a mixture of anti-mouse Ig/negative control beads (BD). The CD45+ cells were gated, after which the CD3+ T lymphocytes, CD3- myeloid cells (also harboring B lymphocytes) or CD14+CD15- monocytes were analyzed for the geomean (as measure for the intensity) of γ-H2AX.

Statistical analysis

All parameters were tested for normality using a Kolmogorov-Smirnov test, with Bonferroni adjustment when evaluated in subgroups. Normality distributed parameters, if necessary after log transformation, were summarized as mean (and standard error (SE)) and compared using an independent samples *t*-test for independent groups or paired *t*-test for paired groups. The non-normally distributed parameters were summarized as median (and range) and compared using a Mann-Whitney test for independent groups or Wilcoxon signed rank test for paired groups. Data of different patients and different cycles were combined to test differences between time points and treatment groups. All tests were 2-tailed with a significance level of 0.05. All data were analyzed using IBM SPSS Statistics for Windows (Version 20.0. Armonk, NY: IBM Corp).

Results

Patient characteristics

From May 2011 until December 2012, thirteen women with early BC were included and randomized into the STF (n=7) or non-STF group (n=6). Patient characteristics are summarized in Table 1. In the STF arm, 42.9% of the patients had stage III disease compared to 16.7% of patients in the non-STF arm. Estrogen receptor status was negative for one patient in the STF group (14.3%) and half of the patients in the non-STF group. Three patients had a Bloom-Richardson grade III tumor in the STF group and one in the non-STF group. One patient could not be graded due to the neoadjuvant chemotherapy. None of these patient characteristics was significantly different between the two groups.

Patients were motivated to fast and the STF was well tolerated. Two patients in the STF arm withdrew from fasting after the third chemotherapy cycle: one due to pyrosis and one due to recurrent febrile neutropenia. In both patients, the side effects persisted on a normal diet during cycles 4-6. All patients finished 6 cycles of TAC. There were no significant differences in chemotherapy-related adjustments between the two groups.

Table 1: Patient characteristics.

	STF (n = 7)	Non-STF (n = 6)	*P* Value
Median Age (range), Years	51 (47-64)	52 (44-69)	1.00
Median Body Mass Index (SEM), kg/m²	25.5 (3.3)	23.8 (2.4)	0.53
WHO-status			
Grade 0	6 (85.7%)	6 (100%)	0.34
Grade 1	1 (14.3%)	0 (0.0%)	
Treatment			
Adjuvant	5 (71.4%)	3 (50.0%)	0.43
Neo-adjuvant	2 (28.6%)	3 (50.0%)	
T-classification			
T1	3 (42.9%)	2 (33.3%)	0.94
T2	3 (42.9%)	3 (50.0%)	
T3	1 (14.3%)	1 (16.7%)	
N-classification			
N0	2 (28.6%)	2 (33.3%)	0.85
N+	5 (71.4%)	4 (66.7%)	
Stage			
II	4 (57.2%)	5 (83.3%)	0.31
III	3 (42.9%)	1 (16.7%)	
ER-status			
ER-	1 (14.3%)	3 (50.0%)	0.16
ER+	6 (85.7%)	3 (50.0%)	
PR-status			
PR-	3 (42.9%)	4 (66.7%)	0.39
PR+	4 (57.1%)	2 (33.3%)	
Grade (BR)			
1	1 (14.3%)	1(16.7%)	0.44
2	2 (28.6%)	4(66.7%)	
3	3 (42.9%)	1(16.7%)	
Unknown	1 (14.3%)	0 (0.0%)	
Chemotherapy related adjustment			
No	3 (42.9%)	3 (50.0%)	0.80
Yes	4 (57.1%)	3 (50.0%)	

STF; short-term fasting, SEM; standard error of the mean, ER; estrogen receptor; PR; progesterone receptor, BR; Bloom-Richardson.

Toxicity

The most frequently observed side effects, were grade I/II and the percentage of occurrence of each side effect is recorded in Table 2. No significant differences were observed between the two patient groups. The total incidence of grade III/IV side effects that occurred in both groups is given in Table 2. The observed grade III/IV side effects were neutropenic fever, fatigue and infection (pneumonia and neutropenic enterocolitis (typhlitis)). There was no significant difference in incidence of grade III/IV side effects between the STF and non-STF group. No grade V toxicity occurred during the chemotherapy in either group.

Table 2: Grade I/II and grade III/IV toxicity during 6 cycles of TAC in both groups.

	Grade I/II	
	STF	**Non-STF**
Fatigue	5 (71%)	6 (100%)
Infection	3 (43%)	1 (17%)
Mucositis	4 (57%)	4 (67%)
Neuropathy	5 (71%)	3 (50%)
Diarrhea	5 (71%)	2 (33%)
Dizziness	3 (43%)	3 (50%)
Nausea	7 (100%)	4 (67%)
Eye complaints	4 (57%)	2 (33%)
Constipation	4 (57%)	2 (33%)
	Grade III/IV	
Total	6	3
Neutropenic fever	2 (29%)	2 (33%)
Fatigue	2 (29%)	0 (0%)
Infection	2 (29%)	1 (17%)

All side effects were scored according CTCAE4.03. Each side effect was scored maximal once per patient during the course (the highest grade of occurrence was scored).
STF; short-term fasting.

Metabolic, endocrine and inflammatory parameters

Metabolic and endocrine parameters at randomization (maximum 2 weeks before first chemotherapy cycle) and the mean or median (depending on distribution) of the day 0 values (immediately before chemotherapy infusion, when patients in the STF group had fasted for 24 hours) were compared (Table 3). As no baseline values were available for three patients, no paired *t*-test could be performed, hence the deviating N values. In the STF and non-STF groups, median blood glucose values were significantly increased between the two time points ($P=0.042$ and $P=0.043$, respectively). There was no significant difference in median insulin level between the two time points in the STF group, but in the non-STF group, the insulin level was significantly increased ($P=0.043$). Mean IGF-1 levels were significantly decreased ($P=0.012$) in the STF group; no change was observed in the non-STF group. IGF-BP3 levels did not change in either group. TSH was significantly reduced ($P=0.034$) in the non-STF group, but not in the STF group. The FT4 did not change significantly over time in patients in either group.

Table 3: Metabolic and endocrine parameters at baseline (before randomization) and day 0 (immediately before chemotherapy infusion during the use of prophylactic dexamethasone).

Parameter	N	Baseline Median (range)	Day 0 (with DEX) Median (range)	In/decrease	*P* value
Glucose (3.1-6.4mmol/L)	STF (n = 5)	5.2 (4.3-5.5)	6.8 (5.6-9.0)	↑	**0.042**
	Non-STF (n = 5)	4.8 (4.7-6.7)	7.0 (6.1-8.8)	↑	**0.043**
Insulin (0-20mU/L)	STF (n = 5)	14.0 (2.0-40.0)	13.0 (6.0-36.0)	=	0.500
	Non-STF (n = 5)	2.0 (2.0-9.0)	16.0 (9.0-63.0)	↑	**0.043**
Parameter	**N**	**Baseline Mean (SE)**	**Day 0 (with DEX) Mean (SE)**	**In/decrease**	***P* value**
IGF-1 (5.4-24.3nmol/L)	STF (n = 4)	23.7 (2.9)	19.6 (3.3)	↓	**0.012**
	Non-STF (n = 5)	17.5 (3.5)	16.8 (2.8)	=	0.634
IGF-BP3 (2.2-5.8mg/L)	STF (n = 4)	5.0 (0.5)	4.2 (0.3)	=	0.212
	Non-STF (n = 5)	4.5 (0.2)	3.9 (0.3)	=	0.122
TSH (0.3-4.8mU/L	STF (n = 3)	1.38 (0.26)	0.61 (0.08)	=	0.065
	Non-STF(n = 5)	1.49 (0.14)	0.42 (0.06)	↓	**0.034**
FT4 (12-22pmol/L)	STF (n = 3)	15.4 (0.92)	13.9 (0.94)	=	0.117
	Non-STF (n = 5)	15.0 (0.54)	14.0 (0.34)	=	0.149

Bold value indicates that p<0.05. Abbreviations: DEX; dexamethasone, IGF-1; Insulin-like growth factor 1, IGF-BP3; insulin- like growth factor binding protein 3, TSH; thyroid-stimulating hormone; FT4;,free thyroxine; STF; short-term fasting, SE; standard error.

Figure 1 shows the mean, log transformation of the mean or the median (dependent of the distribution) of day 0 metabolic, endocrine and inflammatory parameters of all cycles compared between STF and non-STF subjects. The FT4 levels were significantly higher (*P=0.034,* 95% CI 0.08-1.91) in the STF group compared to the non-STF group. Glucose and insulin levels appeared to be lower in the STF group compared to the non-STF group, but the difference was not statistically significant. IGF-1, IGF-BP3, TSH and T3 showed similar levels in STF and non-STF patients.

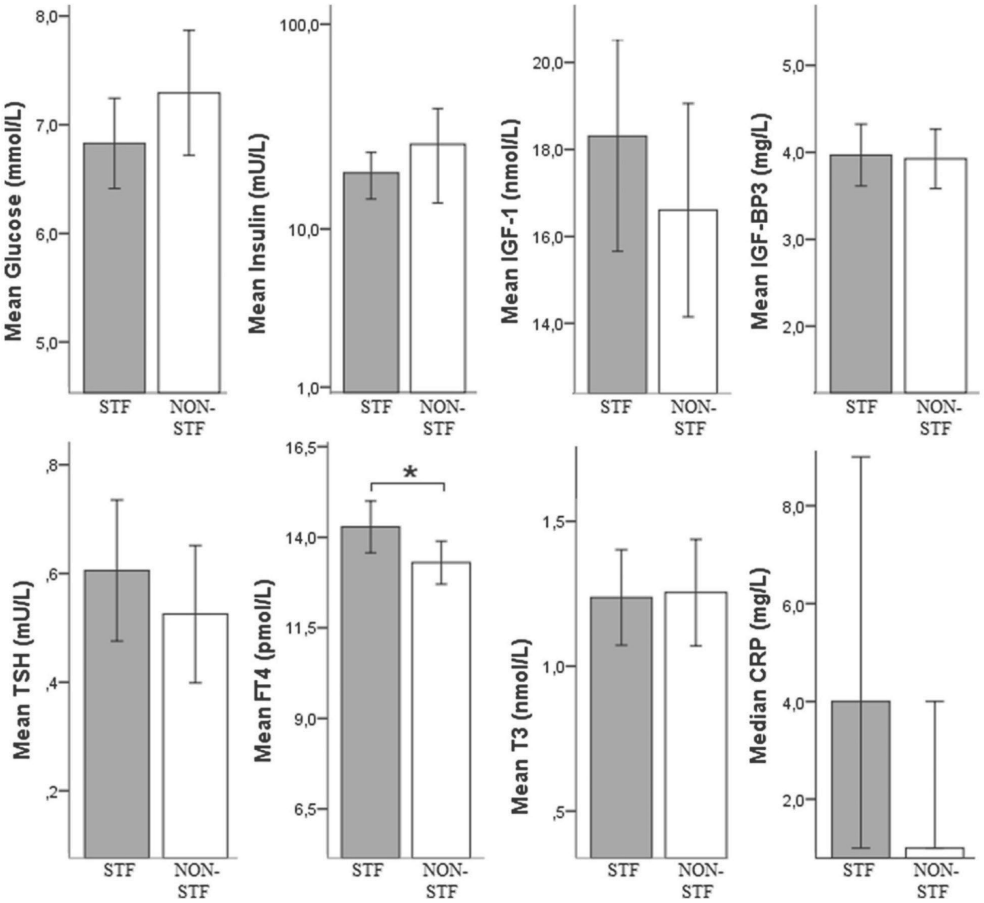

Figure 1: Metabolic, endocrine and inflammatory parameters on day 0 compared between STF and non-STF subjects. Values are measured on day 0 immediately before chemotherapy infusion (during the use of prophylactic dexamethasone). Mean values of different patients of different cycles(1-6) are combined to test differences between both treatment groups. * P value <0.05. Reference values: glucose 3.1-6.4mmol/L; insulin 0-20mU/L; IGF-1 5.4-24.3nmol/L; IGF-BP3 2.2-5.8mg/L; TSH 0.3-4.8mU/L; FT412-22pmol/L, T31.1-3.1nmol/L; CRP 0.0-5.0mg/L;. IGF-1; Abbreviations: STF: short-term fasting, IGF-1:Insulin-like growth factor 1, IGF-BP3: insulin- like growth factor binding protein 3, TSH: thyroid-stimulating hormone; FT4:,free thyroxine; T3: CRP; C-reactive protein.

Hematologic parameters

Hematologic parameters measured on day 0 (i.e., immediately before chemotherapy infusion, when the STF group had fasted for 24 hours), were similar in the two groups. Erythrocyte counts were significantly higher in the STF group during chemotherapy treatment at day 7 (*P=0.007,* 95% CI 0.106-0.638) and at day 21 (*P=0.002,* 95% CI 0.121-0.506) compared to the control group (Figure 2). Thrombocyte counts were only significantly higher at day 7 (*P=0.00007,* 95% CI 38.7-104) in the STF arm compared to the non-STF arm. For leukocytes and neutrophils, no significant difference in counts was observed, both at day 7 and day 21 between STF and non-STF patients (not shown).

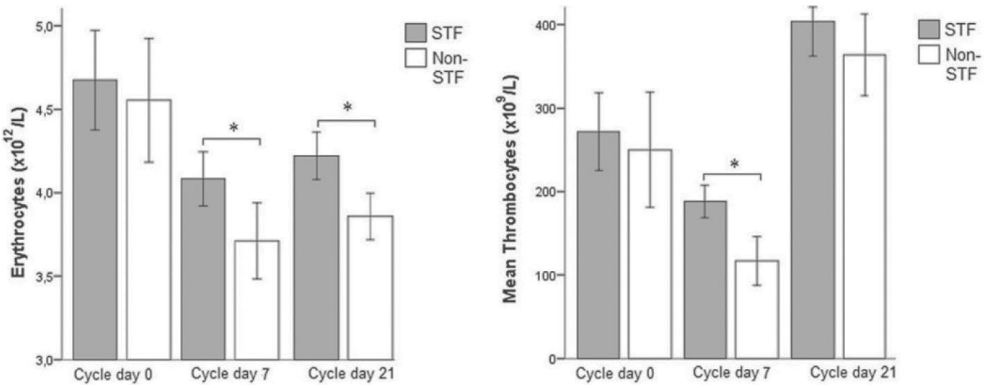

Figure 2: Hematologic parameters compared between both groups. Values are measured on day 0 of cycle 1 immediately before the chemotherapy infusion, on day 7 of cycle 1-5 combined and day 21 of cycle 1-5 combined. * *P* value <0.05. STF; short-term fasting, Reference values: erythrocytes 4-5*10^{12}/L; thrombocytes 150-400*10^{9}/L

DNA damage in PBMCs

No cumulative effect on DNA damage of chemotherapy was seen during the 6 cycles of TAC in CD45+CD3+ lymphocytes, CD45+CD14+CD15- monocytes and CD45+CD3- myeloid as no significant differences in γ-H2AX intensity were seen throughout 6 cycles, (see Supplemantary table 1). Therefore, the measured γ-H2AX intensity from each cycle at the same time point (before chemotherapy, after 30 minutes, and after 7 days) was combined for analysis. The level of γ-H2AX intensity (given as geomean) measured by flow cytometry in CD45+CD3+ lymphocytes, CD45+CD14+CD15- monocytes and CD45+CD3- myeloid are given in Table 4. γ-H2AX intensity was increased after chemotherapy infusion in the CD45+CD3+ lymphocytes 30 minutes after chemotherapy infusion in both groups and in the non-STF group after 7 days as well. In the CD45+CD14+CD15- monocytes no difference in γ-H2AX intensity was seen after 30 minutes, but after 7 days, a significant increase was seen in both groups. In the CD45+CD3- myeloid cells, a significantly increase was seen in γ-H2AX intensity at 30 minutes post-chemotherapy only in the non-STF group. γ-H2AX intensity was consistently higher in CD45+CD14+CD15- monocytes than in CD45+CD3+ lymphocytes and CD45+CD3- myeloid cells.

Table 4: γ-H2AX intensity in CD45+CD3+ lymphocytes, CD45+CD14+CD15- monocytes and CD45+CD3-myeloid cells.

Parameter	N	Before CT Day 0 Mean (SE)	30 minutes after CT Day 0 Mean (SE)	Increase	*P* value
CD45+CD3+ lymphocytes	STF (n = 14)	75.5 (4.7)	89.5 (6.5)	↑	**0.020**
	Non-STF (n = 6)	78.8 (5.6)	95.7 (5.9)	↑	**0.001**
CD45+CD14+CD15- monocytes	STF (n = 12)	162.2 (11.9)	192.5 (14.3)	=	0.055
	Non-STF (n = 6)	180.8 (15.6)	206.2 (20.8)	=	0.051
CD45+CD13- myeloid cells	STF (n =14)	104.0 (7.0)	109.5 (8.4)	=	0.594
	Non-STF (n = 6)	109.0 (7.8)	123.0 (6.7)	↑	**0.009**
Parameter	**N**	**Before CT Day 0 Median (range)**	**7 days after CT Median (range)**	**Increase**	***P* value**
CD45+CD3+ lymphocytes	STF (n =16)	75.5 (49-157)	83.0 (64-141)	=	0.109
	Non-STF (n = 9)	78.0 (47-102)	90.0 (71-114)	↑	**0.015**
CD45+CD14+CD15- monocytes	STF (n = 14)	157.0(114-231)	186.5 (132-295)	↑	**0.035**
	Non-STF (n = 8)	203.5 (116-273)	258.5 (183-319)	↑	**0.021**
CD45+CD13- myeloid cells	STF (n = 16)	106.0 (71-173)	84.0 (65-145)	=	0.379
	Non-STF (n = 9)	88.0 (49-137)	88.0 (74-119)	=	0.477

Paired comparison between pre- and post- chemotherapy (30 minutes and 7 days; median of 6 cycles of TAC) for the different cell types. γ-H2AX intensity is given as mean and median depending on the distribution.
Bold value indicates that $p<0.05$. 95% CI; 95% confidence interval. P values are given for differences of intensity of γ-H2AX between pre- and post-chemotherapy

Discussion

This is the first randomized pilot study to explore the effects of 48 hours STF on the side effects of chemotherapy in early BC patients. Only one study to date[20] has examined the effects of fasting on chemotherapy-induced side effects in cancer patients, but therein the patients served as their own controls and had various tumor types and treatment protocols. The main findings of our study were that STF was well-tolerated, safe and had beneficial effects on hematologic toxicity and possibly on DNA damage in healthy cells (lymphocytes and myeloid cells). Additionally, we found that dexamethasone, when administered during the fasting period, causes an increase in metabolic values.

Although STF was well tolerated, two patients withdrew from STF after 3 cycles of chemotherapy after experiencing a side effect (pyrosis and recurrent febrile neutropenia, respectively). Since these side effects persisted in both patients during the subsequent 3 cycles of chemotherapy without STF, they may not be related to STF. All patients finished their treatment schedule of 6 cycles of TAC and no significant difference in occurrence of chemotherapy-related adjustments were found between the two groups. The side effect profile of the TAC protocol seen in this study was consistent with the existing literature[32-34]. STF had no beneficial effect on patient-reported side effects in this study. This may be explained by the large variability of side effects between patients, which may be attributable to occurrence of symptom clusters and pharmacogenomics[35,36]. This

may have masked any beneficial effects of STF. Additionally, the relatively short period of fasting (48 hours) may explain the lack of benefit in terms of side effects: previous studies have shown that a longer fasting period is required to cause major changes in IGF-1 levels[20,37]. Reduction of plasma IGF-1 levels is a critical mediator of differential stress resistance in response to nutrient restriction (see below).

γ-H2AX phosphorylation indicates the presence of double-strand DNA breaks and could serve as a marker for chemotherapy toxicity in healthy cells, as seen in a phase I/II trial with patients treated with chemotherapy and belinostat[38]. We measured the induction of chemotherapy-induced DNA damage in PBMCs by phosphorylation of H2AX (i.e. γ-H2AX). The level of γ-H2AX in CD45+CD3+ lymphocytes was increased after 30 minutes in both groups. After 7 days, γ-H2AX accumulation remained increased in the non-STF group only, suggesting that STF promotes the recovery of chemotherapy-induced DNA damage in these cells. In CD45+CD3- myeloid cells, the level of γ-H2AX was increased after 30 minutes in the non-STF group, but not in the STF group, suggesting STF protected these cells against the induction of DNA damage by chemotherapy. As these myeloid cells may harbor the antigen-presenting cells required for induction of an effective anti-tumor immune response, this result warrants further study[39]. Moreover, the relation of this finding with the clinical benefit of STF still needs to be established.

The significantly higher erythrocyte and thrombocyte counts observed after chemotherapy in the STF group could be explained by decreased breakdown of circulating cells and/or less severe bone marrow suppression. This supports the hypothesis that STF may protect against chemotherapy-associated hematological toxicity. No significant difference in leukocyte and neutrophil counts was seen. This could be explained by the use of pegfilgrastim, which acts to increase the production of white blood cells in bone marrow and may therefore prevent a decrease in leukocyte counts in response to chemotherapy.

Plasma glucose levels increased and insulin levels remained constant in response to STF. The use of dexamethasone may explain this phenomenon[40-42]. Dexamethasone was administered for anti-emesis, reduction of fluid retention and dampening of hypersensitivity reactions in response to docetaxel[43]. However, the metabolic effects of dexamethasone may have attenuated the benefits of STF. In the absence of dexamethasone, STF reduces circulating glucose, insulin and IGF-1 levels[19,44]. A decrease in IGF-1 affects other factors (e.g. Akt, Ras and mammalian target of rapamycin (mTOR)) to down-regulate cell growth and proliferation[45-47]. Reduction of IGF-1 is one of the key mediators of the protective effects of STF in healthy cells[44]. Although fasting modestly reduced plasma IGF-1 concentrations in the current trial, the concomitant use of dexamethasone probably attenuated the decline and thereby probably counteracted the

beneficial impact of the dietary intervention.

Our study has some limitations. The most obvious limitation of our study is the small sample size, which may have limited the power of the study and precludes firm statistical conclusions. Moreover, as high dose dexamethasone induces insulin resistance, compensatory hyperinsulinemia and hyperglycemia, its prophylactic use may have counteracted the beneficial effects of STF. Therefore the use of this drug warrants further study for future clinical trials with STF. Finally, as DNA damage is repaired rapidly[48], our protocol may not be rapid enough to obtain a reliable quantification. Therefore, a consistent and rapid protocol for the isolation and fixation of PBMCs immediately after blood withdrawal should be applied in future studies to allow for reliable quantification of damage induced by chemotherapy.

Larger randomized trials such as the DIRECT study (NCT02126449) are now ongoing to evaluate the impact of STF on tolerance to and efficacy of neoadjuvant chemotherapy in women with stage II or III BC. Because it is likely that the positive effects of STF will be enhanced if the period of fasting is prolonged[37,49], a very low calorie, low protein fasting mimicking diet (FMD) is used to ease the burden of prolonged fasting[50]. Prophylactic dexamethasone will be omitted in the FMD arm during the first 4 chemotherapy cycles to reduce its potentially counteractive metabolic effects. Moreover, blood will be processed immediately after sampling to prevent potential recovery of DNA damage.

Conclusions

We demonstrate for the first time that STF is feasible for a period of 48 hours during chemotherapy in a homogeneous group of patients with early breast cancer. This study provides evidence that STF attenuates bone marrow toxicity in these patients and reduces chemotherapy-induced DNA damage in PBMCs and/or accelerate its recovery. A larger trial with a longer fasting period is ongoing to investigate the possible benefits of STF during chemotherapy.

List of abbreviations

BC:	breast cancer
CRP :	C-Reactive Protein
DSBs:	double-strand breaks
DSR:	differential stress resistance
FT4:	free thyroxine
G-CSF:	granulocyte-colony stimulating factor
IGF-1:	insulin growth factor 1
IGF-BP3:	insulin growth factor binding protein 3
PBMCs:	peripheral blood mononuclear cells
STF:	short-term fasting
T3:	triiodothyronine
TAC:	docetaxel, doxorubicin and cyclophosphamide
TSH:	thyroid-stimulating hormone
UNL:	upper limit of normal

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Supplementary material

Supplementary Table 1: Median of γ-H2AX geomean intensity in CD45+CD3+ lymphocytes, CD45+CD14+CD15- monocytes and CD45+CD3- myeloid cells among the six cycles tested with the median test, testing for differences of γ-H2AX between cycles.

	CD45+CD3+ lymphocytes		CD45+CD14+ CD15- monocytes		CD45+CD3- myeloid cells	
Time point	**Median γ-H2AX intensity**	***P* value***	**Median H2AX intensity**	***P* value***	**Median γ-H2AX intensity**	***P* value***
before CT Day 0	78.0	0.265	156.5	0.147	106.0	0.953
30 minutes after CT Day 0	97.0	0.931	177.5	0.502	123.0	0.931
7 days after CT	86.0	0.440	181.5	0.602	84.0	0.514

P values* given for differences of intensity of γ-H2AX tested among the six cycles.

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Chapter 4

Fasting mimicking diet as an adjunct to neoadjuvant chemotherapy for breast cancer in the multicentre randomized phase 2 DIRECT trial

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Abstract

Short-term fasting protects tumor-bearing mice against the toxic effects of chemotherapy while enhancing therapeutic efficacy. We randomized 131 patients with HER2-negative stage II/III breast cancer, without diabetes and a BMI over 19kg m^{-2}, to receive either a fasting mimicking diet (FMD) or their regular diet for 3 days prior to and during neoadjuvant chemotherapy. Here we show that there was no difference in toxicity between both groups, despite the fact that dexamethasone was omitted in the FMD group. A radiologically complete or partial response occurs more often in patients using the FMD (OR 3.168, $P=0.039$). Moreover, per-protocol analysis reveals that the Miller&Payne 4/5 pathological response, indicating 90-100% tumor-cell loss, is more likely to occur in patients using the FMD (OR 4.109, $P=0.016$). Also, the FMD significantly curtails chemotherapy-induced DNA damage in T-lymphocytes. These positive findings encourage further exploration of the benefits of fasting/FMD in cancer therapy.

Background

Extensive preclinical evidence suggests that short-term fasting and fasting mimicking diets (FMDs) can protect healthy cells against the perils of a wide variety of stressors, including chemotherapy, simultaneously rendering cancer cells more vulnerable to chemotherapy and other therapies[1-5]. Essentially, fasting causes a switch in healthy cells from a proliferative state towards a maintenance and repair state. Malignant cells, in contrast, seem to be unable to enter this protective state because of oncoprotein activity, and therefore fail to adapt to nutrient scarce conditions. Instead, fasting deprives proliferating cancer cells of nutrients, growth and other factors, which renders them more sensitive to cancer therapy and increases cell death[1,3]. The phenomenon by which normal but not cancer cells become protected to toxins is termed differential stress resistance (DSR)[2,3] whereas the specific sensitization of cancer cells to stress is called Differential Stress Sensitization (DSS)[1,6].

Declines of plasma levels of insulin like growth factor-1 (IGF-1), insulin and glucose are among the mediators of the effects of fasting on cancer cells, as these factors can promote growth and prevent apoptosis[1-4,6,7]. Fasting periods of at least 48 hours are required to induce a robust decrease in circulating glucose, IGF-1 and insulin levels[6,8]. A very low calorie, low protein FMD was developed for its ability to cause metabolic effects on various starvation response markers similar to those caused by water-only fasting, while reducing the burden associated with a water only fast[9,10].

Small clinical studies showed that fasting as an adjunct to chemotherapy is safe and well tolerated, while it may reduce its toxicity[11-14]. This multicentre, open label, randomized DIRECT study was designed to evaluate the impact of an FMD on toxicity as well as on the radiological and pathological response to chemotherapy for breast cancer.

Methods

Study design and patients

This is a randomized, controlled, observer-blind study. Eligible patients from 11 Dutch centers had histologically confirmed diagnosis of HER2-negative, stage II/III (cT1cN+ or ≥T2 any cN, cM0) early breast cancer, adequate bone marrow reserve (white blood counts >3.0 x 10^9/L, absolute neutrophil count ≥1.5 x 10^9/l and platelet count ≥ 100 x 10^9/l), adequate liver function (bilirubin ≤1.5 x upper limit of normal (UNL) range, ALAT and/or ASAT ≤2.5 x UNL, Alkaline Phosphatase ≤5 x UNL), adequate renal function (calculated creatinine clearance ≥50 mL/min), normal cardiac function, a WHO performance state 0-2, age ≥ 18 years, BMI > 19kg m^{-2}, absence of diabetes mellitus, absence of allergies for FMD content, and signed informed consent. The

study (NCT02126449) was conducted in accordance with the Declaration of Helsinki (October 2013) and approved by the Ethics Committee of the Leiden University Medical Center in agreement with the Dutch law for medical research involving human subjects.

Drugs

Women received 8 cycles of neo-adjuvant AC-T chemotherapy (4 cycles doxorubicin 60 mg m^{-2} and cyclophosphamide 600 mg m^{-2} intravenously (i.v.)), followed by 4 cycles of T (docetaxel 100 mg m^{-2} i.v.), or 6 cycles of neo-adjuvant FEC-T chemotherapy, consisting of 3 cycles of 5-fluorouracil, epirubicin and cyclophosphamide at a dose of 500, 100 and 500mg m^{-2} i.v., respectively), followed by 3 cycles of T (docetaxel 100 mg m^{-2} i.v.), all q 3 weeks. The anti-emetic agents granisetron (1 mg i.v.) or ondansetron (8 mg i.v.) were administered prior to chemotherapy. Dexamethasone (8mg i.v.) was administered shortly before chemotherapy for all cycles in the control group, whereas it was omitted during the AC or FEC courses in the FMD group, as dexamethasone may counteract the endocrine and metabolic effects of dietary intervention in the FMD group[19].

Intervention

Women were randomized in a 1:1 ratio to receive the FMD (Xentigen™) or regular diet for 3 days prior to and on the day of each cycle of chemotherapy. The FMD is a 4-day plant-based low amino-acid substitution diet, consisting of soups, broths, liquids and tea (Supplementary figure 3). Calorie content declined from day 1 (~1200kcal), to days 2-4 (~200kcal). Moreover, the carbohydrates/proteins/fats energy ratio was approximately 3.5/1/2 on the first day, while complex carbohydrates were the main macronutrient (>80 energy%) the other subsequent 3 days. Patients were allowed to eat the diet components at any time of the designated day.

Randomization, masking and data storage

Patients were centrally randomized at the LUMC datacenter through block randomization with various block sizes stratified by stage (II versus III), estrogen receptor status (positive versus negative), BMI (<25kg m^{-2} versus >25kg m^{-2}) and chemotherapy regimen (AC-T versus FEC-T). The web based relational database management system ProMISe (https://www.msbi.nl/promise/ProMISe.aspx) was used for data storage and exchange.

Blood sampling

Venous blood samples were drawn prior to each chemotherapy administration (pre-chemotherapy on day -1 or day 0). Compliance with the diet was estimated by the following parameters: insulin, glucose, and IGF-1 (measured in a 9mL serum-separating tube). All samples were analyzed by the accredited clinical laboratories of the participating centers.

The effect of FMD on chemotherapy-induced DNA damage in peripheral blood mononuclear cells (PBMCs) was examined in a side study. Sodium heparinized venous blood samples (9mL) were collected for prior to the first cycle of chemotherapy and three hours after start of chemotherapy.

Toxicity and efficacy

The primary endpoint of the phase II and phase III parts of the study were grade III/IV toxicity and pathological complete response (pCR), respectively. Toxicity was documented by the physician and graded according to the Common Terminology Criteria for Adverse Events version 4.03 (CTCAE v.4.03). pCR was defined as the absence of residual invasive cancer within the breast and lymph nodes[16], excluding isolated tumor cells (ITC).

Secondary endpoints included radiological response and pathological response according to the Miller and Payne (supplementary Table 1)[16]. Histopathology was centrally revised by one pathologist (DC), who was blinded to which treatment the patient received. Clinical response was measured by MRI or ultrasound of the breast halfway and at the end of therapy, according to RECIST1.1[20].

Quality of life (QoL)

Global health was assessed with the EORTC QLQ-C30[21] before therapy (after randomization), halfway therapy, at the end of therapy and at six months follow-up. Higher scores (0-100 scale) on the functional scales indicate a better QoL.

Psychosocial distress was measured with the distress thermometer[22], with an 11-point range from 0 (no distress) to 10 (extreme distress). Patients were asked to circle the number that best described the overall distress they experienced in the past week at 3 timepoints: halfway therapy, at the end of therapy and at six months follow-up.

DNA damage: isolation of PBMCs and γ-H2AX staining

PBMCs were isolated using Ficoll-Amidotrizoaat (Pharmacy LUMC) gradient centrifugation according to the standard operating procedure of the Medical Oncology department of LUMC. Isolated PBMCs were carefully resuspended and 3 times washed in PBS (B. Braun, Melsungen, Germany). Samples were fixed in 1.5% formaldehyde and permealized in ice-cold pure methanol. Cells were washed 3 times in staining buffer (PBS with 5% bovine serum albumin (BSA, Sigma, St Louis, USA)) and stained for 30 minutes on ice with anti-CD45-PerCP-Cy5.5 (BD Bioscience, Breda, the Netherlands), clone 2D1 anti-CD3-PE (BD, clone SK7), anti-CD14-AF700 (BD, clone M5E2), anti-CD15-PE CF594 (BD, clone W6D3) and anti-γ-H2AX-AF488 (Biolegend, clone 2F3), followed by another washing step and resuspension in PBS. Per experiment we used

1,000,000 cells or more when available. The cell acquisition was performed immediately after the staining procedure on the flow cytometer (BD LSR Fortessa Flow Cytometer analyzer, BD Bioscience, Breda, The Netherlands) and data were analyzed using BD FACS Diva Software version 6.2. The CD45+ cells were gated, after which the CD3+ T-lymphocytes, CD3- non-T cells (also harboring B lymphocytes) or CD14+CD15- monocytes were analyzed for the geomean (as measure for the intensity) of γ-H2AX (Supplementary figure 2).

Statistical analysis

The primary endpoint of phase II of the study was grade III/IV toxicity. Based on trials with similar neo-adjuvant chemotherapy[17,23,24], the statistical power analysis revealed that a total number of 128 patients (64 patients in each arm) was required to be able to detect a 50% reduction of grade III/IV adverse effects with 80% power using a nominal significance level of 3.06% .

The primary endpoint of the phase III part of the study was pCR. We estimated the overall pCR rate to amount to 18%, based on studies examining similar third generation chemotherapy[17,18,23]. Our sample size calculation revealed that we would require a total number of 212 patients (106 per treatment arm).

An interim analysis, focusing on feasibility and adverse events, was planned after completion of the phase II part of the protocol by 128 patients and was approved by the Ethics Committee of the Leiden University Medical Center. Early stopping rules included significantly more or unacceptable adverse events in either group. A data safety monitoring board conducted the interim analysis. Survival data will be reported after 5 years follow-up.

All parameters were tested for normality using a Kolmogorov-Smirnov test, with Bonferroni adjustment when evaluated in subgroups. Normally distributed parameters, if necessary after log transformation, were summarized as mean (and standard error of the mean (SEM)) and compared using independent or paired samples *t*-tests when appropriate. The non-normally distributed parameters were summarized as median (and 25th and 75th percentiles) and compared using a Mann-Whitney test for independent groups or Wilcoxon signed rank test for paired groups. The effect of FMD on efficacy of chemotherapy was analyzed using logistic regression, yielding univariate and multivariate odds ratios (ORs), 95% confidence intervals (CIs), and *P*-values. Multivariate analyses were adjusted for stratification factors[25]. ER status, BMI, stage of disease and chemotherapy regimen. The Armitage's trend test was used to test an association between an ordinal variable and two categories. Mean changes in QoL from baseline to halfway, end of therapy and 6 months follow-up were assessed in linear

mixed models with 95% CIs. All tests were 2-tailed with a significance level of 0.05. All data were analyzed using IBM SPSS Statistics for Windows (Version 23.0. Armonk, NY: IBM Corp).

Results

Patient characteristics

From February 2014 to January 2018, 131 patients were randomized (see consort diagram, Figure 1). One patient withdrew informed consent before starting with chemotherapy and one patient was ineligible because of liver metastases, which were diagnosed a day after randomization. Of the 129 patients, 65 received FMD as an adjunct to chemotherapy and 64 patients used their regular diet. Thirty patients received FEC-T chemotherapy and 99 AC-T. Patient characteristics were equally distributed between groups (Table 1 and supplementary Table 2).

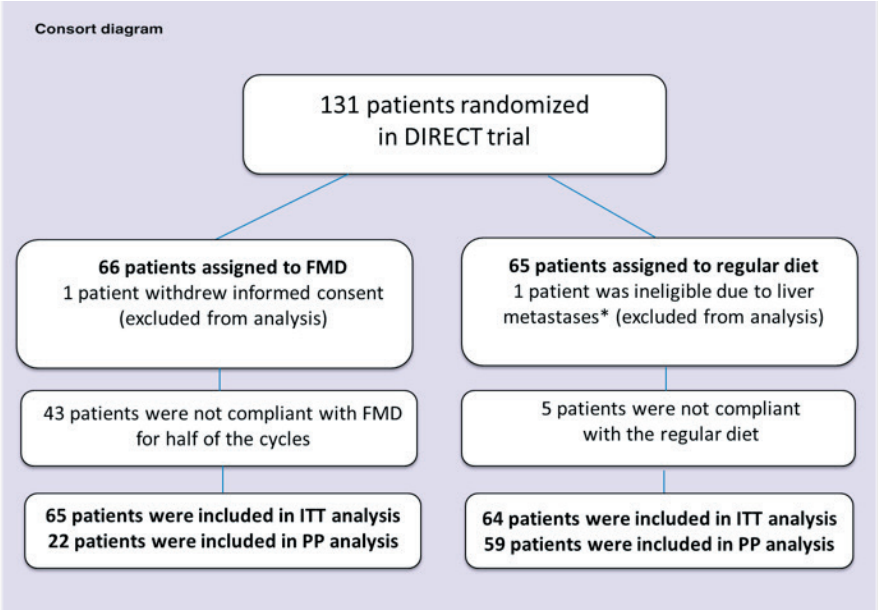

Figure 1: Consort diagram of the DIRECT study. This figure shows reasons for exclusion from the study and the numbers of patients included in the PP and ITT analyses. Abbreviations: FMD: fasting mimicking diet, ITT: Intention to treat, PP: Per protocol. * diagnosed the day after randomization.

Table 1: Patient characteristics.

	FMD (N=65)	Regular diet (N=64)
Median Age (range), Years	49.0 (31-71)	51.0 (27-71)
Median Body Mass Index (range), kg/m²	25.7 (19.8-41.2)	26.0 (19.7-39.0)
WHO-status		
Grade 0	61 (93.8%)	60 (93.8%)
Grade 1	3 (4.6%)	4 (6.3%)
Unknown	1 (1.5%)	0 (0%)
Menopausal status		
Pre/Peri	27 (41.5%)	31 (48.4%)
Post	38 (58.5%)	31 (48.4%)
Unknown	0 (0%)	2 (3.1%)
T-classification		
T1	5 (7.7%)	6 (9.4%)
T2	42 (64.6%)	41 (64.1%)
T3	17 (26.2%)	15 (23.4%)
T4	1 (1.5%)	2 (3.1%)
N-classification		
N0	29 (44.6%)	33 (51.6%)
N1	28 (43.1%)	26 (40.6%)
N2	7 (10.8%)	4 (6.3%)
N3	1 (1.5%)	1 (1.6%)
Stage		
I (ineligible)	0 (0%)	1 (1.6%)
II	51 (78.5%)	48 (75.0%)
III	14 (21.5%)	15 (23.4%)
HR-status		
ER-/PR-	14 (21.5%)	7 (10.9%)
ER-/PR unknown	0 (0%)	1 (1.6%)
ER+/PR-	9 (13.8%)	9 (14.1%)
ER+/PR+	42 (64.6%)	47 (73.4%)
Chemotherapy regimen		
AC-T	52 (80.0%)	47 (73.4%)
FEC-T	13 (20.0%)	17 (26.6%)
Grade (BR)		
I	2 (3.1%)	2 (3.1%)
II	43 (66.2%)	42 (65.6%)
III	20 (30.8%)	19 (29.7%)
Unknown	0 (0%)	1 (1.6%)
Tumortype		
Ductal	53 (81.5%)	49 (76.6%)
Lobular	9 (13.8%)	13 (20.3%)
Other	3 (4.6%)	2 (3.1%)

Patient characteristics. Abbreviations: FMD: Fasting mimicking diet, HR: hormone receptor, AC-T: doxorubicin/cyclophosphamide followed by docetaxel, FEC-T: Fluorouracil/epirubicin/cyclophosphamide followed by docetaxel, BR: Bloom Richardson, ER: estrogen receptor, PR: progesterone receptor.

Interim analysis

Because the overall (both arms) pCR turned out to be significantly lower (11.7%) than anticipated (which would require the recruitment of twice as many participants to be able to detect the hypothesized pCR difference between both arms in a subsequent phase III study), in addition to the worse-than-expected compliance, the Data Safety Monitoring Board advised to dispense with the phase III study. Therefore, we here present the results of the phase II study.

Compliance

Fifty three out of 65 patients (81.5%) completed the first FMD cycle, whereas over 50% completed 2 FMD cycles, which could be sufficient to impact the tumor response to chemotherapy in view of the effects of only one or a few FMD cycles in enhancing the efficacy of chemotherapy in mice [15]. 22 out of 65 patients (33.8%) used the FMD for at least 4 cycles (all AC or FEC cycles), and 20.0% of the patients complied during all cycles of chemotherapy (supplementary Table 3). The main reason for non-adherence to the FMD was dislike of distinct components of the diet, perhaps induced by chemotherapy. In the regular diet group, 5 (7.8%) patients were not compliant (they decided to fast during one or more cycles of chemotherapy).

Intention to treat (ITT) analysis

Data on toxicity are shown in supplementary Table 4. Grade III/IV toxicity, scored during all cycles of chemotherapy, was not significantly different between the FMD group (75.4%) and the regular diet group (65.6%). No grade V toxicity occurred. The percentage of patients who discontinued chemotherapy did not significantly differ between groups (27.7% FMD *vs* 23.8% control, *P=0.580)*. Notably, while side effects were similar in both arms, patients in the FMD arm did not receive dexamethasone before the AC chemotherapy cycles.

The radiological response and pathological response according to Miller and Payne are shown in Figure 2 and supplementary Table 5. The overall pCR rate was 11.7% and did not differ between the two groups (10.8% in FMD group versus 12.7% in control group; OR 0.830, 95% CI 0.282-2.442, *P=0.735*). Interestingly, the radiologically complete or partial response, as measured by MRI or ultrasound before surgery, occurred approximately 3 times more often in the FMD group compared to the control group in univariate (OR 2.886, 95% CI 1.012-8.227, *P=0.047*) and multivariate (OR 3.168, 95% CI 1.062-9.446, *P=0.039*) analyses. Accordingly, the proportion of patients with stable or progressive disease was 2.5 fold lower in the FMD group (11.3%) than in the control group (26.9%, Figure 2).

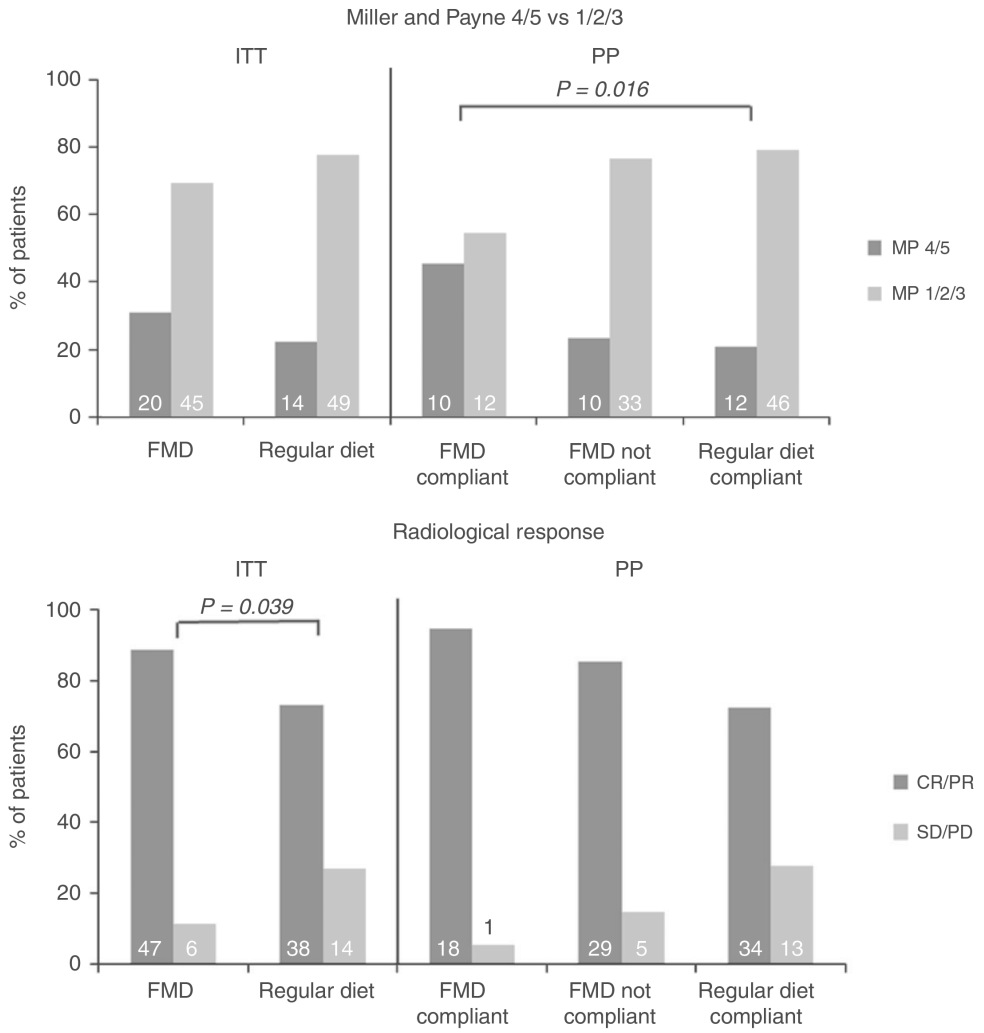

Figure 2: Tumor response data for the ITT and PP analysis. The pathological response was given for Miller and Payne pathological response score 4/5 (90-100% tumor cell loss) vs. 1/2/3 (less than 90% tumor cell loss). The radiological response was scored according RECIST1.1 and given for complete response + partial response vs. stable disease + progression disease. Abbreviations: FMD: fasting mimicking diet, ITT: Intention to treat, PP: Per protocol, MP: Miller and Payne, CR: complete response, PR: partial response, SD: stable disease, PD: progression disease. Logistic regression was used (2-sided).

The FMD affected various metabolic and endocrine parameters in the ITT analysis (Supplementary Table 6). At day -1/ 0 pre-chemotherapy, plasma insulin was significantly lower in the FMD group (*P*=0.004), while a trend for lower plasma glucose levels was observed in the FMD group (*P=0.062*). Urine ketone bodies were higher in the FMD group versus the control group (*P<0.0001*).

Data on global QoL and the distress thermometer are shown in Figure 3 and supplementary Figure 1, respectively. QoL was not significantly different between both groups in terms of global QoL (*P=0.841)* and overall distress *(P=0.674)*.

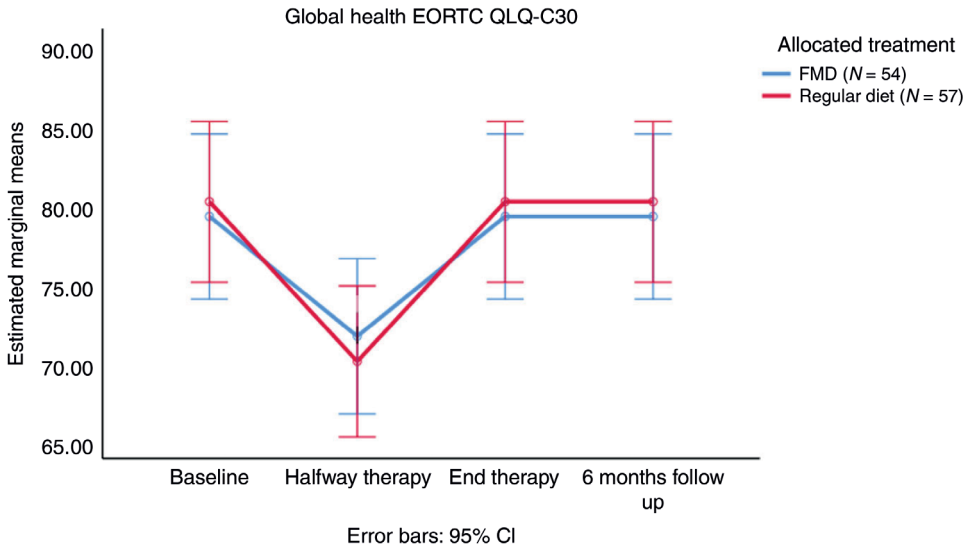

Figure 3: EORTC QLQ-C30 global health domain given for 4 timepoints: before therapy (after randomization), halfway therapy, at the end of therapy and at six months follow-up. Error bars indicate the 95% CI of the mean. Abbreviations: EORTC: European Organization for Research and Treatment of Cancer, FMD: fasting mimicking diet. CI: confidence interval. FMD N=57 and regular diet N=54. Armitage's trend test was used.

Per protocol (PP) analysis

A PP analysis was done to substantiate the effects of FMD on toxicity and efficacy of chemotherapy. Specifically, patients who were compliant with the FMD for at least half of the cycles were compared with those who were less compliant, and with the compliant control patients (i.e. the patients in the control group who did not fast on their own initiative).

Toxicity data of the PP analysis are shown in Table 2. Grade III/IV toxicity did not differ between FMD compliant patients (n=22) *vs.* control (n=59) group

Table 2: Grade III/IV toxicity in both groups (ITT) and in patients who were compliant with the FMD for at least half cycles of CT vs. control patients who did not fast on their own initiative (PP).

Grade III/IV	FMD (N=65)	FMD-C (N=22)	FMD-NC (N=43)	Control (N=64)	*P*-value (ITT)	*P*-value (PP)
Total	31 (47.7%)	11 (50.0%)	20 (46.5%)	36 (56.3%)	0.331	0.539
Neutropenic fever	5 (7.7%)	1 (4.5%)	4 (9.3%)	5 (7.8%)	0.980	0.548
Neutropenia	19 (29.2%)	6 (27.3%)	13 (30.2%)	18 (28.1%)	0.890	0.777

Grade III/IV side effects were scored according CTCAE4.03. Each side effect was scored maximal once per patient during the course. FMD; fasting mimicking diet, C: compliant, NC: Not compliant, ITT intention to treat, PP per protocol, CT: chemotherapy.

In the PP analysis, the pCR rate did not differ between the compliant FMD patients (13.6%) and controls (12.1%, OR 1.150, 95% CI 0.269-4.911, *P=0.850*, supplementary Table 5). However, the Miller and Payne pathological response 4/5 (90-100% tumor cell loss) occurred 3-4 times more often in patients using FMD in both univariate (OR 3.194, 95% CI 1.115-9.152, *P=0.031*) and multivariate analyses (OR 4.109, 95% CI 1.297-13.02, *P=0.016*, Figure 2) than in the control group. Furthermore, the more FMD cycles completed, the more patients had either a complete or partial radiological response to therapy (*P for trend = 0.035*, Figure 4). Both analyses were adjusted for hormone receptor status, TNM stage, BMI and chemotherapy regimen.

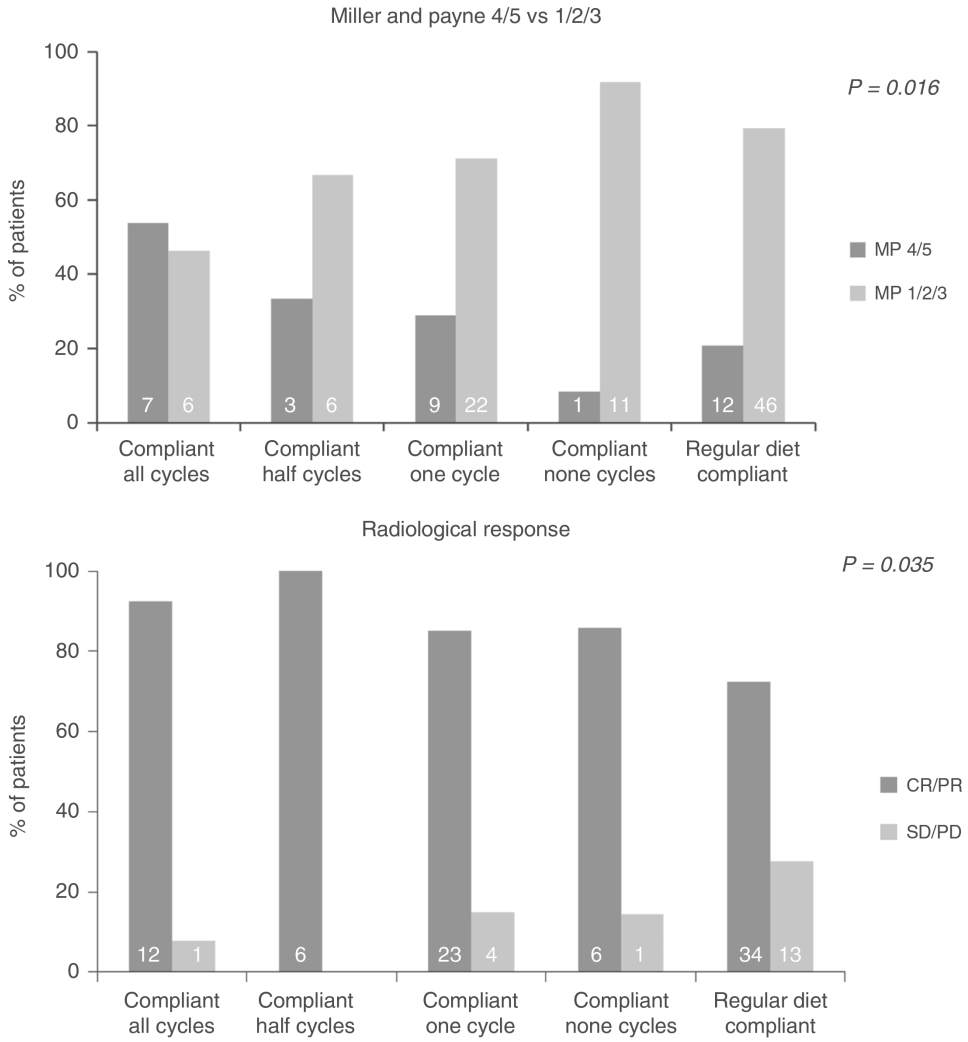

Figure 4: Tumor response data per number of cycles completed. The pathological response was given for Miller and Payne pathological response score 4/5 (90-100% tumor cell loss) vs. 1/2/3 (less than 90% tumor cell loss). The radiological response was scored according RECIST1.1 and given for complete response + partial response vs. stable disease +progression disease. P value is given for Armitage's trend test (2-sided). Abbreviations: FMD: fasting mimicking diet, MP: Miller and Payne, CR: complete response, PR: partial response, SD: stable disease, PD: progression disease.

In the PP analysis (Figure 5 and Supplementary Table 6), glucose was significantly lower in the compliant FMD group compared with the regular diet group before the first cycle and halfway therapy (*P=0.006* and *P=0.042,* respectively). Insulin was significantly lower in the compliant FMD group compared with the control group before the first cycle and halfway therapy (*P=0.001* and *P<0.001*, respectively*)*. IGF-1 was significantly lower halfway therapy in patients who were compliant to the FMD in comparison to control patients (*P=0.025*). Ketone bodies were positive in most of the compliant FMD patients (93.3%) and rarely positive in the compliant control group (8.1%, *P<0.0001*).

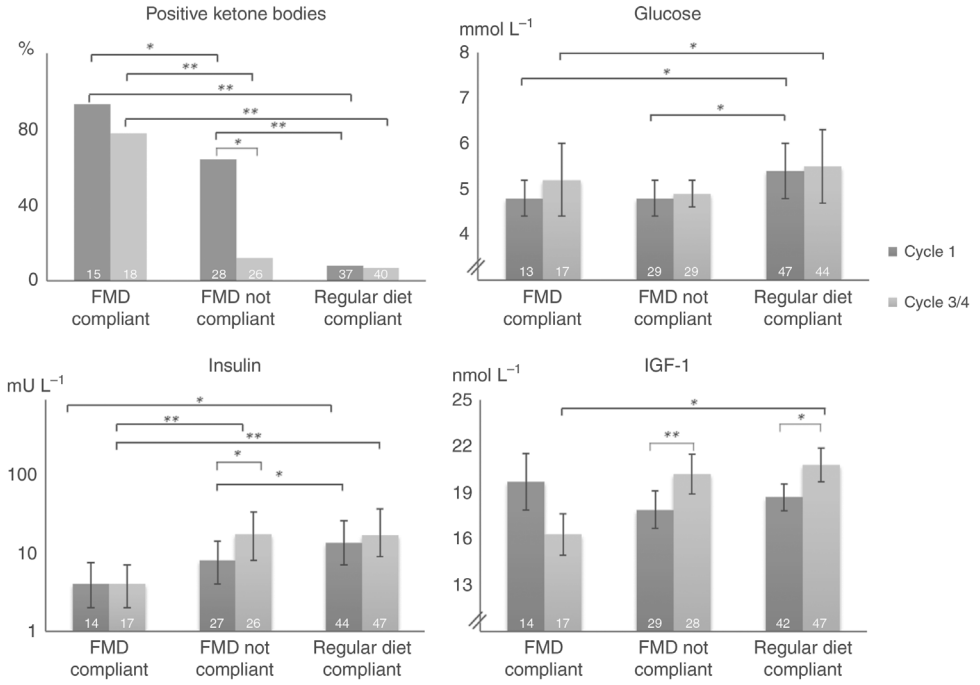

Figure 5: Metabolic and endocrine parameters before chemotherapy compared between compliant and non-compliant patients halfway therapy of the FMD group and the regular group. Values are measured on day -1 or day 0 before cycle 1 and halfway therapy. * P value <0.05, ** P value <0.001 (2-sided). Error bars indicate the standard error of the mean (if data was normally distributed) or the 25% and 75% percentiles of the median (if data was non-normally distributed). Independent t-tests and Mann-Whitney tests were used. Reference values: glucose 3.1-6.4 mmol/L; insulin 0-20 mU/L; IGF-1 5.4-24.3 nmol/L. Abbreviations: FMD: fasting mimicking diet, IGF-1: Insulin-like growth factor 1.

The level of γ-H2AX intensity are reported in supplementary Table 7. Only compliant patients were included. γ-H2AX intensity increased 3 hours after chemotherapy in both groups for each cell type due to chemotherapy. The increase in DNA damage after chemotherapy was significantly less in CD45+CD3+ T-lymphocytes from patients who had FMD as compared to patients using regular diet *(P=0.045*, Figure 6*).*

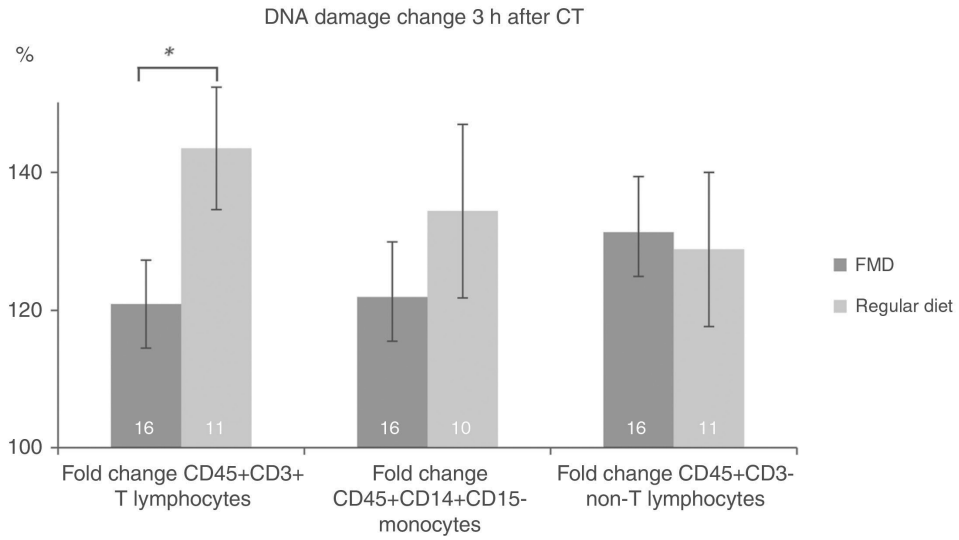

Figure 6: Difference of γ-H2AX intensity in CD45 + CD3+ lymphocytes of each patient before cycle 1 and 3 hours after chemotherapy, given as a percentage increase. Error bars indicate the standard error of the mean. Independent t-tests were used (2-sided). Abbreviations: FMD: fasting mimicking diet. * P=0.045.

Discussion

This is the first randomized controlled study evaluating the effects of an FMD on toxicity and efficacy of chemotherapy in patients with cancer. The results suggest that an FMD significantly reinforces the effects of neoadjuvant chemotherapy on the radiological and pathological tumor response in patients with HER2 negative early breast cancer. The ITT analysis reveals an increase of patients with a radiologically complete or partial response and a reduction of patients with radiologically stable/progressing disease in the FMD group compared to the control group. The PP analysis shows a beneficial effect of the FMD on the pathological response according to Miller and Payne. The more cycles of FMD were adhered to, the higher the percentage of Miller and Payne scores 4/5 (documenting > 90% tumor cell loss) in the surgical specimen (Fig. 4).

By chance, the percentage of patients with a triple negative tumor randomized to receive the FMD was double the percentage of those in the control group (Table 1). pCR is more likely to occur in case of triple negative tumors[16, 17]. However, triple negative tumors were significantly less common in patients who complied with the FMD than in those who did not, while the response of the tumor to chemotherapy was clearly more favorable in compliant patients (Supplementary table 2). Moreover, the positive effects of the FMD persisted after adjustment for the receptor status of the tumor. These facts suggest that it was the FMD rather than the hormone receptor status which determined the better response of the tumor in agreement with the extensive pre-clinical data.

Patients using the FMD as an adjunct to chemotherapy did not experience more grade III/IV adverse events than patients who did not follow a diet, despite the fact that they were not prescribed dexamethasone in concert with FEC/AC. This suggests that the FMD may obviate the need for dexamethasone in the prevention of the side effects of chemotherapy. Importantly, DNA damage in T-lymphocytes was less in patients who received the FMD in combination with chemotherapy compared to those receiving chemotherapy while on a regular diet, suggesting that the FMD protected these cells against the induction of DNA damage by chemotherapy.

The study was meant to be a phase II/III study to evaluate the effects of the FMD on toxicity and efficacy of chemotherapy, respectively. However, a pre-defined interim analysis revealed a lower than anticipated overall pCR rate in the combined arms (albeit similar to the pCR rate in a similar trial of the same BOOG group[18], necessitating the recruitment of twice as many participants to reliably judge the impact of the FMD on this primary outcome measure. Because this would prolong the study period and require additional funds, the DSMB advised to stop and report the results at the completion of phase II. Remarkably, the phase II study, involving only 131 patients, was sufficient to show benefits of the FMD in sensitizing breast cancer cells to chemotherapy, with efficacy demonstrated both at the clinical and pathology levels.

Pre-clinical data, that has been accumulating for over 10 years, indicates that fasting can protect cancer-bearing mice against the side effects of chemotherapy[3], while sensitizing the tumor to its toxic effects[1, 2]. Even one or a few cycles of FMD by itself can inhibit the progression of a wide variety of cancers and increase the therapeutic efficacy of chemotherapy in mice[1,15] but can also prime breast cancer and other tumor cell types to an attack by immune cells[15]. Accordingly, in spite of the fact that many patients could not adhere to the dietary regimen during all cycles of chemotherapy, our intention to treat analysis reveals benefits in terms of tumor response.

Only a few, generally small clinical studies have evaluated the potential of fasting to improve cancer treatment[11-14], primarily focusing on feasibility and toxicity of treatment. Just two of these trials were randomized[11-14], but the results were in line with those of the current study. Previously, we reported reduced hematological toxicity and DNA damage in circulating mononuclear cells in a small group of women who fasted for 24 hours prior to (neo)adjuvant chemotherapy for breast cancer[11]. A second randomized study revealed improved QoL and less fatigue in breast- and ovarian cancer patients, who fasted for 60 hours around the time of chemotherapy[14]. Yet another study reported a trend towards less grade 3-4 neutropenia and reduced DNA damage in leucocytes in patients who fasted for 48-72 as compared to 24 hours around the time of platinum-based chemotherapy for a variety of malignancies[12]. Finally, fasting for variable time

periods may reduce adverse events of chemotherapy, which was suggested by a case series of 10 patients with various cancer types[13]. These data agree with the current data, showing that the FMD is safe and effective as an adjunct to chemotherapy, at least in patients with a normal body mass index at inclusion.

Our data should be cautiously interpreted, particularly those of the PP analysis, which bears the risk of selection bias. However, the ITT analysis confirms the positive impact of the FMD on the radiological response, whilst clearly showing a trend in support of the PP positive effect on the pathological response. Moreover, due to self-selection bias patients in the control group decided to fast on their own initiative, which may have decreased the positive impact of the FMD in the ITT analysis.

In conclusion, the results of this study are the first to suggest that FMD cycles are safe and effective as an adjunct to chemotherapy in women with early breast cancer. These findings together with preclinical data encourage further exploration of the benefits of fasting/FMD in patients receiving a wide range of cancer therapies.

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Supplementary material

Supplementary Table 1: Miller-Payne criteria for grading pathological response after neoadjuvant chemotherapy.

MP Grade	Pathological characteristics of the primary tumor
1	No change or some alteration to individual malignant cells but no reduction in overall cellularity
2	A minor loss of tumor cells but overall cellularity still high; up to 30% loss.
3	Between an estimated 30% and 90% reduction in tumor cells.
4	A marked disappearance of tumor cells such that only small clusters or widely dispersed individual cells remain; more than 90% loss of tumor cells
5	No malignant cells identifiable in sections from the site of the tumor; only vascular fibroelastotic stroma remains often containing macrophages. However, ductal carcinoma in situ may be present

Supplementary Table 2: Patient characteristics of both groups, and of patients who were compliant with the FMD for at least half cycles of CT or those who were not and control patients who did not fast on their own Initiative.

	FMD (N=65)	FMD-C (N=22)	FMD-NC (N=43)	Regular diet (N=64)	Regular diet –C (N=59)
Median Age (range), Years	49.0 (31-71)	51.0 (44-69)	49.0 (31-71)	51.0 (27-71)	51.3 (27-71)
Median Body Mass Index (range), kg m-²	25.7 (19.8-41.2)	25.9 (20.9-39.1)	25.4 (19.8-41.2)	26.0 (19.7-39.0)	26.3 (19.7-35.7)
WHO-status					
Grade 0	61 (93.8%)	20 (90.9%)	41 (95.3%)	60 (93.8%)	56 (94.9%)
Grade 1	3 (4.6%)	1 (4.5%)	2 (4.7%)	4 (6.3%)	3 (5.1%)
Unknown	1 (1.5%)	1 (4.5%)	0 (0.0%)	0 (0%)	0 (0%)
Menopausal status					
Pre/Peri	27 (41.5%)	11 (50.0%)	16 (37.2%)	31 (48.4%)	29 (49.2%)
Post	38 (58.5%)	11 (50.0%)	27 (62.8%)	31 (48.4%)	28 (47.5%)
Unknown	0 (0%)	0 (0%)	0 (0%)	2 (3.1%)	2 (33.9%)
T-classification					
T1	5 (7.7%)	1 (4.5%)	4 (9.3%)	6 (9.4%)	6 (10.2%)
T2	42 (64.6%)	16 (72.7%)	26 (60.5%)	41 (64.1%)	36 (61.0%)
T3	17 (26.2%)	4 (18.2%)	13 (30.2%)	15 (23.4%)	15 (25.4%)
T4	1 (1.5%)	1 (4.5%)	0 (0%)	2 (3.1%)	2 (3.4%)
N-classification					
N0	29 (44.6%)	8 (36.4%)	21 (48.8%)	33 (51.6%)	30 (50.8%)
N1	28 (43.1%)	10 (45.5%)	16 (41.9%)	26 (40.6%)	24 (40.7%)
N2	7 (10.8%)	3 (13.6%)	4 (9.3%)	4 (6.3%)	4 (6.8%)
N3	1 (1.5%)	1 (4.5%)	0 (0%)	1 (1.6%)	1 (1.7%)
Stage					
I (ineligible)	0 (0%)	0 (0%)	0 (0%)	1 (1.6%)	1 (1.6%)
II	51 (78.5%)	16 (72.7%)	35 (81.4%)	48 (75.0%)	43 (75.0%)
III	14 (21.5%)	6 (27.3%)	8 (18.6%)	15 (23.4%)	15 (23.4%)
HR-status					
ER-/PR-	14 (21.5%)	3 (13.6%)	11 (25.6%)	7 (10.9%)	6 (10.1%)
ER-/PR unknown	0 (0%)	0 (0%)	0 (0%)	1 (1.6%)	1 (1.7%)
ER+/PR-	9 (13.8%)	3 (13.6%)	6 (14.0%)	9 (14.1%)	9 (15.3%)
ER+/PR+	42 (64.6%)	16 (72.7%)	26 (60.5%)	47 (73.4%)	44 (74.6%)
Chemotherapy regimen					
AC-T	52 (80.0%)	18 (81.8%)	34 (79.1%)	47 (73.4%)	44 (74.6%)
FEC-T	13 (20.0%)	4 (18.2%)	9 (20.9%)	17 (26.6%)	15 (25.4%)

Grade (BR)					
I	2 (3.1%)	0 (0.0%)	2 (4.7%)	2 (3.1%)	2 (3.4%)
II	43 (66.2%)	15 (68.2%)	28 (65.1%)	42 (65.6%)	37 (62.7%)
III	20 (30.8%)	7 (31.8%)	13 (30.2%)	19 (29.7%)	19 (32.2%)
Unknown	0 (0%)	0 (0%)	0 (0%)	1 (1.6%)	1 (1.7%)
Tumortype					
Ductal	53 (81.5%)	18 (81.8%)	35 (81.4%)	49 (76.6%)	44 (74.6%)
Lobular	9 (13.8%)	4 (18.2%)	5 (11.6%)	13 (20.3%)	13 (22.0%)
Other	3 (4.6%)	0 (0.0%)	3 (7.0%)	2 (3.1%)	2 (3.4%)

Patient characteristics. Abbreviations: CT: chemotherapy FMD: Fasting mimicking diet, C: compliant, NC: Not compliant, HR: hormone receptor, AC-T: doxorubicin/cyclophosphamide followed by docetaxel, FEC-T: Fluorouracil/epirubicin/cyclophosphamide followed by docetaxel, BR: Bloom Richardson, ER: estrogen receptor, PR: progesterone receptor.

Supplementary Table 3: Compliance in both arms.

	FMD (N=65)	**Regular diet (N=64)**
Compliant – all cycles and surgery		
Yes	10 (15.4%)	59 (92.2%)
No	55 (84.6%)	5 (7.8%)
Compliant – all cycles*		
Yes	13 (20.0%)	
No	52 (80.0%)	
Compliant – half of cycles**		
Yes	22 (33.8%)	
No	43 (66.2%)	
Compliant – first cycle		
Yes	53 (81.5%)	
No	11 (16.9%)	
Unknown	1 (1.5%)	
Reason for early stop FMD		
Taste	26 (51.0%)	
Nausea	10 (15.4%)	
Hunger	5 (9.8%)	
Stop chemotherapy	2 (3.9%)	
Other	8 (35.3%)	

Compliance given per group. * Compliance is defined as patients complied the FMD or regular diet all cycles of their treatment arm. ** Compliance is defined as patients complied to the FMD or regular diet half of the cycles of their treatment arm. Regular diet patients were not compliant if they were fasting for at least one cycle on their own. Abbreviations: FMD: Fasting mimicking diet.

Supplementary Table 4: Grade III/IV toxicity during 8 cycles of AC-T/FEC-T in both groups.

Grade III/IV	**FMD (N=65)**	**Control (N=64)**	***P*-value (ITT)**
Total	49 (75.4%)	42 (65.6%)	0.224
Neutropenic fever	22 (33.8%)	12 (18.8%)	0.052
Neutropenia	33 (50.8%)	22 (34.4%)	0.060

Grade III/IV side effects were scored according CTCAE4.03. Each side effect was scored maximal once per patient during the course. FMD; fasting mimicking diet, C: compliance, ITT: intention to treat. Crosstabs were used.

Supplementary Table 5: Associations between tumor, patient characteristics, treatment arm and efficacy of chemotherapy.

Parameter		N	pCR	Univariate analysis	*P* value	Multivariate analysis	*P* value
BMI	< 25kg m-2	59	11 (18.6%)	1.000	**0.032**	1.000	0.067*
	≥ 25kg m-2	69	4 (5.8%)	0.269 (0.081-0.895)		0.308 (0.087-1.085)	
HR receptor status	ER +	106	8 (7.5%)	1.000	**0.003**	1.000	**0.006***
	ER -	22	7 (31.8%)	5.717 (1.809-18.07)		5.626 (1.624-19.48)	
Stage	I/II	100	13 (13.0%)	1.000	0.402		
	III	28	2 (7.1%)	0.515 (0.109-2.430)			
CT schedule	AC-T	98	14 (14.3%)	1.000	0.136		
	FEC-T	30	1 (3.3%)	0.207 (0.026-1.643)			
ITT	FMD	65	7 (10.8%)	0.830 (0.282-2.442)	0.735	0.581 (0.174-1.942)	0.378
	Regular diet	63	8 (12.7%)	1.000		1.000	
PP	FMD C	22	3 (13.6%)	1.150 (0.269-4.911)	0.850	1.074 (0.216-5.344)	0.816
	FMD NC	43	4 (9.3%)	0.747 (0.204-2.734)	0.660	0.478 (0.113-2.017)	0.315
	Regular diet C	58	7 (12.1%)	1.000		1.000	
Parameter		**N**	**MP 4/5 vs. 1/2/3**	**Univariate analysis**	***P* value**	**Multivariate analysis**	***P* value**
BMI	< 25kg m-2	59	22 (37.3%)	1.000	**0.013**	1.000	**0.016***
	≥ 25kg m-2	69	12 (17.4%)	0.354 (0.157-1.801)		0.354 (0.152-0.825)	
HR receptor status	ER +	106	24 (22.6%)	1.000	**0.032**	1.000	0.082*
	ER -	22	10 (45.5%)	2.847 (1.096-7.396)		2.437 (0.894-6.646)	
Stage	I/II	100	30 (30.0%)	1.000	0.105		
	III	28	4 (14.3%)	0.389 (0.124-1.218)			
CT schedule	AC-T	98	26 (26.5%)	1.000	0.988		
	FEC-T	30	8 (26.7%)	1.007 (0.399-2.540)			
ITT	FMD	65	20 (30.8%)	1.556 (0.703-3.441)	0.275	1.526 (0.655-3.556)	0.328
	Regular diet	63	14 (22.2%)	1.000		1.000	
PP	FMD C	22	10 (45.5%)	3.194 (1.115-9.152)	**0.031**	4.109 (1.297-13.02)	**0.016**
	FMD NC	43	10 (23.3%)	1.162 (0.449-3.006)	0.757	0.926 (0.328-2.614)	0.884
	Regular diet C	58	12 (20.7%)	1.000		1.000	
Parameter		**N**	**Radiological response CR+PR vs. SD+PD**	**Univariate analysis**	***P* value**	**Multivariate analysis**	***P* value**
BMI	< 25kg m-2	50	43 (86.0%)	1.000	0.214		
	≥ 25kg m-2	55	42 (76.4%)	0.526 (0.191-1.448)			
HR receptor status	ER +	83	67 (80.7%)	1.000	0.907		
	ER -	22	18 (81.8%)	1.075 (0.320-3.614)			
Stage	I/II	86	71 (82.6%)	1.000	0.376		
	III	19	14 (73.7%)	0.592 (0.185-1.893)			
CT schedule	AC-T	82	68 (82.9%)	1.000	0.334		
	FEC-T	23	17 (73.9%)	0.583 (0.195-1.742)			
ITT	FMD	53	47 (88.7%)	2.886 (1.012-8.227)	**0.047**	3.168 (1.062-9.446)	**0.039**
	Regular diet	52	38 (73.1%)	1.000		1.000	
PP	FMD C	19	18 (94.7%)	6.882 (0.832-56.91)	**0.074**	6.365 (0.759-53.37)	0.088
	FMD NC	34	29 (85.3%)	2.218 (0.706-6.963)	0.172	2.715 (0.785-9.390)	0.115
	Regular diet C	47	34 (72.3%)	1.000		1.000	

Regression models are used to measure associations. The radiological response was scored according RECIST and given for complete response + partial response vs. stable disease +progression disease. Abbreviations: pCR: pathological complete response, BMI: body mass index, HR: hormone receptor status, FMD: fasting mimicking diet, C: Compliant, NC: Not compliant, ITT: Intention to treat, PP: Per protocol, MP: Miller and Payne, CR: complete response, PR: partial response, SD: stable disease, PD: progression disease. * Result from ITT multivariate analysis.

Supplementary Table 6: Metabolic and endocrine parameters first cycle.

Parameter	FMD (N=65)	FMD-C (N=53)	FMD-NC (N=11)	Regular diet (N=64)	Regular diet-C (N=59)	*P*-value (ITT)	*P*-value (PP)
Glucose (3.1-6.4mmol/L)	5.1	4.9	6.4	5.7	5.9	**0.062**	**0.006**
Insulin (0-20mU/L)	6.0	4.0	19.0	12.0	13.5	**0.004**	**<0.0001**
IGF-1 (5.4-24.3nmol/L)	17.4	17.3	20.0	17.9	18.0	0.925	0.602
IGF-BP3 (2.2-5.8mg/L)	3.7	3.7	3.8	4.0	4.0	0.601	0.570
CRP (<5.0mg/L)	3.0	3.5	3.0	2.0	2.0	**0.007**	**0.010**
Ketone bodies						**<0.0001**	**<0.0001**
Pos	32 (74.4%)	14 (93.3%)	10 (35.7%)	5 (12.5%)			
Neg	11 (25.6%)	1 (6.7%)	18 (64.3%)	35 (87.5%)			

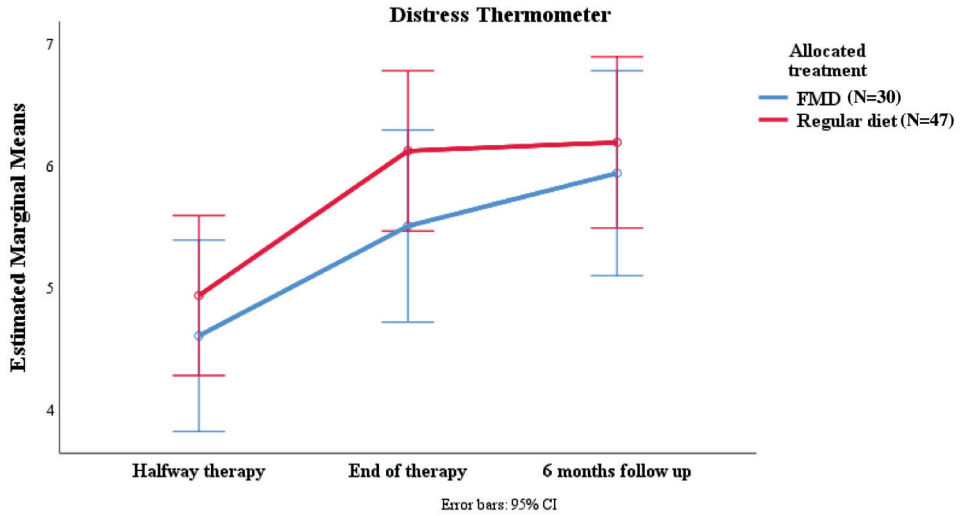
Insulin-like growth factor 1, IGF-BP3 insulin- like growth factor binding protein 3. ITT: intention to treat, PP: per protocol. Independent t-tests and Mann-Whitney tests were used.

4

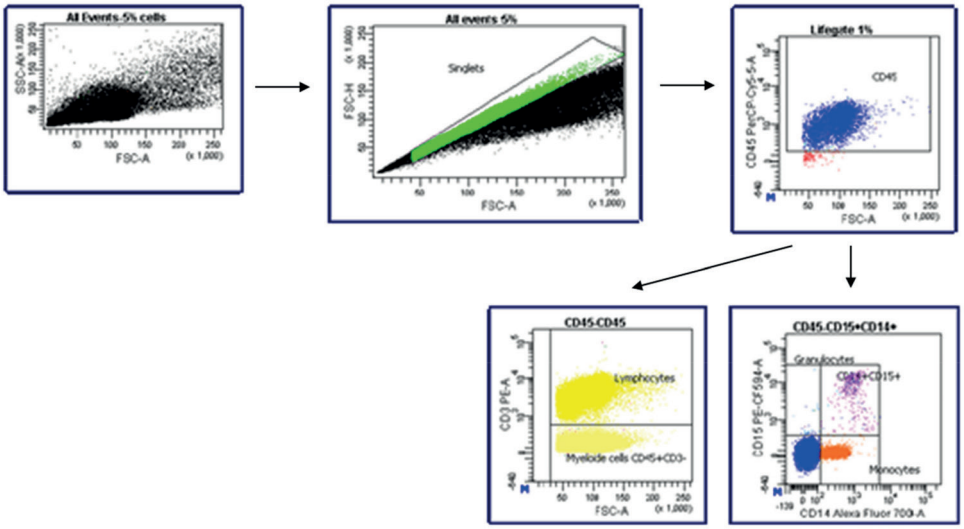
Supplementary Table 7: γ-H2AX intensity in peripheral blood mononuclear cells.

Parameter	N	Before CT (SE)	3 hours after CT (SE)	*P* value each group	*P* value between groups
CD45+CD3+ T-lymphocytes	FMD (n = 16)	143.4 (10.0)	173.4 (16.4)	**0.011**	**0.045**
	Regular diet (n = 11)	147.7 (8.6)	206.8 (14.6)	**0.001**	
CD45+CD14+CD15- monocytes	FMD (n = 16)	229.1 (16.0)	273.2 (21.5)	**0.025**	0.388
	Regular diet (n = 10)	230.7 (11.1)	309.5 (30.9)	**0.021**	
CD45+CD3- non-T cells	FMD (n = 16)	175.7 (10.6)	231.0 (21.3)	**0.025**	0.856
	Regular diet (n = 11)	185.6 (9.4)	237.8 (23.9)	**0.037**	

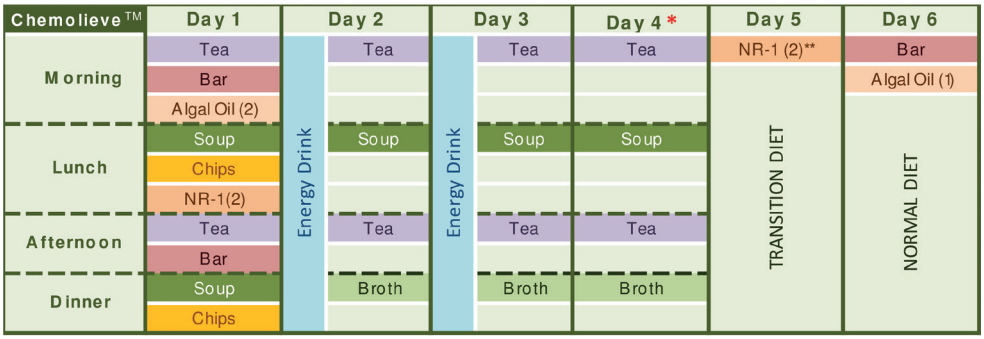
Paired comparison between pre- and 3 hours post- chemotherapy for different cell types in peripheral blood mononuclear cells. γ-H2AX intensity is given as mean. FMD; fasting mimicking diet. Independent and paired t-tests were used.



Supplementary figure 1: Psychosocial distress given for 3 timepoints: halfway therapy, at the end of therapy and at six months follow-up. Error bars indicate the 95%CI of the mean. FMD: fasting mimicking diet. CI: confidence interval. FMD N=30 and regular diet N=47.



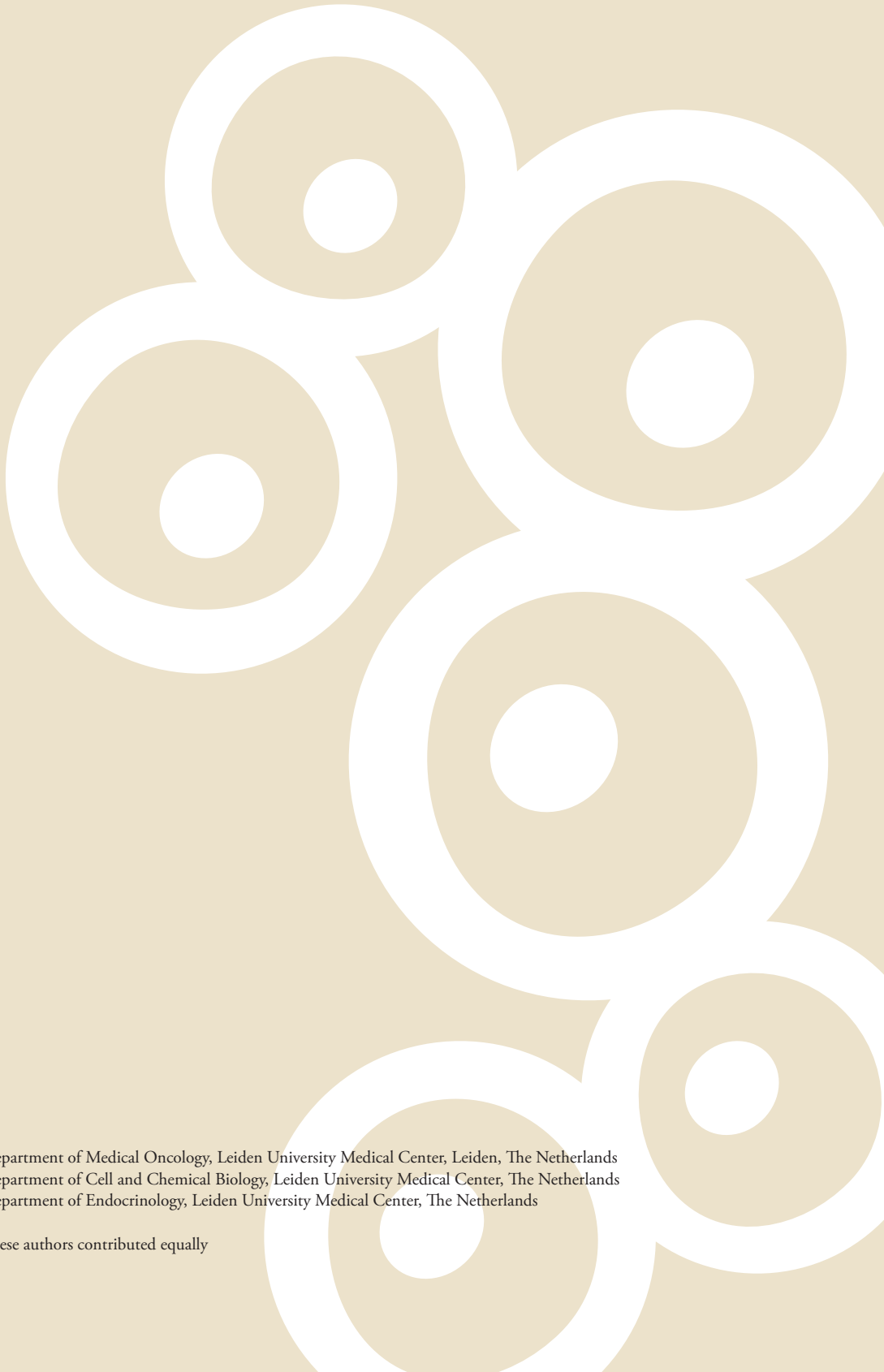
Supplementary figure 2: Gating strategy: The CD45+ cells were gated, after which the CD3+ T-lymphocytes, CD3- non-T cells (also harboring B lymphocytes) or CD14+CD15- monocytes were analyzed for the geomean (as measure for the intensity) of γ-H2AX.



Supplementary figure 3: Chemolieve fasting mimicking diet schedule.

Part II

IGF-1 and insulin pathway in cancer treatment



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Chapter 5

Unraveling the Resistance of IGF-Pathway Inhibition in Ewing Sarcoma

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Abstract

Insulin-like growth factor one receptor (IGF1R) inhibitors are effective in preclinical studies, but so far, no convincing benefit in clinical studies has been observed, except in some rare cases of sustained response in Ewing sarcoma patients. The mechanism of resistance is unknown, but several hypotheses are proposed. In this review, multiple possible mechanisms of resistance to IGF-targeted therapies are discussed, including activated insulin signaling, pituitary-driven feedback loops through growth hormone (GH) secretion and autocrine loops. Additionally, the outcomes of clinical trials of IGF1-targeted therapies are discussed, as well as strategies to overcome the possible resistance mechanisms. In conclusion, lowering the plasma insulin levels or blocking its activity could provide an additional target in cancer therapy in combination with IGF1 inhibition. Furthermore, because Ewing sarcoma cells predominantly express the insulin receptor A (IRA) and healthy tissue insulin receptor B (IRB), it may be possible to synthesize a specific IRA inhibitor.

Introduction

Insulin-like growth factor-1 (IGF1) and other members of the IGF1 receptor (IGF1R) pathway have been associated with the development, progression and metastasis of cancer and resistance to anticancer therapies[1,2]. Convincing preclinical evidence supporting the efficacy of IGF1R pathway inhibition in the treatment of cancer has led to the development of many IGF1R pathway inhibitors[3-7], which have been investigated in numerous clinical trials in breast cancer; Ewing sarcoma and various other types of solid tumors, like non-small cell lung cancer, hepatocellular, gastric and esophageal carcinoma (Table 1)[8-27]. Unfortunately, no convincing benefit of IGF1R pathway inhibitors has been found in these studies[28,29], except in some rare cases of a sustained response in patients with Ewing sarcoma and adrenocortical carcinoma[10,30]. In Ewing sarcoma, IGF1R was early identified as a possible target for the treatment, of which the preclinical results were published, where Scotlandi et al. showed the proliferative effect of IGF1 on Ewing sarcoma cell lines[31]. However, later clinical trials exhibited disappointing results due to therapy resistance to IGF1R inhibition (Table 1).

Possible resistance mechanisms that could explain the disappointing results of IGF1R inhibitors in the clinical setting are: (1) inadequate inhibition of the pathway downstream of IGF1R, as this pathway can be activated through the IGF1R but, also, through the insulin receptor A (IRA) or hybrids between the two receptors[32-34]; (2) the disruption of negative feedback loops in the pituitary, whereby the IGF1R ligands IGF1, IGF2 and insulin, and other endocrine-signaling molecules such as the growth hormone (GH) and glucose, increase by inhibiting the receptors, leading to tumor growth[35,36]; (3) the existence of autocrine or paracrine feedback loops in the tumor, through which the IGF1R pathway is continuously activated, perhaps via intracellular routes[12,37] and (4) tumor growth and survival due to other driver mutations downstream of the IGF1R pathway or in other oncogenic pathways, which makes inhibiting IGF1R irrelevant[38,39].

This review covers the current knowledge of IGF1R pathway inhibitors and their possible resistance mechanisms that may explain the disappointing results of IGF1R pathway inhibitors in the clinical setting. We also discuss potential strategies to overcome the resistance mechanism to guide future IGF1R inhibitor research and therapy.

Table 1: IGF1 receptor (IGF1R) pathway inhibitor monotherapy in clinical studies involving patients with Ewing sarcoma and other types of solid tumors.

IGF1R Antagonist Monoclonal Antibodies					
Study	**Trial**	**Patients**	**Compound**	**Endocrine Side Effects and Biomarkers**	**Clinical Response**
Haluska et al., 2007 [8]	Phase I	24 patients with distinct solid tumors or sarcoma	Figitumumab (CP-751, 871)	• Hyperglycemia, increase of insulin, GH and IGF-1	7/12 SD, 1 long responder
Tolcher et al., 2009 [9]	Phase I	53 patients with distinct tumors and sarcoma	Ganitumab (AMG 479)	• 5 patients with hyperglycemia • IGF1 levels increase during treatment • patients with complete response possess IGF1R in metastases	1 CR, 2 PR
Olmos et al., 2010 [10]	Phase I	29 patients with distinct sarcoma (16 Ewing sarcoma)	Figitumumab (CP-751, 871)	• 5 patients with hyperglycemia	1 CR, 1 PR (both Ewing sarcoma), 8 SD
Kurzrock et al., 2010 [11]	Phase I	35 patients with distinct solid tumors or sarcoma (9 Ewing)	Teprotumumab (R1507, RO4858696)	• 2 patients with hyperglycemia • IGF1 serum levels increase during treatment	2/33 PR, 14/33 SD
Juergens et al., 2011 [12]	Phase I/II	31 (phase 1) and 107 (phase 2) patients with distinct sarcoma (16 and 107 Ewing, respectively)	Figitumumab (CP-751, 871)	• 3 patients with grade 3 hyperglycemia • IGF1 baseline levels were prognostic for survival, higher levels were associated with better survival • Highest IGF1 level showed a reduced clinical benefit • Increase of serum levels of IGF1, GH and insulin during treatment	15/106 PR, 25/106 SD
Malempati et al., 2012 [13]	Phase I/II	47 patients with distinct solid tumors or sarcoma (35 Ewing)	Cixutumumab (IMC-A12)	• 14/44 patients hyperglycemia • Increase in serum levels IGF-I and IGFBP-3 • No change in serum levels IGF-II and IGFBP-2	3/25 PR, 5/25 SD (Ewing sarcoma), 2/13 SD (Other)
Murakami et al., 2012 [14]	Phase I	19 patients with distinct solid tumors	Ganitumab (AMG 479)	• IGF1 and IGFBP3 increased after administration, GH not • IGF1 and IGFBP3 were not predictive or prognostic for a response of treatment	7/19 SD
Tap et al., 2012 [15]	Phase II	38 patients with distinct sarcoma (22 Ewing sarcoma)	Ganitumab (AMG 479)	• 5/38 hyperglycemia (2 pts grade III) • IGF1 serum level increased	2/35 PR, 21/35 SD
Schöffski et al., 2013 [16]	Phase II	113 patients with distinct sarcoma (18 Ewing sarcoma)	Cixutumumab (IMC-A12)	• 22/111 hyperglycemia (6 patients, grade III)	2/111 PR, 44/111 SD
Pappo et al., 2014 [17]	Phase II	163 patients with distinct sarcoma	R1507	• 15/163 hyperglycemia (4 patients, grade III)	4/163 PR, 42/163 SD
Abou-Alfa et al., 2014 [18]	Phase II	24 patients with hepatocellular carcinoma	Cixutumumab (IMC-A12)	• 24/24 hyperglycemia (11 patients, grade III)	7/24 SD
Frappaz et al., 2016 [27]	Phase II	20 patients with distinct sarcoma (6 Ewing sarcoma)	Dalotuzumab (Mk-0646)	• 3/20 hyperglycemia	1 PR

IGF1R/IR Dual Inhibitors					
Study	**Trial**	**Patients**	**Compound**	**Endocrine Side Effects and Biomarkers**	**Clinical Response**
Puzanov et al., 2014 [19]	Phase I	95 patients with distinct solid tumors and sarcoma	Linsitinib (OSI-906)	• 4 patients with hyperglycemia • Efficacy independent of KRAS mutation • Increase of IGF1 serum levels	30/95 SD
Jones et al., 2015 [20]	Phase I	97 patients with distinct solid tumors and sarcoma	Linsitinib (OSI-906)	• 37% hyperglycemia • Increase of IGF1 serum levels	2/66 PR, 27/66 SD
Fassnacht et al., 2015 [21]	Phase III	90 patients with adrenocortical carcinoma	Linsitinib (OSI-906)	• 2 patients with grade III hyperglycemia • IGF1 serum levels increase	3/90 PR
Barata et al., 2018 [22]	Phase II	17 patients with metastatic castrate-resistant prostate cancer	Linsitinib (OSI-906)	• 8 patients with hyperglycemia	1/17 PR, 8/17 SD
Chiappori et al., 2016 [23]	Phase II	29 patients with small cell lung cancer	Linsitinib (OSI-906)	• 7/29 hyperglycemia (1 patient grade III)	1/29 SD
IGF1/2 Neutralizing Antibody					
Study	**Trial**	**Patients**	**Compound**	**Endocrine Side Effects and Biomarkers**	**Clinical Response**
Haluska et al., 2014 [24]	Phase I	43 patients with distinct solid tumors (1 Ewing)	Medi-573	• 1 Patient with hyperglycemia • No elevation of insulin or GH • IGF1 and IGF2 suppressed	13/39 SD
Iguchi et al., 2015 [25]	Phase I	10 patients with distinct solid tumors	Medi-573	• 1 patient with hyperglycemia • IGF1/2 decreased	4/10 SD
De Bono et al., 2020 [26]	Phase I	125 patients with distinct solid tumors and sarcoma	Xentuzumab	• 2 patients with grade III hyperglycemia • IGF bioactivity decreased, total levels did not decrease • No effects on IGF2	2/125 PR, 55 SD

SD: stable disease, PR: partial response and CR: complete response. IGF1/2: Insulin-like growth factor-1 and 2. GH: growth hormone.

IGF1/Insulin Pathway and Cancer

The IGF1/insulin pathway regulates growth in normal tissues and is associated with cancer development and reduced cancer survival rates. The pathway has been extensively described before[7,40]. Briefly, IGF1R, IRA or hybrid receptors can be activated by binding IGF1, IGF2 and insulin ligands, which leads to activation of the RAS/MAPK and PI3K/AKT downstream pathways (Figure 1)[41,42]. Each ligand has a specific affinity for each receptor[43,44]. The IGF1R and IRA are both frequently overexpressed in distinct types of cancers, including breast, colorectal and prostate carcinoma[44]. Additionally, although recurrent activating mutations in the IGF1R are unknown, single-nucleotide polymorphisms with unknown significance have been described[45].

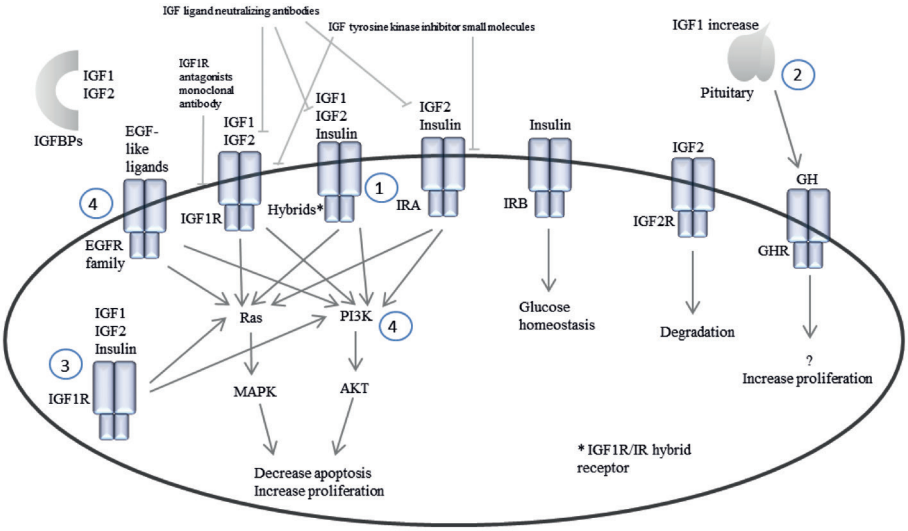

Figure 1: IGF and insulin signaling. Receptor hybridization, activation and downstream signaling of IGF, insulin and GH receptors. IGF1/2 = insulin-like growth factor 1/2, IGF1R = insulin-like growth factor 1 receptor, IGF2R = insulin-like growth factor 2 receptor or mannose 6-phosphate receptor, IR(A/B) = insulin receptor (A/B), GH = growth hormone, GHR = growth hormone receptor, EGF = epidermal growth factor and EGFR = epidermal growth factor receptor.

Epidemiological studies have shown a relationship between high circulating IGF1 levels and cancer incidence[1,46]. Counterintuitively, high levels of IGF1 in the plasma and cytoplasm of the cancer cells seem to be a prognostic for the improved survival in cancer patients[12,17,47,48]. High baseline IGF1 was counterintuitively associated with improved event-free survival (EFS) in Ewing sarcoma patients. Additionally, in Ewing sarcoma, patients with metastatic disease exhibited lower IGF1 levels when compared to the localized disease, suggesting that further progression of the disease negatively modulates IGF1 levels, which would explain the higher EFS in patients with higher IGF1 levels[47]. However, during systemic treatment, increases of the levels of IGF1 seem to not be predictive for the treatment outcome[48,49].

Insulin Receptor and Insulin

The IR, also known as INSR, has two different isoforms: IRA and insulin receptor B (IRB)[44]. The difference between these two receptor variants is that IRA is 12 amino acids shorter than IRB, due to the alternative splicing of exon 11[44]. This difference results in a distinct affinity for their ligands. Insulin and IGF2 can bind the IRA, while IRB only binds insulin with high affinity, whereby different downstream pathways are activated: IRB fulfills an important role in glucose homeostasis, whereas IRA, the embryological splice variant, is the dominantly expressed isoform in many cancer cells[32]. The IRA activates proliferation and antiapoptotic pathways, and its expression is associated with resistance to cancer treatment[50]. Interestingly, reduced insulin levels as a result of a very low-caloric diet and weight loss are associated with a relative IRB mRNA increase, without affecting the total gene expression of IR in adipose tissue[51]. Hyperinsulinemia, in the context of obesity, insulin resistance or type 2 diabetes mellitus, is also associated with an increased risk of cancer and cancer therapy resistance[52,53].

IGF2R and IGF Binding Proteins

IGF2 can bind to the IGF1R and IRA, but it can also bind to the IGF2 receptor (IGF2R), which is also known as the Cation-Independent Mannose-6-Phosphate Receptor. This receptor is considered a tumor suppressor, as both the ligand and receptor are internalized and degraded after binding, reducing the bioavailability of IGF2 and, thus, inhibiting the proliferative effects of IGF2[54,55]. Moreover, six IGF-binding proteins (IGFBPs) exist, which can bind and inactivate IGF1 and IGF2 by blocking them from binding to their receptor while protecting them from degradation and increasing their half-life[56,57]. Interestingly, these binding proteins simultaneously enhance IGF signaling locally and increase IGF availability for eventual binding to the IGF1R[57]. Some data suggest that higher levels of IGFBP3, the main IGF-binding protein, are associated with an increased risk of cancer[46], while others support an inverse association[47,58]. For example, a significant correlation between increased IGFBP mRNA expression in tumor tissues and increased patient survival has been reported. Furthermore, an increase in IGF1R signaling in response to IGFBP3 downregulation has been indicated as a possible resistance mechanism in cancer treatment[59]. Therefore, IGFBPs may be a possible tumor suppressor in tumors with active IGF1R signaling[59]. Apart from the endocrinal function, IGFBPs also have functions in a variety of other processes. IGFBPs can bind to cell-surface receptors and internalize into the cell. After internalization, the IGFBPs can induce apoptosis and change transcriptional regulation[59]. However, the exact role of the IGFBPs in different cancers remains unclear and requires more study.

IGF1R Pathway Inhibitors and Resistance

IGF1R pathway inhibitors possess different properties to inhibit the IGF pathway and can be classified into three groups: (1) IGF1R antagonist monoclonal antibodies, (2) IGF tyrosine kinase inhibitor small molecules and (3) IGF ligand neutralizing antibodies[7,40].

IGF1R Antagonist Monoclonal Antibodies

IGF1R antagonist monoclonal antibodies bind selectively to IGF1R with high affinity and block the interaction of IGF1R with its ligands, inducing the internalization and degradation of IGF1R[6,60]. The IGF1R antagonist monoclonal antibodies ganitumab (AMG-479)[14], dalotuzumab (MK-0646)[28], cixutumumab (IMC-A12)[13], teprotumumab (R-1507)[17] and figitumumab (CP-751871) were tested in clinical studies (Table 1)[61]. These inhibitors induced downregulation of the IGF1R homodimers and hybrid receptors (e.g., IGF1R/IRA), while the integrity of IRA homodimers and their activation by insulin or IGF2 was not influenced[61]. Indeed, Schmitz et al. found decreased IGF1R expression in patients treated with figitumumab but the absence of the inhibition of AKT, leading to the hypothesis that the downstream pathway remains activated[61]. In clinical studies, figitumumab and other IGF1R antibody antagonists were shown to increase circulating IGF1 and growth hormone (GH) levels, as well as glucose and insulin plasma levels[12,24,61]. Thus, the activation of downstream pathways and an increase of several growth factors despite the IGF1R blockade may explain the failure of these compounds in the clinical setting. Patients whose tumors express IGF1R but not IRA may, however, benefit from IGF1R inhibitors, which might explain why these compounds caused long-lasting tumor response in two cases in clinical trials (Table 1)[10].

IGF1R Tyrosine Kinase Inhibitor Small Molecules

IGF tyrosine kinase inhibitor small molecules, such as linsitinib (OSI-906), BMS-754807 and KW-2450, target both the IGF1R and the insulin receptor (IR) and their hybrid receptors[19,62]. Puzanov et al. found that linsitinib decreased phosphorylation of the IGF1R and IR in peripheral blood mononuclear cells (PBMC)[19]. Accordingly, hyperglycemia and hyperinsulinemia are common side effects of these agents due to cross-reactivity with the insulin receptor B (IRB), which is involved in glucose metabolism[19]. This can lead to the discontinuation of treatment and may also cause resistance to this kind of inhibitor. The IGF tyrosine kinase inhibitor small molecules did not show a survival benefit in advanced or metastatic adrenocortical carcinoma in a large phase 3 trial[21] and showed disappointing results in other (small) clinical trials (Table 1)[19,20,22,23].

IGF Ligand Neutralizing Antibodies

The IGF ligand neutralizing antibodies dusigitumab (MEDI-573) and xentuzumab (BI 836845) inhibit the IGF1R and the IRA by binding and neutralizing both IGF1

and IGF2 ligands[24]. In contrast with the IGF1R antagonist monoclonal antibodies and IGF1R tyrosine kinase inhibitors, these compounds do not cause hyperglycemia, as they do not compromise insulin action[24,25]. However, as insulin can also activate the IRA and hybrid receptors, the IGF1R pathway may not be adequately inhibited. Only a few small clinical trials in heavily pretreated patients have been performed to date that showed only a few partial responses (Table 1)[10,25,26,30,63].

Strategies to Overcome Resistance Mechanisms of IGF1R-Inhibitors

Several strategies have been proposed to overcome the mechanisms of resistance to the different IGF1R inhibitor types.

Activation of IRA and/or Hybrid Receptors

As previously described, the pathway downstream of the IGF1R may be inadequately inhibited by all three distinct inhibitors. While the IGF1R itself is appropriately inhibited, the IRA and its hybrid receptors may still be activated by its ligands (IGF1, IGF2 and/or insulin)[32-34]. This indicates that signaling through the IRA may be an important resistance mechanism to anti-IGF1R treatment. In support of this, it has been shown that IGF1R inhibition can lead to compensatory IR activation in colorectal cancer, ovarian carcinoma, and Ewing sarcoma in vitro[64]. The addition of a specific IRA inhibitor would be required to overcome this, as nonspecific IR inhibitors (e.g., the compound S961) and IGF1R tyrosine kinase inhibitor small molecules cause hyperglycemia and compensatory hyperinsulinemia[19,65]. However, specific IRA inhibitors are not yet available. Developing a specific IRA antagonist may serve as a novel treatment option combined with IGF1 inhibitors, as this may be an option with knowledge about the crystal structure[66]. Alternatively, (short-term) fasting during treatment with an IGF1R inhibitor may have similar effects, as it causes a significant decrease in insulin serum levels[67,68]. Longer periods of dietary restriction are required to significantly reduce IGF2 levels[67], which could still activate downstream pathways through IRA activation. Therefore, more studies are needed to evaluate the efficacy of (short-term) fasting as an adjunct to IGF1R treatment in patients with cancer[68-70].

Disruption of Negative Feedback

Another mechanism of resistance to IGF1R pathway inhibition in solid tumors is the increase of plasma GH due to the lack of negative feedback by IGF1 both in the pituitary and hypothalamus, which enables a higher release of GH [71]. This phenomenon is seen in clinical trials with IGF1R antagonist monoclonal antibodies and IGF1R tyrosine kinase inhibitor small molecules, which may blunt the efficacy of these drugs. Additionally, independent potentiating effects of GH that are not mediated by IGF1 have been demonstrated on breast cancer cells [72-74]. For example, GH induces

tumor growth without increasing IGF1[74]. This is supported by the fact that several cancers express GH receptors (GHR), and GHR positivity is predictive of a worse outcome[75-78]. However, our preclinical data did not show a stimulatory effect of GH on Ewing sarcoma cells in vitro (Appendix A, Figure A1). Additionally, GH diminishes the anti-IGF1R tumor inhibition activity, suggesting that increased GH is a plausible cause of IGF1R inhibitor failure in the clinic [74]. Another preclinical study showed that GH causes chemoresistance despite the presence of an IGF1R antagonist monoclonal antibody. In this study, the cancer cells became chemosensitive again in the presence of the GH antagonist pegvisomant[79]. Increased GH levels cause increased IGF1 levels, hyperinsulinemia, insulin resistance and, ultimately, hyperglycemia[36]. Accordingly, patients with acromegaly, who have high GH plasma levels, show a higher incidence of cancer [80,81], while patients with Laron syndrome who are resistant to GH due to a defective GHR and patients with GH deficiency have reduced cancer susceptibility [82,83].

It is proposed that high levels of IGF1 cause resistance to IGF1R inhibitors due to a competitive affinity to the IGF1R receptor. For example, an excess of IGF1 reverses the inhibitory effect of figitumumab in preclinical studies, which is presumed to be due to their similar affinity for the IGF1R [84]. A solution to overcome this may be to increase the dose of the IGF1R inhibitor or to decrease the IGF1 serum levels by adding a GH antagonist, such as pegvisomant [36,85]. In a clinical phase I study (NCT00976508), two patients with Ewing sarcoma had partial responses to the combination treatment of figitumumab and pegvisomant [63]. Unfortunately, the study was stopped prematurely due to the cessation of figitumumab production.

Furthermore, increased insulin secretion activates the IR and may explain the suboptimal therapeutic benefits. Again, an IRA inhibitor and/or short-term fasting in combination with IGF1 inhibitors may be an effective approach to decrease insulin signaling and adequately inhibit the downstream pathway. Our preclinical data supports that insulin clearly stimulates cell growth and blocks the apoptosis (Appendix A, Figure A1) of Ewing sarcoma cells in vitro. Additionally, stimulation with insulin reversed an increase in PARP cleavage, a marker for apoptosis, induced by IGF1R blocking. Furthermore, stimulation with insulin increased AKT phosphorylation in cells treated with an IGF1R inhibitor. This indicates that lowering insulin levels or blocking the IRA may increase the efficacy of IGF1R inhibitors. Additionally, IGF1R blocking can induce hyperglycemia and hyperinsulinemia in patients[8], which could activate the IRA in response to IGF1R inhibition. However, the exact mechanism for the observed hyperglycemia as a side effect of IGF1 inhibitors is unclear, but cross-reactivity with the IRB, which is involved in the glucose metabolism, is likely to be an important factor [19].

Autocrine Loops in the Tumor

Autocrine activation by the tumor is described in preclinical studies, whereby both the IGF1R and one of its ligands are expressed by the tumor or surrounding tumor stroma[12,37]. This would continuously activate the IGF1R pathway, perhaps even via an intracellular route, making it impossible to inhibit with an antibody-based approach. In line with this, lowering the serum GH and/or IGF1 by somatostatin analogs does not always have antitumor effects in clinical studies in breast cancer[75].

Activation or Mutation of Other Pathways

Finally, resistance may occur when the IGF1R pathway is activated through downstream mutations of the pathway (such as PTEN) or in bypassing oncogenic pathways (such as epidermal growth factor receptor (EGFR))[38,39].

To overcome these resistance mechanisms, it may be necessary to utilize combination therapies to simultaneously block all pathways contributing to tumor growth[40,86]. Combination therapies with IGF1 inhibitors are extensively reviewed elsewhere[40,86]. IGF ligand neutralizing antibodies are good candidates due to the lack of side effects, such as hyperinsulinemia and hyperglycemia, and may be combined with EGFR family inhibitors, Cyclin-dependent kinase (CDK) inhibitors, endocrine therapy or immune checkpoint inhibitors[40].

Use of Biomarkers

If IGF1R is not (overly) expressed by the tumor, it is probably not meaningful to use IGF inhibitors, as the pathway is probably not involved in tumor genesis, growth and therapy resistance. In these cases, it is necessary to determine biomarkers, such as secondary mutations, receptor levels and isoform identification of the IR to select patients who may benefit from treatment. It is particularly important to make use of biomarkers such as the expression levels of IGF1 and IGF2 in tumors with autocrine loops to predict if a patient will benefit from treatment with an IGF inhibitor.

The described resistance mechanisms and potential strategies to combat them are summarized in Table 2 and Figure 1.

Table 2: Resistance mechanisms.

Resistance Mechanism	Example	Proposed Solution
Activation of the pathway trough IRA or hybrid receptors	IGF1R is inhibited, but IRA and hybrids receptors still activate the downstream pathway	Add an IRA inhibitor Short-term fasting
Abrogation of negative feedback	High levels of IGF1 still activate the receptor due to a competitive affinity	Increase dose of IGF1-inhibitor Decrease IGF1 levels by adding GH antagonist [36, 79]
	High levels of insulin activate IRA and hybrid receptors	Add an IRA inhibitor Short-term fasting
	High levels of glucose	Short-term fasting
	High levels of GH activate the GHR and causes an increase in IGF1 serum levels	Adding GH antagonist
Autocrine loops in the tumor	Expression of the receptor and ligand by the tumor Expression of the receptor by the tumor and the ligands by stroma	IGF1 inhibitors not effective, biomarker studies necessary to select patient who does not benefit from treatment
Other pathways mutated	Other drivers likeEGFR) or secondary mutations (PI3K or PTEN)	Combination therapy [40] IGF1 inhibitors not effective, biomarker studies necessary to select patient who does not benefit from treatment

Ewing Sarcoma vs. Other Solid Tumors

Ewing sarcoma is a rare cancer[87] that is characterized by a translocation that increases the bioactivity of IGF1[47,88]. In 85% of cases, the somatic translocation t(11;22) results in the aberrant product of the Ewing sarcoma breakpoint region 1 (EWSR1) gene and Friend leukemia virus integration 1 (FLI1) gene[89] and other variants of the involved gene families in the remaining cases[90]. The product is the EWSR1-FLI1 fusion protein, which binds—amongst other things—to the IGFBP3 promoter, which leads to a dramatic reduction in the expression of IGFBP3[47,58,88] without inhibiting the availability of IGF1[91-93]. IGF1R was early identified as a target in Ewing sarcoma, as the IGF1R was highly expressed in Ewing sarcoma cell lines in addition to the expression of IGF1, which may thus signal in an autocrine loop[31]. Additionally, the IGF1R inhibition experiments reduced the growth of Ewing sarcoma both in vitro[94] and in vivo[95]. However, in clinical trials, IGF1R inhibitory compounds have not shown the same efficacy (Table 1). Nonetheless, a few patients with Ewing sarcoma experienced a long-term response to IGF1R inhibitor therapy[10,30]. It is not clear why only these few patients showed a clinical benefit. The activation of the IRA may cause resistance to specific IGF1R inhibitors as resistant cells switch from IGF1-IGF1R signaling to IGF2/insulin/IRA signaling, activating the same proliferative downstream pathways[32]. This may indicate that these responding patients with Ewing sarcoma did not have active IRA signaling. However, there is no data to support this, but it should be investigated further. Additionally, a meta-analysis of five clinical trials by Amin et al. showed a potential synergistic effect of mechanistic

Target of Rapamycin (mTOR) inhibitors and IGF1R monoclonal antibodies in Ewing sarcoma patients[96]. Since mTOR signaling is a downstream target of both the IGF1R and the IRA, inhibiting mTOR might indeed be a viable treatment option, in addition to IGF1R inhibition. Therefore, the lack of a response in patients with Ewing sarcoma may reflect alterations in pathways that are not disrupted by IGF1R inhibition and/or the other resistance mechanisms mentioned above. Garofalo et al.[97] identified multiple functional pathways associated with IGF1R inhibition resistance. Of the pathways identified, the MAPK kinase pathway and, again, the IGF2/insulin/IRA pathways seem to be important for the resistance to IGF1R inhibition, in addition to a variety of other pathways. Furthermore, the IGF2 and IRA expression increased in vitro in response to IGF1R inhibition with figitumumab[97]. Together, this indicates that, for a better efficacy of IGF1R inhibition in the clinic, either better IRA inhibition is needed and a better understanding of other pathways involved in resistance to IGF1R inhibition like mTOR or the pathways outlined by Garofalo et al. are required[97]. Through this, we can begin to better understand the pathways that could be co-targeted in conjunction with IGF1R inhibition to avoid the IGF1R inhibition resistance. Additionally, the role of the IGF2 mRNA-binding protein 3 (IGF2BP3) in IGF1R and IRA signaling needs to be better understood. In Ewing sarcoma, this oncofetal protein can mediate IGF1R loss and subsequent compensatory IRA and IGF2 activation in some cell lines[98]. In line with this, cell lines with a decreased expression of IGF2BP3 exhibited a higher sensitivity to OSI-906, which means that IGF2BP3 could be a biomarker for IGF1R inhibition[98].

Discussion

In this review, we summarized several hypotheses of mechanisms of resistance that may explain the disappointing results of IGF1R pathway inhibitors in clinical studies.

First, in the clinical setting, IGF1R inhibition with IGF1R antagonist monoclonal antibodies or IGF tyrosine kinase inhibitor small molecules causes hyperglycemia and subsequent hyperinsulinemia due to cross-reactivity with the IRB and hybrid receptors[21,36]. Therefore, activation of the IGF1/insulin pathway through insulin could be an important resistance mechanism that prevents IGF1 inhibition from achieving clinical efficacy. This indicates that lowering the plasma insulin levels or blocking its activity could provide an additional target in cancer therapy and may be effective in combination with IGF1 inhibition. This is supported by our data (Appendix A, Figure A2), which showed that insulin reverts the inhibitory effect of OSI-906 on Ewing sarcoma cells in vitro. Short-term fasting may also be a valuable addition to IGF1R inhibition, as it dramatically lowers the insulin and IGF1[68,70,99].

Second, as the IRA is expressed in Ewing sarcoma cell lines and other solid tumors[44],

blocking the IGF1R alone could be insufficient to achieve clinical benefit. Therefore, IGF1 inhibition with a receptor antagonist or a tyrosine kinase inhibitor could be combined with an IR inhibitor[100]. A specific IRA inhibitor would be an optimal addition to the IGF tyrosine kinase inhibitor small molecules to prevent metabolic side effects caused by inhibiting IRB and the subsequent therapy resistance. Given the Ewing sarcoma cell lines predominantly express the IRA variant, and the 12 amino acid differences in the extracellular domains of IRA and IRB[44,50], the specific inhibition of IRA may in itself be an effective treatment of Ewing sarcoma.

Third, it was postulated that an increase of GH through an inhibited feedback loop by blocking IGF1 signaling might induce cell growth and resistance to IGF1 inhibition. However, our results suggest that GH has no effect on Ewing sarcoma cells in vitro (Appendix A, Figure A1). A combination treatment of IGF1R inhibition with pegvisomant, a growth hormone receptor antagonist, has been tried in a phase 1 trial[63], but the final results are not published yet.

Finally, we propose that autocrine loops and other secondary mutations could be the reason for the failure of IGF1R inhibitors in Ewing sarcoma and other solid tumors. Therefore, it is necessary to measure biomarkers such as universal secondary mutations (e.g., TP53, STAG2, IGF2BP3 and the CDKN2A/CDKN2B status in Ewing sarcoma patients)[101]; IGF1 and IGF2 ligand levels and IGF1R, IR and IRA receptor expression in select patients who may benefit from treatment with IGF1R inhibitors. In addition, it may be possible to personalize the treatment with combined treatment strategies based on these biomarkers[40]. Tumors of patients included in phase I trials may be resistant to IGF1R inhibition treatment due to secondary mutations caused by (extensive) pretreatment, and IGF1R inhibition might be more effective as a first-line treatment. However, driver mutations are still positive in 92% of pretreated patients with different tumor types[102].

Conclusions

The failure of IGF1R inhibitors in clinical studies may be caused by resistant tumors due to secondary mutations in pretreated patients. The complexity of the IGF1R pathway may also play a role in their failure, as pathway activation may not be adequately inhibited due to the insulin and IGF2 activation of IRA, as supported by our preclinical data. Future research should aim to assess the efficacy of combination treatments utilizing IGF1R inhibition and IRA inhibition, lowering insulin and the use of personalized treatments based on tumor biomarkers and ligand levels in patients with solid tumors and, in particular, in patients with Ewing sarcoma.

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Appendix A

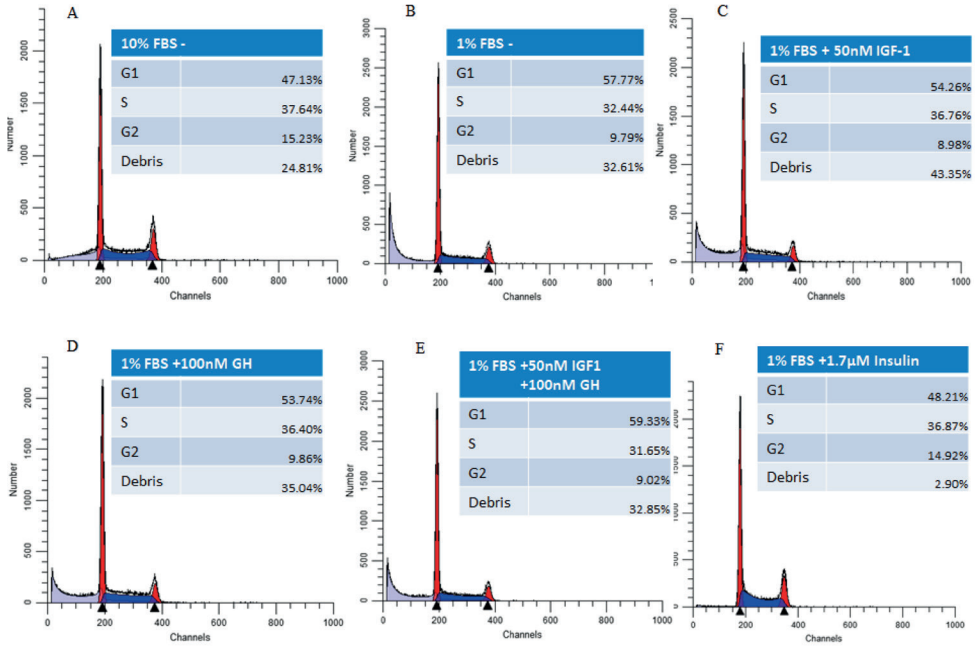

Figure A1: In vitro tumor growth of the Ewing sarcoma cell line with various growth factors. Flow cytometry results for the SKNMC cell line after 72-h incubation in different conditions. (**A**) Cells cultured in standard 10% Fetal bovine serum (FBS) medium. (**B**) Cells cultured in 1% FBS medium. (**C**) Cells cultured in 1% FBS medium with 50-nM IGF1. (**D**) Cells cultured in 1% FBS medium with 100-nM GH. (**E**) Cells cultured in 1% FBS medium with 50-nM IGF1 and 100-nM GH. (**F**) Cells cultured in 1% FBS medium with 1:100 insulin-transferrin/selenium (ITS), which corresponds to 1.7-µM insulin. G1, S and G2 percentages are shown as % counts of viable cells, while the debris % shown is the % of the total counts. The figures show an increased proportion of cells in the S phase when compared to 1% FBS control for IGF1, GH and insulin. However, only insulin increased the fraction of cells in the G2 phase. Additionally, the cells treated with insulin showed less cell debris, a marker for cell death.

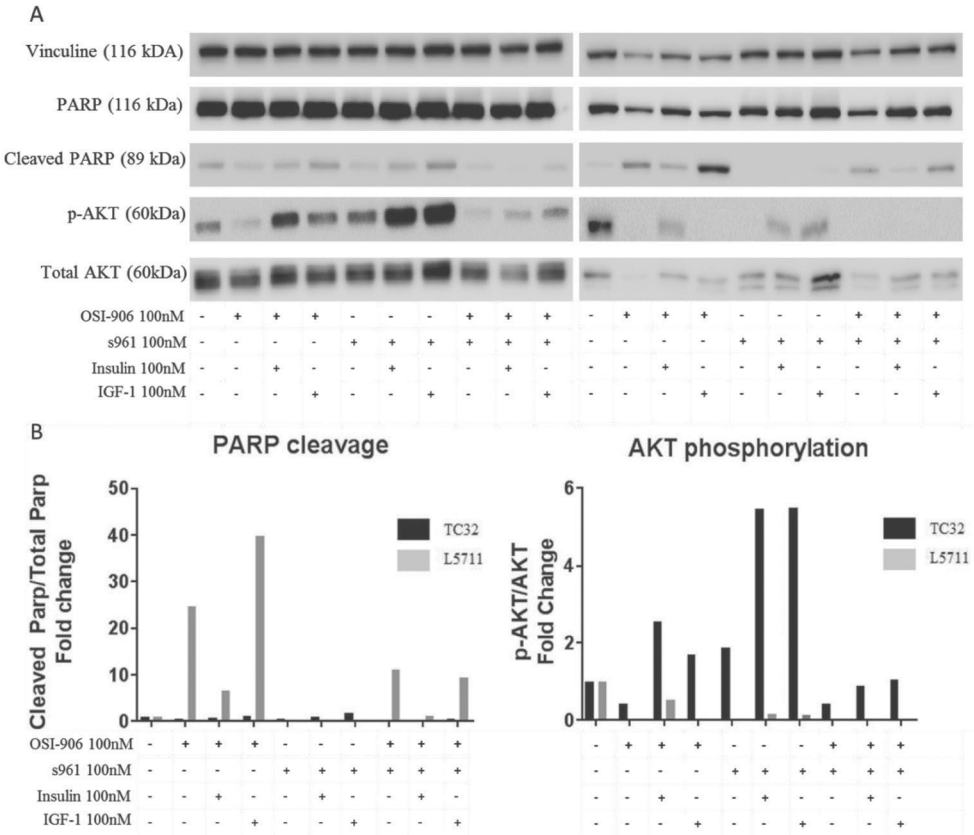

Figure A2: Induction of PARP cleavage and the reduction of AKT phosphorylation by OSI-906 saved by insulin in Ewing sarcoma cell lines (n = 1). (**A**) Western blot with PARP; p-AKT and total AKT with s961, insulin and IGF1. (**B**) Quantification of Western blots displayed as fold changes with the untreated control set to 1. OSI-906 was overall more effective in reducing AKT phosphorylation and inducing PARP cleavage in the L5711 cell line than the TC32 cell line. This susceptibility may be explained because L5711 is a TP53, CDKN2A/B and STAG2 wild type, while TC32 has a homozygous loss of the CDKN2A/B locus. The addition of insulin reduced PARP cleavage and increased AKT phosphorylation in the presence of the IGF1R inhibitor OSI-906 in the L5711 cell line.

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Chapter 6

Insulin-like growth factor 1 receptor expression and IGF1R 3129G>T polymorphism are associated with response to neoadjuvant chemotherapy in breast cancer patients: results from the NEOZOTAC trial (BOOG 2010-01)

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Abstract

Introduction: The insulin-like growth factor 1 (IGF-1) pathway is involved in cell growth and proliferation and is associated with tumorigenesis and therapy resistance. This study aims to elucidate whether variation in the IGF-1 pathway is predictive for pathologic response in early HER2 negative breast cancer (BC) patients, taking part in the phase III NEOZOTAC trial, randomizing between 6 cycles of neoadjuvant TAC chemotherapy with or without zoledronic acid.

Methods: Formalin-fixed paraffin-embedded tissue samples of prechemotherapy biopsies and operation specimens were collected for analysis of IGF-1 receptor (IGF-1R) expression (n=216) and for analysis of 8 candidate single nucleotide polymorphisms (SNPs) in genes of the IGF-1 pathway (n=184) using OpenArray® RealTime PCR. Associations with patient and tumor characteristics and chemotherapy response according to Miller and Payne pathologic response were performed using chi-square and regression analysis.

Results: During chemotherapy, a significant number of tumors (47.2%) showed a decrease in IGF-1R expression, while in a small number of tumors an upregulation was seen (15.1%). IGF-1R expression before treatment was not associated with pathological response, however, absence of IGF-1R expression after treatment was associated with a better response in multivariate analysis ($P=0.006$) and patients with a decrease in expression during treatment showed a better response to chemotherapy as well ($P=0.020$). Moreover, the variant T allele of 3129G>T in *IGF1R* (rs2016347) was associated with a better pathological response in multivariate analysis ($P=0.032$).

Conclusions: Absent or diminished expression of IGF-1R after neoadjuvant chemotherapy was associated with a better pathological response. Additionally, we found a SNP (rs2016347) in *IGF1R* as a potential predictive marker for chemotherapy efficacy in BC patients treated with TAC.

Introduction

Insulin-like growth factor 1 (IGF-1) and other members of the IGF-1 pathway have been associated with development, progression and metastasis of several cancers[1,2]. Additionally, epidemiologic studies have shown a relation between high circulating IGF-1 levels, breast density[3] and risk of breast cancer (BC)[4]. Increased IGF-1 levels are associated with an elevated BC mortality[5] and with inherent resistance to anti-tumor treatments in preclinical models[6-9]. Furthermore, the IGF-1 receptor (IGF-1R), a transmembrane tyrosine kinase, is frequently upregulated in BC[10,11]. The biological activity of IGF-1 and IGF-2 depends on binding with the insulin-like growth factor binding proteins (IGF-BPs), mainly IGF-BP3[12,13]. Both IGFs bind the IGF-1R and activate the Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/AKT pathways, through which cell proliferation is stimulated and apoptosis is inhibited, respectively[14,15]. Additionally, the IGF-1R and the estrogen receptor (ER) have been shown to work synergistically, whereby activated ER binds to the promoter regions of *IGF1R* to promote transcription and IGF-1 is able to activate unliganded ER[16,17].

Previous research has shown that low IGF-1R expression in the tumor is predictive for pathological complete response (pCR) in ER+ positive tumors[10] and that upregulation of IGF-1R during chemotherapy predicts a poor outcome in a relative small, heterogeneous group of BC patients[18]. Moreover, genes encoding members of the IGF-1 pathway are known to harbor several single nucleotide polymorphisms (SNPs) that influence the activity of the pathway. SNPs associated with IGF-1 and IGF-BP3 plasma levels and breast density are described[19,20] as well as SNPs associated with therapy resistance and outcome[21,22].

Neoadjuvant chemotherapy has been demonstrated to be equivalent to adjuvant chemotherapy for BC survival. This treatment has the advantage of more frequent breast-conserving therapy[23] and offers the opportunity for translational research of molecular predictors of tumor response. Additionally, the Miller and Payne (MP) histological grading system can be used to assess response to neoadjuvant chemotherapy because it is associated with patients' disease-free and overall survival[24,25]. This study evaluates the expression of the IGF-1R of the tumor before and after neoadjuvant chemotherapy and whether it predicts pathological response according to MP classification after neoadjuvant chemotherapy in human epidermal growth factor receptor 2 (HER2) negative early BC patients treated in the NEOZOTAC trial[26]. Moreover, we aim to identify SNPs, which have been described to influence the activity of the IGF-1 pathway, to predict chemotherapy efficacy in this cohort. In addition, these SNPs are tested for association with the occurrence of side effects.

Patients and methods

Study population

From July 2010 until April 2012 250 women participated in the multicenter phase III NEOZOTAC trial, randomizing between TAC chemotherapy (75mg/m^2 of docetaxel, 50mg/m^2 of doxorubicin and 500mg/m^2 of cyclophosphamide) with or without zoledronic acid (4mg within 24 hours after chemotherapy). Eligible patients had a histologically confirmed diagnosis of HER2 negative stage II or III BC. Other inclusion and exclusion criteria have been described elsewhere[26]. Tumor regression was scored according to the MP classification[24]. pCR was defined as the absence of residual invasive cancer within the breast and lymph nodes[24]. Side effects and hematological toxicity were graded according to the Common Terminology Criteria for Adverse Events version 4.0 (CTCAE v.4.0)[27]. All patients gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki (2008) and approved by the Ethics Committee of the Leiden University Medical Center in agreement with the Dutch law for medical research involving humans.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples of prechemotherapy biopsies and operation specimens were collected for analysis of IGF-1R expression using immunohistochemistry (IHC). From each FFPE tumor tissue sample one section of 4µm was cut and deparaffinized with xylene, rehydrated through graded alcohol and rinsed in distilled water. After blocking of endogenous peroxidase activity with 0.3% H_2O_2 for 20 minutes, heat induced antigen retrieval was performed in the EnVision TM Flex Target Retrieval Solution in PT Link (Dako, Glostrup, Denmark) at low pH. After blocking with 5% normal goat serum to reduce aspecific binding by the primary antibody, the sections were incubated overnight at room temperature in a humidified chamber with the IGF-1R antibody (IGF-1 receptor β, D4O6W, rabbit monoclonal, Cell Signaling Technology, Danvers, MA, USA) diluted in phosphate-buffered saline (PBS)/bovine serum albumin (BSA) 1% at a dilution of 1:200. After the primary antibody incubation, the sections were washed with PBS and incubated with a secondary anti-rabbit antibody EnVision™ (Dako, Glostrup, Denmark) for 30 minutes and visualized using liquid DAB+ (Dako, Glostrup, Denmark). Eventually, sections were counterstained with Mayer's hematoxylin, dehydrated and subsequently permanently mounted with Pertex (Histolab, Gothenburg, Sweden). Breast and placenta sections that had previously been identified to express the IGF-1R were used as positive controls and sections that underwent the IHC staining procedure without application of primary antibodies served as negative controls. Membranous IGF-1R expression was scored on a scale of 0 - 3+ (see Figure 1). Samples were considered negative if 0 or 1+ was scored, and positive if 2+ and 3+ was given. The staining was scored by two independent researchers (SdG and ALM).

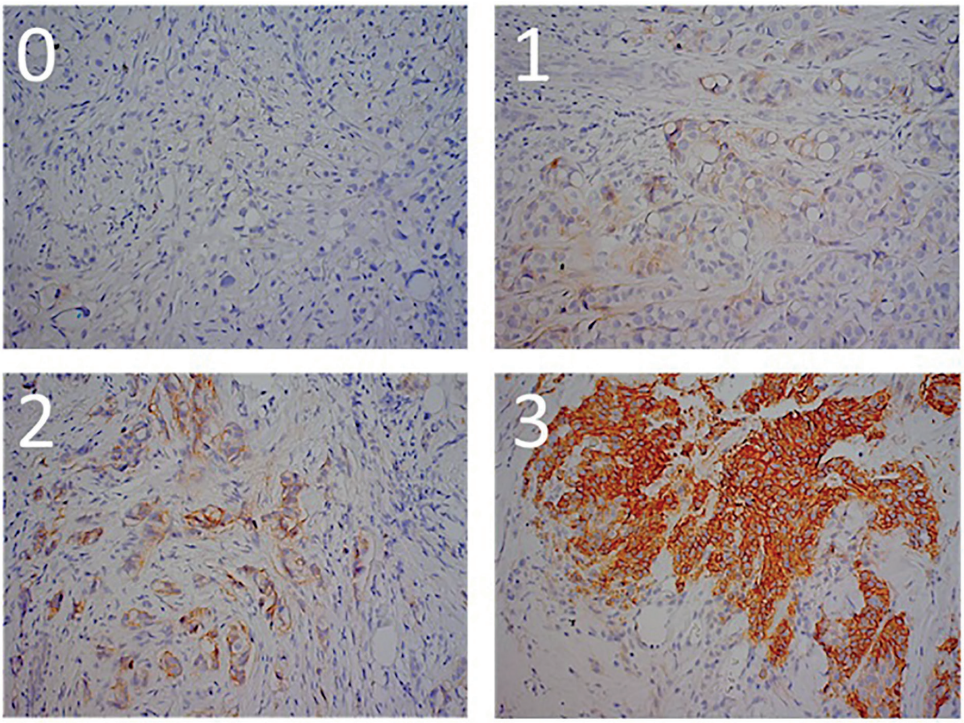

Figure 1: Examples of the membranous IGF-1R staining in breast tumor tissue sections. Score 0: Staining is observed in <10% of the tumor cells. Score 1+: An incomplete staining is observed in >10% of the tumor cells, Score 2+: A weak or moderate complete staining is observed in >10% of the tumor cells, Score 3+: A strong complete staining is observed >10% of tumor cells. Samples were considered negative if 0 or 1+ was scored, and positive if 2+ and 3+ was given.

SNP selection

To select relevant SNPs in the IGF-1 pathway, a PubMed search with the keywords "IGF-1", "IGF-2", "IGF-BP3", "IGF-1R", "single nucleotide polymorphism", "breast cancer" and/or "clinical outcome", was conducted in July 2013. SNPs that were associated with IGF-1 or IGF-BP3 plasma levels, BC risk or clinical outcome in cancer patients treated with chemotherapy, were selected. SNPs with a minor allele frequency (MAF) >0.01 in a Caucasian population according to the HapMap project database and with a potential functionality according to the literature review or using national institutes of health functionality database were selected[28]. To minimize the number of tested associations, tagging SNPs were selected for SNPs that were in linkage disequilibrium (r^2 >0.7). The selected SNPs in the *IGF1, IGF2, IGFBP3* and *IGF1R* genes are summarized in Table 1.

Table 1: Selected SNPs in IGF-1 pathway.

RS number	Gene	Alleles (major > minor)	Position in gene and functionality	Clinical influence of polymorphism
rs10735380	IGF1	A>G	Transcription factor binding site, intronic	Variant G allele associated with increased serum IGF-1 level[20,35,41]
rs1520220	IGF1	C>G	Intronic	Variant G allele associated with increased serum IGF-1 level[35,42] and BC risk[42]
rs6220	IGF1	A>G	3'-untranslated region, microRNA binding site	Variant G allele associated with increased serum IGF-1 level and increased BC risk[42]
rs2946834	IGF1	G>A	3'-untranslated region	Variant A allele associated with increased serum IGF-1 level[35,42] and with worse outcome in BC[21]
rs2270628	IGFBP3	C>T	Downstream	Variant T allele associated with decreased serum IGF-BP3 level[20,35,36]
rs2854746	IGFBP3	G>C	Non-synonymous in exon 1 (Ala32Gly)	Variant C allele associated with increased serum IGF-BP3 level[20,35,36,43] and with better outcome in advanced gastric cancer treated with CT[44]
rs4320932	IGF2	T>C	Transcription factor binding site, intronic	Variant C allele associated with worse outcome in ovarian cancer and worse response to CT[45]
rs2016347	IGF1R	G>T	3'-untranslated region, microRNA binding site	Variant T allele associated with better outcome in ER+ BC[22]

SNPs selected on basis of literature research and the clinical influence. IGF1: insulin-like growth factor 1, IGF2 insulin-like growth factor 2, IGFBP3 insulin-like growth factor binding protein 3, IGF1R; insulin-like growth factor 1 receptor, CT: chemotherapy.

DNA isolation and preamplification

DNA was extracted from FFPE tissue samples. Preferentially, tissue from tumor-negative breast tissue and tumor-negative lymph nodes was used (N=95); however, when this was unavailable or unclear from the pathology report tissue from tumor-containing blocks was used. Three sections of 4µm were incubated overnight at 50°C in 500µl lysis buffer (NH_4CL 8.4g/L, $KHCO_3$ 1.0g/L, proteinase K 0.25mg/ml). Next, 300µl was taken to extract DNA using Maxwell forensic DNA isolation kit (Promega, Leiden, The Netherlands) according to the manufacturer's protocol. DNA isolated from FFPE tissue is cross-linked and fragmented into pieces with a length of a few hundred base pairs. To make DNA isolated from FFPE tissue more suitable for genotyping, pre-amplification was accomplished for enrichment of the target DNA[29]. The preamplification step consisted of a PCR reaction with 8 diluted Taqman assays (LifeTechnologies, Nieuwerkerk aan den IJssel, the Netherlands) and was performed using the following protocol; to 2.5 µL of DNA, 1 µL of a dilution of 8 Taqman assays (pooled at a final concentration of 0.2x) and 3.5 µL HotStarTaq DNA polymerase was added and amplified on a conventional PCR machine. The following PCR conditions were used; 10 minutes at 95°C followed by 18 cycles each consisting of 15 seconds at 95°C and 4 minutes at 60°C. The mixture

was diluted 15-times and 2 µL was used for Real Time PCR analysis. The selected SNPs were analyzed using TaqMan OpenArray® technology (Life Technologies); however, in case of low call rate, missing samples were re-analyzed separately using Viia7 RealTime PCR system (Life Technologies).

Statistical analysis

Possible associations between parameters were analyzed using Pearson's chi-square test and logistic regression. Univariate and multivariate odds ratios (ORs), 95% confidence intervals (CIs), and *P* values were derived from logistic regressions. IGF-1R expression and clinical variables, which have been reported to be associated with pCR, were tested in univariate analysis (e.g. hormone receptor (HR) status and clinical T status). The association between IGF-1R expression and MP classification were tested using a logistic ordinal regression where MP classification groups were treated as ordered. In multivariate analyses parameters were adjusted for covariates with $P < 0.1$. We also reanalyzed the latter model using linear regression to check for linearity of relationship between IGF-1R expression and MP classification.

Genotype distributions were tested for adherence to Hardy-Weinberg equilibrium and SNPs significant at the 0.05 level after Bonferroni correction were excluded from the analysis. Genotypes found to be (borderline) significant in the univariate logistic regression models were carried forward to the multivariate model, adjusting for covariates with $P < 0.1$. To correct for multiple testing a global score test including all SNPs was performed[30]. The score test assumes that the regression coefficients of the SNPs are normally distributed and tests whether the variance of this distribution is bigger than zero. In that case at least one regression coefficient has to be unequal to zero. To investigate the individual, relative contribution of SNPs, a classification and regression tree (CART) was computed (Statistical Package for Social Sciences (SPSS): classify, tree). A ROC curve and AUC were computed for the predicted probabilities of the CART. The global *P* value was computed using package *globaltest* in R version 3.1.3. All other analyses were computed using SPSS software™ 20.0 (IBM Corp., Armonk, NY, USA). A significance level of 0.05 was used for all tests.

Results

Patient characteristics

Patients of both study arms, chemotherapy with or without zoledronic acid, were included in this study, as no differences were found between both arms regarding pathological response[26]. FFPE tissue was available from 216 (86.4%) of 250 patients. Clinical characteristics of the 216 patients are given in Table 2, which are comparable with the characteristics of the entire cohort of the NEOZOTAC trial[26]. Almost 12% of the patients had a pCR.

Table 2: Patient characteristics.

	Patients (N=216) NEOZOTAC	
Median Age	Years (range),	49.5 (28-70)
Median BMI	kg/m^2 (range),	26.2 (18.3-42.0)
cT stage	cT1 or cT2 cT3 or cT4	123 (56.9%) 93 (43.1%)
cN stage	cN0 cN+	101 (46.8%) 115 (53.2%)
Tumor type	Ductal Lobular Other Unknown	128 (59.3%) 38 (17.6%) 18 (8.4%) 32 (14.8%)
HR status	ER+ and/or PR+ ER- and PR-	180 (83.3%) 36 (16.7%)
Allocated treatment	TAC TAC+ZA	109 (50.5%) 107 (49.5%)
pCR breast and LN	Yes No Unknown	25 (11.6%) 184 (85.2%) 7 (3.2%)
MP breast	1 2 3 4 5 Unknown	33 (15.3%) 56 (25.9%) 41 (19.0%) 42 (19.4%) 35 (16.2%) 9 (4.2%)

HR: hormone receptor, ER: estrogen receptor, PR: progesterone receptor, TAC: Docetaxel, doxorubicin and cyclophosphamide, ZA: zoledronic acid, pCR: pathologic complete response, LN: lymph nodes, MP: Miller and Payne.

IGF-1R expression

FFPE breast tumor tissue from 216 patients was available for analyzing at least one condition (biopsy and/or operation specimen), while both samples were available for 106 cases. Data of available tissue are summarized in the consort diagram (Figure 2). Representative tissue examples with different scoring values can be found in Figure 1. High IGF-1R expression in the prechemotherapy biopsy was associated with ER expression ($P=0.001$) and the progesterone receptor (PR) expression ($P=0.035$). ER and/or PR positive tumors showed positive IGF-1R on the membrane in 78.0% of the cases, while triple negative tumors showed positivity for IGF-1R in only 50.0% of the cases.

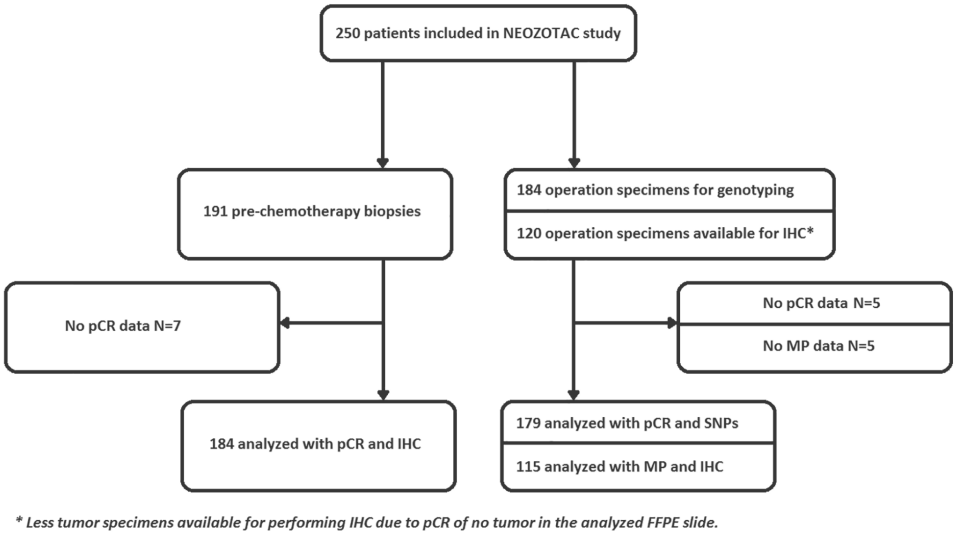

Figure 2: Consort diagram.

During chemotherapy, a significant subset (47.2%), of tumors showed a decrease in IGF-1R expression while in a small subset of tumors the IGF-1R was upregulated (15.1%). IGF-1R expression before treatment was not associated with pathological response (Figure 3). However, the absence of IGF-1R expression (45%) after treatment in the postchemotherapy operation specimens was associated with a better pathological response comparing ordinal MP classification response in univariate analysis (OR 2.60, 95% CI 1.31-5.18, *P=0.006*) (Figure 3). This result remained significant in multivariate analysis when adjusting for HR status and clinical N stage (OR 2.64, 95% CI 1.32-5.31, *P=0.006*). With linear regression *P=0.008,* indicating that the relationship between MP classification and IGF-1R expression is almost linear. Additionally, patients with a decrease in expression during treatment showed a better response to chemotherapy as well (OR 2.64, 95% CI 1.17-5.98, *P=0.020* in multivariate analysis). Treatment with zoledronic acid had no influence on the IGF-1R expression in the operation specimen after treatment (*P=0.620*) nor on diminished IGF-1R expression during treatment (*P=0.830*) (data not shown).

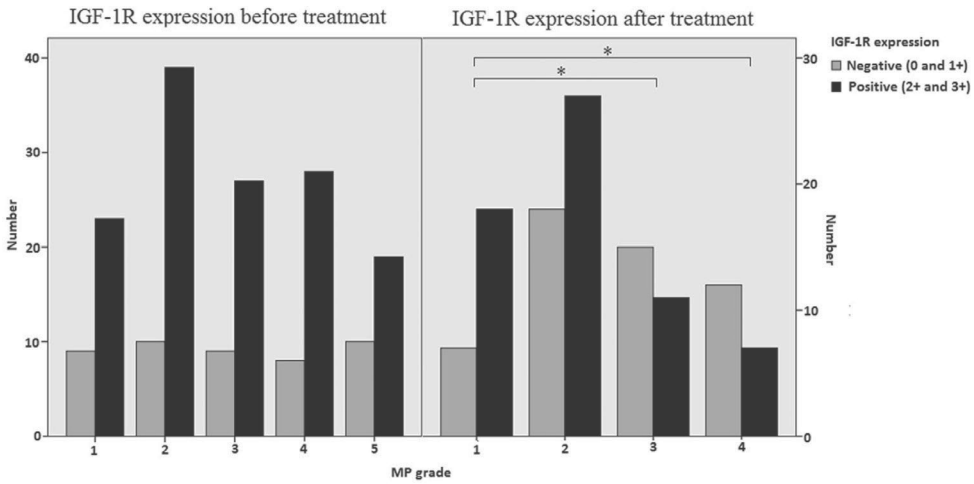

Figure 3: Membranous IGF-1R expression before and after treatment and the association with pathological response. MP: Miller and Payne. * P<0.05.

IGF-1R pathway SNPs

FFPE tissue samples from 184 (74%) of 250 patients were available for analysis of IGF-1 pathway polymorphisms (preferentially tumor-negative tissue, see Methods). Data of available tissue are summarized in the consort diagram (Figure 2). Of the eight genotyped SNPs, two significantly deviated from the Hardy-Weinberg equilibrium (rs2946834 and rs1520220). After correction for multiple testing rs2946834 still significantly deviated from the Hardy-Weinberg equilibrium and was therefore excluded from the analysis. The genotype frequencies of rs1520220 did not differ from those observed in a publicly available database of European subjects (e.g. from the HapMap project)[27]. All 8 SNPs had a callrate above 85%, which is shown in Table 3. Clinical T stage, clinical N stage and HR status were associated with pCR, wherefore is adjusted in multivariate analyses (Table 4). The variant T allele of 3129G>T in *IGF1R* (rs2016347) was associated with pCR in multivariate analysis (4.4% for GG vs. 16.7% GT/TT, *P=0.032*) and the variant C allele of rs2854746 in *IGFBP3* tended to be associated with pCR in multivariate analysis (7.3% for GG vs. 18.1% GC/CC, *P=0.058*). The global *P* value used for multiple testing correction for all eight SNPs together was *P=0.0095* for the dominant model (global score test). The CART derived from these SNPs is shown in Figure 4. The corresponding AUC was 0.613 (95% CI 0.518-0.707, *P=0.040*).

Table 3: Distribution of genotypes of the investigated SNPs.

SNP	Allele	N=184 (%)	HWE X^2	P value	Call rate
rs10735380 IGF1	AA	110 (54.3)	2.1	0.144	94%
	AG	68 (37.0)			
	GG	5 (2.7)			
	NE	11 (6.0)			
rs1520220 IGF1	CC	115 (62.5)	4.4	**0.040***	94%
	CG	46 (25)			
	GG	11 (6.0)			
	NE	12 (6.5)			
rs6220 IGF1	AA	91(49.5)	3.3	0.068	89%
	AG	56 (30.4)			
	GG	17 (9.2)			
	NE	20 (10.9)			
rs2946834** IGF1	GG	82 (44.6)	10.1	**0.001***	88%
	GA	53 (28.8)			
	AA	26 (14.1)			
	NE	23 (12.5)			
rs2270628 IGFBP3	CC	105 (57.1)	2.8	0.096	87%
	CT	45 (24.5)			
	TT	10 (5.4)			
	NE	24 (13.0)			
rs2854746 IGFBP3	GG	59 (32.1)	1.9	0.170	90%
	GC	72 (39.1)			
	CC	34 (18.5)			
	NE	19 (10.3)			
rs4320932 IGF2	TT	111 (60.3)	0.04	0.843	96%
	TC	57 (31)			
	CC	8 (4.3)			
	NE	8 (4.3)			
rs2016347 IGF1R	GG	48 (26.1)	1.8	0.185	96%
	GT	96 (52.2)			
	TT	32 (17.4)			
	NE	8 (4.3)			

HWE=Hardy-Weinberg equilibrium, NE=Not Evaluable, despite attempt to genotype, IGF1: insulin-like growth factor 1, IGF2 insulin-like growth factor 2, IGFBP3 insulin-like growth factor binding protein 3, IGF1R; insulin-like growth factor-1 receptor. *Not in HWE, ** SNP excluded from analyses as SNP is significantly deviated from HWE after Bonferroni correction.

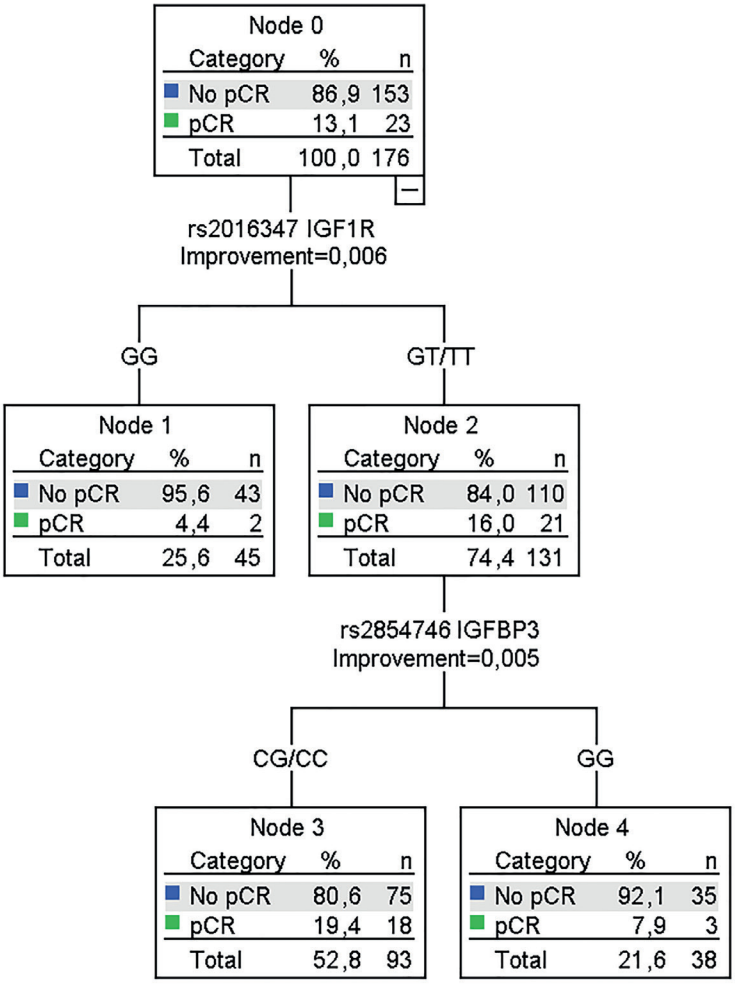

Figure 4: CART analyses of pCR in BC patients treated with TAC chemotherapy. The tree is divided by the SNPs to predict pCR, which has significant prediction score (AUC 0.613 95% CI 0.518-0.707, P=0.040).

Moreover, the variant T allele of C>T in *IGFBP3* (rs2270628) was associated with a higher occurrence of grade III/IV side effects in univariate analysis (OR 2.20, 95% CI 1.04-4.67, *P=0.039,*) and multivariate analysis (18.1% for CC vs. 32.7% CT/TT, OR 2.30, 95% CI 1.06-4.98, *P=0.034*) (not shown). The multivariate analysis was adjusted for body mass index, as it was significantly associated with grade III/IV side effects.

Genotype-phenotype associations

Rs2016347 in *IGF1R* was not associated with IGF-1R expression before chemotherapy (78.3% for GG vs. 65.9% GT/TT, *P=0.115)* or after chemotherapy (50.0% for GG vs. 67.7% GT/TT, *P=0.099).*

Table 4: Associations between tumor and patient characteristics, SNPs and pCR in breast and lymph nodes.

				Univariate analysis			Multivariate analysis		
Parameter		**N**	**% pCR**	**OR**	**95% C.I.**	**P value**	**OR**	**95% C.I.**	***P* value**
cT stage	cT1/cT2	106	17.9	1	ref.		1	ref	
	cT3/T4	73	6.8	0.34	0.12-0.95	**0.039**	0.49	0.16-1.50	0.209
cN stage	cN0	84	21.4	1	ref		1	ref	
	cN+	95	6.3	0.25	0.09-0.66	**0.005**	0.19	0.06-0.58	**0.003**
HR status	ER+ and/or PR+	151	8.6	1	ref.		1	ref	
	Triple negative	28	39.3	6.87	2.66-17.7	**0.00007**	9.35	3.09-28.3	**0.00008**
Allocated treatment	TAC + ZA	87	14.9	1	ref.	0.559			
	TAC only	92	12.0	0.77	0.33-1.83				
Age				0.96	0.89-1.09	0.186			
BMI				0.97	0.88-1.08	0.581			
rs10735380 IGF1	AA	97	13.4	1	ref				
	AG	66	13.6	1.02	0.41-2.55	0.966			
	GG	5	20.0	1.61	0.17-15.6	0.679			
rs1520220 `IGF1	CC	111	15.3	1	ref				
	CG	45	13.3	0.85	0.31-2.32	0.752			
	GG	11	0.0	-	-	-			
rs6220 IGF1	AA	88	11.4	1	ref				
	AG	56	16.1	1.49	.57-3.94	0.418			
	GG	17	17.6	1.67	0.41-6.48	0.475			
rs2270628 IGFBP3	CC	101	11.9	1	ref				
	CT	45	17.8	1.60	0.61-4.24	0.342			
	TT	9	0.0	-	-	-			
rs2854746 IGFBP3	GG	55	7.3	1	ref		1	ref	
	GC	72	16.7	2.55	0.78-8.40	0.124	3.06	0.82-11.4	0.097
	CC	33	21.2	3.43	0.92-12.8	**0.066**	4.02	0.92-17.6	0.065
	GG	55	7.3	1	ref		1	ref	
	GC/CC	105	18.1	2.82	0.91-8.74	**0.073**	3.35	0.96-11.7	0.058
rs4320932 IGF2	TT	106	15.1	1	ref				
	TC	57	12.3	0.79	0.30-2.04	0.623			
	CC	8	12.5	0.80	0.09-6.98	0.843			
rs2016347 IGF1R	GG	45	4.4	1	ref		1	ref	
	GT	94	17.0	4.41	0.97-20.1	**0.055**	5.58	1.08-28.7	**0.040**
	TT	32	15.6	3.98	0.72-22.0	0.113	6.67	1.03-43.1	**0.046**
	GG	45	4.4	1	ref		1	ref	
	GT/TT	126	16.7	4.30	1.00-19.1	**0.056**	5.82	1.17-29.1	**0.032**

HR: hormone receptor, ER: estrogen receptor, PR: progesterone receptor, TAC: docetaxel, doxorubicin, cyclophosphamide, ZA: zoledronic acid, OR: Odds Ratio, C.I.: Confidence Interval, IGF1: insulin-like growth factor 1, IGF2: insulin-like growth factor 2, IGFBP3: insulin-like growth factor binding protein 3, IGF1R: insulin-like growth factor 1 receptor.

Discussion

This translational study showed that IGF-1R expression changed in most of the tumors during treatment in stage II/III HER2 negative BC patients treated with neoadjuvant TAC chemotherapy and that absent or diminished expression after treatment was associated with a better pathological response according to MP classification. Additionally, we found that the variant T allele of 3129G>T in *IGF1R* (rs2016347) was significantly associated with a better pathological response according to MP classification after neoadjuvant chemotherapy.

Changes of IGF-1R expression of the tumor during chemotherapy have been described previously[18,31]. Our study confirms these results in a larger and more homogeneous patient cohort. Moreover, in the current trial a greater part of the tumors showed a decline in IGF-1R expression (47.2%) compared with the prior described 14.0%. This might be explained by the difference in chemotherapy regimens used as well as the absence of HER2 expression in our cohort, as HER2 positive tumors show less IGF-1R expression[10,11]. The decline of IGF-1R expression in the tumor during TAC treatment observed in our study might reflect chemotherapy efficacy, as patients with a decline in IGF-1R expression showed a significantly better pathological response than tumors with no change or an increase in expression. In keeping with this inference, downregulation of IGF-1R during chemotherapy treatment is associated with prolonged survival[18]. Bhargava et al. showed that low IGF-1R expression before treatment was associated with a better response to neoadjuvant chemotherapy in ER+ tumors, but not in triple negative tumors[10]. We could not reproduce this association, but this could be explained by the difference in cohort, e.g. differences in HER2 status and chemotherapy regimen.

In our exploratory analysis of IGF-1 pathway polymorphisms, the variant T allele of 3129G>T in *IGF1R* (rs2016347) was associated with a better pathological response according to MP after neoadjuvant chemotherapy. This is in accordance with studies that associated 3129G>T in *IGF1R* (rs2016347) with cancer prognosis and treatment outcome[22,32,33]. Winder et al.[22] found that the T allele was associated with a better overall survival in colorectal cancer patients treated with cetuximab[33] and a better overall survival in ER positive BC patients treated with tamoxifen. Rs2016347 is localized in 3'UTR region of the *IGF1R* gene, functioning as a microRNA binding site[28]. As microRNA binding sites are important for mRNA translation and degradation, the variant T allele of rs2016347 might disturb binding to this microRNA site[34]. Although the precise functional effect of *IGF1R* rs2016347 is unknown, it would be a plausible explanation that the T allele of rs2016347 may reduce IGF-1R expression. However, in our study rs2016347 in *IGF1R* was not associated with IGF-1R expression.

The variant T allele of C>T in *IGFBP3* (rs2270628) was associated with the occurrence of grade III/IV side effects. Although the mechanism is unclear, several studies have shown that the variant T allele of rs2270628 is associated with decreased serum IGF-BP3 levels[35,36]. IGF-1 activity depends on binding with IGF-BP3[12,13], so it may be that higher activity of IGF-1 due to lower levels of IGF-BP3 causes a higher incidence of toxicity of chemotherapy in our study[6].

Our study has some limitations. Using our approach, we could not investigate the best responders (MP5) after chemotherapy because inherently no tumor tissue was left to measure IGF-1R in the operation specimen. Moreover, the response of the lymph nodes is not evaluated in the MP grading system as it focuses only on the primary tumor. Although, the survival of patients with a partial response is affected by residual lymph node status[37]. Additionally, the number of evaluable triple negative tumors was too small to evaluate for differences in response associated with IGF-1R between HR-positive tumors and triple negative tumors. Our sample size for the explorative genotype-phenotype optional side study was small and this was probably the reason why we could not reproduce the associations between the serum IGF-1 and IGF-BP3 levels and SNPs. However, the results of our study provide further evidence for the importance of patient selection for (co-) treatment with an IGF-1 inhibitor. Until now no convincing benefit of IGF-I pathway inhibitors was found in clinical studies in BC[38-40]. These studies lacked patient selection based on IGF-1 pathway activity. It may be hypothesized that patients with a diminished IGF-1R after chemotherapy will not benefit from an IGF-1R inhibitor, while a patient with upregulated IGF-1R might benefit.

Conclusions

IGF-1R expression in the tumor changed during chemotherapy and absent or diminished expression of IGF-1R after treatment was associated with a better pathological response. Rs2016347 in *IGF1R* was associated with pCR after TAC chemotherapy. These observations may help to predict the efficacy of TAC chemotherapy and to select patients who might benefit from (co-) treatment with an IGF-1 pathway inhibitor.

Abbreviations

BC:	breast cancer
CART:	classification and regression tree
CI:	confindence interval
CTCAE:	Common Terminology Criteria for Adverse Events
ER:	estrogen receptor
FFPE:	Formalin-fixed paraffin-embedded
HR:	hormone receptor
IGF-BPs:	insulin-like growth factor binding proteins
IGF-1:	insulin-like growth factor 1
IGF-1R:	insulin-like growth factor 1 receptor
IHC:	immunohistochemistry
MP:	miller and payne
OR:	odds ratio
pCR:	pathological complete response
PR:	progesterone receptor
SNPs:	single nucleotide polymorphisms

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Chapter 7

Addition of zoledronic acid to neoadjuvant chemotherapy is not beneficial in patients with HER2-negative stage II/III breast cancer: 5-year survival analysis of the NEOZOTAC trial (BOOG 2010-01)

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Abstract

Background: Adjuvant bisphosphonates are associated with improved breast cancer survival in postmenopausal patients. Addition of zoledronic acid (ZA) to neoadjuvant chemotherapy did not improve pathological complete response in the phase III NEOZOTAC trial. Here we report the results of the secondary endpoints, disease free survival (DFS) and overall survival (OS).

Patients and methods: Patients with HER2-negative, stage II/III breast cancer were randomized to receive the standard 6 cycles of neoadjuvant TAC (docetaxel/doxorubicin/cyclophosphamide) chemotherapy with or without 4 mg intravenous (IV) ZA administered within 24 hours of chemotherapy. This was repeated every 21 days for 6 cycles. Cox regression models were used to evaluate the effect of ZA and covariates on DFS and OS. Regression models were used to examine the association between insulin, glucose, insulin growth factor-1 (IGF-1) levels and IGF-1 receptor (IGF-1R) expression with survival outcomes.

Results: Two hundred forty six women were eligible for inclusion. After a median follow up of 6.4 years, OS for all patients was significantly worse for those who received ZA (HR 0.468, 95% CI 0.226–0.967, *P*=0.040). DFS was not significantly different between the treatment arms (HR 0.656, 95% CI 0.371–1.160, *P*=0.147). In a subgroup analysis of postmenopausal women, no significant difference in DFS or OS was found for those who received ZA compared with the control group (HR 0.464, 95% CI 0.176–1.222, *P*=0.120; HR 0.539, 95% CI 0.228–1.273, *P*=0.159, respectively). The subgroup analysis of premenopausal patients was not significantly different for DFS and OS ((HR 0.798, 95% CI 0.369–1.725, P=0.565; HR 0.456, 95% CI 0.156–1.336, *P*=0.152 respectively). Baseline IGF-1R expression was not significantly associated with DFS or OS. In a predefined additional study, lower serum levels of insulin were associated with improved DFS (HR 1.025, 95% CI 1.005–1.045, *P*=0.014).

Conclusions: Our results suggest that ZA in combination with neoadjuvant chemotherapy was associated with a worse OS in breast cancer (both pre- and postmenopausal patients). However, in a subgroup analysis of postmenopausal patients, ZA treatment was not associated with DFS or OS. Also, DFS was not significantly different between both groups. IGF-1R expression in tumor tissue before and after neoadjuvant treatment did not predict survival.

Introduction

Bisphosphonates (BPs) act to suppress bone resorption by inducing osteoclast apoptosis[1,2]. BPs are indicated for treatment and prevention of osteoporosis and prevention of skeletal related events due to metastasis of solid tumors or multiple myeloma[3]. Results of the meta-analysis of the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) showed that adjuvant BPs were associated with decreased fracture rate, as well as improved breast cancer survival and bone metastasis risk. These benefits were only found in postmenopausal (natural or induced) women in a subgroup analysis[4]. The benefits may be explained by the increased bone resorption in postmenopausal patients, as BP prevented tumor growth in bone in a postmenopausal model but not in a premenopausal model[5]. Currently, BPs are considered as a part of the adjuvant breast cancer treatment in postmenopausal patients and patients receiving ovarian suppression therapy[6]. The exact mechanism of the anti-tumor effect of BPs is unknown. However, the following mechanisms have been proposed[7], BPs may: 1) prevent tumors cells from metastasizing to the bone by decreasing bone turnover[8], 2) change the bone micro-environment by reducing growth factors such as insulin-like growth factor- 1 (IGF-1) and insulin, and thereby inhibit proliferation[9-12], 3) have immunomodulatory properties by activating γδ T cells[13,14] and recruiting tumor associated macrophages[15,16], 4) reduce angiogenic factors[17,18] and/or 5) kill dormant disseminated tumor cells[19,20]. BP was reported to improve the tumor response when combined with doxorubicin in an experimental breast cancer model[21]. Moreover, adding a BP to neoadjuvant chemotherapy in breast cancer patients resulted in a significantly lower residual invasive tumor size and a non-significantly higher pathological complete response (pCR) rate in an exploratory evaluation of the AZURE trial[22].

Clinically, in our phase III randomized NEOZOTAC study examining the effect of zolendronic acid (ZA) in addition to neoadjuvant TAC chemotherapy in HER2 negative early breast cancer, ZA did not improve the primary endpoint, pathological complete response (pCR)[23]. A subsequent meta-analysis did not show a significant increase in pCR rate when adding a BP to neoadjuvant chemotherapy in patients with early breast cancer[16,24]. In this paper, we report the secondary endpoints of disease-free survival (DFS) and overall survival (OS) from the NEOZOTAC study[23].

Additionally, we report associations between the IGF-1 receptor (IGF-1R) expression and the concentrations of circulating growth factors such as insulin and IGF-1, and survival. IGF-1R and insulin receptor isoform A (IR-A) are frequently upregulated in breast cancer[25,26]. Both receptors activate the Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/AKT pathways, through which cell proliferation is stimulated and apoptosis is inhibited[27].

Methods

Study population

As previously described[23], from July 2010 until April 2012, 250 women participated in the multi-center phase III NEOZOTAC trial and 246 were evaluated in the study (2 patients were ineligible and 2 patients withdrew informed consent, Figure 1). Eligible patients had a histologically confirmed diagnosis of HER2 negative, stage II or III (T2 any cN, cM0) early breast cancer, adequate bone marrow (i.e. white blood cell count ≥3.0 x 10^9/L, neutrophil count ≥1.5 x 10^9/l and platelet count ≥ 100 x 10^9/l), normal liver function (i.e. bilirubin ≤1.5 x upper limit of normal (UNL) range, ALAT and/or ASAT ≤2.5 x UNL, Alkaline Phosphatase ≤5 x UNL), adequate renal function (i.e. calculated creatinine clearance ≥50 mL/min), adequate cardiac function, WHO performance state 0-2, age ≥ 18 years, absence of pregnancy or current lactation and written informed consent. Menopause was defined as 1 year without menstrual activity, previous bilateral oophorectomy, age older than 60 years or baseline FSH >20 U/l and estradiol <110 pmol/l). The study (NCT01099436) was conducted in accordance with the Declaration of Helsinki (October 2008) and was approved by the Ethics Committee of the LUMC in agreement with Dutch law for medical research involving human subjects.

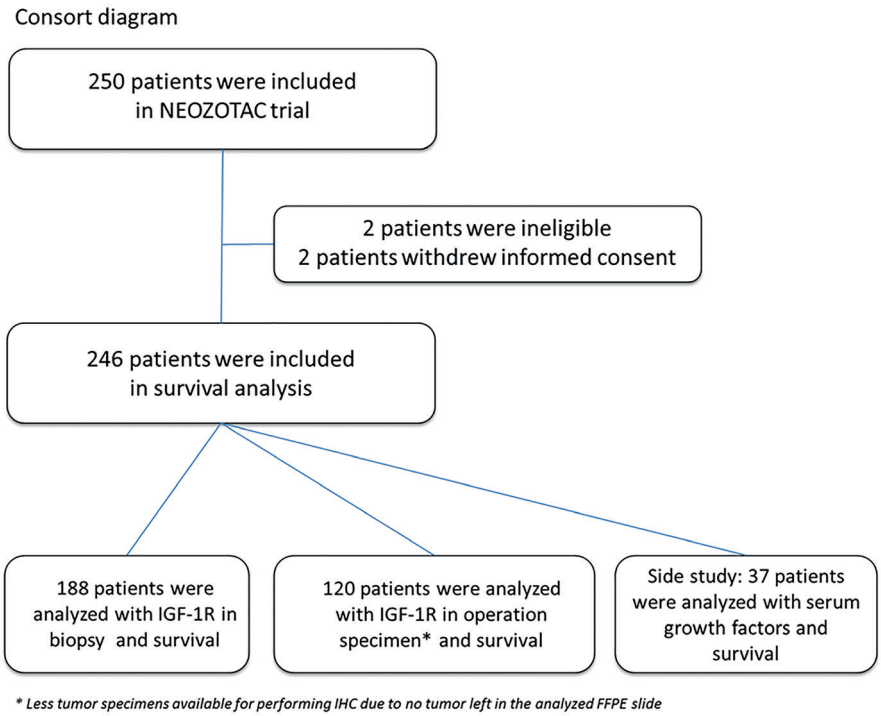

Figure 1: Consort diagram of the trial.

Treatment

Women received 6 cycles of neo-adjuvant TAC chemotherapy (75mg/m^2 of docetaxel, 50mg/m^2 of doxorubicin and 500mg/m^2 of cyclophosphamide) with or without ZA (4mg i.v. in 15 minutes within 24 hours after chemotherapy, repeated every 21 days for 6 cycles). Pegfilgrastim (Neulasta®) was administered as primary prophylaxis (6 mg once per cycle) as a subcutaneous injection 24 hours after chemotherapy for all cycles. ZA therapy was combined with daily supplements of 500mg calcium and 400 IU vitamin D.

Randomization

Patients were randomized at the LUMC Datacenter of the Department of Surgery using Pocock's minimization technique, stratified by center, clinical T-classification, clinical N-classification, and estrogen receptor status. The ALEA randomization program was used.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples of pre-chemotherapy biopsies and surgical specimens were collected for analysis of IGF-1R expression using immunohistochemistry (IHC) (Figure 1). The staining method is described extensively elsewhere[25].

Blood sampling and analysis

Non-fasting blood samples were obtained directly before chemotherapy administration to measure glucose, insulin and IGF-1 levels. Samples were collected and kept on ice directly after drawing. After centrifuging, the supernatant was stored at –80°C, and at the end of the study was sent to the Leiden University Medical Center (LUMC) for analysis. Serum glucose levels were determined by spectrophotometry (Modular P800, Roche Diagnostics, Almere, The Netherlands) and insulin levels were analyzed with the chemiluminescence immunoassay (CIA) (Immulite 2500, Siemens, The Hague, The Netherlands). Serum levels of IGF-1 (IDS-iSYS) were analyzed with immunodiagnostic Systems (Frankfurt, Germany). The IGF-1 assay is traceable to the WHO IS 02/254.17.

Endpoints

The primary endpoint of the study was pCR, and the results of pCR are described elsewhere[23]. PCR was defined as the absence of residual invasive cancer within the breast and lymph nodes according to the Miller and Payne (MP) classification[28]. Secondary endpoints were DFS, defined as the time from date of inclusion until the date of the earliest documented local or distant recurrence, contralateral breast cancer including DCIS, second primary invasive cancer or death from any cause, and OS, defined as the time from inclusion until date of death from any cause. Additionally, we studied the association between insulin, glucose, IGF-1 levels and IGF-1R expression with survival

outcomes.

Statistical analysis

Median follow-up was calculated by applying the reverse Kaplan–Meier methodology. Cox regression models were used to evaluate the effect of ZA and other risk factors on DFS and OS. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were estimated. Relevant risk factors described in the literature or found to have a *P*-value of less than 0.1 in univariate analyses were incorporated in the multivariate model. All tests were two tailed. *P*-values of less than 0.05 were considered significant. All analyses were computed using SPSS software™ 23.0 (IBM Corp., Armonk, NY, USA).

Results

Patient characteristics

The clinical characteristics of the patients included in the study are shown in Table 1 and were described previously[23]. None of these patient characteristics were significantly different between the two groups. Of the total cohort, 39.4% had a postmenopausal status at the start of the study.

Table 1: Patient characteristics.

	TAC + ZA N = 122 (49.6%)	**TAC N = 124 (50.4%)**	**IGF-1R biopsy data available N = 188 (76.4%)**	**Serum data available N = 37 (15%)**
Median Age (range), Years	48.0 (29–67)	49.0 (34–70)	49 (29-70)	49 (34-65)
Median BMI (range), kg/m^2	26.1 (18.5-40.0)	25.0 (18.3-42.0)	25.0 (18.3-42.0)	24.9 (19.4-39.5)
Menopausal status				
Pre/Peri	72 (59.0%)	75 (60.5%)	110 (58.5%)	24 (64.9%)
Post	50 (41.0%)	47 (37.9%)	76 (40.4%)	13 (35.1%)
T-classification				
T1/T2	73 (59.8%)	71 (57.3%)	108 (57.4%)	21 (56.8%)
T3/T4	49 (40.2%)	53 (42.7%)	80 (42.6%)	16 (43.2%)
N-classification				
N0	54 (44.3%)	56 (45.2%)	90 (47.9%)	22 (59.5%)
N+	68 (55.7%)	68 (54.8%)	98 (52.1%)	15 (40.5%)
HR-status				
ER+ and/or PR+	101 (82.8%)	104 (83.9%)	158 (84.0%)	33 (89.2%)
ER- and PR-	21 (17.2%)	20 (16.1%)	30 (16.0%)	4 (10.8%)

TAC: Docetaxel, doxorubicin and cyclophosphamide, ZA: zoledronic acid, BMI: body mass index. HR: hormone receptor, ER: estrogen receptor, PR: progesterone receptor, pCR: pathologic complete response, LN: lymph nodes, MP: Miller and Payne.

Response

The primary endpoint pCR was achieved in 13.3% of the total cohort. This was not significantly different between the two arms (*P=0.980).* As described previously, pCR was also not significantly different between the two arms in a subgroup analysis of postmenopausal women (14.0% versus 8.7%)[23]. The pCR and recurrence rates are shown in table 2. Patients with pCR after neoadjuvant chemotherapy had a numerically longer period of DFS (HR 0.253, 95% CI 0.061–1.041, *P*=0.057), but OS was not associated with pCR (HR 0.389, 95% CI 0.093–1.624, *P*=0.195) (Figure 2a, b).

Table 2: Shortterm and longterm outcome.

Response	TAC + ZA N = 122 (49.6%)	TAC N = 122 (49.6%)	*P* Value
pCR breast and LN			
Yes	16 (13.3%)	16 (13.2%)	
No	104 (86.7%)	105 (86.8%)	0.980
Miller and Payne			
1	19 (15.8%)	18 (14.8%)	
2	35 (29.2%)	31 (25.4%)	
3	24 (20.0%)	25 (20.5%)	
4	21 (17.5%)	25 (20.5%)	
5	21 (17.5%)	23 (18.9%)	0.950
Recurrence			
Total	29 (23.8%)	20 (16.1%)	0.134
Local	5 (4.1%)	5 (4.0%)	0.979
Regional	7 (5.7%)	4 (3.2%)	0.341
Distant	27 (22.1%)	17 (13.7%)	0.085
Second primary tumor	5 (4.1%)	5 (4.0%)	0.979
Death			
Yes	23 (18.9%)	11 (8.9%)	
No	99 (81.1%)	113 (91.1%)	**0.023**
Cause of death			
Breast cancer	22 (95.7%)	11 (91.6%)	0.630
Other	1 (4.3%)	1 (8.3%)	

TAC: Docetaxel, doxorubicin and cyclophosphamide, ZA: zoledronic acid, pCR: pathologic complete response, LN: lymph nodes, MP: Miller and Payne.

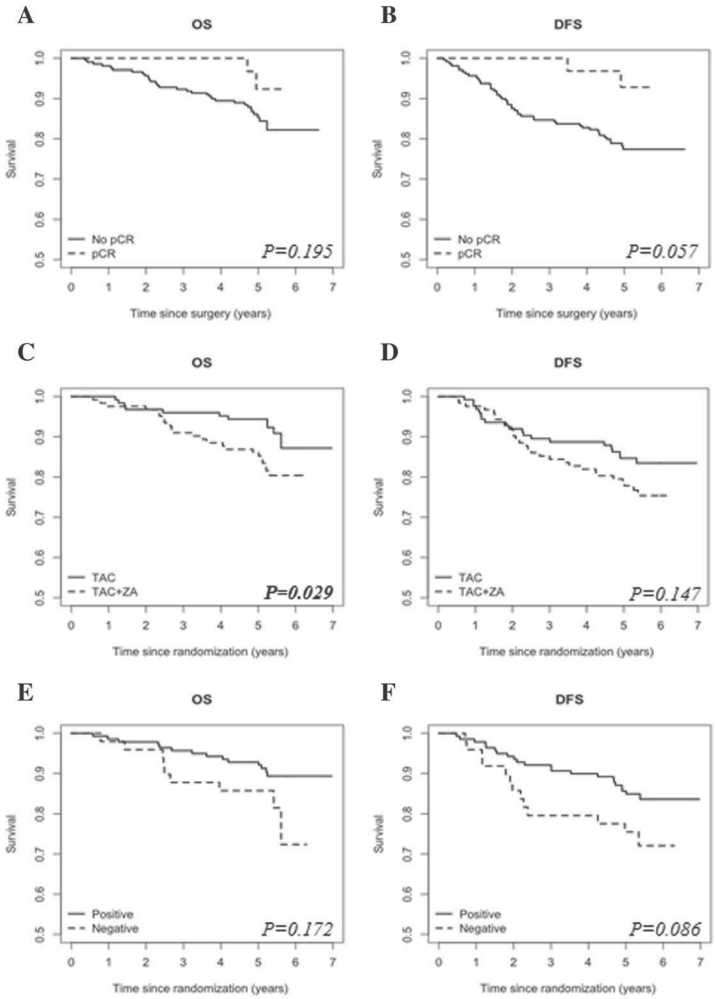

Figure 2: Kaplan–Meier curves of overall survival (left column) and disease free-survival (right column) for pCR (A and B), for treatment with or without zoledronic acid (C and D), and IGF-1R expression before neoadjuvant chemotherapy (E and F). Note: P-values are given for the univariate analyses of the Cox regression analyses. Bold values indicate that P <0.05. Abbreviations: IGF-1, insulin-like growth factor 1; DFS, disease-free survival; OS, overall survival, pCR, pathological complete response.

Survival outcomes

The median follow-up was 6.43 years (95% CI 6.25–6.61). Kaplan–Meier curves of survival rates are shown in Figure 2. Risk factors associated with survival as described in the literature and those with $P<0.1$ in univariate analyses were included in the regression model for multivariate analysis of mortality determinants. A Cox model was used to study the associations between risk factors and survival outcomes. The estimated HRs and associated 95% confidence intervals for univariate and multivariate analyses for OS and DFS are shown in Tables 3 and 4, respectively. Age, hormone receptor status,

T status, N status and menopausal status were adjusted for in the multivariate Cox model. Women who received ZA had a significantly worse OS than women who did not receive ZA in univariate analyses (HR 0.448, 95% CI 0.218–0.919, *P*=0.029) (Figure 2C) and in multivariate analyses (HR 0.468, 95% CI 0.226–0.967, *P*=0.040). DFS did not significantly differ between groups in univariate analysis (HR 0.656, 95% CI 0.371–1.160, *P*=0.147) (Figure 2D). In the ZA arm, one patient died of stage IV lung cancer, and in the control arm, one patient died of unknown causes. The percentage of breast cancer deaths was not significantly different between both arms. In a subgroup analysis of postmenopausal women, addition of ZA to chemotherapy did not affect DFS or OS (HR 0.539, 95% CI 0.228–1.273, *P*=0.159; HR 0.464, 95% CI 0.176–1.222, *P*=0.120, respectively) (Figure 3A and B). There was also no significant difference in survival (DFS or OS) between the two arms in the premenopausal subgroup (HR 0.798, 95% CI 0.369–1.725, *P*=0.565; HR 0.456, 95% CI 0.156–1.336, *P*=0.152 respectively) (Figure 3C and D).

Table 3: Univariate and multivariate Cox models of OS.

	Univariate analysis			**Multivariate analysis**		
	HR	**95%CI**	***P* value**	**HR**	**95%CI**	***P* value**
Age	1.042	0.999-1.086	0.054	1.019	0.962-1.079	0.522
BMI	1.013	0.943.-1.088	0.730			
HR status	2.019	0.942-4.328	0.071	2.104	0.978-4.529	0.057
Menopausal status	2.133	1.081-4.210	0.029	1.768	0.670-4.665	0.250
cN status	3.921	1.624-9.471	0.002	4.060	1.672-9.859	0.002
cT status	1.680	0.857-3.295	0.131	1.1516	0.763-3.011	0.235
Zoledronic acid	0.448	0.218-0.919	0.029	0.468	0.226-0.967	0.040

Bold values indicate that P <0.05, OS overall survival, HR hazard ratio, CI confidence interval, BMI body mass index.

Table 4: Univariate and multivariate Cox models of DFS.

	Univariate analysis			**Multivariate analysis**		
	HR	**95%CI**	***P* value**	**HR**	**95%CI**	***P* value**
Age	1.034	0.998-1.070	0.061	1.036	0.850-2.637	0.043
BMI	0.989	0.930.-1.053	0.739			
HR status	1.698	0.868-3.323	0.122	1.799	0.916-3.536	0.088
Menopausal status	1.393	0.795-2.442	0.247			
cN status	2.724	1.420-5.224	0.003	2.811	1.461-5.407	0.002
cT status	1.569	0.896-2.748	0.115	1.497	0.850-2.637	0.162
Zoledronic acid	0.656	0.371-1.160	0.147			

Bold values indicate that P <0.05, DFS disease free survival, HR hazard ratio, CI confidence interval, BMI body mass index.

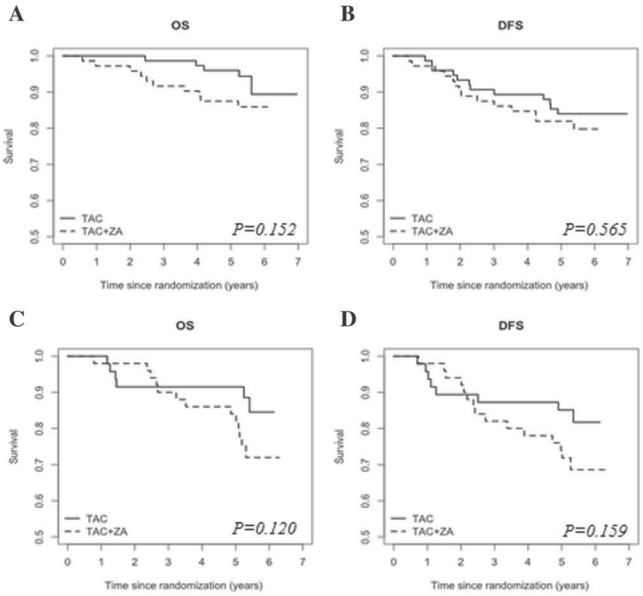

Figure 3: Kaplan–Meier curves of overall survival (left column) and disease free-survival (right column) for pre/perimenopausal women (A and B) and postmenopausal woman (C and D). P-values are given for the univariate analyses of the Cox regression analyses. Abbreviations: DFS, disease-free survival; OS, overall survival.

IGF pathway analysis

IGF-1R expression data was available for 188 patients before and 120 patients after chemotherapy treatment. The clinical characteristics of the patients included in the IGF pathway analysis are shown in Table 1 and were described previously[29]. Presence of IGF-1R expression in the tumor pre-treatment was numerically related to DFS, but this was not significant (HR 0.549, 95% CI 0.276–1.089, *P=0.086*) (Figure 2E), and it was not associated with OS (HR 0.562, 95% CI 0.246–1.285, *P=0.172*) (Figure 2F). In patients with HR-positive breast cancer, presence of baseline IGF-1R tumor expression was associated with a better DFS in univariate analyses (HR 0.433, 95% CI 0.198–0.946 *P=0.036*), but not in multivariate analysis (HR 0.484, 95% CI 0.214–1.096, *P=0.082*). There was no significant association between the IGF-1R receptor and OS (HR 0.433, 95% CI 0.198–0.946 *P=0.120*). Neither presence of IGF-1R expression after neoadjuvant chemotherapy nor decrease in expression during therapy were related to survival. Furthermore, treatment with ZA had no influence on the IGF-1R expression in the surgical specimen after chemotherapy treatment.

In a subgroup of patients (N=37), baseline serum levels of glucose, insulin and IGF-1 were measured. Patient characteristics are reported in Table 1. These were not significantly different compared to the total cohort. Lower serum insulin levels were associated with

improved DFS (HR 1.025, 95% CI 1.005–1.045, *P=0.014*), but not OS (HR 1.073 95% CI 0.953–1.209, *P=0.244*). Glucose and IGF-1 concentrations were not associated with survival.

Discussion

This study found that ZA as an adjunct to neoadjuvant chemotherapy had no beneficial effect in patients with stage II/III HER2-negative breast cancer receiving TAC chemotherapy and, in pre- and postmenopausal patients, was associated with worse OS, but not DFS. Additionally, in a post hoc analysis, there was no beneficial effect of ZA in postmenopausal patients. Interestingly, lower insulin levels were associated with improved DFS, but not with OS.

The negative impact of ZA on OS when used as an adjunct to neoadjuvant chemotherapy was not expected, as several studies have shown a benefit of ZA in the adjuvant setting in postmenopausal women[4]. Our study population may explain the negative impact of ZA on survival, as the majority (59.8%) of patients were premenopausal. Accordingly, the Azure trial showed that ZA in the adjuvant setting was associated with worse DFS and OS in a subgroup of patients younger than 40 years old, who are presumably largely premenopausal[30]. However, we also did not find a benefit in postmenopausal patients.

Moreover, a major difference between adjuvant and neo-adjuvant use of BPs is the length of administration. Neoadjuvant BPs are administered for a shorter time period and therefore may not positively impact survival outcomes. In the JONIE1 trial, ZA did not have a beneficial effect on survival in the neoadjuvant setting[31], although the authors did find a positive association with pCR in previous studies[32]. In keeping with our results, a meta-analysis of four studies did not show any effect of ZA addition to neoadjuvant chemotherapy on pCR rate[16].

In a predefined additional exploratory side study, lower serum insulin levels were associated with improved DFS. In keeping with this result, patients with insulin levels greater than 13 μIU/mL had a twofold increased risk for disease progression compared to patients with insulin levels below this cut-off[33]. Goodwin et al. found that higher fasting insulin levels at baseline in breast cancer patients without diabetes were also associated with worse OS [34]. Higher insulin levels may give the tumor a growth advantage, as most breast tumors express the IGF-1R and IR-A, both of which are involved in proliferation and tumorigenesis and are associated with tumor progression[27,35].

Our study has some limitations. We are aware that the sample size is small and the results should therefore be interpreted with caution. The majority of the patients included in

this study were premenopausal women, but the positive effect of ZA on survival would be expected in postmenopausal women. Our post hoc analyses of postmenopausal women are not statistically powered, making it impossible to draw firm conclusions. Patients using BPs at baseline were excluded, however, the use of adjuvant BPs might have influenced the survival outcome, but this information is not available in our study. The sample size of the additional exploratory study of growth factors was small. However, the results of our study provide further evidence of the importance of the insulin and IGF-1R pathway in breast cancer.

Conclusion

Our study does not support the use of ZA as an adjunct to neoadjuvant chemotherapy.

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Chapter 8

Serum levels of IGF-1 and IGFBP-3 serum levels are associated with event-free survival in adult Ewing sarcoma patients treated with chemotherapy

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Abstract

Background: Activation of the insulin like growth factor-1 (IGF-1) pathway is involved in cell growth and proliferation, and is associated with tumorigenesis, tumor progression and therapy resistance in solid tumors. We examined whether variability in serum levels of IGF-1, IGF-2 and IGF binding protein-3 (IGF-BP3) can predict event-free survival (EFS) and overall survival (OS) in Ewing sarcoma patients treated with chemotherapy.

Patients and methods: Serum levels of IGF-1, IGF-2 and IGF-BP3 of 22 patients with localized or metastasized Ewing sarcoma, treated with six cycles of vincristine/ifosfamide/doxorubicin/etoposide (VIDE) chemotherapy were recorded. Baseline levels were compared with presixth cycle levels using paired t-tests and were tested for associations with EFS and OS. Continuous variables were dichotomized according to the Contal and O'Quigley procedure Survival analyses were performed using Cox regression analysis.

Results: High baseline IGF-1 and IGF-BP3 serum levels were associated with EFS (hazard ratio [HR] 0.075, 95% confidence interval [CI] 0.009-0.602 and HR 0.090, 95% CI 0.011-0.712, respectively) in univariate and multivariate analyses (HR 0.063, 95% CI 0.007-0.590 and HR 0.057, 95% CI 0.005-0.585, respectively). OS was improved but this was not statistically significant. IGF-BP3 and IGF-2 serum levels increased during treatment with VIDE chemotherapy *(P=0.055* and *P=0.023,* respectively).

Conclusion: High circulating serum levels of IGF-1 and IGF-BP3 and the molar ratio of IGF-1:IGF-BP3 serum levels were associated with improved EFS and a trend for improved OS in Ewing sarcoma patients treated with VIDE chemotherapy. These findings suggest the need for further investigation of the IGF-1 pathway as a biomarker of disease progression in patients with Ewing sarcoma.

Introduction

Ewing sarcoma is a rare form of cancer; however, it is the second most common bone sarcoma in children and young adults. The disease is usually primarily localized in the skeleton but may occur in soft tissue. Mortality is high despite intensive multidisciplinary treatment, including surgery, chemotherapy and radiotherapy[1]: overall survival (OS) at five years is 30% in patients with primary metastatic disease and 60% in patients with localized disease.[2] Evidence suggests that the insulin-like growth factor 1 (IGF-1) pathway plays a major role in the pathogenesis of Ewing sarcoma.[3,4] The IGF-1 pathway consists of the IGF-1 receptor (IGF-1R), two ligands (IGF-1 and IGF-2) and six IGF binding proteins (IGF-BP1-6), of which IGF-BP3 is the most abundant.[5-7] The insulin receptor (IR) is highly homologous to the IGF-1R, allowing for the formation of hybrid receptors that can activate the downstream pathway. The A isoform of the IR, which is predominant in cancer cells, can also activate the pathway.[6,8]

Eighty-five percent of Ewing sarcoma tumors are marked by the chromosomal translocation *t*(11;22).[9] This somatic mutation results in aberrant products of the Ewing sarcoma breakpoint region 1 (*EWSR1)* and the Friend leukemia virus integration 1 (*FLI1*) genes. The EWSR1-FLI1 oncoprotein binds to the *IGFBP3* promoter, which inhibits expression of IGF-BP3 (Figure 1).[3,4] The biological activity of IGF-1 is inhibited primarily by binding IGF-BP3.[5,10] Therefore, high levels of bioavailable IGF-1 (and IGF-2) may activate the IGF-1R in Ewing sarcoma to stimulate Ras/MAPK and PI3K/AKT pathways, through which cell proliferation is promoted and apoptosis inhibited.[5,8]

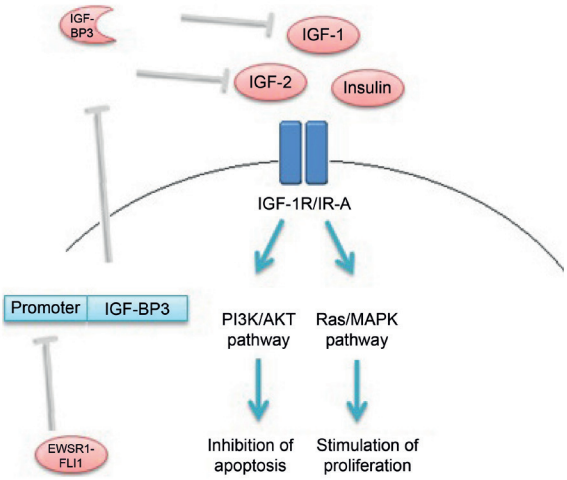

Figure 1: Schematic overview of the effect of EWSR1-FLI 1oncoprotein on IGF-1 pathway in Ewing sarcoma cells. Note: EWSR1-FLI 1 binds the promoter region of IGF-BP3, which inhibits transcription. Abbreviations: EWSR1-FLI 1, Ewing sarcoma breakpoint region 1-Friend leukemia virus integration 1; IGF-1, insulin-like growth factor 1; IGF-BP3, insulin-like growth factor-binding protein 3; IGF-2, insulin-like growth factor 2; IGF-1R, insulin-like growth factor 1 receptor; IR -A, insulin receptor isoform A; PI 3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; MAP K, mitogen-activated protein kinase.

Convincing preclinical evidence of the efficacy of IGF-1 pathway inhibitors in the context of cancer treatment has led to the development of numerous such agents, that have been investigated in clinical trials as treatments for Ewing sarcoma, breast cancer and other solid tumors.[6,11-13] Unfortunately, convincing clinical benefits of these agents have been limited to rare long-lasting effects in a small proportion of patients with Ewing sarcoma.[13] A better understanding of the role of the IGF-1 pathway could therefore help illuminate specific therapeutic targets and potential biomarkers of disease progression.

This observational study examined whether circulating levels of IGF-1 pathway components predict the (event-free) survival of Ewing sarcoma patients treated with (vincristine/ifosfamide/doxorubicin/etoposide) VIDE chemotherapy.

Patients and methods

Study population

Patients with primary localized or metastatic Ewing sarcoma who were treated between 2009 and 2014 with VIDE chemotherapy (1.5mg/m^2 of vincristine on day 1, 3000mg/m^2 of ifosfamide on days 1/2/3, 20mg/m^2 of doxorubicin on days 1/2/3 and 150mg/m^2 of etoposide on days 1/2/3) at the Medical Oncology department of the Leiden University Medical Center were included. For all participants, the diagnosis was established in a multidisciplinary setting and confirmed using molecular analysis. Response of the tumor to chemotherapy was defined according to the Van der Woude classification as 'good' or 'poor', based on pathology and/or radiology reports, where available. [14,15] The study was conducted according the Dutch 'Code of Good Conduct' and was approved by the Ethics Committee of the Leiden University Medical Center . Patient consent was assumed as per the no-objection rule of the 'Code of Good Conduct' (https://www.federa.org/codes-conduct). Biomarker data has been reported in accordance with REMARK criteria.[16]

Blood sampling and assay methods

Fasting blood samples were obtained immediately prior to the first and sixth chemotherapy cycles. Serum levels of IGF-1 (IDS-iSYS, Immunodiagnostic Systems, Frankfurt, Germany) and IGF-BP3 (Immulite 2500, Siemens, The Hague, The Netherlands) were analyzed by chemiluminescence immunoassay (CIA). The IGF-1 assay is traceable to the WHO IS 02/254.[17] The IGF-BP3 assay is traceable to WHO NIBSC Reagent 93/560 according to IFU: IMMULITE 2000 IGF-BP3 (PIL2KGB-14, 2012-06-18).[18] Serum IGF-2 was analyzed by radioimmunoassay after Sep-Pak C18 column extraction calibrated to WHO 96/538 international standard.[19]

Statistical analysis methods

The primary outcome measure was the association of baseline serum levels of IGF-1, IGF-2, IGF-BP3 with event-free survival (EFS). Secondary outcome measures included the association of baseline serum levels of IGF-1, IGF-2 and IGF-BP3 with presixth cycle levels and with OS.

A linear regression model was used to study the association between age and IGF-1. Mean serum levels were compared using a *t*-test for paired groups. To dichotomize continuous covariates for use in a Cox model, the Contal and O'Quigley technique based on the log-rank test statistic was used. This method cutoff point for the covariate of interest at which the largest difference is seen between individuals in two groups. The procedure involves estimation of the cutoff point for the covariate and tests the hypothesis that this covariate in its binary version has no effect on the outcome. Technical details concerning the procedure are described elsewhere [20,21]. In this study, the median was used as the cutoff point for IGF-BP3 serum levels. Since there were no events in one of the groups identified by using the Contal and O'Quigley procedure, a Cox model could not be performed. EFS was defined as time from date of diagnosis until the date of the earliest documented disease recurrence or progression, or death from any cause. OS was defined as time from date of diagnosis until date of death from any cause. Median follow-up was calculated by applying the reverse Kaplan Meier methodology.[22] Cox regression models were used to evaluate the effect of the covariates on EFS and OS. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were calculated. Serum levels of the components of the IGF-1 pathway that were found to be significant (to 1%) in univariate Cox regression models were carried forward to the multivariate model. Relevant risk factors described in the literature or found to have a *P*-value of less than 0.1 in univariate analyses were incorporated in the multivariate model. All tests were 2-tailed. *P* values of less than 0.05 were considered significant. All data were analyzed using Statistical Package for Social Sciences (SPSS) software™ 20.0 (IBM Corp., Armonk, NY, USA) and R version 3.3 (R Foundation for Statistical Computing, Vienna, Austria).[23]

Results

Patient characteristics

The clinical characteristics at diagnosis of the 22 Ewing sarcoma patients included in the study are shown in Table 1. All patients were 16 years or older. Nine patients had metastastic disease and 13 had localized disease at initial diagnosis. One patient received two cycles of etoposide and cisplatin chemotherapy before the diagnosis of Ewing sarcoma was made as small-cell carcinoma was initially suspected. Three patients were diagnosed with Ewing sarcoma only after surgical excision of the tumor. Two of those patients were diagnosed with an incidental extraskeletal Ewing sarcoma of the kidney and one patient of the adrenal gland.

Table 1: Patient characteristics.

Patients (N=22)	
Gender	
Female	6 (27.3%)
Male	16 (72.7%)
Age (Years)	
Range	16-62
< 18	3 (13.6%)
≥ 18-30	10 (45.5%)
≥ 30	9 (40.9%)
Median BMI (range), kg/m^2	22.7 (17.0-36.8)
Chemotherapy before diagnosis	
Yes	1 (4.5%)
No	21 (95.5%)
Surgery before diagnosis	
Yes	3 (13.6%)
No	19 (86.4%)
Extraskeletal	
Yes	5 (22.7%)
No	17 (77.3%)
Metastasis	
With metastasis	9 (40.9%)
Only pulmonary	4 (18.2%)
Skeletal	3 (13.6%)
Bone marrow	2 (9.1%)
Without metastasis	13 (59.1%)
Response to chemotherapy	
Good	14 (63.6%)
Poor	5 (22.7%)
Not applicable	3 (13.6%)

Abbreviation: BMI, body mass index.

IGF pathway serum levels during chemotherapy

Serum samples at baseline and after five VIDE cycles were available for 22 and 13 patients, respectively. For nine patients no samples were collected due to logistical reasons. There was no significant difference in IGF-1 serum levels in patients with metastastic disease versus localized disease (mean values 28.2 versus 34.4 nmol/L (*P=0.227)*, respectively). At baseline, age was significantly associated with IGF-1 values (*P=0.035*) in our cohort. Baseline IGF-1, IGF-2 and IGF-BP3 levels were compared with levels prior to the sixth chemotherapy cycle. IGF-1 levels did not change significantly during chemotherapy. IGF-BP3 and IGF-2 levels increased during chemotherapy (*P=0.055* and *P=0.023,* respectively). Mean values of IGF-1, IGF-2 and IGF-BP3 at baseline and presixth cycle are shown in Figure 2. IGF-1, IGF-BP3 and IGF-2 levels in subjects with good response and poor response were compared according to the Van der Woude classification using data from pathology and/or radiology reports. Three patients underwent surgery before diagnosis, so it was not possible to assess response to chemotherapy. Fourteen patients showed a good response and five patients a poor response to chemotherapy. IGF-1, IGF-BP3 and IGF-2 levels at baseline were not associated with response to chemotherapy (*P=0.702, P=0.912* and *P=0.888,* respectively).

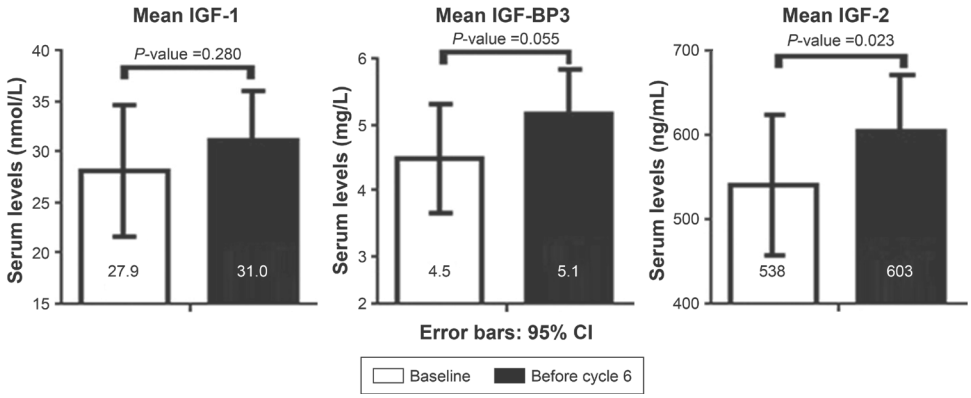

Figure 2: Serum levels of IGF-1, IGF-BP3, and IGF-2 at baseline and after five cycles of VIDE chemotherapy (N=13). Abbreviations: IGF-1, insulin-like growth factor 1; IGF-BP3, insulin-like growth factor binding protein 3; IGF-2, insulin-like growth factor 2; VIDE, vincristine/ifosfamide/doxorubicin/etoposide; CI, confidence interval.

Serum levels and association with survival

The median follow-up was 3.25 years (95% CI 2.25-4.25). For baseline values of IGF-1 and IGF-2, and the molar IGF-1:IGF-BP3 ratio, the cutoff point based on the Contal and O'Quigley procedure was equal to 28.7 nmol/L, 608 ng/mL and 0.21, respectively. The median cutoff point of baseline values of IGF-BP3 was 4.7mg/L. Kaplan Meier curves for IGF-1, IGF-2, IGF-BP3, the molar IGF-1:IGF-BP3 ratio and the number of patients per group are shown in Figure 3. In this analysis, IGF-1, IGF-BP3 and the molar IGF-1:IGF-BP3 ratio are prognostic factors for survival. Risk factors described in the literature to be associated with survival[24] and those with $P < 0.1$ in univariate analyses were included in the regression model for multivariate analysis. A Cox model was used to study the association between risk factors and survival outcomes. The estimate HRs and associated 95% CIs for univariate and multivariate analyses are shown in Table 2. Age and body mass index (BMI) were adjusted for in the multivariate Cox model. IGF-1 was a significant prognostic factor for EFS (HR 0.063, 95% CI 0.007-0.590, *P=0.015*). This was not a significant for OS in this patient group (HR 0.162, 95% CI 0.017-1.496, *P=0.109*). IGF-BP3 was significantly associated with EFS (HR 0.057, 95% CI 0.005-0.585, *P=0.016*), but not OS (HR 0.125, 95% CI 0.011-1.423, *P=0.094*). IGF-2 levels were not significantly associated with EFS (HR 1.064, 95% CI 0.164-6.905, *P=0.948)* or OS (HR 5.297, 95% CI 0.451-62.24, *P=0.185)* (not shown). To investigate the influence of free IGF-1, the molar IGF-1:IGF-BP3 ratio was used as risk factor. The molar IGF-1:IGF-BP3 ratio was associated with EFS (HR 0.103, 95%CI 0.011-0.934, *P=0.043*), but not OS (HR 0.163, 95%CI 0.017-1.546, *P=0.225*) in multivariate analyses. IGF-1, IGF-BP3 and IGF-2 serum alterations were not associated with EFS or OS.

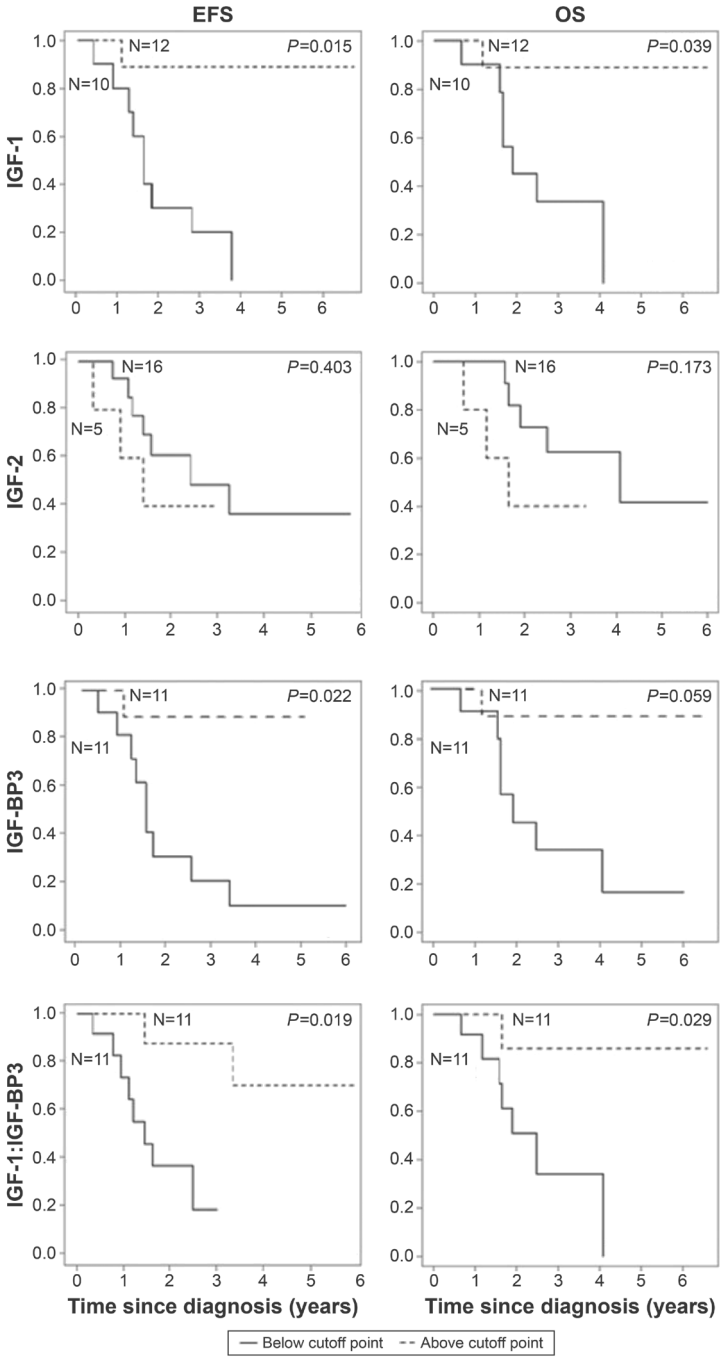

Figure 3: Kaplan–Meier curves of event-free survival (left column) and overall survival (right column) for IGF-1, IGF-BP3, and molar IGF-1 (nm/L):IGF-BP3 (nm/L) ratio levels under or above the cutoff point. Note: P-values are given for the univariate analyses of the Cox regression analyses. Abbreviations: IGF-1, insulin-like growth factor 1; IGF-BP3, insulin-like growth factor-binding protein 3; EFS, event-free survival; OS, overall survival.

Table 2: Univariate and multivariate Cox models of EFS for IGF-1, IGF-BP3, IGF-2 and IGF-1:IGF-BP3.

	Univariate analysis			Multivariate analysis		
	HR	**95%CI**	***P* value**	**HR**	**95%CI**	***P* value**
Metastasis at diagnosis	2.061	0.575-7.380	0.267			
Age	1.021	0.973-1.071	0.396			
BMI	1.158	0.998-1.343	0.053			
IGF-1	0.075	0.009-0.602	**0.015**	0.063	0.007-0.590	**0.015**
IGF-BP3	0.090	0.011-0.712	**0.022**	0.057	0.005-0.585	**0.016**
IGF-2	1.809	0.451-7.254	0.403			
IGF-1: IGF-BP3	0.082	0.010-0.660	**0.019**	0.103	0.011-0.934	**0.043**

Note: Bold values indicate P<0.05. Abbreviations: EFs, event-free survival; HR, hazard ratio; IGF-1, insulin-like growth factor 1; IGF-BP3, insulin-like growth factor-binding protein 3; IGF-2, insulin-like growth factor 2; HR, hazard ratio; CI, confidence interval; BMI, body mass index.

IGF-1 serum levels consistently decline after adolescence in healthy subjects.[17] Therefore, levels were compared for each subject in our cohort to the age- and gender-matched reference value. The measured values were calculated as a percentage of the corresponding reference values. High percentage of measured IGF-1 compared to the reference value predicted EFS (HR 0.614 per 10 percent increase, 95%CI 0.405-0.931, *P=0.021*), but not OS (HR 0.703 per 10 percent increase, 95%CI 0.460-1.075, *P=0.104*) in multivariate analysis.

Discussion

We found that high baseline IGF-1 and IGF-BP3 serum levels and the molar IGF-1:IGF-BP3 ratio in serum were associated with improved EFS and a trend for OS in Ewing sarcoma patients treated with VIDE chemotherapy. IGF-2 serum levels were not significantly associated with EFS or OS. Moreover, IGF-BP3 and IGF-2 serum levels increased during VIDE chemotherapy in this patient group. IGF-1 serum levels did not change during chemotherapy.

It seems counterintuitive that high levels of IGF-1 predict better survival in Ewing sarcoma, as IGF-1 is involved in the development, progression and therapy resistance of several solid tumors.[7] However, low levels of IGF-1 may reflect an endocrine adaptation to (severe) disease.[25] Indeed, low levels of IGF-1 were reported to be associated with greater tumor burden and more aggressive systemic illness in Ewing sarcoma.[26] Additionally, lower levels of IGF-1 were reported in 5 patients with metastatic disease compared to patients with localized disease.[26] Toretsky et al. also reported that Ewing sarcoma patients with metastatic disease had lower circulating IGF-1 levels than patients with localized disease, suggesting that more severe illness downregulates IGF-1 production

in the liver.[27] In keeping with these reports, we observed lower levels of serum IGF-1 in patients with metastatic disease compared to localized disease, but the difference was not statistically significant, perhaps because of the small population size. Interestingly, low levels of circulating IGF-1 predicted worse survival in other solid tumors [28-35] and systemic diseases.[36,37] Borinstein et al. did not find an association between serum IGF-1 levels and treatment outcome in 226 Ewing sarcoma patients[38], but this may be explained by the fact that relatively young patients were included compared to our study (88% of subjects were younger than 18 years), increasing the confounding effect of age-associated growth hormone and IGF-1 release.

In Ewing sarcoma, autocrine loops of IGF-1 signaling have been shown to be crucial for proliferation and cell survival in vitro.[3,39-41] Indeed, serum IGF-1 levels may have little or no influence on tumor growth, as the autocrine production of IGF-1 (and IGF-2) is probably sufficient and decisive. Analogously, autocrine IGF-1 production by the tumor has been shown to be a negative predictor of survival in colorectal cancer patients treated with cetuximab and irinotecan.[42] Presumably, autocrine IGF-1 production by the tumor does not affect circulating levels. Thus, we believe that circulating levels of IGF-related peptides reflect disease severity in various conditions, but cannot guide therapeutic strategies. In contrast, determining of autocrine IGF-1 (and IGF-2) levels and the expression of the IGF-1R in the tumor might help to select patients who could benefit from (co-)treatment with an IGF-1R inhibitor. Moreover, IGF-1R expression may be diminished after treatment with chemotherapy, which has been shown in breast cancer patients[43,44], suggesting that the efficacy of an IGF-1R inhibitor after chemotherapy might be diminished.

In our study, high IGF-BP3 serum levels predicted better EFS. IGF-BP3, the synthesis of which is primarily stimulated by growth hormone, inhibits the bioavailability of IGF-1 and has direct antitumor effects.[8,45] In Ewing sarcoma cells, binding of the EWS-FLI oncoprotein to the *IGF-BP3* promoter inhibits autocrine IGF-BP3 production.[3,4] Therefore, perhaps high circulating levels of IGF-BP3 lead to better survival of Ewing sarcoma patients due to the direct apoptotic and growth inhibitory effects in Ewing sarcoma cells. Alternatively, a decline of IGF-BP3 (along with IGF-1) may reflect an endocrine adaptation to severe disease.

We found that IGF-BP3 and IGF-2 serum levels increased during treatment with VIDE chemotherapy in Ewing sarcoma patients. Similarly, Kümmel et al. observed that IGF-BP3 serum levels increase during anthracycline and taxane chemotherapy in breast cancer patients,[46] and Gallego et al. reported that IGF-BP3 serum levels increase during FOLFOX (Folinic acid, Fluorouracil and Oxaliplatin) or FOLFIRI (Folinic acid, Fluorouracil and Irinotecan) chemotherapy in colorectal cancer.[34] Interestingly, in the

latter study, IGF-BP3 serum levels increased when the tumor was stable or responding to therapy but declined upon disease progression.[34] Although IGF-2 is three times more abundant in serum than IGF-1, our understanding of this protein is limited, [47] but it is possible that the increase in serum IGF-BP3 and IGF-2 levels during treatment is a systemic reflection of (partial) disease recovery.

The sample size of our study is small, and the results clearly need validation in a larger cohort. However, our data suggest that high circulating levels of IGF-1 pathway components predict longer EFS in patients with Ewing sarcoma treated with VIDE chemotherapy. This observation apparently contrasts with data showing that autocrine production of IGF-1 by tumor tissue promotes tumor growth.[5,8,47] A possible unifying explanation is that perhaps low circulating levels of the IGF-1 pathway components reflect an endocrine response to severe disease in contrast to the autocrine production of IGF-1 by tumor tissue that primarily determines tumor growth.[34,48] This theory is consistent with findings, including those of this study, of increased circulating IGF-BP3/IGF-2 levels in the course of chemotherapy for various tumor-types.[34,46]

Conclusion

High circulating levels of IGF-1 and IGF-BP3 at baseline appear to be a favorable indicator of outcome in Ewing sarcoma patients treated with chemotherapy. These findings suggest the need for further investigation of the value of the IGF-1 pathway as biomarker for treatment outcome and treatment target in Ewing sarcoma.

Acknowledgement

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Chapter 9

General discussion and future perspectives

Discussion

This chapter discusses the main findings of the studies described in the eight previous chapters in the context of the current literature. The thesis is divided into two parts as described in the introduction (**Chapter 1**); the first part focusses on the effects of short-term fasting on chemotherapy outcome in patients with breast cancer and the second part on the IGF-1 and insulin pathway as a target for cancer therapy and as a biomarker for chemotherapy outcome.

Part I. Short-term fasting and fasting mimicking diets as an adjunct to chemotherapy

In this part of the thesis the effects of short-term fasting and fasting mimicking diets on toxicity and efficacy of chemotherapy is investigated.

In **Chapter 2**, preclinical research is evaluated, which shows that short-term fasting during chemotherapy is effective in vitro in a wide variety of tumors, such as breast cancer, ovarian cancer, melanoma, lung cancer and colorectal cancer[1,2]. Moreover, data suggest that short-term fasting enhances the effects of radiotherapy and tyrosine kinase inhibitors (TKIs) as well[3-5]. Preclinical studies also show that short-term fasting simultaneously protects mice from chemotoxicity[1,6]. The mechanisms behind the distinctive response of healthy and cancer cells, which is described as "Differential Stress Resistance" (DSR), to short-term fasting are not fully unravelled[7,8]. However, it is clear that IGF-1 and insulin are important factors, as a decrease of these growth factors increase stress resistance in healthy cells to chemotherapy agents like doxorubicin or cyclophosphamide, but not in cancer cells[9]. Autophagy and glucose metabolism are also proposed mechanisms behind the DSR, due to nutrient deprivation, whereby tumors with diminished autophagy are highly sensitive to short-term fasting[7]. Although preclinical results are promising, the application of short-term fasting in cancer patients is not obvious. The metabolic differences between mice and humans for example may cause that humans need to fast for much longer periods to have similar effects as seen in mice[10]. Clinical research is in its infancy, however, the few small clinical studies to date show that short-term fasting as an adjunct to chemotherapy in humans is safe[11-15], as only mild side effects as hunger and dizziness were seen[12,14-16].

The effects of short-term fasting on chemotherapy-induced side effects and quality of life (QOL) in humans is not evident yet, however, there are some indications that patients may benefit. In a case series a reduction in fatigue, weakness, vomiting and diarrhea was seen when patients with distinct chemotherapy schedules fasted during the chemotherapy cycles compared to chemotherapy cycles without fasting[12]. Bauersfeld

et al. concluded that short-term fasting led to a better tolerance to chemotherapy with less compromised QOL and reduced fatigue in the 8 days following chemotherapy[15]. In another pilot trial from our hospital (**Chapter 3**), no difference in side effects were found, however, mean erythrocyte and thrombocyte counts 7 days post-chemotherapy were significantly higher in patients who fasted 24 hours before chemotherapy compared to patients in the control group[11]. In our DIRECT trial (**Chapter 4**), however, we found no difference in chemotherapy-induced side effects or QOL[16]. Moreover, the increase of levels of γ-H2AX in PBMCs, a marker of chemotherapy induced DNA damage in healthy cells, were lower in patients who fasted or used a fasting-mimicking diet compared to patients in the control group[11,14]. Reduction of side effects would improve QOL and potentially reduce expenses of hospitalization and the use of drugs such as anti-emetics. Moreover, short-term fasting may broaden the therapeutic window of cancer treatments, allowing for an increase of the dosage of (chemo) therapeutic agents, thereby enhancing their efficacy.

Although the effects of fasting on hormones and growth factors are studied in healthy subjects and it is known that glucose, insulin and IGF-1 levels decrease dramatically during short-term fasting[17-19], the exact effects of short-term fasting on these mediators in cancer patients during chemotherapy were unknown. In our pilot study we found evidence that during docetaxel, doxorubicin and cyclophosphamide (TAC) chemotherapy plasma glucose levels increased and insulin levels remained constant despite short-term fasting[11]. The use of concomitant dexamethasone for anti-emesis, reduction of fluid retention and dampening of hypersensitivity reactions in response to docetaxel may explain these findings, as it induces insulin resistance, compensatory hyperinsulinemia and hyperglycemia[20]. Therefore, the use of dexamethasone or other corticosteroids may counteract the beneficial impact of short-term fasting and fasting mimicking diets on chemotherapy tolerability and efficacy. In the DIRECT trial dexamethasone was omitted in the fasting mimicking diet arm during the first half chemotherapy cycles to reduce its potentially counteractive metabolic effects. As expected, a large decrease in insulin and glucose was found in the patients who were compliant to the fasting mimicking diet compared to the patients with a regular diet[16].

Preclinical studies show that chemotherapy efficacy can be enhanced by short-term fasting or fasting mimicking diets[1]. The first (small) clinical studies were predominantly focused on safety and the effects on chemotherapy-induced toxicity[11,15], although the effects on efficacy of chemotherapy may be more interesting. In the randomized DIRECT study, we found the first evidence of increased efficacy as a result of a fasting mimicking diet on radiological and pathological response according Miller and Payne in early breast cancer patients treated with doxorubicin, cyclophosphamide followed by docetaxel (AC-T) or 5-FU, epirubicin, cyclophosphamide followed by docetaxel (FEC-T). However, the,

pathological complete response after neo-adjuvant chemotherapy (Miller and Payne 5), was not different between the fasting mimicking diet group and the control group. Data on survival are not available yet[16]. Therefore, more research is needed to establish the effects of short-term fasting on chemotherapy efficacy and to research in which tumors short-term fasting may be effective as an adjunct to cancer treatment.

In the DIRECT study, the compliance to follow a 72-hour fasting mimicking diet during the chemotherapy cycles was high for 1 cycle but decreased with subsequent cycles. In other (small) studies usually a higher compliance rate for 3 or more cycles is seen[12,15]. The disappointing compliance rate in the DIRECT study may be caused by aversion to distinct components of the diet in combination chemotherapy, the amount of chemotherapy cycles and lacking support by a dietician which was not standard offered. Therefore, close monitoring of patients by nutritionists with expertise in low calorie diets may increase compliance. As well as diets with a more variable and fresh taste may be needed to increase compliance and successfully examine the impact on chemotherapy tolerability and efficacy. Although, one or two cycles of short-term fasting may be enough to increase efficacy of chemotherapy as seen in preclinical studies[1]. Additionally, ketone bodies measurements in urine appeared to be a good objective marker for compliance.

An important remark on safety is that effects of short-term fasting in patients at risk for malnutrition or cachexia are unknown and further limitation of nutrient intake in these patients may be unsafe, even for a short period of time. However, in fit patients, with a normal or high BMI treated with (neo)-adjuvant chemotherapy and without diabetes mellitus, short-term fasting emerges as a promising strategy to enhance the efficacy and tolerability of chemotherapy and more research is needed to firmly establish clinical effects.

Part II. IGF-1 and insulin pathway in cancer treatment

In the second part of the thesis the IGF-1 and insulin pathway in cancer treatment are investigated as a target for therapy and its predictive role. First, **Chapter 5** describes the IGF-1 pathway as an treatment target[21] and subsequently describes the pathway as biomarker for chemotherapy efficacy[21-23].

Clinical research of IGF-1R inhibitors has shown that IGF-1R inhibitors have no convincing benefit in clinical studies, except for a few patients with Ewing sarcoma[24,25]. We hypothesize that the failure of IGF-1R inhibitors in clinical studies may be caused by the complexity of the IGF-1R pathway. The pathway activation is not adequately inhibited by the distinct inhibitors, as IGF-1R inhibition causes hyperglycemia and subsequent hyperinsulinemia due to cross-reactivity with the insulin receptor isoform B (IR-B) and hybrid receptors and the ligand IGF-2 is not inhibited as well [21].

Therefore, the ligands insulin and IGF-2 still activate IR-A and hybrid receptors and stimulate tumor proliferation and survival via the same downstream pathway[26,27]. Additionally, in our preclinical experiments we found evidence that insulin causes resistance to IGF-1 inhibition, as it exhibits proliferative and survival effects in Ewing cell lines[21]. Therefore, activation of the IGF-1/insulin pathway through insulin could be an important resistance mechanism. Lowering insulin, perhaps with a short-term fasting intervention, or blocking its activity entirely with an IR-A inhibitor serves as a possible target in cancer therapy and may be effective in combination with IGF-1R inhibition[7,28]. Moreover, measuring biomarkers, such as ligand levels and receptor expression seems necessary to select patients who may benefit from treatment with IGF-1R inhibitors and urge for further research.

In **Chapter 6** we aimed to identify single nucleotide polymorphisms (SNPs), which influence the IGF-1R pathway activity, to predict chemotherapy efficacy in patients with HER2 negative early breast cancer treated in the NEOZOTAC trial. We found that variation in the *IGF-1R* gene was associated with pathological response, which may be explained by diminished IGF-1R activity [22]. Additionally, we evaluated if pathological response according Miller and Payne could be predicted by IGF-1R expression in the tumor before and after TAC chemotherapy in the same cohort [22]. A decline of IGF-1R expression in the tumor during treatment was associated with a better pathological response, in line with the study of Heskamp et al. where downregulation of IGF-1R during chemotherapy treatment was associated with prolonged survival[29]. Therefore, the IGF-1R seems to play an important role in therapy resistance in breast cancer patients. However, in our cohort diminished IGF-1R expression during treatment was not associated with better survival. (**Chapter 7**).

Additionally, in a small side study of the NEOZOTAC trial lower serum insulin levels were associated with improved disease-free survival [30]. Accordingly, Feroni et al. found that patients with breast cancer with higher levels of insulin had an increased risk for disease progression [31] and Goodwin et al. found that higher fasting insulin levels at baseline in breast cancer patients without diabetes were associated with worse overall survival [32]. Higher insulin levels may give the tumor a growth advantage by activating the IGF-1/insulin pathway [33].

The last chapter of this thesis (**Chapter 8**) describes how high baseline IGF-1 serum levels were associated with improved event-free survival and a trend for overall survival in patients with Ewing sarcoma treated with VIDE chemotherapy. Although counterintuitive, low levels of IGF-1 may reflect an endocrine adaptation to severe disease [34]. Supporting results for this hypothesis are for example that low levels of IGF-1 were associated with more aggressive systemic illness in Ewing sarcoma [35] and that lower

levels of IGF-1 were found in patients with metastatic disease compared to patients with localized disease [35]. Moreover, low levels of circulating IGF-1 predicted worse survival in other solid tumors [36-43] and systemic diseases [44,45] as well.

Additionally, serum IGF-1 levels may have little or no influence on tumor growth, as the autocrine production of IGF-1 (and IGF-2/insulin) is probably sufficient and decisive in most patients with Ewing sarcoma [46-49]. Therefore, determining of autocrine IGF-1 (and IGF-2/insulin) levels as well as the expression of the IGF-1R in the tumor might help to select patients who could benefit from (co-) treatment with an IGF-1R inhibitor.

Conclusion and future perspectives

Although the first small clinical studies of short-term fasting as adjunct to chemotherapy are promising in terms of decreased toxicity and enhanced efficacy [15,16,50], the exact mechanism and effects are not established yet. Besides, it has not been proven that short-term fasting provokes a better overall survival. More studies and a longer follow-up are needed to prove this. Therefore, fasting interventions should only be applied in the context of clinical research in patients with cancer until there is robust evidence for the safety and benefits. For future studies some recommendations can be done. First, tumors in which insulin or IGF-1 pathway are known to cause chemotherapy resistance, such as breast cancer [22,29,51], may be good candidates for future studies of short-term fasting as an adjunct to chemotherapy. Second, an adequate decrease in insulin, glucose and IGF-1 are probably needed to have beneficial effects on treatment outcome, thus the fasting intervention should have an adequate length. The 72-hour fasting mimicking diet used in the DIRECT study seems an good approach as insulin decreased dramatically, however, the compliance was unexpectedly low [16]. Therefore, in future studies more variable diet options and close monitoring of patients by nutritionists with expertise in fasting may be needed. Besides fasting interventions to increase tolerability and efficacy of chemotherapy, future studies should also address other therapies as targeted therapy and immunotherapy. Another approach for future studies may be to combine short-term fasting with physical therapy, as exercise during chemotherapy can improve treatment outcome as well and increase muscle strength and patients quality of life[52-54]. Additionally, as there is increasing evidence that hyperglycemia and hyperinsulinemia worsen the outcome in cancer patients [55], the wide-use of corticosteroids during chemotherapy may be debated [56] and studies as the REDEX trial (NCT02776436), to decrease the use of these agents may be of huge interest. Moreover, the disappointing results of clinical studies of IGF-1R inhibitors may be caused by the complexity of the IGF-1R pathway, whereby pathway activation is not adequately inhibited due to the ligands insulin and IGF-2 and activation of IR-A. Therefore, good strategies for future research would be to combine IGF-1R inhibition with IR-A inhibition and/or lowering insulin with short term fasting, based on biomarkers and ligand levels.

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Chapter 10

Summary and appendices

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Dankwoord

Nederlandse samenvatting

Kanker is na hart- en vaatziekten de ziekte met de meeste doden wereldwijd en het voorkomen ervan neemt nog steeds toe. Daarom zijn preventieve maatregelen om kanker te voorkomen en betere behandelingsmethodes om kanker te genezen nodig.
Het eerste deel van dit proefschrift beschrijft onderzoek naar de effecten van calorie restrictie en kortdurend vasten op kanker en de behandeling daarvan. In **hoofdstuk 2** wordt de bestaande literatuur samengevat en kritisch bediscussieerd. Langdurige calorierestrictie zorgt bij proefdieren voor vermindering van het ontstaan van kanker. Eveneens zorgt kortdurend vasten voor verbetering van het effect van anti-kankertherapie in proefdieren en kan het de bijwerkingen verminderen. De hypothese is dat vasten gezonde cellen beschermt en kankercellen gevoeliger maakt voor chemotherapie. Gezonde cellen gaan tijdens vasten energie investeren in herstel en behoud van de cel en niet in groei. Kankercellen daarentegen zijn niet meer in staat groei af te remmen door mutaties en hebben de nutrieten juist nodig om te kunnen delen en zijn daarom mogelijk juist gevoeliger voor chemotherapie tijdens vasten. In **hoofdstuk 3** wordt een klinische pilot studie beschreven waarin vasten werd toegepast bij13 patiënten die chemotherapie kregen voor borstkanker. De studieresultaten zijn gebruikt voor het opzetten van een grotere klinische studie, de DIRECT trial, waarin het effect van een dieet waar vasten mee wordt nagebootst werd bestudeerd in deze categorie patiënten. De DIRECT-studie wordt beschreven in **hoofdstuk 4**. De resultaten van de klinische trials laten zien dat kortdurend vasten, of het gebruik van een dieet waar vasten mee wordt nagebootst, in fitte kanker patiënten die behandeld worden met chemotherapie veilig is. Er ontstonden niet meer ernstige bijwerkingen van de chemotherapie dan in de controle groep. Wel was er minder DNA schade in de witte bloedcellen van de patiënten die het dieet volgden vergeleken met die van de controlegroep. Tevens zijn er aanwijzingen dat in de groep patiënten die het vasten nabootsten met een dieet, de chemotherapie beter werkte dan in de groep patiënten die een normaal dieet volgde. Deze resultaten zijn veelbelovend, maar er is meer onderzoek nodig om dit in meer patiënten aan te tonen en om precies uit te zoeken wat de effecten zijn. Het vasten zorgde in de klinische trials, mits er geen corticosteroïden (corticosteroïden zijn vaak onderdeel van de medicatie tegen door chemotherapie veroorzaakte misselijkheid) werden gegeven, voor een daling van groeifactoren zoals glucose, insuline en insuline achtige groeifactor 1 (IGF-1).

In het tweede deel van het proefschrift wordt verder gekeken naar de effecten van insuline en IGF-1, beiden onderdeel van de complexe IGF-1 pathway (ook wel signaleringsroute). Deze route is onder andere betrokken bij celgroei en het ontstaan van kanker. In **hoofdstuk 5** wordt de literatuur samengevat over remmers van de IGF-1 route, die tot op heden geen goede effectiviteit in klinische trials hebben laten zien. Een hypothese is dat door alleen delen van de route te remmen, andere delen juist op-

reguleren. Derhalve zouden verschillende remmers gecombineerd moeten worden of zou zoals reeds in het eerste deel van dit proefschrift beschreven, kortdurend vasten de gehele route kunnen remmen, doordat insuline en IGF-1 gelijktijdig verlaagd worden tijdens vasten. In **hoofdstuk 6, 7 en 8** is gekeken of IGF-1 en insuline levels en variatie in de IGF-1 receptor de uitkomsten kunnen voorspellen van de behandeling van chemotherapie bij kankerpatiënten. In de NEOZOTAC studie waarin patiënten met borstkanker worden behandeld met neoadjuvante chemotherapie, werd in de patiënten met een goede response een afname van de IGF-1 receptor gezien. Deze resultaten zouden een aanwijzing kunnen zijn dat de IGF-1 route zorgt voor verminderde werking van chemotherapie. Tevens werd gezien dat patiënten met lagere insuline levels een betere ziekte vrije overleving hadden, wat ook door resultaten van andere onderzoekers wordt ondersteund. Verder werd gezien dat in een groep Ewing sarcoom patiënten lagere levels IGF-1 geassocieerd waren met een slechtere overleving. Ook deze bevinding wordt ondersteund door andere onderzoekers. De hypothese is dat tumoren zelf IGF-1 aanmaken en dat de waarden die worden gemeten in het bloed, een reflectie zijn van de ernst van de ziekte, waarbij lagere waarden worden gemeten in patiënten met meer ziektelast. Door het meten van IGF-1 in de tumor zouden patiënten kunnen worden geselecteerd die mogelijk effect hebben van een IGF-1 remmer.

In het laatste hoofdstuk van dit proefschrift worden aanbevelingen gedaan voor de toekomst. Zo is er meer onderzoek nodig naar de effecten van kortdurend vasten tijdens de behandeling met chemotherapie bij kankerpatiënten. Er moet gezocht waren naar een toepassing van vasten welke patiënten goed kunnen volhouden en daar moet voldoende begeleiding bij zijn. Tevens is er onderzoek nodig naar het gebruik van hoge dosis corticosteroïden tijdens de behandeling van kankerpatiënten, omdat mogelijke schadelijke effecten niet goed onderzocht zijn en deze middelen verhoogde glucose en insuline spiegels veroorzaken, welke in verband worden gebracht met slechtere uitkomsten van patiënten met kanker. Tot slot is het zinvol om de effecten van de combinatie van verschillende IGF- route remmers te onderzoeken, omdat door de complexiteit van de route, remming van een enkel onderdeel niet zinvol is gebleken.

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Curriculum Vitae

Stefanie de Groot was born on June 9th 1985 in Rotterdam, The Netherlands. She grew up with her parents and brother Anthonie in 's-Gravendeel. She attended the Johan de Witt gymnasium in Dordrecht and obtained her gymnasium graduation in 2003. Afterwards, she started studying chemistry and subsequently medicine in 2006 at Leiden University. She obtained her bachelor's degree for chemistry in 2009 and her doctoral degree for medicine in 2012. During the clinical rotations she performed her final scientific and clinical internship at the department of Clinical oncology at the LUMC in Leiden under supervision of prof. dr. Hans Gelderblom. After a short period working in the clinic as a non-training resident in internal medicine, she started her PhD project described in this thesis in 2013 under supervision of dr. Judith R. Kroep, prof. dr. Hanno Pijl and prof. dr. ir. Jacobus J.M. van der Hoeven at the LUMC in Leiden. In 2015 she started her clinical training to become an internist at the Haga hospital in the Hague. After 2.5 years she temporarily paused her training to finish this thesis, after which she resumed her training in the LUMC with the intention to become internist with the subspecialty medical oncology. Stefanie lives in the Hague with her partner René and their son Felix.

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Short term fasting,

IGF/insulin-axis and therapy outcome in patients with cancer

S. de Groot