Proliferation in operable breast cancer

Aspects of prognostication and relevance of carbohydrate metabolism

Tone Hoel Lende

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Thesis for the degree of Philosophiae Doctor (PhD)
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Stavanger, November 2019

Tone Hoel Lende

Abbreviations

AI: Aromatase Inhibitor

ASIR: Age-standardized incidence rate

ATP: adenosine triphosphate

AUC: Area under the curve

BC: Breast cancer

BCSS: Breast cancer-specific survival

BCT: Breast conservative therapy

CAMS: Cell-to-cell adhesion molecules

CI: Confidence interval

CISH: Chromogenic in situ hybridization

CTC: Circulating tumor cell

CV: Coefficient of variance

DCIS: Ductal carcinoma in situ

EGFR: Epidermal growth factor receptor

EMF: Electromagnetic field

EMT: Epithelial mesenchymal transition

ER: Estrogen receptor

ERAS: Enhanced Recovery After Surgery

FISH: Fluorescence in situ hybridization

GG: Guanine-guanine gene variant

GLOBOCAN: Global Cancer Observatory

HER-2: Human epithelial growth factor receptor 2

HES: Hematoxylin-eosin-saffron staining

HGH: Human growth hormone

HR: Hazard rate

HR-MAS: High resolution magic angle spinning

IDC: Invasive ductal carcinoma

IGF-R: Insulin-like growth factor receptor

IGF-1: Insulin-like growth factor 1

IGF-1R: Insulin-like growth factor 1 receptor

IHC: Immunohistochemistry

Invasive carcinoma NST: Invasive carcinoma no special type

IPA: Ingenuity Pathway Analysis

IR: Insulin receptor

LOH: Loss of heterozygosity

MAI: Mitotic activity index

MC: Metabolic cluster

MET: Mesenchymal to epithelial transition

MINDACT: Micro array In Node Negative and 0-3 Positive Lymph Node Disease May Avoid Chemotherapy Trial

MMMCP: Multicenter Morphometric Mammary Carcinoma Project

MRI: Magnetic resonance imaging

NBCG: Norwegian Breast Cancer Group

NSD: Norwegian Center for Research Data

OR: Odds ratio

OS: Overall survival

PAM50: Prediction Analysis of Microarray 50 (Prosigna)

PCA: Principal component analysis

PLS: Partial lest square

PLS-DA: Partial least squares discriminant analysis

PPH3: Phosphorylated phospho-histone 3

PR: Progesterone receptor

PROM: Patient-reported outcome measure

QoL: Quality of Life

RCT: Randomized controlled trial

REK: Regional ethical committee

RFS: Relapse-free survival

ROC: Receiver operating characteristic

ROR: Risk of recurrence

ROS: Reactive oxygen species

RTK: Receptor tyrosine kinase

SEER: Surveillance, Epidemiology and End Results

SN: Sentinel node

TCA: Tricarboxylic acid cycle

TDLU: Terminal ductal lobular unit

TIL: Tumor infiltrating leukocyte

VIP: Variable Importance in Projection score

WHO: World Health Organization

Abstract

Breast cancer is the most common malignant disease among women in the Western world. In Norway and the Netherlands, the incidence has more than doubled in the last 50 years, likely due to increased estrogen exposure, higher levels of alimentary carbohydrates and fat, and reduced physical activity, the so-called Western lifestyle. Treatment of breast cancer is based on an additive multimodal approach comprising surgery, radiation therapy, and adjuvant systemic therapy (i.e., chemotherapy, anti-estrogen therapy, and biological therapy). However, the disease is heterogeneous with different molecular gene expression profiles, phenotypes, and risk profiles. Thus, it is important to optimize treatment to avoid over- and under-treatment; to achieve this, prognostic and predictive factors must be explored further.

The first study is a retrospective population-based study in which we used the original Multicenter Morphometric Mammary Carcinoma Project (MMMCP) data set from the Netherlands and introduced new exposure variables. We compared the prognostic power of tumor proliferation to classical prognostic factors in treatment-naïve patients with lymph node-negative BC aged < 55 years. Several tools are available for clinicians making decisions regarding adjuvant systemic treatment. Among these tools, we used the Norwegian Breast Cancer Group treatment guidelines from 2010 and Adjuvant! Online v 8.0. Compared to the grouping obtained by these tools, MAI-3 identified 40% of the patients as under-treated and 20% of the patients as over-treated, which shows the importance of proliferation as a prognostic and predictive factor that should be included in the decision-making process for treatment. Later, Ki-67 was added to the Norwegian Breast Cancer Group (NBCG) guidelines with changes to the treatment regimen for luminal breast cancer patients.

The second study is a randomized controlled trial in which we examined the influence of pre-operative carbohydrate load compared to standard fasting procedures regarding tumor proliferation and clinical outcome in operable breast cancer patients. The

Enhanced Recovery After Surgery (ERAS) protocol is used for patients going through major surgery with pre-operative carbohydrate loading. To the best of our knowledge, no studies have explored the effects of such carbohydrate loading in tumor tissue. In our study, we observed an increase in the number of luminal breast cancer patients with MAI≥10 in the group receiving pre-operative carbohydrate load. The proportion of PR-negative patients was also increased in the carbohydrate group. No differences were found regarding the well-being of patients after surgery. Both relapse-free survival (RFS) and breast cancer-specific survival (BCSS) were inferior among the ER+/T2 patients in the carbohydrate group.

In the third study, we performed an explorative study based on the patient material from the second study. The metabolic consequences were explored in the tumor and liquid biopsies from operable breast cancer patients receiving pre-operative carbohydrate loading. Pre-operative carbohydrate loading increased the systemic lactate and pyruvate content in patients with high-proliferation tumors. Tumor tissue with high proliferation had high glutathione content, which is an intratumoral protection factor. The metabolic signature or pathway is the same as in the Warburg effect. Regarding microRNA involved in endocrine resistance, four out of seven microRNAs were recruited after carbohydrate loading. High levels of systemic lactate and pyruvate and tissue glutathione were associated with decreased RFS, BCSS, and overall survival (OS). Integrated pathway analysis in serum revealed the activation of five major anabolic metabolic networks contributing to proliferation and growth. These findings agree with previous studies showing that metabolic profiling of serum samples can provide prognostic information in operable breast cancer.

Future research comprises the calibration of MAI against the gold standard PAM-50 (Prosigna) test through the nationwide EMIT study endorsed by the NBCG, which has added the Prosigna test to their 2020 treatment guidelines. However, the inexpensive and standardized MAI may serve as a good proxy for costly gene expression methods, especially in developing countries. We also suggest performing a new randomized

controlled trial in ER+/T2 patients with a metformin arm, a metformin + endocrine therapy arm and placebo arm. The metformin or metformin + endocrine therapy should be introduced after diagnostic biopsy, but before surgery. Moreover, glutathione should be pursued for the purpose of targeted therapy. The above-mentioned strategies seem to be only a small step for biomarker research but will undoubtedly bring the clinical knowledge regarding the relevance of metabolic networks a giant leap forward.

List of publications

Lende TH, Janssen EAM, Gudlaugsson E, Voorhorst F, Smaaland R, Van Diest P, Søiland H, Baak JPA. (2010) In Patients Younger Than Age 55 Years With Lymph Node–Negative Breast Cancer, Proliferation by Mitotic Activity Index Is Prognostically Superior to Adjuvant! *Journal of Clinical Oncology* 2010: 29(7), 852-8.

Lende TH, Austdal M, Varhaugvik A, Skaland I, Gudlaugsson E, Kvaløy JT, Akslen LA, Søiland H*, Janssen EAM*, Baak JPA*. (2019). Influence of pre-operative oral carbohydrate loading vs. standard fasting procedure on tumor proliferation and clinical outcome in breast cancer patients — a randomized trial. *BMC Cancer* 2019 Nov 8; 19:1076

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List of Contents:

Scientific environment 2
Acknowledgments 3
Abbreviations 6
Abstract 9
List of publications 12
List of Contents: 13
List of figures 17
List of tables 18
1.0 Introduction 19
1.1 History of Breast Cancer 19
1.2 Breast Cancer Epidemiology 22
1.2.1 The World 22
1.2.2 The Netherlands 23
1.2.3 Norway 23
1.3 Etiology and Risk Factors in Breast Cancer 24
1.3.1 Target: the breast 24
1.3.2 Genetic factors 26
1.3.3 Environmental factors 27
1.3.4 Lifestyle factors 27
1.3.5 Endocrine factors 28
1.3.6 Integrative models of risk factors in breast cancer 29
1.4 Tumor Biology —Hallmarks of Cancer 30
1.5 Carcinogenesis of Breast Cancer 37
1.6 Treatment of Operable Breast Cancer 38
1.7 Prognostic and Predictive Factors in Breast Cancer 39
1.7.1 Tumor size 42
1.7.2 Axillary lymph nodes 43
1.7.3 Morphological subtypes 44
1.7.4 Histological grading 45
1.7.5 Hormonal receptors: Estrogen receptor (ER) and progesterone receptor (PR) 46
1.7.6 HER-2 48
1.7.7 Biomarkers of proliferation 50
1.7.8 Adjuvant! Online: A web-based tool to integrate prognostic and predictive factors 55
1.7.9 Molecular subtyping 57

1.7.10 A useful candidate gene test: Prosigna™ (PAM50) 59
1.8 Insulin, Insulin-c-peptide, IGF-1, and IGFBP3 in Breast Cancer Risk, Progression, and Prognosis 61
1.8.1 Insulin 61
1.8.2 Insulin-C-peptide 62
1.8.3 IGF-1 64
1.8.4 IGFBP-3 65
1.9 The ERAS Concept 65
1.10 The Warburg Effect: Deregulation of Cellular Energetics and Metabolic Reprogramming as Putative Prognostic and Predictive Factors in Breast Cancer 67
1.11 Metabolomics in Breast Cancer 69
1.11.1 Metabolomic studies of carbohydrate metabolism in cancer cells 70
1.11.2 Metabolomic studies of lipid and fatty acid metabolism in cancer cells 71
1.11.3 Metabolomic studies of amino acid metabolism in cancer cells 71
1.11.4 Metabolomic profiling of breast cancer 72
1.11.5 Brief overview of tools and methods used in analysis of metabolomic data 75
1.12 Endocrine Resistance — The Ultimate Hallmark of Breast Cancer 78
1.13 Rationale for the Present Thesis 81
2.0 Objectives, Aims, and Hypotheses of the Present Study/Thesis 83
2.1 Overall Objectives 83
2.1.1 83
2.1.2 83
2.1.3 83
2.2 Specific Aims & Hypotheses 83
2.2.1 Paper I 83
2.2.2 Paper II 83
2.2.3 Paper III 84
3.0 Synopsis of the Studies 85
3.1 Paper I 86
3.2 Paper II 89
3.3 Paper III 90
4.0 Discussion of the main findings 95
4.1 MAI Predicts Under- and Over-treatment in Breast Cancer 95
4.2 Increased MAI in the carbohydrate group. 98
4.3 Progesterone Receptor as a Concomitant biomarker of Increased Proliferation, Increased IGF Signaling, and Increased Endocrine Resistance in Luminal Breast Cancer 99

4.4 Insulin-related Pathways and Metformin in Breast Cancer....99
4.5 Increased Tumor Size in the Carbohydrate Group....101
4.6 Metabolomic Changes after Carbohydrates and Fasting....101
5.0 Validity and Methodological Considerations....105
5.1 Factors Contributing to Internal Validity....105
5.1.1 Research designs....105
5.1.2 Sample size and statistical power....107
5.1.3 Representativeness....107
5.1.4 Random and systematic errors....108
5.1.5 Randomization....109
5.1.6 Data collection....110
5.1.7 Survival analysis....113
5.1.8 Bias....114
5.1.9 Handling of data and statistical analyses....115
5.2. External Validity....121
5.2.1. Relations between external and internal validity....121
5.2.2 Participation bias....122
5.3 Ethical Considerations....123
6.0 Future Perspectives....125
7.0 Conclusions....131
7.1 Individual papers....131
7.1.1. Paper I....131
7.1.2 Paper II....131
7.1.3 Paper III....131
7.2 Overall Conclusions....132
8.0 References....133
9.0 Errata....157
10.0 Appendix....159
Appendix 1. Simplified TNM-Classification in Breast Cancer....160
Appendix 2. Primary Treatment of Operable (early) Breast Cancer....161
Appendix 3: NBCG guidelines June 2009 to February 2012....162
Appendix 4. NBCG recommendations for AST with Gene profiling test....163
Appendix 5. NBCG recommendations for AST without gene test.....164
Appendix 6. Endocrine Treatment of Breast Cancer Patients....165
Appendix 7. PROM of Well-being....166

Appendix 8. EMIT Protocol 167
11.0 Paper I-III 169
Paper I 169
Paper II 169
Paper III 169

List of figures

Figure 1. Evolution of Breast Cancer knowledge 19
Figure 2. Worldwide breast cancer incidence 22
Figure 3.Overview of cancer types and breast cancer in Norway 24
Figure 4. Anatomy of the breast 25
Figure 5. Overview of breast cancer susceptibility loci and genes. 26
Figure 6. An integrative model of risk factors in breast cancer 29
Figure 7. Hallmarks of cancer 30
Figure 8. Signaling networks and pathways 36
Figure 9. Main steps in carcinogenesis of breast cancer 37
Figure 10. Algorithm of 38
Figure 11. Prognostic and 39
Figure 12. Interpretation of prognostic and predictive factors 40
Figure 13. Tools in adjuvant decision making 41
Figure 14. Prognostic information from tumor size 42
Figure 15. Prognostic information from lymph node status 43
Figure 16. Morphological features of six invasive breast carcinomas 44
Figure 17. Estrogen and progesterone receptor expression in breast cancer 47
Figure 18. HER-2 protein expression and gene amplification 48
Figure 19. Overview of HER-2, EGFR and ER signaling transduction 49
Figure 20. Cell cycle 50
Figure 21. Ki-67 in cell cycle 51
Figure 22. PPH3 in cell cycle 52
Figure 23. MAI in cell cycle 53
Figure 24. MAI assessment in tumor periphery. 54
Figure 25. The Adjuvant! Online algorithm 56
Figure 26. Molecular subtyping 57
Figure 27. Long term prognostic information of molecular subtypes 58
Figure 28. The Prosigna Score Chart 60
Figure 29. The effect of insulin, IGF-1 and IGFBP3 on breast cancer cells 63
Figure 30. Enhanced Recovery After Surgery 66
Figure 31. The Warburg effect 68
Figure 32. MR spectrometer 70
Figure 33. MR spectrum from HR-MRS and HR-MAS-MRS 72
Figure 34. Endocrine resistance of breast cancer 79
Figure 35. Overview of the three studies in the thesis 85
Figure 36. Prognostic information of MAI-3 88
Figure 37. Relapse free survival in ER+ and ER+/T2 tumors. 90
Figure 38. PCA and VIP sore from the serum samples 92
Figure 39. Relapse free survival in ER+ patients 93
Figure 40. Overview of the main steps in the IHC method 111
Figure 41. ROC curve 120
Figure 42. Overview of the Preoperative Metformin Study in ER+/T2 tumors 128

List of tables

Table 1. Cornerstones in the history of breast cancer. 19
Table 2. The algorithm of histological grading. 45
Table 3. Molecular structure and their corresponding NMR spectrum. 74
Table 4. Overview of variables representing low and high risk BC 87
Table 5. Influence of Adjuvant! and MAI-3 on NBCG 88
Table 6. Overview of Relapse free survival and Breast cancer specific survival 92
Table 7. Key steps in the IHC process 110
Table 8. Components of the ROC statistics 118

1.0 Introduction

1.1 History of Breast Cancer

The first written report on breast cancer dates to around 3000 BC (1). The Egyptian architect and physician Imhotep described the findings in a male breast cancer patient: "*[I]f thou puttest thy hand upon his breast upon these tumors, (and) thou findest them very cool, there being no fever at all therein when thy hand touches him; they have no granulation, they form no fluid, they do not generate secretions of fluid, and they are bulging to thy hand. Thou shouldst say concerning him, 'One having tumors. An ailment with which I will contend.' Treatment: There is no treatment*." (1).

Importantly, this knowledge has paved the way for an understanding of the inner biology of malignancy and provided new ways to treat breast cancer, 5000 years after Imhotep (Fig. 1).

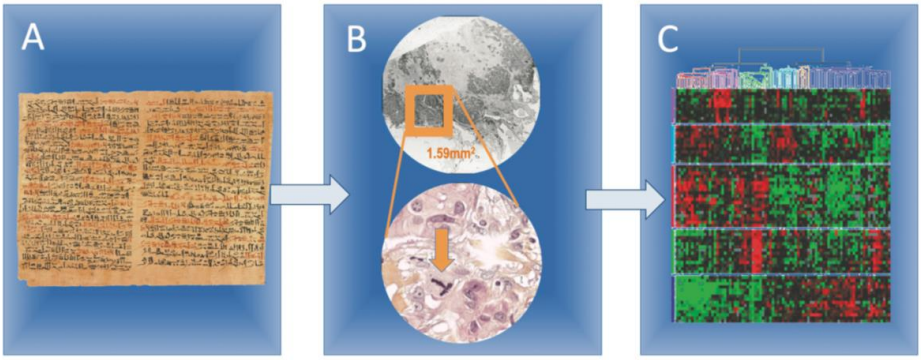


Figure 1. Evolution of Breast Cancer knowledge

The 5000-year long road from Imhotep's Surgical Syntax (A) via Jan Baak's Mitotic Activity Index (B) to Therese Sørlie's Molecular Codex (C) — and towards a modern understanding of breast cancer.

Over the years, breast cancer has been a disease in which new scientific knowledge has been applied and tested first. This is probably due to the easy access to the tumor and a yearning to heal a mother/wife/sister/daughter from a dreadful ailment. Therefore, breast cancer can be regarded as a 'model disease' — always being in the front row when new treatment concepts are to be tested (Table 1).

Table 1. Cornerstones in the history of breast cancer.

Line	When	Who	What	Ref
1	460 - 375 BC	Hippocrates	Breast cancer: due to surplus of black bile. First written description of the natural course of breast cancer. Father of the 'additive treatment principle' in breast cancer.	(2)
2	300 BC	Leonides of Alexandria	'Karkinoma': cancer is like the crab, bites itself onto the surrounding tissue. Difficult to remove.	(2)
3	1580 AD	Cervinius	Importance of the axillary nodes and first case of their removal.	(2)
4	1630	Nicolas Tulp	Surgery: 'The sole remedy is a timely operation'.	(2)
5	1655	Johann Schulteus	Painful, swift amputation of the breast.	(2)
6	1838	Johannes Muller	First microscopic view of cancer cells within the breast tumor.	(2)
7	1889	Stephen Paget	Paradigm: Soil and seed hypothesis for metastasis. Certain cancer cells need certain 'soil' to seed and grow. e.g., breast cancer cells and skeleton	(2)
8	1894	William Halstead	Centrifugal Paradigm: Breast cancer is a local disease that spreads centrifugally. First mastectomy in general anesthesia.	(2, 3)
9	1895	Wilhelm Conrad Roentgen	Discovery: X-rays.	(3)
10	1896	George T Beatson	Ovarian ablation leads to tumor reduction in locally advanced breast cancer.	(4)
11	1919	EB Krumbhaar & HD Krumbhaar	Discovery: Cytotoxic effect of custard-based and phosgene-based war gases used in battle in World War 1.	(5, 6)
12	1925	Otto Warburg	Discovery: Cancer cells ferment glucose to lactate despite the presence of oxygen.	(7)
13	1925	RB Greenough	Level of differentiation and mitosis matter in categorization of malignancy in cancer.	(8)
14	1926	Stafford L Warren	Mammography	(9)
15	1957	Bloom HJ & Richardson WW	Histological grading of breast tumors.	(10)
16	1957-1959	Dora Richardson	Commenced the synthesis of triphenyl ethylene, which ends up with tamoxifen.	(11)
17	1965	Roar Nissen Meyer	Randomized study of peri-operative chemotherapy (cyclophosphamide) for breast cancer patients.	(3, 12)

18	1966	Elwood Jensen	Discovery: Estrogen receptor.	(13)
19	1967	Bernard Fischer	Systemic Paradigm: Breast cancer regarded as a systemic disease.	(14)
20	1972	V. Craig Jordan	Tamoxifen first used in breast cancer trials.	(15)
21	1973	U. Veronesi B. Fischer	Commenced trials of breast conservative therapy.	(16)
22	1980s	Several	Combined Paradigm: Loco-regional control of primary tumor + systemic adjuvant treatment.	
23	1990	NCI (National Cancer Institute)	Approval of breast conservative treatment.	
24	1994	PA Friedman & PA Futeral	Detection of BRCA 1 germ line mutation transmission and risk of breast and ovarian carcinoma.	(17, 18)
25	1995-97	AE Guilliano	Establishment of sentinel node biopsy in breast cancer staging.	(19)
26	1998/2006	Several	Trastuzumab approved as anti-HER-2 agent. In 2006, approved in treatment of HER-2-positive breast cancer patients.	(20-22)
27	2000 - 2001	Chuck Perou et al Terese Sørli et al	Molecular subtyping of breast cancer.	(23, 24)
28	2005	Jan PA Baak	Mitotic Activity Index (MAI-10) strongest prognostic factor in LN negative breast cancer patients (after > 25 years of research).	(25)
29	2006	Emiel AM Janssen	MAI-3 predicts effect of chemotherapy	(26)
30	2015-2018	Peter Schmid et al.	Immunotherapy success in triple-negative breast cancer.	(27)
31	2018	PI: Bjørn Naume	Embarking on the first study in Norway using the gene expression test PAM-50 (Prosigna) for decision-making in adjuvant treatment (EMIT-study).	(28)

1.2 Breast Cancer Epidemiology

1.2.1 The World

Breast cancer is the most frequent female malignancy worldwide. Globally, breast cancer accounted for approximately 2.1 million new cases (11.6% of all cancer types) and 0.63 million deaths (6.6% of all cancer deaths) in 2018 (Fig. 2) (29). The incidence is highest in Western Europe, North America, and Australia (30), with standard incidence rates of 92.6 per 100,000 persons, 84.8 per 100,000 persons, and 94.2 per 100,000 persons, respectively (29). In 2018, the number of new cases in Europe was 562,500 (31), with the standard incidence rates varying from 22.6 per 100,000 inhabitants/year in Uzbekistan to 113 per 100,000/year in Belgium (32). Thus, breast cancer is a major challenge to the women and families who are affected, but also for society to arrange diagnostic units and handle the treatment burden. Therefore, it is of utmost importance to have reliable biomarkers to optimize treatment and develop new treatment methods and regimens.

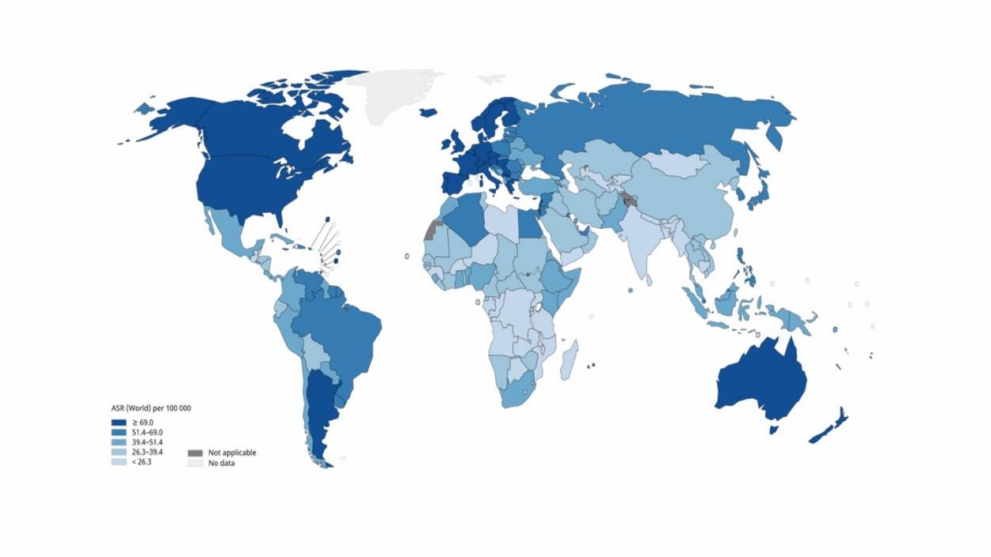


Figure 2. Worldwide breast cancer incidence

Estimated age-standardized worldwide incidence rates for female breast cancer at all ages in 2018 (29).

1.2.2 The Netherlands

The Netherlands has the fourth highest incidence of breast cancer worldwide, with an age-standardized incidence rate (ASIR) of 105.9 per 100,000 inhabitants (33). There is no pronounced spatial occurrence of breast cancer in the Netherlands (34). From 1989 to 2003, the breast cancer incidence increased from 73 to 91 per 100,000 in habitants/year. In addition, during this period, there was a trend of spatial difference towards higher incidence in rural areas, but no temporal trend differences were observed (35).

1.2.3 Norway

In Norway, the breast cancer incidence has more than doubled over the last 60 years, and the ASIR reached 87.7 per 100,000 inhabitants/year in 2018 (34) (i.e., 131.0/100,000/year according to the Norwegian Standard; Fig. 3A).

The median age of breast cancer patients in Norway is 62 years, with a peak age of 65 years (Fig. 3B) (36). The prevalence of a recent or former breast cancer diagnosis was 47,568 women in 2017, 21,363 of whom survived more than 10 years (36). In 2017, 629 deaths were attributed to breast cancer, whereas the overall relative survival during the period 2013-2017 was 90.4% (36). However, the Norwegian Breast Cancer Registry does not report long-term survival (i.e., >10 years). In 2017, this incidence translated into 3589 women and 34 men affected with breast cancer in Norway. There is a substantial spatial distribution of new cases in Norway (Fig. 3C).

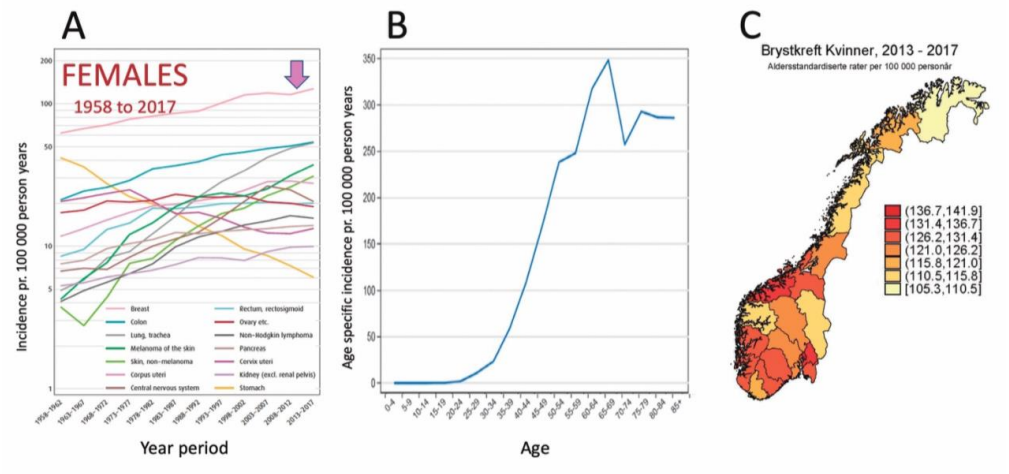


Figure 3.Overview of cancer types and breast cancer in Norway

A. Incidence of various cancer types in Norwegian women from 1958 to 2017. Time in 5-year intervals on the X-axis and incidence rate (number of new cases per 100,000 inhabitants/5-year period) on the Y-axis. Breast cancer is denoted by a pink arrow.

B. Age-specific incidence rates of breast cancer in Norwegian women. Notably, the incidence curve has a steep increase in the mid-50s, when women normally enter menopause. Years of women on the x-axis and age-adjusted incident rate on the Y-axis (number of cases per 100,000 inhabitants/year).

C. Spatial distribution of the estimated age-standardized incidence rates in Norway for female breastcancer at all ages in 2018 (29).

1.3 Etiology and Risk Factors in Breast Cancer

1.3.1 Target: the breast

The breast develops when the embryo is 4-5 mm long in the 5th-6th week of gestation, with formation of an ectodermal fold on the ventral side of the embryo, the so-called milk rim (37). Formation of the breast buds occurs on this rim by stem cells that start to sprout and form the breast bud. In human embryos, only the thoracic bud remains and develops into a specialized apocrine sweat gland. Thus, the stem cells in terminal ductal lobular units (TDLUs) may be influenced by hormonal (i.e., estrogen) changes in utero, during development in adolescence, and during the mature life of the woman (Fig. 4A).

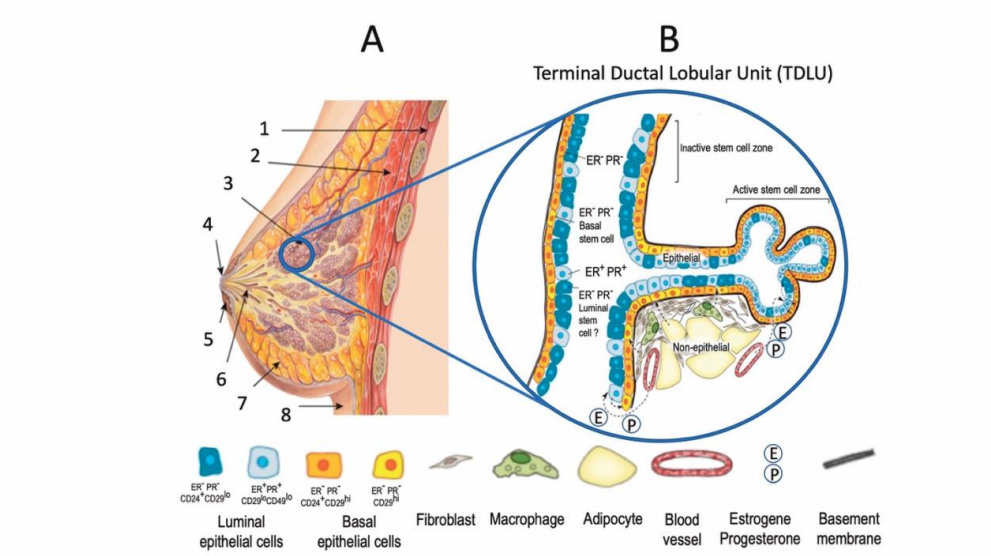


Figure 4. Anatomy of the breast

A. Sagittal section of the female breast. 1. Intercostal muscles; 2. M. pectoralis major; 3. parenchymal tissue, with approximately 18 lobes in each breast; 4. nipple; 5. areolar skin; 6. large milk collection sinuses; 7. subcutaneous and intra mammary adipose tissue; 8. inferior mammary fold (38).

B. Microscopic details of a breast lobe showing the smallest functional unit in the breast, the terminal ductal lobular unit (TDLU), where the luminal and basal stem cells reside. ER, estrogen receptor; PR, progesterone receptor. Modified from (39).

In the TDLUs, luminal stem cells are more numerous than other stem cells (Fig. 4B). Moreover, the fibroblasts, macrophages, and adipocytes in the microenvironment of the TDLU are co-actors in carcinogenesis and the promotion of breast cancer (39).

1.3.2 Genetic factors

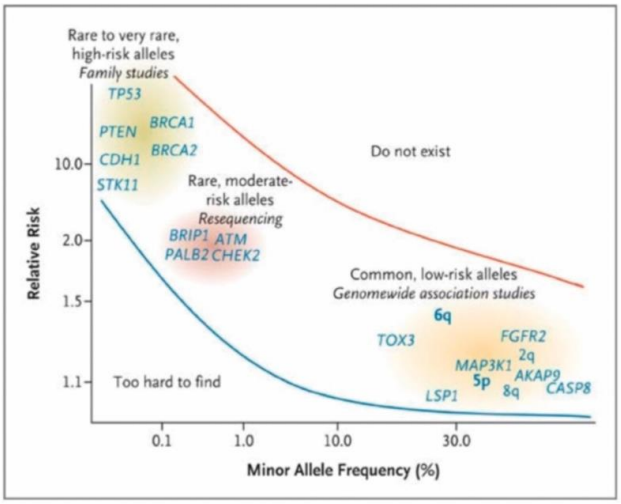


Figure 5. Overview of breast cancer susceptibility loci and genes.

Upper left: Low frequency/high risk. Lower right: High frequency/ low relative (46)

For many years, the estimated fraction of new breast cancers arising from germline mutation has been reported to be 5-10% (40, 41). Recently, next generation sequencing technology has detected up to 13% germline mutations in breast cancer (42). Women with a genetic risk for developing breast cancer are divided into three groups (43).

(The first group compriseswomen who are at the absolute highest risk of developing breast cancer during their lifetime. These women carry the highly penetrant BRCA-1 or BRCA- 2 mutations, which comprise 20-30% of all hereditary breast cancers. The BRCA-1 and -2 proteins are involved in the repair of double DNA strand lesions. Thus, the penetrance is quite high, with a lifetime risk of developing breast cancer of 50% to 70% (44). Consequently, younger breast cancer patients < 40 years of age account for more of these mutations (43). In the second group, there are several low-frequency and less penetrant genes, such as ATM, CHEK2, PALB2, CDH 1, and STK11 (Fig. 5) (45). The third group comprises patients with a family history of breast cancer only, with no gene mutations in genetic tests (45).

1.3.3 Environmental factors

Women who have undergone thoracic ionization radiation against mediastinal lymphoma are at higher risk of developing breast cancer (46). In addition, living under elevated high-voltage cables is associated with a higher risk of breast cancer due to exposure to electromagnetic fields (EMFs). A Norwegian study reported a 39% increase in breast cancer risk when living closer than 40 m from a 33 kV cable and 300 m from a 420 kV cable (47). Furthermore, light exposure at night is known to raise the risk of breast cancer among night workers, 8% to 40% depending on the study design (48-50). The effects of both EMFs and 'light at night' are probably mediated through the melatonin signaling axis, which converges on a gain in estradiol synthesis and ER production (51). Environmental pollution (air and food) with organochlorides (i.e., polychlorinated biphenyls [PCPs], dioxins, and aromatic hydrocarbons) are known to create DNA adducts and mimic estradiol at the ER level (i.e., endocrine disruptors) (52, 53).

1.3.4 Lifestyle factors

Ecological studies suggest that lifestyle factors play an important role in breast cancer carcinogenesis and a complex interaction between nutrients, hormones, and genetic factors (54). Women who moved from Asia to the USA and adapted to the American lifestyle have been shown to have a several-times increased risk of breast cancer. Moreover, postmenopausal weight gain is a known risk factor, probably through increased estrogen synthesis in adipose tissue (55, 56).

On the other hand, postmenopausal weight loss leads to a reduction in breast cancer risk, with an odds ratio (OR) of 0.88 (95% CI 0.78 to 0.98) (57). In addition, breast cancer risk is related to adipocyte-derived adipokines (e.g., leptin and adiponectin) and inflammatory cytokines, which all contribute to carcinogenesis through the activation of leptin receptor on the breast parenchymal cell and through microenvironment signaling (58-60). Approximately one in five Norwegian women are obese (body mass index (BMI) $\geq$ 30

kg/m2) (61) and at higher risk of developing breast cancer (62, 63). There is also an association between daily fat intake and the lethality of breast cancer (54). Furthermore, alcohol consumption is known to increase breast cancer risk in both pre- and post-menopausal women (64). The modern Western lifestyle comprises a high intake of carbohydrates, which increases the levels of insulin and insulin-like growth factor 1 (IGF-1) (65), which has a proliferative effect on the stem cells in the TDLUs. Lessons learned from an ecological study in 1995 indicated that Asian emigrants to USA have a 50% to 75% lower risk of breast cancer than US-born Asian American women (66). In 2010, however, only Chinese and Philippine-born women had lower breast cancer risk than their US-born counterparts (67). In a recent study from 2019, Asian immigrants who have lived 50% of their life in the US had a higher breast cancer risk than their US-born counterparts (68). Thus, diet is an important risk factor for both developing breast cancer and prognosis in breast cancer patients. Recently, high intake of meat was shown to increase the risk of acquiring breast cancer, whereas fruit intake reduced breast cancer risk in a large UK cohort study (69). Women with diabetes mellitus type 2 have an increased risk of getting breast cancer, but the disease mechanisms may vary in different subgroups (70). Importantly, physical activity decreases the risk of breast cancer in both pre- and post-menopausal women (64). Furthermore, prebiotics (i.e., dietary fiber, short chain fatty acids) assist the growth of beneficial microbiota in the gut (71), which in turn may reduce breast cancer progression, including preventing the formation of metastatic niches and metastases (72). Combined with physical activity and weight management, a healthy diet is an important way to reduce breast cancer risk (73).

1.3.5 Endocrine factors

Endocrine risk factors are connected to the total accumulated estrogen impact on stem cells in TDLUs. In breast cancer etiology, the molecular mechanism of estrogens includes two routes: the 4-OH quinine metabolites of estrogens create DNA adducts and mutations (74), and/or estrogens activate the ER, which is involved in the transcription of more than 1000 genes involved in cell proliferation, growth, and reduced apoptosis. This dual action is unique for estrogens. Estrogens pass the placental barrier and affect the mammary

epithelium of the fetus in utero. Early menarche (<12 years), first pregnancy > 30 years of age, no breast feeding, nulliparous status, and late natural menopause (> 55 years of age) increases the number of years with high circulating estrogen levels (64). The development of breast cancer is also associated with greater birthweight, adult attained height (via HGH axis (Fig. 6), and adult weight gain and obesity, which are proxies for increased intrauterine estrogen exposure and early endogenous estradiol production, respectively (74).

1.3.6 Integrative models of risk factors in breast cancer

Notably, most of the above-mentioned risk factors converge towards estrogen or estrogen receptor as a central factor (75). This underscores the importance of controlling the ER pathway in both the prevention and treatment of breast cancer (Fig. 6).

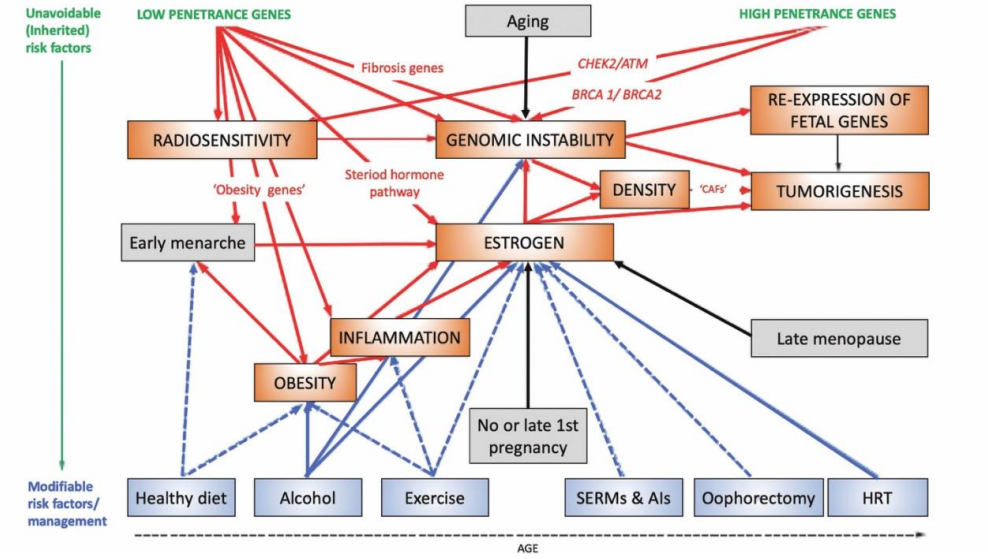


Figure 6. An integrative model of risk factors in breast cancer

1.4 Tumor Biology —Hallmarks of Cancer

Despite the various types of cells, genes, and effector molecules that act in concert in the development of cancer, some common features have emerged during the last two decades, such as in the research by Hanahan & Weinberg in 2000 (76) and 2011 (77) (Fig.7).

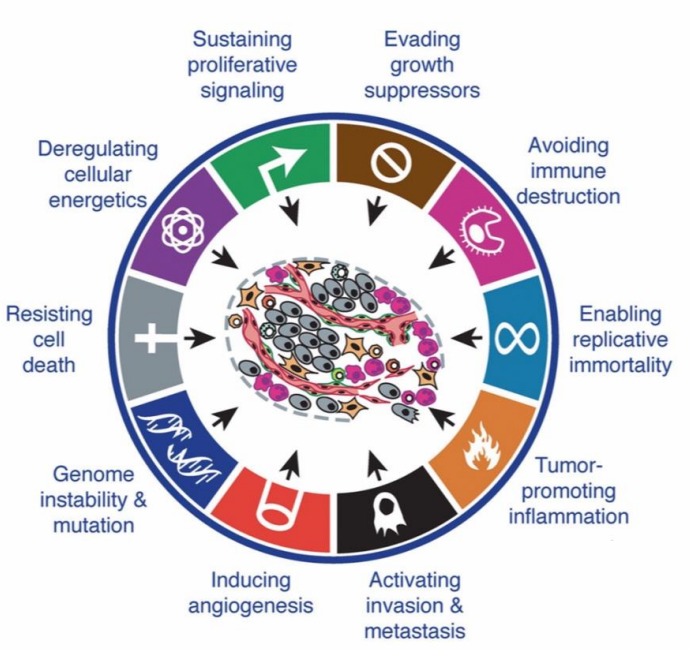


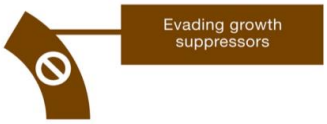
Figure 7. Hallmarks of cancer

The extended version of Hallmarks of Cancer from 2011(77).

These converging characteristics have been the basis of an increased focus on cellular and subcellular mechanisms for what was previously only seen with a clinical eye. These '10 commandments of cancer' both explain the nature of cancer and point out weaknesses that can be utilized as treatment targets.

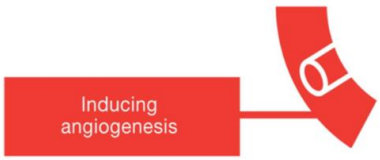
1.4.1 Alteration of the genome of neoplastic cells is the underlying factor in the various hallmarks of cancer. Certain mutant genotypes confer a selective survival advantage of subclones and enable them to become dominant in a local tissue environment. Three basic ways by which a gene can be changed are known: direct action on the DNA (e.g., single base alteration, deletion, addition, or frameshift mutation); a change in whole genes (e.g., copy number amplification or chromosome translocation), which will lead to an increased number of key factors in various signaling pathways; transcription of genes, which may be influenced by epigenetic changes (i.e., methylation or demethylation of histones, leading to silencing or activation of DNA transcription, respectively). Importantly, defects in the DNA repair machinery result in the accumulation of genomic changes and lead to genomic instability.

1.4.2. Cells are normally dependent on a stimulus from the external environment to divide, grow, and move. Cancer cells sustain their own growth signals and become independent of the exterior surroundings. In breast cancer, overproduction of estrogen receptor (ER) and the transmembrane tyrosine kinase receptor human epithelial growth receptor HER-2 by amplification of their genes, *ESR-1* and *HER-2-neu*, respectively, are typical examples of this hallmark. The widely used drugs tamoxifen and trastuzumab block the ER and HER-2 pathways, respectively. Furthermore, all stimulatory signal pathways are counteracted by an inhibitory system to avoid overstimulation. Cancer cells often have defects in such important suppressor mechanisms. Mutations in PTEN leads to a defect, removal of PIP3, by failing to attenuate the PI3-kinase. This important signaling pathway is downstream of Receptor Tyrosine Kinases (RTKs), and upstream activation of Protein Kinase B (Akt) prepares the cancer cell to grow and thrive by increasing protein synthesis. In endocrine-resistant breast cancer, a PTEN mutation may be one of the underlying mechanisms.



1.4.3 In normal tissue, multiple anti-proliferative signals operate in concert to maintain cellular quiescence and tissue homeostasis. The retinoblastoma (Rb) and TP53 proteins comprise central prototypical tumor suppressors and represent two complementary regulatory circuits that decide whether the cell will proliferate, senescence will be activated, or the apoptotic program will be induced. Mutations in the *TP53* gene will lead to a failure in the induction of apoptosis or cellular senescence, increasing cancer cell longevity. Mutation of *TP53* may occur up to 50% (30 out of 69) of Norwegian breast cancer patients (23). Moreover, breast cancer with *TP53* mutation are more prone to respond to platinum cytotoxic drugs given as pre-operative down-staging in T3 breast cancer tumors.

1.4.4 Evasion of programmed cell death is another way to increase cancer cell longevity. The apoptotic machinery consists of both sensory receptors and effector signal pathways. The sensors monitor the intracellular and extracellular conditions and act as sentinels of the cell fate. Important life-promoting systems are the insulin-like growth factors (IGF-1/IGF-2) and the corresponding receptor IGF-1R. These survival factors are frequently expressed in breast cancer cells. External death signals are mediated through the FAS-ligand/FAS-receptor and TNFα/TNF-R1 systems, whereas nuclear DNA and mitochondrial damage (cytochrome C release) are strong internal cellular sentinel pathways. The ultimate apoptotic effectors are the potent proteolytic caspases, which induce cell autophagy without any necrotic responses. Both chemotherapy and radiation therapy induce apoptosis through such mechanisms.



1.4.5 All cells require nutrients and oxygen. Initially, cancer cells rely on diffusion, but they soon demand a larger and more reliable nutritional supply via their own blood vessels. Cancer cells release vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs), which skew the 'angiogenic shift equilibrium' in the endothelial cells and pericytes towards the formation of new blood and lymphatic vessels. In breast cancer, VEGF is released from pre-invasive ductal carcinoma in situ lesions, which stimulates adjacent blood vessels to proliferate prior to invasion. Moreover, neo-angiogenic vessels are more leaky than normal ones. This is utilized in MRI and contrast-enhanced digital mammography (CEDM) to detect the presence of immature vessels in breast cancer based on leakage of the contrast material.

1.4.6 In contrast to apoptosis, necrosis leads to tumor cell 'explosion' and the release of contents into the local tissue environment. This process recruits inflammatory cells, which release tumor-promoting signals, such as IL-1α, into the tumor microenvironment. Such recruitment of immune cells will do more damage than good to the patient.

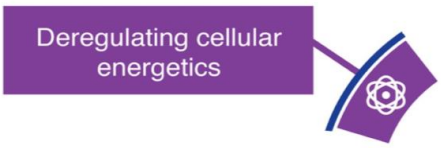
1.4.7 Most of the hallmarks of cancer lead to an uncoupling of the cell growth program. All mammalian cells carry an inherited intrinsic mammalian cell-autonomous program that limits the replicative potential of the cell. Disruption of this trait leads to limitless replicative potential and growth of the tumor to clinically detectable sizes. Telomeres on the end of the chromosomes secure replication, whereas a lack of telomeres activates the senescence and apoptosis machinery in the cell. Telomeres

are kept viable and maintained by a specialized DNA polymerase, telomerase, which is up-regulated in virtually all types of cancer cells. Targeting the telomerase is a potential treatment option in cancer.

1.4.8 During cancer development, some cells become programmed to leave the primary tumor, travel to distant sites, and grow new distant metastases. A complex interplay between various cell-to-cell adhesion molecules (CAMs), such as integrins, b-catenin, and E-cadherin, is activated to achieve metastatic ability. The metastatic process comprises three processes: 1) epithelial to mesenchymal transition (EMT), which transforms the broad and large epithelial cancer cell into a smooth muscle cell-like cell type, which can move between cells like a parasitic worm; 2) the journey in the peripheral blood or lymphatic vessels to distant sites and leaving the vessel; and 3) mesenchymal to epithelial transition (MET), which is the opposite of the EMT process.

1.4.9 Normally, the immune system will destroy incipient cancer cells, but natural selection towards weakly immunogenic cancer cells over time is thought to create cancer cells that avoid detection by immune cells. Other mechanisms for avoiding immune detection include the secretion of immunosuppressive factors and recruitment of immune cells that suppress the action of cytotoxic lymphocytes. Both the innate and adaptive cellular arms of the immune system are involved in tumor cell surveillance. Two main strategies are exploited in immunotherapy: 1. Checkpoint inhibitors (e.g., PD-L1, PD1, CTL4 inhibitor therapy in breast cancer), which allow the antigen-presenting cell to detect cancer cells, and 2. CAR

T-cells, which are ex-vivo chimera-modulated cytotoxic T cells that attack the targeted epitopes.



1.4.10 Cancer cells need both ATP, for the various cellular reactions, and DNA-building elements such as ribonucleic acids. The so-called Warburg effect is a metabolic switch that allows aerobic production of lactate and the production of ribose via the pentose phosphate shunt for the synthesis of all DNA and RNA nucleotides (See 1.10 and Fig. 31 for further details).

1.4.11 Integrative signaling pathways – the functional circuits

The above-mentioned hallmarks of cancer are all based on various cellular signaling pathways working in concert (Fig. 8A). Such cascades work together to form functional circuits assigned to specific tasks (e.g., proliferation and motility) (Fig. 8B) (76). Furthermore, considerable crosstalk is seen between these functional units to increase the autonomy of the cancer cell (77).

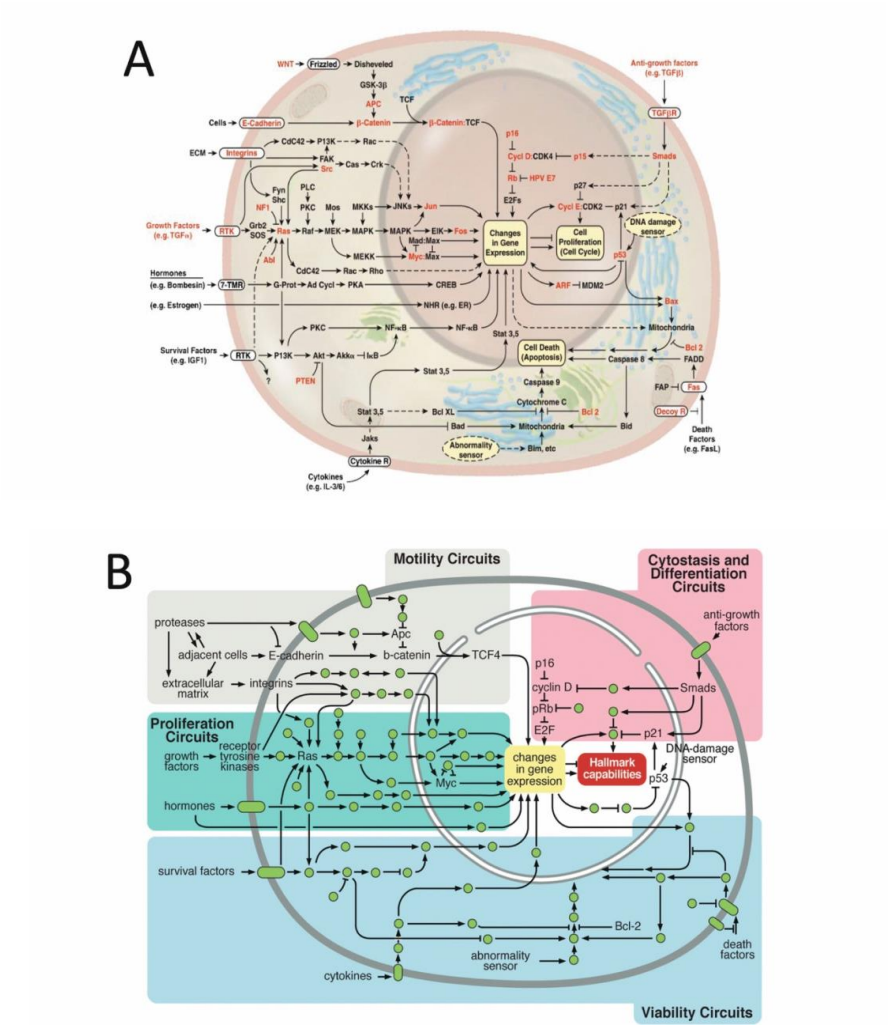


Figure 8. Signaling networks and pathways

A. Overview of autonomous cellular signaling networks in cancer cells. Signal molecules with functionally altered genes are marked in red (76).

B. Signaling pathways from Fig. 8A work in concert forming functional circuits, such as proliferation, motility, viability and cytostasis, and differentiation (77).

1.5 Carcinogenesis of Breast Cancer

Carcinogenesis is a multistep process in which the inherited genetic susceptibility and cumulative acquired influence of carcinogens on the stem cells in TDLUs over many years produce a cancer stem cell (Fig. 9). These cancer stem cells harbor the various hallmarks of cancer discussed in chapter 1.3. In breast cancer, amplification of c-myc and c-erbB2 are typical genetic changes that make the stem cell insufficient in growth factors. Inactivation of tumor suppressor genes (TP53) and repair genes (BRCA-1) by mutation or loss of heterozygosity (LOH) is an important carcinogenic step. Obviously, if a woman has a germline mutation in BRCA-1 (i.e., first hit) she will be much more vulnerable to various genetic events during life (e.g., LOH; the second hit) that may end result in a non-functional BRCA protein in the cell (78). As double-strand DNA repair depends on a functional BRCA-1 molecule, all kinds of DNA damage start to accumulate in the cancer stem cell (79); a critical mutation level may be achieved earlier in life compared to women without this germline mutation (80). Finally, epigenetic changes, such as methylation or demethylation of the promoter region of various genes, may silence tumor suppressor genes

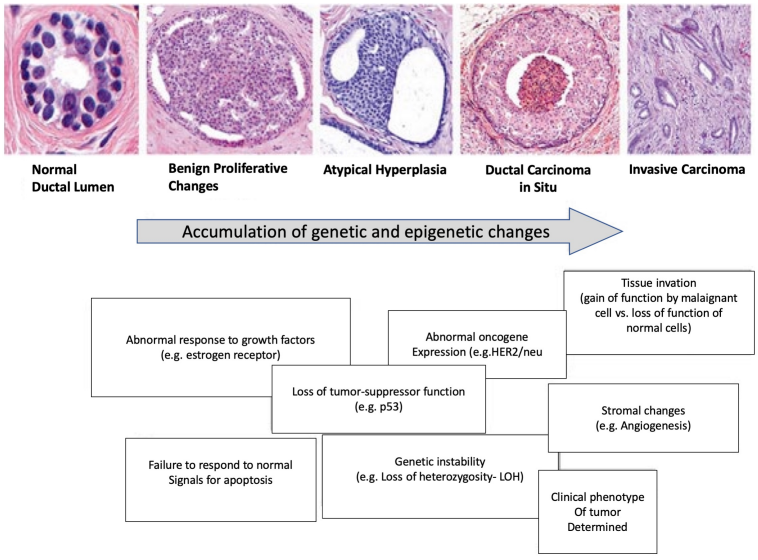


Figure 9. Main steps in carcinogenesis of breast cancer (80)

and activate oncogenes (81). The cancer stem cells in TDLUs create new cell populations that may grow into cellular arrangements that are possible to detect on mammography and are visible under a light microscope (Fig. 9) (78). Typically, such cellular alterations are benign proliferative changes, columnar cell changes, columnar cell hyperplasia, atypical hyperplasia, flat epithelial atypia, ductal carcinoma in situ (DCIS) type I, II, and III, and various invasive carcinomas (78, 82-85).

1.6 Treatment of Operable Breast Cancer

Treatment of operable (i.e., early) breast cancer has one ultimate goal: cure through removal of the primary tumor and eradication of putative minimal residual disease outside the breast (86)(91). The current regimens for early breast cancer 'stand on the shoulders' of two important historical paradigms: the loco/regional (Table 1, pt. 8) and systemic paradigm (Table 1, pt. 19). Thus, the primary treatment has two main components with a common objective: to achieve locoregional control in the breast and axilla (surgery and adjuvant radiotherapy), and to eradicate any systemic minimal residual disease at the time of diagnosis (systemic adjuvant treatment) (Fig. 10).

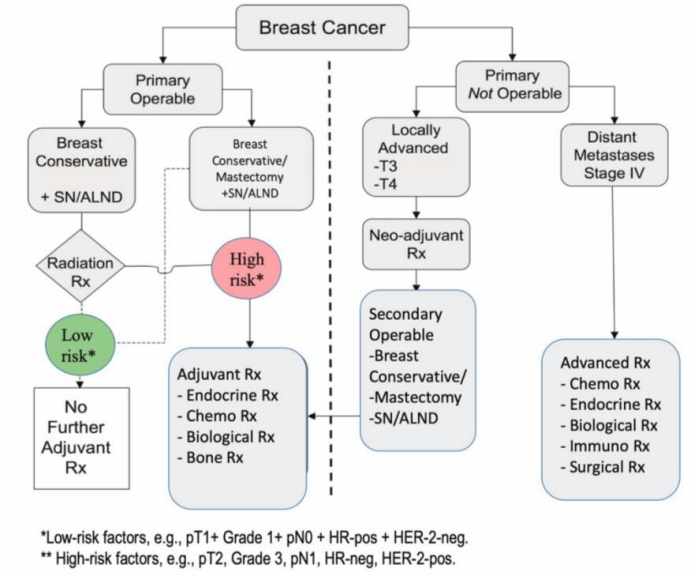


Figure 10. Algorithm of breast cancer treatment

1.7 Prognostic and Predictive Factors in Breast Cancer

Treatment of breast cancer may create both life-threatening and cumbersome side effects, which may last many years. On the other hand, breast cancer is a potentially lethal disease that requires potent treatment options to eradicate it. Thus, it is of utmost importance to avoid both over- and under-treatment. Prognostic and predictive factors help clinicians in the decision-making process (Fig. 11) (87). A prognostic factor identifies subgroups with an inferior/good prognosis (Fig. 12A and 12B) and is available at the time of surgery, correlating with the natural course of the disease (88). Moreover, such factors must be decided using materials from treatment-naïve patients, i.e., they have not received any active treatment besides surgery (87).

Therefore, prognostic factors will indicate who needs adjuvant treatment (Fig. 11). In contrast, predictive factors foretell which subgroup will respond to a certain treatment and which will not (Fig. 12C and 12D). Thus, research on predictive factors must be based on a solid biological hypothesis (87). Taken together, the prognostic and predictive factors form the basis for personalizing the treatment of breast cancer. In particular, there is a need for more reliable predictive factors, as more treatment options are available than predictive factors (Fig. 11) (86). From (89) based on (87).

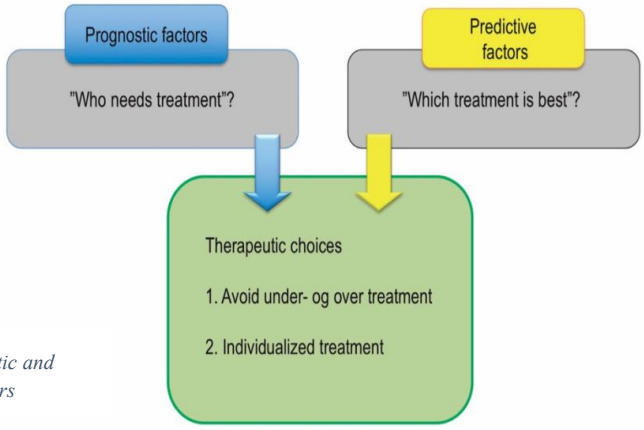


Figure 11. Prognostic and predictive risk factors

Unifying algorithm of the use of prognostic and predictive factors in the management of breast cancer patients (89).

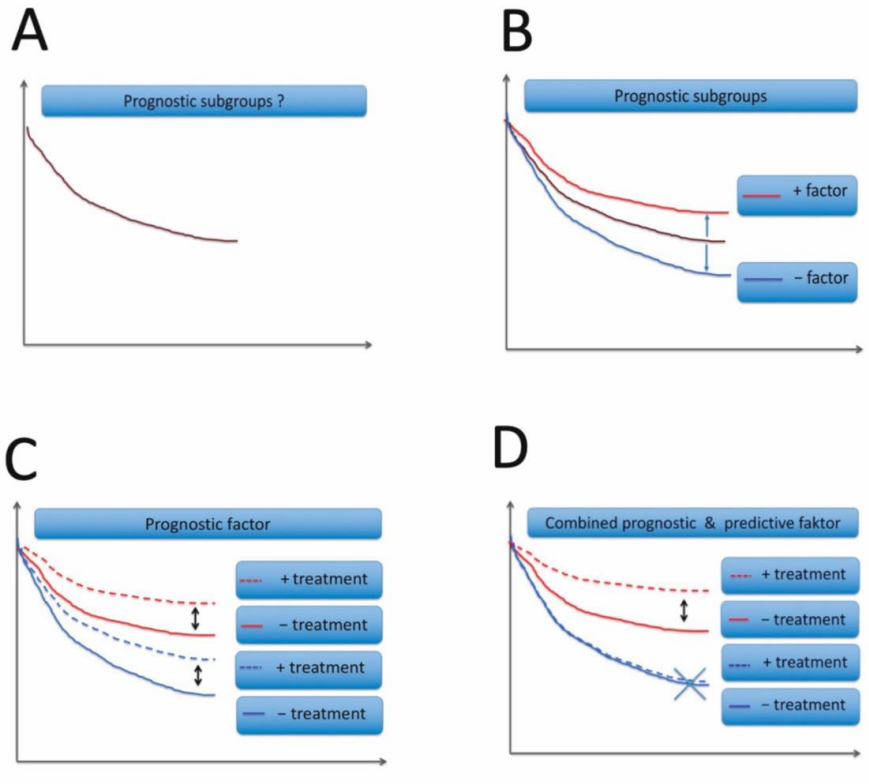


Figure 12. Interpretation of prognostic and predictive factors (91)

A. Kaplan-Meier survival curve in a certain patient population that did not receive any adjuvant treatment. More than 50% of the population relapsed/died during the observation period. Prognostic subgroups?
B. Same population as in A, but a prognostic factor divides the main population (brown) into a better (red) and worse (blue) prognostic subgroups (e.g., axillary lymph node metastases in breast cancer).
C. Treatment is given to both prognostic subgroups as in B. Prognoses are improved in both subgroups, indicating that this factor is a pure prognostic factor (e.g., axillary lymph node metastases in breast cancer).
D. Treatment is given to both prognostic subgroups in B, but only the red subgroups respond, whereas the prognosis for the blue group remains unchanged. This factor is both prognostic and predictive. (e.g., only estrogen receptor-positive breast cancers respond to tamoxifen or aromatase inhibitors, not the estrogen receptor-negative subgroup).

To find the 'correct' balance between escalating or de-escalating treatment strategies in breast cancer, international consensuses provide helpful insight (90). National guidelines are based on such agreement (Fig. 13). An overview of the treatment details in Norway is provided in the Appendix (Appendixes 1-5). Furthermore, the various prognostic and

predictive factors and information on comorbidity may be utilized by web-based tools to evaluate the contribution of each treatment option to the estimated 10-year survival (Fig. 13). Gene expression tests provide both prognostic and predictive information, whereas the classical factors provide overlapping or uncertain information (Fig. 13) (91).

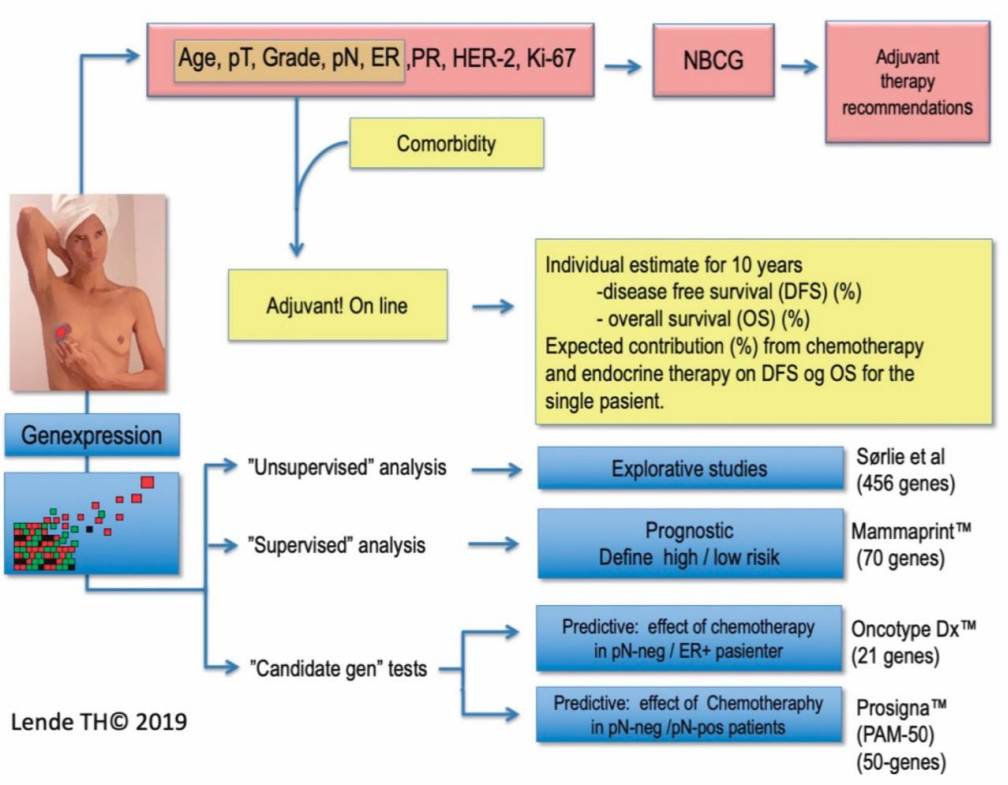


Figure 13. Tools in adjuvant decision making

Overview over prognostic and predictive methods available in decision making process of breast cancer patients.

1.7.1 Tumor size

In breast cancer, tumor size and lymph node metastases are the two strongest prognostic factors (TNM classification; Appendix 1, (92)). Tumor size measured in the surgical specimen (pT) correlates with both lymph node status (lymph node negative vs. positive) and the number of metastatic lymph nodes (93). In treatment-naïve patients, there is a linear relationship between tumor size and the risk of distant metastases (93, 94). In one cohort, 20-year relapse-free survival (RFS) was 88% for tumors <10 mm, 72% for those 11-30 mm, and 59% for tumors with diameters between 31 and 50 mm (95). Median time to progression also decreased with increasing tumor size. In another cohort, the 20-year disease-free survival was 50% for all pT1 tumors and 30% for all pT2 tumors (Fig. 14A) (93).There is a logistic relationship between tumor size and the percent of patients with positive lymph nodes (Fig. 14B)(96).For lymph node-negative patients, pT is particularly important, as it alone can trigger recommendations for systemic adjuvant treatment when other factors are unchanged (91). This is also reflected in the risk classification according to the St. Gallen criteria, in which one sees that, to belong to the low-risk group, the tumor must be <2 cm (90, 97).

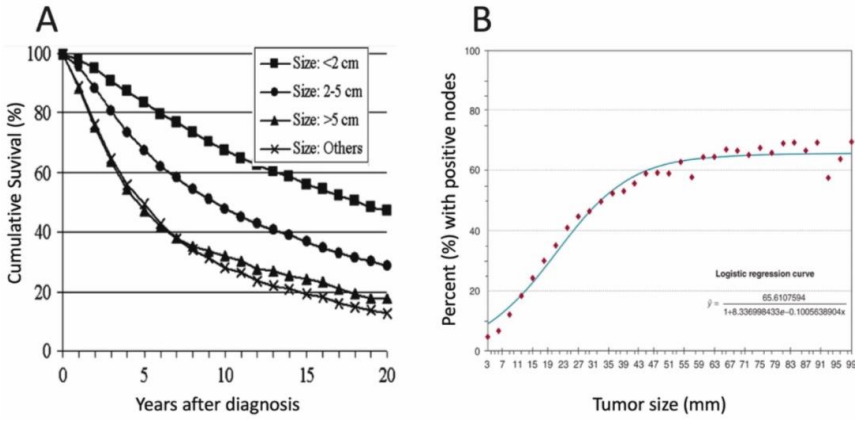


Figure 14. Prognostic information from tumor size

A. Prognostic information from tumor size as analyzed in treatment naïve BC- patients in the Netherlands (93).
B. The logistic correlation between tumor size in mm (X-axis) and percent of patients with positive lymph nodes (Y-axis) (96).

1.7.2 Axillary lymph nodes

The strongest prognostic factor is pathological lymph node status (pN). A strong correlation exists between the number of lymph nodes with metastasis and survival (93). Metastasis to regional lymph nodes (i.e., pN-positive status) always triggers adjuvant systemic treatment. The 5-year survival was 82.8% in treatment-naïve patients without proven metastases to axillary lymph nodes (pN-negative), 73% for pN1-3, 45.7% for pN4-12, and 28.4% for pN≥13 (98). However, the 20-year disease-free survival is remarkably worse in treatment-naïve patients (Fig. 15A) (93).

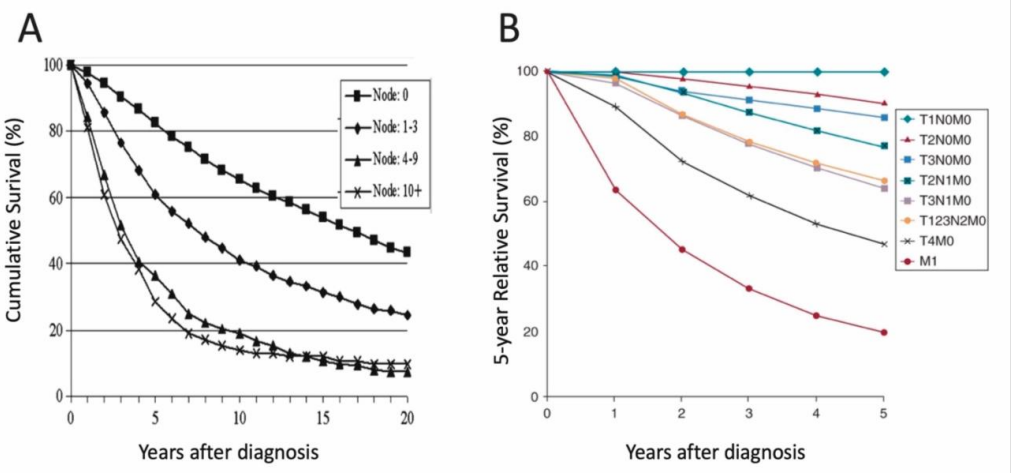


Figure 15. Prognostic information from lymph node status

A. Prognostic information from number of positive lymph node categories in treatment naïve BC-patients in the Netherlands (93).
B. The prognostic effect of tumor size on various groups of lymph node categories (96).

As pN is such a strong prognostic factor, it is important to establish robust prognostic and predictive factors for pN-negative (N0) patients (e.g. tumor size in Fig. 15B(96)) in order to avoid over- and under-treatment.

1.7.3 Morphological subtypes

The various histological subtypes (Fig. 16) (99-104) provide prognostic information in univariate analysis. However, in multivariate analysis, they exhibit no independent prognostic value, with the exception of the lobular subtype (105), but they have no predictive value. Invasive tubular cancer has the best prognosis. Lobular and mucinous carcinomas have a fairly good prognosis, whereas the invasive 'no special types' (NST), medullary carcinomas, and signet cell carcinomas have an inferior outcome (106). The reason why the histological subtypes, other than lobular, exhibit no independent prognostic information is due to sharing some overlapping features that are much stronger prognosticators, such as histological grading and proliferation. Moreover, breast cancer lacking both ER, PR and HER-2 expression are called triple negative breast cancers. These cancers have a particular inferior prognosis the first five years of follow up, due to lack of treatment targets (107).

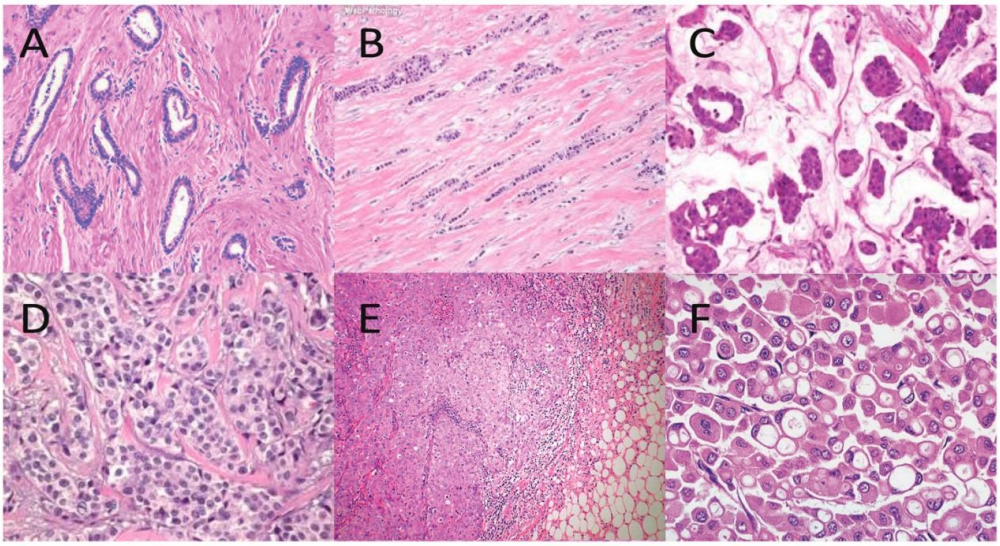


Figure 16. Morphological features of six invasive breast carcinomas

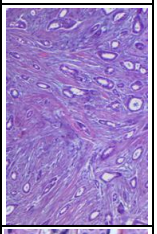
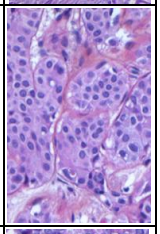
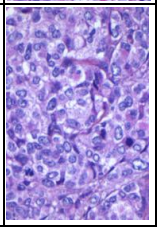
A. Invasive tubular carcinoma (101), B. Invasive lobular carcinoma (102), C. Invasive mucinous carcinoma (105),

D. Invasive carcinoma, no special type (NST) (103), E. Invasive medullary (104), F. Invasive signet cell carcinoma (106).

1.7.4 Histological grading

Histological subtyping focuses on differences between the tumors, but three common features may stratify breast tumors according to the degree of tumor differentiation i.e. tubular formation, nuclear pleomorphism, and mitotic count (Table 1, pt. 15 and Table 2) (10).

Table 2. The algorithm of histological grading. (Based on (10))

Key feature	**Single scores**	**Adding scores**	**Sum score**	**Histological grade**	**Differ-entiation**	**Typical picture**
Tubular formation		Σ	3-5 points	Grade 1	High/good 'the good'	
>75% of the tumor	1point					
10%-75% of the tumor	2points					
<10% of the tumor	3points					
Nuclear pleomorphism			6-7 points	Grade 2	Intermediate 'the bad'	
Uniform	1point					
Some variation	2points					
Marked variation	3points					
Mitotic Index (10 HPF, 0.44 mm)			8-9 points	Grade 3	Low/poor 'the ugly'	
0-5 mitoses	1point					
6-10 mitoses	2points					
≥ 11 mitoses	3points					

The histological grade serves as a proxy for proliferation (7). In multivariable analysis, only the mitotic index component of the histological grade remains in the final model. Thus, histological grade is a good prognosticator (108) and serves as an important predictive factor when the decision-making process between luminal A vs. luminal B cannot be aided by other factors (Appendix 5) (91). A drawback of histological grading is the poor reproducibility (kappa = 0.44 to 0.69) due to great individual assessment of tubular

formation and nuclear pleomorphism, as well as where and how to count mitoses. Grades 1 and 3 have good reproducibility, whereas grade 2 is poorly reproducible. Multivariable analysis has shown that the mitotic count has prognostic and predictive ability, not the other two features (25). Thus, tubular formation and nuclear pleomorphism 'dilute' the prognostic power of the mitoses in the histological grade. Interestingly, gene expression analysis revealed that histological grade 2 tumors are mixtures of grade 1 and grade 3 cancers (109).

1.7.5 Hormonal receptors: Estrogen receptor (ER) and progesterone receptor (PR)

The two hormonal receptors, ER and PR, are members of the nuclear receptor family and have versatile effects on gene regulation that belong to several hallmarks of cancer (110). The prognostic information they provide is based on the degree of tumor differentiation. Grade 1 tumors often follow strong expression of both ER and PR (Fig. 17A+ 17C), whereas poorly differentiated tumors may lack one or both two hormone receptors (Fig. 17 B). Expression of these two receptors also provide information on endocrine sensitivity and thus predict the efficacy of endocrine treatment (i.e., tamoxifen and aromatase inhibitors (AI)) (Fig. 12D). There is a correlation between the amount of ER and response to anti-estrogen therapy, but tamoxifen and AIs have the same effect for all levels of ER expression. Thus, there is no level of ER that can distinguish between a better predictive effect of AI or tamoxifen (111, 112). The degree of ER positivity (ER+) may determine when endocrine therapy can be given alone, or when one needs to be supplemented with chemotherapy (Appendix 5).

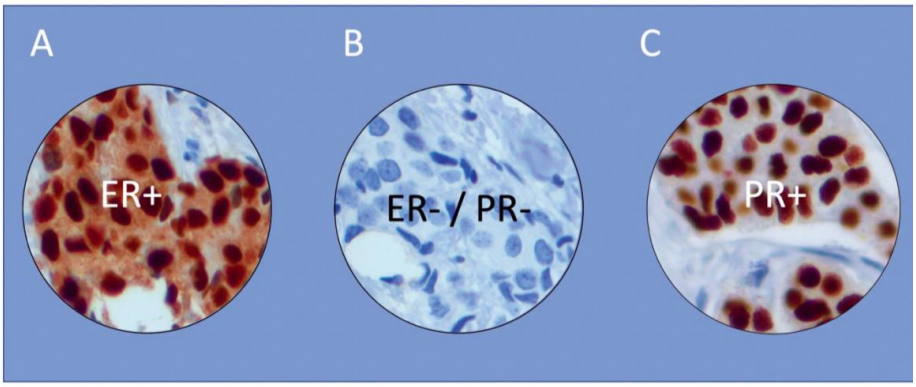


Figure 17. Estrogen and progesterone receptor expression in breast cancer

A. Estrogen receptor positive. B. Estrogen and Progesteron receptor negative. C. Progesterone receptor positive (91).

As ER transcribes the PR, the presence of PR is a marker of a functional ER receptor pathway and endocrine sensitivity (Fig. 19, pkt. 11). The response to tamoxifen in the ER+/PR+ subgroup is 50%-70%, but it decreases to 30-40% when PR expression disappears (88). The mechanisms underlying endocrine resistance (endocrine switch) is phosphorylation of ER, mutations in the ESR1 gene (113), perturbation of the equilibrium between co-activators (CoAs)/co-repressors (CoRs), hyperactivity in the Cycline D/CDK4/6 (114) or a switch in signaling through the ER/PR pathway to membrane-bound tyrosine kinase activating receptors (e.g., EGFR/IR/IGF) (Fig. 19 pkt. 3 and 7) (115-118). In the clinical setting, it is important to identify whether such resistance is present, as this has direct implications on the choice of treatment. Thus, reliable predictive markers for endocrine resistance need to be identified (see 1.12).

1.7.6 HER-2

HER-2 is a typical example of breast cancer cells becoming enough for their own growth signals. It is a transmembrane receptor with an extracellular domain and an intracellular tyrosine kinase unit capable of activating downstream signaling pathways through the ras-Raf-Mek-Erk cascade and with cross-linking to the Akt/m-TOR pathway (Fig. 19). Approximately 15% of women with breast cancer over-express HER-2 at the genomic and/or protein level. HER-2 protein is detected in tissues by immunohistochemistry, whereas situ hybridizations (FISH/CISH) detects amplification on the gene level (17q12) (Fig. 18).

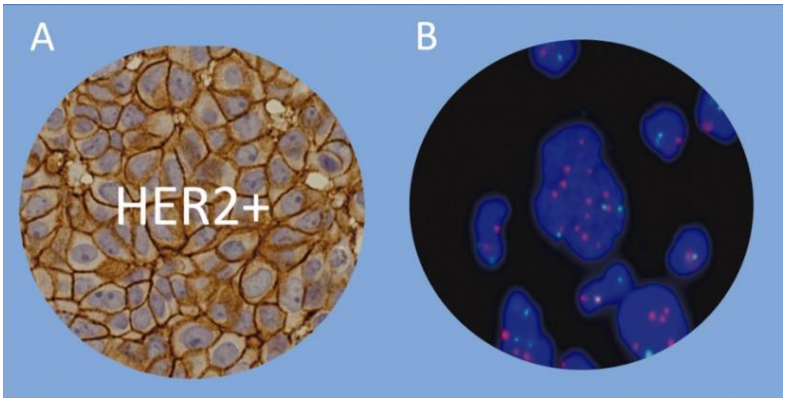


Figure 18. HER-2 protein expression and gene amplification

A. HER-2 highly over expressed on the protein level located in the outer cytoplasmic membrane. Detected by IHC with a score of +++.

B. Amplification of the HER-2 gene at 17q12 (red dots) detected by FISH. Up to 6 x amplification. Reference are the centrosomes stained as green dots.

Gene amplification results in 10-100 times more HER-2 molecules per breast cancer cell. As a result, signal transmission is enhanced and leads to increased proliferation, growth, and survival. In addition to be a prognosticator, HER-2 is also an independent predictive factor regarding the effect of chemotherapy and monoclonal antibody trastuzumab (22, 119, 120) and tyrosine kinase inhibitor lapatinib (121).

In Fig. 19, the downstream intracellular signal pathways from HER2, EGFR and ER/PR are depicted. Also, the targets of various drugs toward these pathways are indicated.

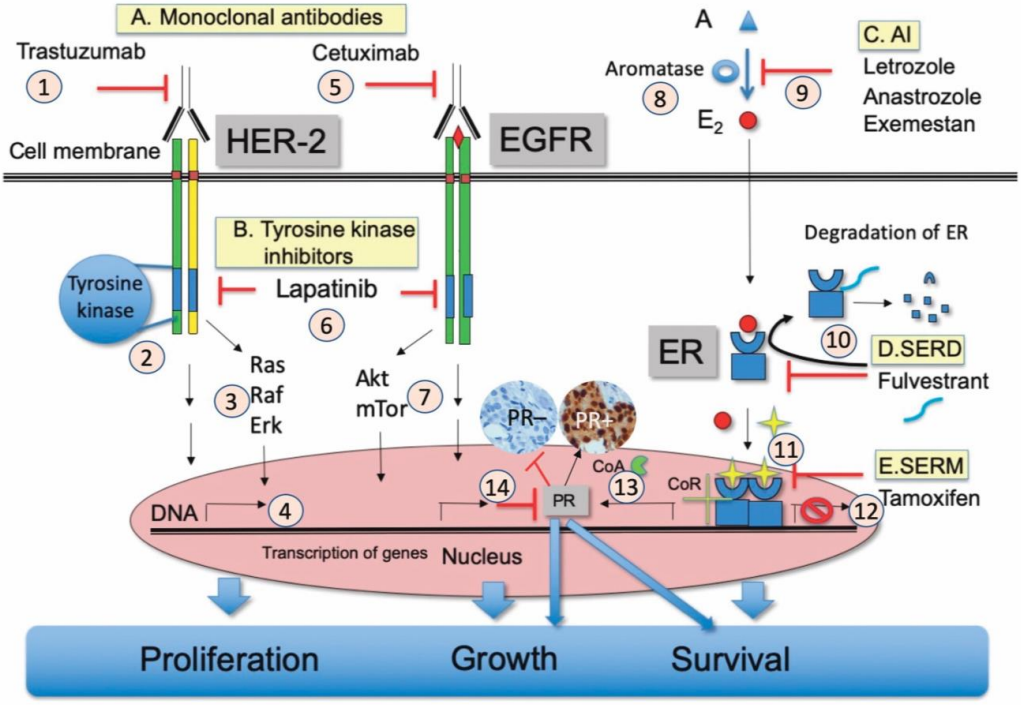


Figure 19. Overview of HER-2, EGFR and ER signaling transduction

Overview of signaling transduction through the HER-2, EGFR, and estrogen receptor (ER) pathways. E2, estradiol, normal endocrine responsive signal pathway. Developed from (116) and (89).

1. Trastuzumab binds to the extracellular domain of HER-2 receptor, which does not have a natural ligand.
2. Intracellular tyrosine kinase autophosphorylates downstream targets.
3. Signal transduction via various intracellular pathways, such as the Ras-Raf Erk pathway.
4. Downstream signaling from both HER 2 and EGFR ends up in the nucleus, where gene transcription is stimulated.
5. Cetuximab inhibits EGFR signaling by binding to extracellular domain.
6. Lapatinib inhibits the intracellular tyrosine kinase of both HER 2 and EGFR.

7. Signal transduction via various intracellular pathways, such as the Akt/mTOR pathway.
8. Aromatase converts androgens to estrogens.
9. Aromatase inhibitors (C) shut down this conversion.
10. Selective estrogen receptor degrader (SERD) (D) stimulates proteasomal degradation of ER.
11. Selective estrogen receptor modulator (SERM) (E) modulates the binding of co-activators (CoAs) and co-repressors (CoRs) to decide whether ER transcribes genes or inhibits such gene transcription.
12. SERM has bound CoR to the ER, which inhibits transcription.
13. ER has bound CoA, which activates transcription, especially Progesterone Receptor (PR) as a sign of endocrine sensitivity.
14. EGFR and HER-2 signaling inhibit PR transcription. Thus, a low or negative PR is a sign of endocrine resistance.

1.7.7 Biomarkers of proliferation

Proliferation is one of the most important features of a mammalian cell, replacing cells thatdie during the life cycle. The proliferation rate is much higher in cancer than in normal tissue. The cell cycle is divided into five phases: G_0 (non-dividing/functional state/senescence), G_1 (Gap1), S (synthesis and doubling of DNA content), G_2 (Gap 2), and the M (mitosis) phase (Fig. 20). Histological grade is the oldest surrogate marker of proliferation. Here, mitotic index is built into the algorithm (Table 2). Immunohistochemistry (IHC) detects biomarkers at the protein level, which are surrogate biomarkers for gene expression on the mRNA level (122). In the Western World, the luminal subgroups comprise 75% of all breast cancers. Proliferation is the feature that best separates luminal A from luminal B cancers. In situations in which genetic tests (e.g. Prosigna/ PAM50) are not available, surrogate markers of proliferation are used to distinguish between luminal A

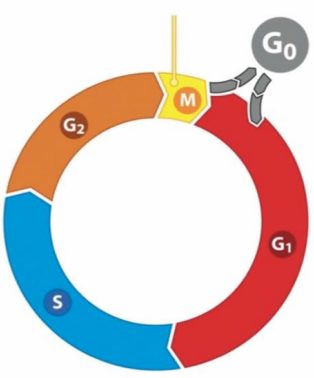


Figure 20. Cell cycle

and luminal B cancer. As the luminal B cancer status is predictive of the effect of chemotherapy, it is important to accurately draw a line between luminal A and luminal B tumors in order to avoid under- or over-treatment of breast cancer patients (Fig. 26).

1.7.7.1 Ki-67

In 1983, in Kiel, a protein was detected in well #67 of a myeloma cell culture that correlated with proliferation (123) and was called 'Ki-67'. It is found in all phases of the cell cycle except G_0 (Fig. 21). Ki-67 protein expression has been regarded as a good proxy for all proliferation-related genes in the genetic tests (PAM50/Oncotype). It is measured as the percentage of positive tumor cells among all cells in the measurement area. Over the last decade, Ki-67 has become the most used marker of proliferation in breast cancer cells (124). It has also been included in the St. Gallen treatment guidelines (90, 97, 125, 126). Using this biomarker, several pitfalls have been found in various clinical settings (127-130), various technical laboratory settings (131, 132), and when deciding the threshold between 'low' and 'high' proliferation (133-135). However, it has been one of the most important biomarkers in the NBCG guidelines for distinguishing between luminal A and B cancers and the use of chemotherapy in luminal breast cancers (91). The great between-lab variation in Ki-67 has led to a vague definition of luminal A vs. B. The recent St. Gallen guidelines recommend using the median value from the local laboratory as the reference value. Low proliferation is defined by a value lower than the median value -10%, and high proliferation as a value higher than the median value + 10%. In between values are

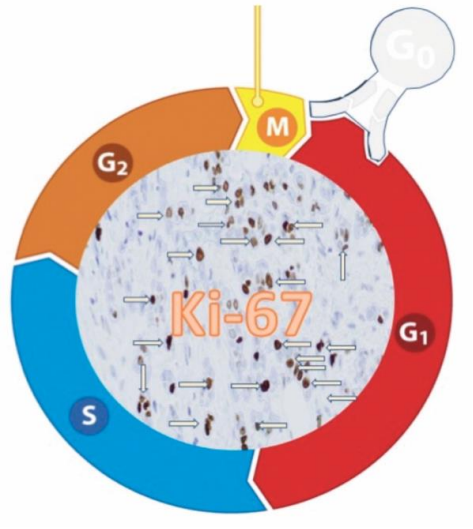


Figure 21. Ki-67 in cell cycle

KI-67 is present in all phases of the cell cycle, except the G_0 phase (shadowed area). Center: Immunohistochemistry of Ki-67 with brown nuclei expressing Ki-67 (arrows).

'intermediate' scores (90). In such cases, the pT and histological grade are useful for finding the most probable luminal status (136).

1.7.7.2. PPH3

The phosphorylated form of phosphohistone 3 (PPH3) correlates well with cells being in the G_2M phase (Fig. 22A). On IHC sections, the cancer cells stand out, and it is easy to determine which cells should be counted.

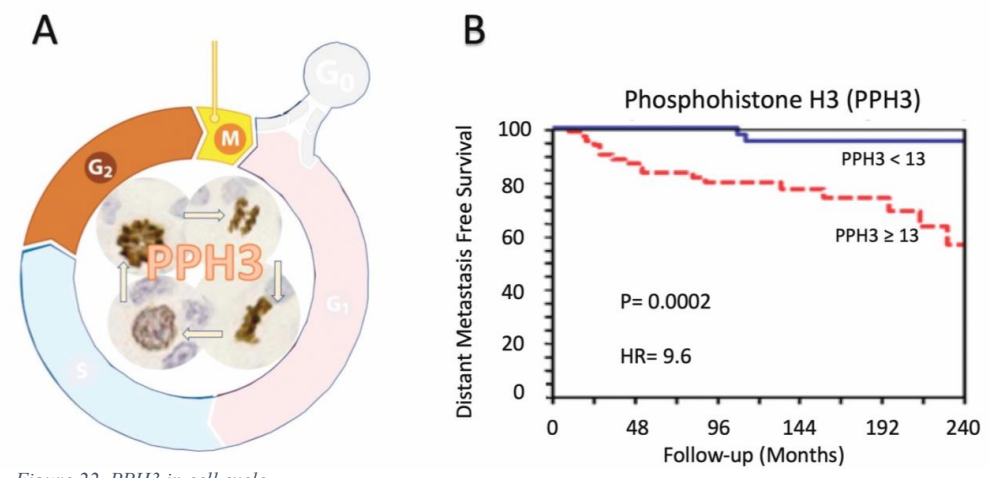


Figure 22. PPH3 in cell cycle

A. PPH3 is present in G_2 and M phase only (G_0, G_1 and S-phases are shadowed) (135).
B. Prognostic information form PPH3 </≥ 13.

Additionally, apoptotic figures do not express PPH3, which make the differentiation between mitotic figures and apoptotic figures very easy (137). A cut-off of 13 positive PPH3 cells per 1.59 mm^2 in the invasive front of the tumor (see 1.8.8.3) (138) has been validated (139) as being highly prognostic in all breast cancer subtypes (Fig. 22B). However, international consensus has not been reached for its inclusion in any treatment algorithm or recommendations.

1.7.7.3 MAI

The Mitotic Activity Index (MAI) is a reproducible proliferation marker that selectively assesses the mitotic activity in the periphery (i.e., invasive front) (Fig. 23A) of the tumor. The measurement protocol is strictly standardized. Briefly, an area of 3 to 5 mm in the invasive front of the tumor is carefully selected. The MAI is the number of all unequivocal mitoses in 10 adjacent high-power fields in a total area of 1.59 mm^2. A MAI of 3 means 3 mitoses per 1.59 mm^2.

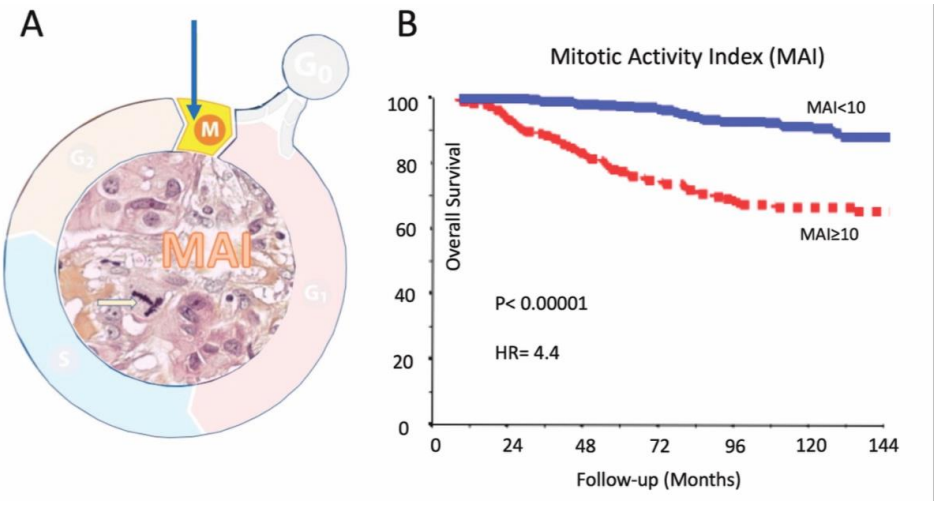


Figure 23. MAI in cell cycle

A. MAI exclusively assesses cells in M-phase (arrow) All other phases are shadowed. Center: HE-staining of whole section from a tumor with one unequivocal mitotic figure (arrow).
B. Prognostic information from MAI </≥10.

Obviously, only cells in the M-phase should be counted (Fig. 23A). To avoid sources of error, one should stick to the check list below (140). The threshold of 10 mitoses/1.59 mm^2 is highly prognostic in both lymph node-negative (25, 140, 141) (Fig. 23B) and lymph node-positive breast cancers. It is also the surrogate marker that best separates luminal A tumors from luminal B tumors (142). Moreover, using the threshold of an MAI </> 3 is a good predictor of the effect of chemotherapy (26).

Rules for MAI assessment (Fig. 24) (140)

1. General

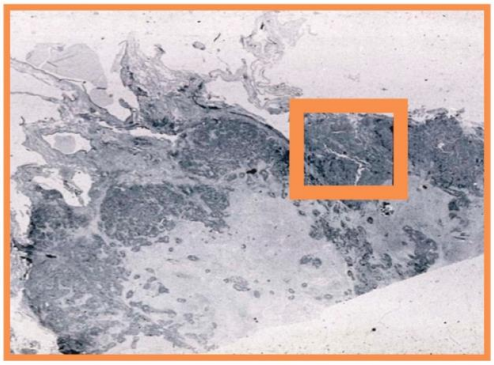


Figure 24. MAI assessment in tumor periphery.

A. Never count under time pressure

B. Carefully adjust the microscope (light, condenser, diaphragm) for optimal microscopic images

C. Carefully define the objective and field diameter, and apply a calculation factor when deviating from 450 µm

D. Disregard poor quality sections.

E. Ignore carcinoma in situ and micro-invasive cancer < 2 mm

F. Count mitoses in the periphery of the tumor only, disregard the center of the tumor

G. In the periphery, disregard

   a. Necrotic areas
   b. Inflamed areas
   c. Areas close to the skin

H. In the tumor periphery, at low magnification select the area with the highest subjective mitotic activity

I. Demarcate this area (minimally 2 x 2 mm, maximally 5 x 5 mm) as the measurement area

2.Within the measurement area

J. Change to the 40x objective (field diameter 450 µm/0.45 mm)

K. Count all unambiguous mitotic figures in this field of vision (FOV)

L. Move to the neighboring field of vision and repeat the procedure until 10 FOVs have been analyzed

M. The MAI is the total number of mitotic figures in 1.59 mm^2 of breast cancer tissue

3. Pitfalls resulting in erroneous MAI values

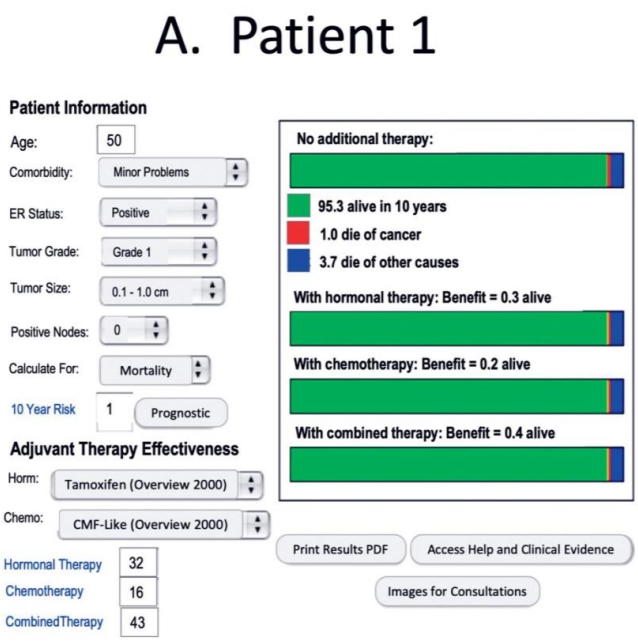
N. Random counts

O. Inaccurate selection and demarcation of the measurement area

1.7.8 Adjuvant! Online: A web-based tool to integrate prognostic and predictive factors

Adjuvant! (version 8.0) is available online at http://www.adjuvantonline.com (143). The data sources, assumptions, calculations, and theoretical background have been described previously (144).

Estimated prognoses are calculated based on the population-based Surveillance, Epidemiology, and End Results (SEER) registry from the United States. Age, comorbidity, tumor size (pT), histological grade, ER status, LN status, and treatment were entered into the database program. From this information, the Adjuvant! program calculates the predicted outcome, breast cancer death rate, and breast cancer–specific survival (BCSS; 100 – breast cancer death rate). RFS and overall survival may also be calculated (Fig. 25).



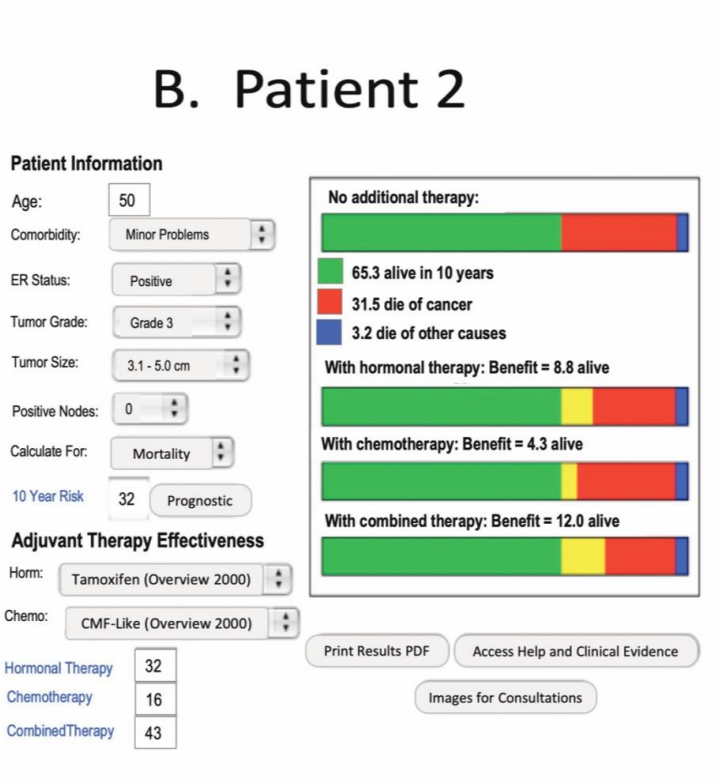


Figure 25. The Adjuvant! Online algorithm

Fig. 25 shows two examples of how two different patients are scored in the Adjuvant! Online program. Age, comorbidity, ER status, Tumor grade (histological grade), tumor size, nodal status for each patient are punched into the program. Then the program calculates the various outcomes.
Green bar indicates the chance of being alive in 10 years.
Red bar represents the risk of dying from breast cancer.
Blue bar shows the risk of dying of other causes.
Yellow bar depicts the reduction in risk of dying of breast cancer if chemotherapy, hormonal therapy, or combination of the two are administered.

A. Patient 1 with a good 10-year prognosis (95,3%) without adjuvant therapy. However, hormonal or chemotherapy will not improve the prognosis significantly (i.e. 0.3% and 0.2% respectively).

B. Patient 2 with a clearly reduced 10-year survival (65,3%) Importantly, in this patient hormonal therapy will improve 10 -year survival with 8.8%, chemotherapy with 4.3 % and the combination will improve the 10-year prognosis with 12 %.

1.7.9 Molecular subtyping

Through unsupervised hierarchical cluster analysis of the molecular profile of breast cancers (24), with a completely new taxonomy, breast cancer patients may be grouped according to the molecular clustering patterns: luminal A, luminal B, HER 2-positive, basal, and normal-like subgroups (Fig. 26A) (23). This new paradigm has also increased our understanding of prognosis (Fig. 26B) (23), treatment prediction, and the clinical management of breast cancer patients (145). Luminal A and B tumor cells express ER/PR and comprise 75% of all breast cancers in the Western World. Similarly, HER-2-positive breast cancers comprise 15% of tumors. The basal type and normal-like are the triple-negative subgroup (i.e., they lack ER/PR and HER-2) and comprise 10% of cancers in the Western World.

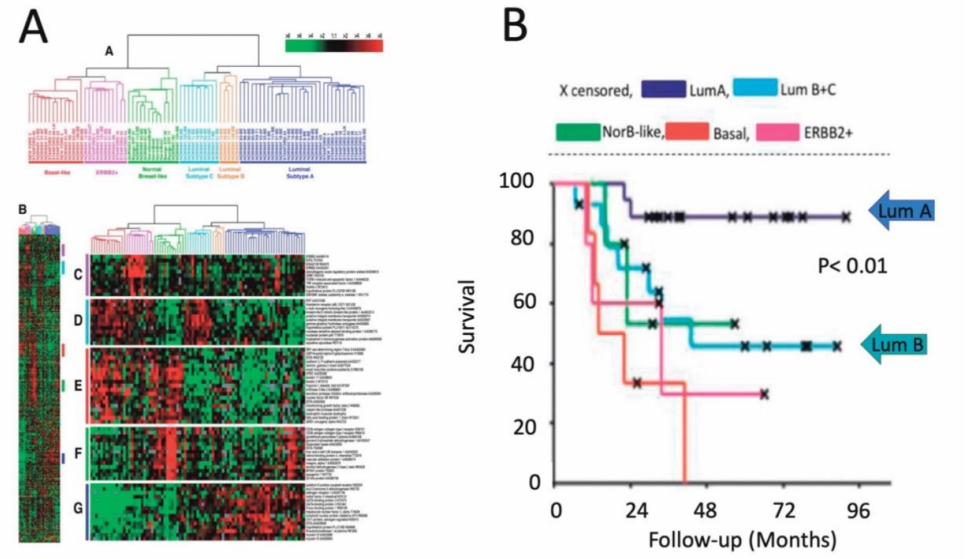


Figure 26. Molecular subtyping

A. Molecular subtyping of breast cancer by unsupervised hierarchal cluster analysis into luminal A, luminal B, HER-2 (ERBB2+), normal-like, and basal genotype (23).

B. Relapse-free survival of the molecular subtypes described in A (23).

Both HER-2 and basal-like subtypes indicate poorer outcomes than the luminal groups (Fig. 27) in these HER-2 treatment naïve patients. The triple-negative breast cancers have a higher incidence in younger women and BRCA-1 mutation carriers (146).

In a Norwegian long-term observational study (147) in which the patients were grouped according to the new molecular paradigm, the luminal A subgroup had an inferior prognosis over a median 20 years and up to 50 years of follow-up. Due to this long timeframe, there is a heterogeneity in treatment schedules. Some patients are adjuvant systemic treatment naïve while others have received adjuvant systemic treatment according to the national guidelines at time. Even though this study uses surrogate markers at the protein level, the report clearly indicates that luminal A and B tumors should be treated (Fig. 27). Furthermore, one of the recurrent questions in the last decade was how to distinguish between luminal A and luminal B cancers. Studies have shown that luminal B patients are good responders to chemotherapy, whereas luminal A cancers gain no benefit from it. The most accurate approach for separating luminal A from luminal B tumors is large-scale tumor gene expression profiling by microarray technology (see 1.7.7) (23, 24). Further development of this analytical principle has resulted in an assay using a reduced gene set built into a classifier, termed PAM50 (148-150).

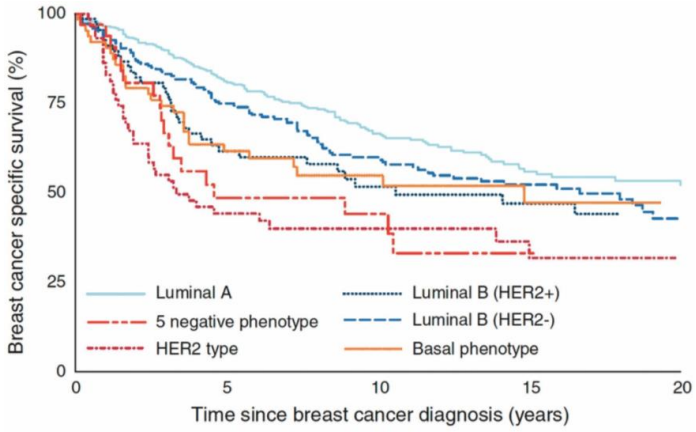


Figure 27. Long term prognostic information of molecular subtypes

Long-term prognosis in treatment-naïve breast cancer patients in a Norwegian historical study cohort. (136)

1.7.10 A useful candidate gene test: Prosigna™ (PAM50)

The centroid-based Prediction Analysis of Microarray 50 (PAM50) method utilizes 50 classifier genes and 5 housekeeping genes out of the 456 genes from the original cluster analysis (24) to classify various tumors into luminal A, luminal B, HER-2, or basal type (triple-negative). Moreover, based on the proliferation-related genes included in the PAM50, a prognostic model (risk of recurrence (ROR) score) (Fig. 28) is available to estimate clinical outcome. The ROR score may be utilized as a continuous parameter that indicates the percentage risk of systemic (distant) recurrence over a 10-year period, or to define three distinct risk groups: low risk (ROR < 40), intermediate risk (ROR = 41-60), and high risk (ROR > 60). These scores may be used in treatment guidelines when considering chemotherapy (Appendix 2). In several studies, the ROR score was superior to conventional parameters, IHC-based assays, and other multigene expression tools (151-154). Data from the TransATAC study comparing Clinical Treatment Score, IHC4, Recurrence Score (Oncotype DX™), EPclin, BCI, and Prosigna™, indicates that EPclin and Prosigna™ are the strongest predictors of distant recurrence in both node-positive and node-negative, hormone receptor-positive breast cancer patients. In particular, these markers are promising for identifying patients at low risk of distant recurrence (155) (see Appendix 2). The various candidate gene tests share a common trait, the proliferation genes that make up the basis for calculating the various recurrence scores (155, 156). Thus, in situations in which such gene tests are not available, various surrogate markers may be used.

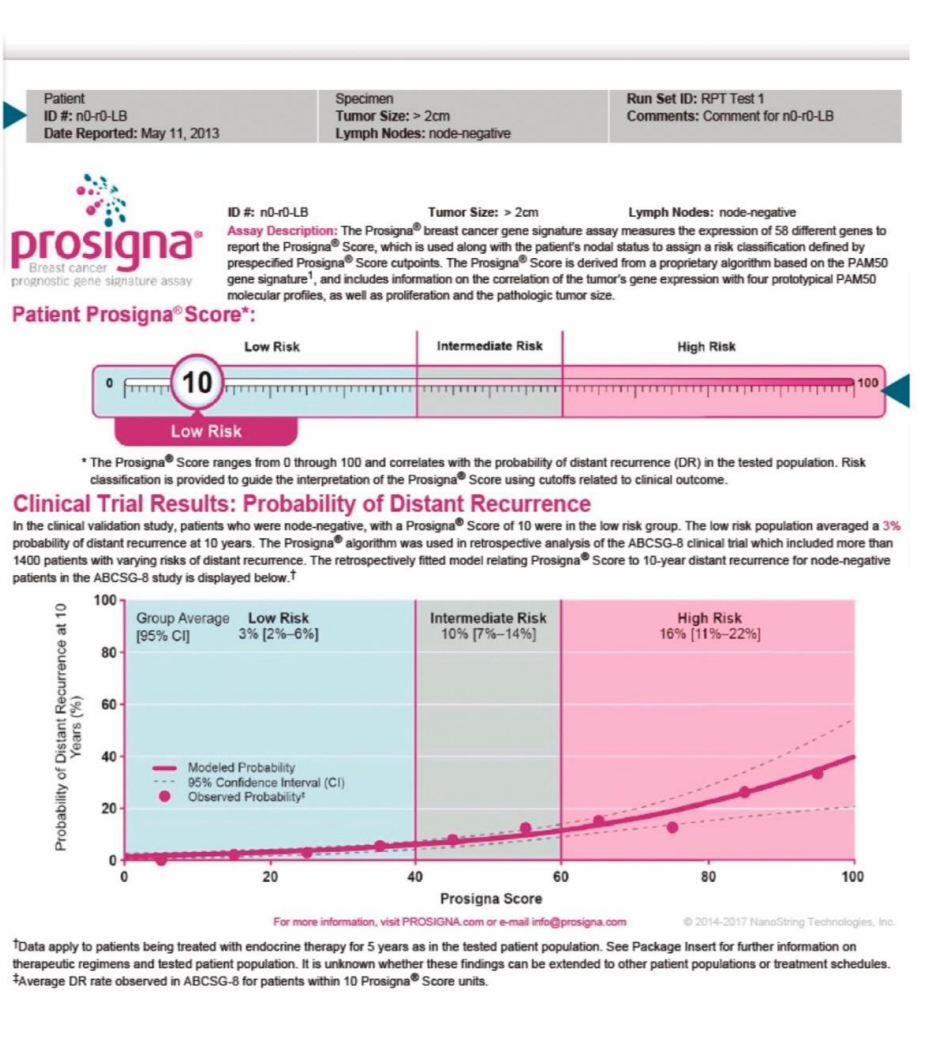


Figure 28. The Prosigna Score Chart

Upper and lower scale: The patient is scored based on a 'risk of recurrence' (ROR) and grouped into low risk (0-40), intermediate risk (40-60), and high risk (60-80). On the lower scale, the estimated absolute 10-year probability of distant recurrence in the various risk groups: low risk=2-6%, intermediate risk=7-14%, and high risk=11-22%. In addition, the Prosigna test provides the molecular subtype: luminal A, luminal B, HER-2, and basal (TNBC). (157)

1.8 Insulin, Insulin-c-peptide, IGF-1, and IGFBP3 in Breast Cancer Risk, Progression, and Prognosis

The most important analytes related to the regulation of blood sugar are insulin, insulin-c-peptide, IGF-1, IGFBP-3, lactate, pyruvate, and fructosamine (158). They have a complex interaction as explained below.

1.8.1 Insulin

Insulin is produced in the beta cells of the Langerhans islets in the pancreas. This peptide hormone is regarded as the most important anabolic hormone in the body (159) and facilitates the transportation of glucose into the cells through the GLUT4 glucose transporter in the outer cell membrane (160, 161). Inside the cells, insulin stimulates the formation of glycogen, lipogenesis, and protein synthesis, confirming the true anabolic nature of this hormone (159, 161). The production is a multistep process starting with transcription of INS, a gene located on chromosome 11p15.5, forming the poly peptide 'pre-proinsulin' (162). Pre-proinsulin is anchored to the rough endoplasmic reticulum by a signal peptide for its transportation to the Golgi apparatus. Here, the signal peptide is cleaved to form proinsulin, which adopts its tertiary structure through three disulfide bridges (163, 164). Proinsulin consists of three peptide chains coupled together: the α-chain, β-chain, and connecting peptide (c-peptide) between the α- and β-chains. Proinsulin is stored in intracellular vesicles in the beta cells (164). At this point, the production of insulin is independent of the blood sugar level. When the blood glucose concentration increases, an enzyme cleaves the c-peptide from proinsulin to form the biologically active insulin and c-peptide. Both are excreted into the portal vein in equimolar amounts (Fig. 29) (163, 164).

Insulin binds to the transmembrane homodimer insulin receptor (IR), which has intracellular tyrosine kinase activity that activates the downstream Akt (PKB) pathway

(Fig. 29). The IR is also targeted by IGF-1/2 signaling molecules. Insulin release from the pancreas oscillates with a period of 3–6 minutes. This oscillation prevents downregulation of IR in the target organs (165). Recently, a meta-analysis concluded that blood glucose levels in non-diabetic women are associated with a small increase in breast cancer risk (RR=1.11; 1.0 – 1.23) (166).

1.8.2 Insulin-C-peptide

C-peptide is the connecting peptide between the α- and β-chains in proinsulin. This polypeptide consists of 31 amino acids and is cleaved from proinsulin, forming insulin and c-peptide in equimolar amounts (164). The c-peptide has two main functions. First, it serves as a marker of the endogenous production of insulin, as c-peptide is much more stable and has a longer half-life in serum than insulin. Second, it has its own biological activities, including activating G-protein coupled receptors to release intracellular Ca^{++}, which targets downstream PKC and MAP kinase (Raf/ERK 1/2) (Fig. 29) (167). Reduction of apoptosis is one putative action in breast cancer cells.

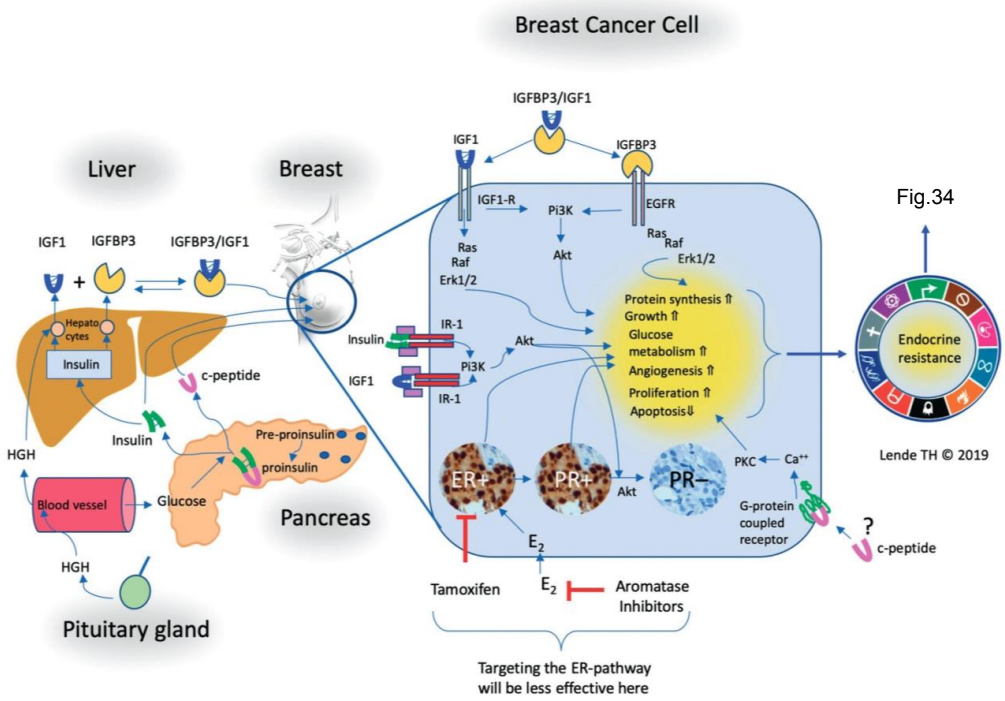


Figure 29. The effect of insulin, IGF-1 and IGFBP3 on breast cancer cells

Production of insulin by the Langerhans islets (blue dots) in the pancreas, and IGF1 and IGFBP3 in the hepatocytes of the liver. Insulin, IGF1, IGFBP3, and insulin c-peptide are all active in the breast cancer cell, promoting several of the hallmarks of cancer.

HGH, human growth hormone; IGFBP3, insulin growth factor binding protein 3; IR-1, insulin receptor 1; IGF1-R, insulin growth factor 1 receptor; EGFR, epidermal growth factor receptor; PI3K, phosphatidyl inositol 3 kinase; Akt, protein kinase B; ER, estrogen receptor; E_2, estradiol. Ras, Raf, and Erk1/2 are mitogenic activating phosphatase (MAP) kinases downstream of IGF1R and EGFR. Ca++, Calcium ions; PKC, protein kinase C (Ca-dependent protein kinase). In breast cancer cells with fully developed IRs and IGF1Rs, the intracellular pathways will overrule the blocking effect of endocrine treatment (tamoxifen and aromatase inhibitors). Insulin-C-peptide has been shown to activate PKC through G-protein coupled receptors in several types of cells and to inhibit apoptosis. It is likely, but not yet proven, that this is the case in breast cancer cells. Based on (128-167).

In line with this, c-peptide is associated with all-cancer mortality (168) and the outcome of breast cancer (169). Serum levels of c-peptide seem to increase the risk of breast cancer in postmenopausal women (170, 171), but not in pre-menopausal women (172),(173).

1.8.3 IGF-1

Insulin-like growth factor 1 (IGF-1) is a 70-amin-acid peptide hormone resembling insulin. It is produced in the hepatocytes after transcription of IGF1 on 12q23.2 (174), which is stimulated mainly by insulin and the human growth hormone (HGH) (Fig. 29). IGF-1 is responsible for sulfating the glycosaminoglycan matrix in the growing cartilage, bone, and connective tissue (175). Like insulin, it is an anabolic hormone involved in the growth process in the body. IGF-1 is produced throughout life, from in utero until old age (176). Serum levels of IGF-1 are dependent on insulin levels, genetic make-up, the time of day, age, sex, exercise status, stress levels, nutrition level and body mass index (BMI), disease state, ethnicity, and estrogen status (177).

Approximately 98% of the IGF-1 is bound to one of the transporter proteins insulin-like growth factor-binding protein (IGFBP)-1 to -6, of which IGFBP-3 is the most abundant (178, 179). On the target cells, IGF-1 binds primarily to IGF-1R, and to IR with a 0.1-times affinity compared to IGF-1R. Downstream from these receptors, the Akt-pathway is activated, which induces cell proliferation and growth, and strongly inhibits apoptosis (180, 181). IGF-1 is also involved in angiogenesis (182). In breast cancer, IGF-1 seems to play a role in both carcinogenesis (183-186) and prognosis (187), and may be a novel way of treating breast cancer patients (188). Notably, IGF-1 plays a role in estrogen receptor-positive cancers only (189). Both vegan diets and intermitting fasting (5:2 diet) downregulate circulating IGF-1 levels (190).

1.8.4 IGFBP-3

IGFBP-3 is the most abundant transporter protein among the six isoforms produced in the liver. Enhanced by insulin and HGH (ref), it transports IGF-1 and IGF-2 from the liver to the target cells (191). IGF-1/IGFBP-3 may reflect the bioavailability of IGF-1. At the cellular level IGFBP-3 can reduce available free IGF-1, interact with cell surface proteins, and enter the nucleus to bind to nuclear hormone receptors (192). In breast cancer, IGFBP-3 may bind to and activate EGFR. High levels of IGFBP-3 within tumors are associated with increased cancer severity (or worse outcome) for some cancers, but decreased severity or better outcome for others.

Estrogen inhibits IGFBP-3 production, and its tissue levels are lower in ER-positive breast cancers than in ER-negative cancers. In breast cancer, IGFBP-3 is also involved in DNA repair mechanisms (193) and thought to increase the longevity of cancer cells. Moreover, IGFBP-3 seems to have a differential effect on various cell types. In breast cancer, IGFBP-3 may interact directly with EGFR and stimulate proliferation (194). It can also trap IGF-1 at the outer cell membrane and increase signaling through IGF-1R (195). Furthermore, the presence of IGFBP-3 in breast cancer tissue has been correlated with an inferior prognosis (196). However, this may vary between various tissue types (197).

1.9 The ERAS Concept

Enhanced Recovery After Surgery (ERAS) has been introduced in peri-operative care in patients undergoing longstanding surgery (198). In gastrointestinal surgery, ERAS reduces both recovery time and postoperative complications (199). The ERAS concept comprises almost 20 different elements distributed over the pre-operative, intra-operative, and post-operative phases (Fig. 30) (200). Pre-operative fasting and fasting during surgery bring the body under systemic stress, which leads to the release of adrenaline, noradrenaline, and cortisol (201).

These hormones are catalytic in action (198), i.e. they stimulate the degradation of glycogen depots, proteins, and triglycerides to produce energy (ATP). Insulin counteracts all the above-mentioned actions by modifying the activity of numerous enzymes (164). The actions of insulin on the global human metabolism include increased cellular intake of certain substances, most prominently glucose in muscle and adipose tissue (approximately two-thirds of body cells) (202). Insulin also inhibits fatty acid release by hormone-sensitive lipase in adipose tissue and increases DNA replication and protein synthesis via control of amino acid uptake (203). Thus, one of the most important components in ERAS is to avoid pre-operative fasting, which leads to low insulin levels and turns on the catalytic state of the body. By giving the patients two doses of carbohydrates and glucose-enriched juice pre-operatively, insulin levels increase and the surgery occurs while the patient is in an anabolic state (200).

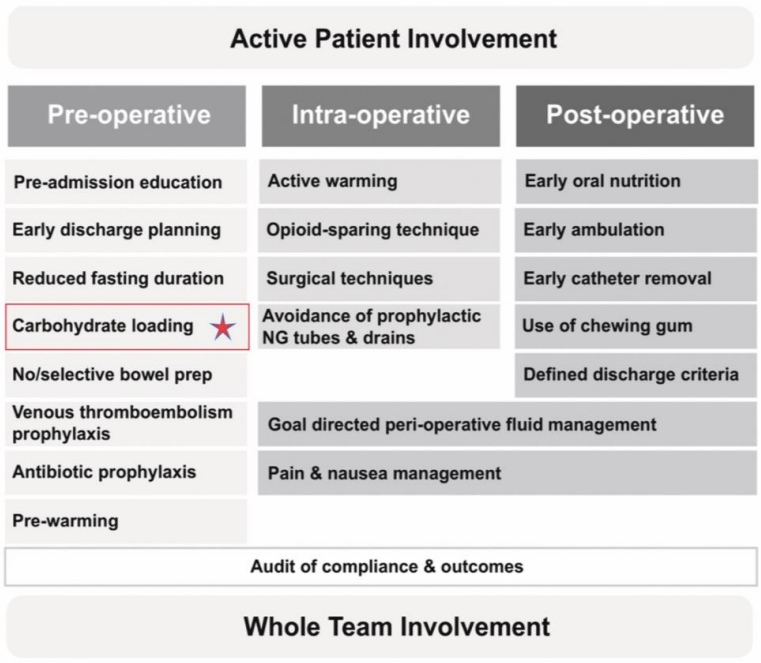


Figure 30. Enhanced Recovery After Surgery

The concept of Enhanced Recovery After Surgery (ERAS) includes carbohydrate loading (red asterix) (From 198).

1.10 The Warburg Effect: Deregulation of Cellular Energetics and Metabolic Reprogramming as Putative Prognostic and Predictive Factors in Breast Cancer

The Warburg effect described in 1924 (Table 1, pt. 12) comprises several metabolic changes that occur simultaneously (7, 204-208), including changes in carbohydrate, lipid, and protein metabolism (Fig. 31). The Warburg effect fulfills many of the metabolic requirements that a proliferating cancer cell demands (209). The Warburg effect and metabolic reprogramming are emerging hallmarks of cancer (see 1.4.10) (77). Cancer cells mutate to alter several metabolic pathways to their advantage, ultimately leading to malignancy and mortality for the host. In rapidly proliferating cells, such as cancer cells, some metabolic pathways may be enhanced or inhibited to facilitate growth, mutation, and resistance to apoptosis. This characteristic of cancer makes it uniquely suited for metabolic analyses (210). The Warburg phenomenon is due, at least in part, to the upregulation of genes coding for glucose transporters and glycolytic and regulatory enzymes mediated by the increased activity of transcription factors c-MYC and HIF-1 in cancer cells, and the coordinated loss of regulatory proteins due to the loss of p53 function. Loss of p53 function also leads to the activation of GLUT-3 transcription via NFκB (Fig. 31) (208).

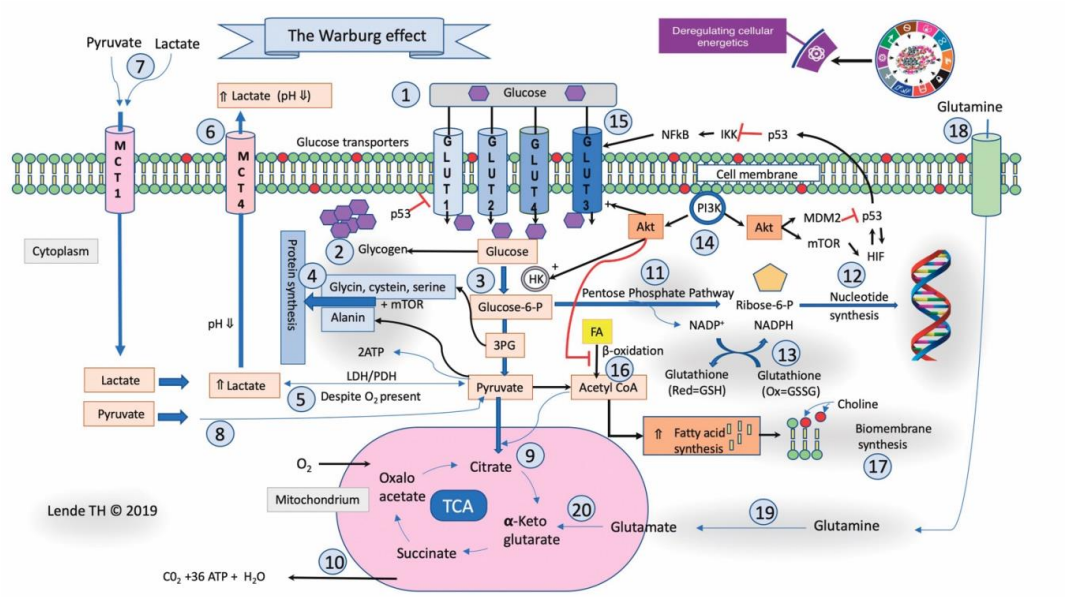


Figure 31. The Warburg effect

Overview of the various aspects of the Warburg effect in a breast cancer cell regarding deregulated cellular energetics, an important hallmark of cancer. Glucose metabolism is altered in cancer cells. First, the amount of glucose transport molecules in the cell membrane is up regulated. Normally, oxidative phosphorylation follows glycolysis and yields CO_2 and 36 adenine triphosphate (ATP) via the tricarboxylic acid (TCA) cycle. Lactate production occurs only in the presence of a lack of oxygen (O_2). In the Warburg effect, glycolysis is up regulated and reduction of pyruvate to lactate occurs despite the presence of O_2. This shift in the equilibrium of the various steps of glycolysis backwards (red arrows), which opens the PPP and initiates the production of ribose-6-P for nucleotide synthesis. MCT1, mono-carboxylate transporter no. 1 (i.e., lactate, pyruvate, etc.); GLUT1-2-3-4, glucose transporter molecule 1-2-3-4; TCA, tri-carboxyl acid; ATP, adenosine triphosphate; 3PG, glyceraldehyde-3-P; FA, fatty acid; HIF, hypoxia induced factor. Based on (7, 196, 204-208).

1. In cancer cells, glucose transporters are up-regulated.
2. Glucose is converted to the polymer glycogen for intracellular storage.
3. Glycolysis yields two ATP molecules for energy demanding biochemical processes.
4. Glycolysis creates precursors for amino acid synthesis (alanine, glycine, and several other amino acids for protein synthesis).
5. Despite the presence of oxygen, pyruvate is converted to lactate.
6. Lactate is excreted from the tumor cells through MCT-4 ports.
7. Cancer cells may take up lactate and pyruvate from other sources via the MCT-1 ports.
8. LDH creates an equilibrium between lactate and pyruvate.
9. Pyruvate and acetyl CoA enter the mitochondrion to start the tri carboxyl acid (TCA) cycle.
10. TCA cycle yields 36 moles of ATP per mole of glucose trough the electron transport chain.

11. Glucose-6-P functions in the pentose phosphate pathway (PPP) to produce ribose.
12. Ribose is a vital part of the nucleotides comprising the backbone of both DNA and RNA.
13. In the PPP, NAD+ and NADP+ are reduced to NADH and NADPH, which are used to reduce glutathione (GSSG) to GSH, protecting against ROS.
14. PI3K is an important pathway to balance glucose intake in the cancer cell and glycolysis, glycogen storage, and the PPP.
15. Regulation of glucose uptake via GLUT3 ports and the PI3K-> Akt-> p53 axis.
16. Fatty acid metabolism through the beta-oxidation pathway is stopped.
17. Synthesis of fatty acids and formation of bio-membrane are stimulated, and choline transport for phospholipid synthesis (211).
18. Uptake of glutamine via special ports.
19. Glutamine is converted to glutamate, which enters the mitochondrion.
20. Glutamate feeds into the TCA cycle at the α-ketoglutarate level.

1.11 Metabolomics in Breast Cancer

Metabolomics is the study of small molecules, such as substrates, intermediates, and end products of cellular metabolism, including carbohydrates, amino acids, and small organic acids. Metabolomics studies can give valuable insight into breast tumor biology, the metabolic status of the patient, and how the metabolic status of the patient is affected by treatment (212-215). The metabolic state of cancer cells is substantially altered compared to normal cells (77, 216), a fact that can be utilized for diagnostic purposes. Specific metabolic signatures in tumor tissue have already been coupled to breast cancer subtypes and prognosis (210). Earlier metabolomic analyses of primary tumors found several factors relating to prognosis, treatment response, and survival (213, 214). For example, increased tumor lactate and glycine levels are related to poor prognosis in patients with ER-positive cancer (212).

High-resolution magnetic resonance spectroscopy (HR MRS) can be used to measure the concentrations of metabolites in biological samples, such as biofluids and

Figure 32. MR spectrometer
MR Spectrometer for high-throughput analysis of tissue and biofluids. Photo Geir Mogen

tumor tissue (217). MRS offers precise identification of substances and high throughput, automated quantitative analysis. HR MRS analysis in this thesis was performed at the MR Core Facility, Dept. of Circulation and Medical Imaging, NTNU (Fig. 32). The MR Core Facility hosts two new state-of-the-art spectrometers with ultra-shielded magnets operating at 600 MHz for proton detection. One spectrometer is dedicated to high-throughput biofluid analysis, with a sample jet enabling automated sample loading, barcoded sample tracking, and cold storage (4°C) and capable of running up to 100 samples a day.

Approximately 30 metabolites can be detected and quantified in serum spectra, and approximately 60 in urine spectra. Another spectrometer is dedicated to tissue analysis by high-resolution magic angle spinning (HR MAS) MRS for increased sensitivity in semisolid samples. In tissue samples, approximately 30 metabolites can be detected and quantified. The MR Core Facility collaborates with the vendor (Bruker BioSpin GmbH, Germany) to ensure efficient and validated acquisition protocols, making the method feasible for high-throughput analysis and implementation in the clinic.

1.11.1 Metabolomic studies of carbohydrate metabolism in cancer cells

Metabolic reprogramming of glucose metabolism in cancer comprises glycolytic fermentation to pyruvate and, despite the presence of oxygen, conversion to lactate (Fig. 31, pt. 5). This upregulation of aerobic glycolysis leads to increased cellular acidity, providing resistance to acid-induced cell toxicity and a powerful growth advantage (216). Moreover, upregulation of glucose transporters in the outer cell membrane via the Akt-pathway enhances glucose consumption, which is utilized in positron emission transmission (PET) imaging. The hexokinase conversion step from glucose to glucose-6-P is the rate-

determining step in glycolysis. Activation of PI3K and Akt stimulates formation of the hexokinase enzyme, which increases glucose-6-P that enters the pentose phosphate pathway. This shunt leads to production of pentose ribose-6-P for nucleotide synthesis (Fig. 31, pt.11 and 12).

1.11.2 Metabolomic studies of lipid and fatty acid metabolism in cancer cells

Lipid metabolism is also altered in tumor cells (218) (Fig. 31, pt. 16 and 17). The beta-oxidation of fatty acids is blocked, and circulating lipoproteins are also affected by cancer (219, 220). Serum lipoprotein sub-fractions have been associated with breast cancer characteristics, such as proliferation (221). An increase in long-chain fatty acids is a marker of increased biosynthesis of bilayer cellular membranes. Thus, a cancer cell has increased levels of various fatty acids and precursors of the phospholipid bilayer membrane (e.g. choline) (222).

In breast cancer cells previous findings have shown metabolic alterations of choline and choline containing compounds (222). Choline is a precursor for phosphatidylcholine, a phospholipid in cellular membranes, and a strong correlation between choline metabolites and cellular turnover and tumor growth is detected (223). Metabolites containing choline act as signal transducers and second messengers for growth factors in the ras-raf-MAPK pathway and protein kinase C pathway. Choline phospholipids seem to be regulated by receptor tyrosine kinase pathways.

1.11.3 Metabolomic studies of amino acid metabolism in cancer cells

A rapidly proliferating cell requires intracellular proteins for vital cellular processes. Glycolysis also provides precursors for amino acid production (Fig. 31, pt. 4), for glycine and alanine. Metabolomic analysis of the tumor tissue can measure the levels of lactate, glycine, cysteine, serine, arginine, glutamine, proline, asparagine, and aspartate (222). Glutamine, a non-essential amino acid, is in addition to glucose an energy source in cancer cells. It is hydrolyzed to glutamate and ammonium by the enzyme glutaminase (GLS). High GLS expression is found in breast cancer associated with high grade and metastatic disease.

Glutamate acts in protein synthesis, enters the tricarboxylic acid cycle (TCA) for ATP production and is a precursor for glutathione, an important cellular antioxidant. Oxidative stress is important for the cancer cell to achieve the various hallmarks of cancer including angiogenesis, invasion and proliferation (224). However, glutathione, a tripeptide of glutamate, cysteine and glycine, is furthermore important for breast cancer cells to resist external attacks comprising superoxides and oxidative stress overload associated with rapid metabolism according to the following generic equation 2 GSH + O_2^- → GSSG + H_2O (225).

1.11.4 Metabolomic profiling of breast cancer

Ex vivo MRS provides insight into the underlying biochemical processes associated with the malignant transformation of normal cells or tissue. While MRI presents tumor morphology, HR-MRS provides the biochemical information about the sample to understand the physiology and metabolism of the disease (226) (Table 3). The readout spectra from such analyses reveal component-specific peaks in which the spectral regions on the x-axis indicate the substance. The area under the peaks represents the concentration of the component in the medium (Fig. 33) (222).

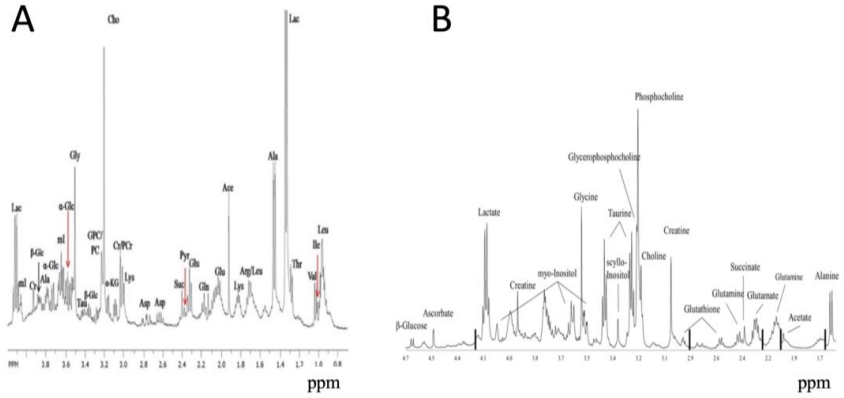


Figure 33. MR spectrum from HR-MRS and HR-MAS-MRS

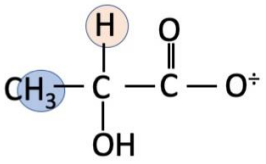
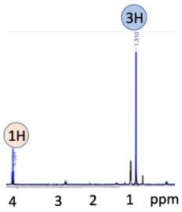
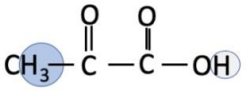
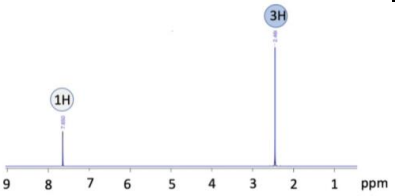
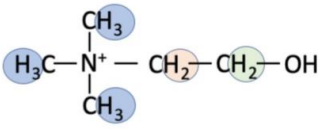
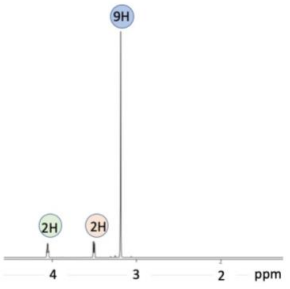
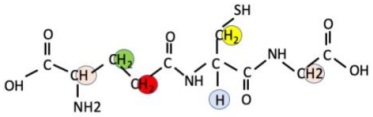
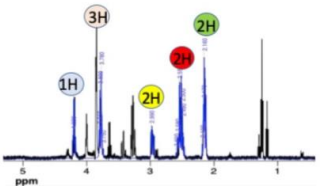
A. Spectrum from high-resolution magnetic resonance spectroscopy (HR-MRS) analysis of body fluids (e.g. serum). The spectral region on the x-axis is from 0.8 to 3.8 ppm (226).

B. Spectrum from high-resolution magic angle spin magnetic resonance spectroscopy (HR-MAS-MRS) analysis of breast tissue with a spectral region of 3.3 to 3.6 (222).

Both plots: The spectral region (ppm) on the X-axis indicates the substance, whereas the area under the curve represents the amount/concentration of each component.

Table 3. Molecular structure and their corresponding NMR spectrum.

The relationship between the molecular structure and the NMR specter of some key components in the present thesis (227).

Component	Molecular structure	NMR specter
Lactate		
Pyruvate		
Choline		
Glutathione		

Lactate and glycine are promising biomarkers in ER-positive breast cancer, with tumors containing high lactate and glycine levels having the worst prognosis (228). Comparing information from metabolomics, proteomic and genetic analyses has shown that

metabolic expression correlates to protein expression, while genetic subtypes were evenly distributed across metabolic subgroups. Consequently, metabolic aberrations may present additional information to explain breast cancer heterogeneity. (222)

1.11.5 Brief overview of tools and methods used in analysis of metabolomic data

Metabolomics is the study of small molecules, i.e. < 1000DA. The metabolites are affected by medication, diseases, sex, age, diet among others. They represent the downstream of genes, RNA, and proteins, and are regarded as the link between the genotype and phenotype of the cells (222). MRS metabolomics of BC has shown different metabolite profiles between breast cancer patients and healthy controls, and certain metabolite profiles are linked specifically to breast cancer.

1.11.5.1 Technical aspects

The MR spectroscopy technique is based on what happens with specific nuclei in a magnetic field (223). The spin configuration of the nuclei depends on the number of protons and neutrons the nucleus harbors. The Larmor frequency is the frequency (Measured in Hz) that is specific for different nuclei, dependent on the magnetic field (Tesla) that is applied. The nuclei are excited by a radio frequency that is the same as the frequency related to the nucleus that is of interest. When the radio frequency is switched off, the nucleus loses energy and the spectrum obtained is dependent on the chemical surroundings to the nucleus and the amount of sample that is examined (229).

In tissue the molecules are anisotropic: they are less mobile than in liquid, giving broad and overlapping peaks. Magic Angle spinning mimics the molecular motion found in liquids, giving spectra with narrower peaks.

The signal from the protons in different molecules are distinctly separated in MR specter, plotted as a function of the resonance frequency.

Chemical shift is the difference of resonance frequency (of the molecule of interest) and the reference frequency (TSP – trimethylsilylpropionic acid). The resonance difference is often 1 million times less than the actual resonance frequency and is therefore expressed as parts per million (ppm) (Fig. 33). PH and temperature might change the chemical shift.

1.11.5.2 Preprocessing of the metabolomic data

The reference compound has chemical shift zero by definition. The signal in NMR spectrum is proportional to the concentration of molecules of which the nuclei producing the signal is portion of. That means that the components also can be quantified.

There are a lot of variables that must be handled in metabolomics. This requires preprocessing of the raw data material to get a proper format for statistical analysis. Multivariate analyses in Paper III were performed in R V.3.5 and MetaboAnalyst. Dependent of pH and temperature the peaks might shift left and right. These and other challenges require preprocessing of the data. The baseline is adjusted, the peaks are aligned, the samples are normalized to account for dilution or sample weight differences, and auto scaled (mean-centered and divided by variance) to account for fold- and variance differences between the metabolites (230).

1.11.5.3 Statistical analysis of the metabolomic data

PCA is an unsupervised method used to find naturally occurring patterns. By PCA new variables and coordinate systems are generated to reduce the dimensions. In a PCA biplot, PC scores of samples (one dot for each sample) and loading variables (vectors) are obtained. These show what kind of influence each score and loading has on PC1 and PC2. PC1 represents the maximum of variation between the data points on the rotation line, with minimal error. PC2 represents the second most variation between the datapoints. The angle between the vectors in loading plots shows how the different variables are correlated to each other. Small angle means positive correlation, an angle 90 of degree means no correlation and an angle > 180 means a negative correlation.

To find differences in metabolic profiling amongst categories Partial Least Squares Discriminant analysis (PLS-DA) was used. Partial least squares regression (PLS-R) was used to find correlations between metabolic profile and continuous variables.

One challenge in multivariate analysis is overfitting that means that latent variables that represent noise are included, leading to prediction models which will not fit new data. This is the most serious problem. If too few numbers of latent variables are included in the model, problem with underfitting will arise.

To validate the results regarding quality and robustness, cross validation and permutation testing were performed. Cross validation is a resampling technique. The dataset is divided into new groups or "folds", and one by one the folds are excluded while the test is run. By permutation test the classes or groups are rearranged before being entered in the model, to simulate the "null hypothesis" that no difference exists between the groups. If equal or better models can be created by random data, the conclusion is that the model is not valid.

1.11.5.4 Deriving of the metabolomic data into functional pathway analysis

Metabolite Set Enrichment Analysis (MSEA) is a web-based tool that makes it possible to investigate metabolites and by help of this get suggested biological pathways or disease conditions that can be explored (231). It was used to investigate the metabolic pathways affected by the carbohydrate intervention.

Ingenuity pathway analysis (IPA) is also a platform that is available to identify biological pathways (232). In addition, this program makes it possible to identify key regulators and activity to explain expression patterns, predict downstream effects on biological and disease processes and provide targeted data on genes, proteins, chemicals and drugs. By using this tool, we identified the top five functions enriched in the dataset, and the metabolites connected to four microRNAs involved in tamoxifen resistance (Paper III).

1.12 Endocrine Resistance — The Ultimate Hallmark of Breast Cancer

Relapse in a luminal breast cancer patient occurs due to the development of endocrine resistance in micro-metastases, leading to a failure of endocrine treatment to eradicate these cells. The reasons for a reduced effect of endocrine treatment in breast cancer can be grouped into three main categories: 1) metabolic: inherited genetic polymorphisms in the drug metabolism of tamoxifen, leading to low serum concentrations of active tamoxifen metabolites; 2) intrinsic/de-novo/primary: inborn resistance to tamoxifen by the cell before tamoxifen is administered; and 3) acquired resistance: gradual reduction of the effect of tamoxifen/aromatase inhibitor, leading to increased estrogen sensitivity under ongoing treatment with tamoxifen/aromatase inhibitor.

Intrinsic and acquired resistance may share some common pathways/mechanisms, with a difference in time of appearance. Acquired resistance is likely a multigene phenomenon, involving a network of inter-related signaling pathways. Endocrine resistance in breast cancer may be regarded as a separate hallmark and is the cause of development of distant metastases in luminal breast cancer. Endocrine resistance increases the defiance of the cancer cells against endocrine treatment (i.e., tamoxifen and aromatase inhibitors). It also involves a shift in cellular vulnerability from endocrine treatment to chemotherapy. Thus, endocrine resistance is about identifying the luminal B subtype, which will benefit from chemotherapy up-front. Therefore, it is important to understand the main mechanisms of endocrine resistance, which are depicted in Fig. 34. The numbers in the following text correspond to the numbers in Fig. 34.

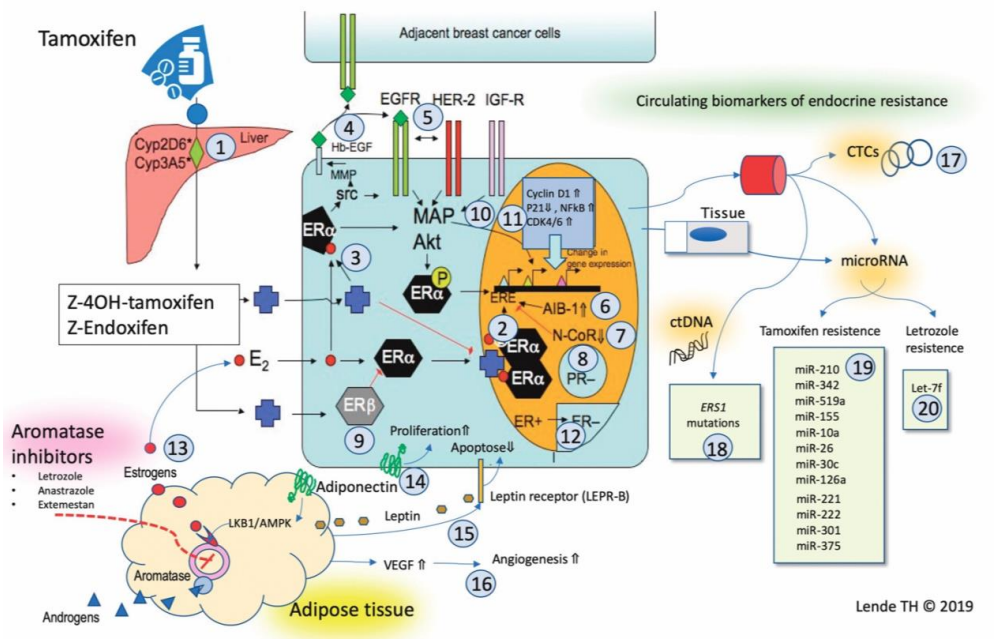


Figure 34. Endocrine resistance of breast cancer

Overview of the mechanisms of endocrine resistance. See the text for explanation. *, gene polymorphism; E2, estradiol; ERα, estrogen receptor α; ERβ, estrogen receptor-β; AIB-1, amplified in breast cancer-1; N-CoR, nuclear receptor co-repressor; Z-endoxifen, Z-4-OH-N-desmethyl tamoxifen; MMP, matrix metalloproteinase; IGF-R, insulin growth factor receptor; Tam, tamoxifen; RbBP8, retinoblastoma binding protein 8; PR, progesterone receptor; VEGF, vascular epithelial growth factor; ERS1, estrogen receptor 1 gene; CTC, circulating tumor cell; miR, micro-RNA; ctDNA, circulating tumor DNA. The red cylinder depicts a blood vessel; rectangular shape with a blue oval ring depicts an object glass for microscopic analysis of tissue by immunohistochemistry, etc. Numbers in small circles correspond to the text.

1. Metabolic resistance: Both polymorphisms of certain alleles in the activation of tamoxifen (CYP2D6 and CYP3A5) and the inactivation of enzymes SULT-1A1 will lead to greater inter-individual variation in the concentrations of active metabolites (4-OH tamoxifen and 4-OH-N-desmethyl-tamoxifen). Reduced disease-free survival is seen in patients with CYP2D6 *4, *5, *10, and *40 alleles (233).

2. Change in ERα protein and/or the ERα responsive element (ERE) in the target genes, increasing the sensitivity for estradiol (116).

3. Activation of the MISS-ERα pathway. Tamoxifen may act as an agonist of ER, causing cross-talk between the MAP and Akt pathway. These pathways phosphorylate ERα, with activation and stimulation of cell proliferation as a consequence (234).

4. MISS-ERα signaling activates src, which activates matrix metalloproteinases (MMPs), releasing epidermal growth factor from heparin binding epidermal growth factor (Hb-EGF). The released EGF activates EGFR (234).

5. Amplification of HER-2 has two effects: increased activation of MAP/Akt, which gives the same effect as in 3. Mutations in HER-2 receptor may lead to downstream activation of pathways (e.g., PIK3CA) (235). Increased levels of AIB -1 are also seen.

6. Increased AIB-1/SRC1 (co-activator) leads to increased ERα genomic signaling. If this co-activator becomes amplified at high levels, it can activate ERα, even if tamoxifen is bound to ERα. Thus, tamoxifen acts as an agonist instead of an antagonist (236).

7. Decreased levels of co-repressor NCoR in the nucleus will reduce the antagonistic effect of tamoxifen on ERα (116, 117).

8. Lack of PR expression, a marker of intrinsic resistance, probably due to altered ERα signaling (see Fig. 29) (237).

9. Tamoxifen has an agonistic effect on ERβ, which counteracts ERα. ERβ1 in the cell will improve disease-free survival and overall survival (116, 234).

10. Ligand-independent activation of ERα through cross-talk with other RTKs, such as EGF, IR, and IGF-1R. Activation of ERα occurs via phosphorylation (117, 238-240).

11. Change in gene expression in multiple genes, e.g., the cyclin D1-retinblastoma axis (e.g., RbBP8), p21, NFkB, and c-fos, and the activation of CDK4/6 kinases (114).

12. Shift from ER+ to ER– cells in the tumor, which may include population remodeling or transcriptional repression of ER. The latter includes mutation (241) or splice variants of ERα, resulting in a non-functional ER (115).

13. Conversion of androgens from the adrenals into estrogens by aromatase in adipose tissue (both located peripherally and adjacent to the tumor cells) (242).

14. Adiponectin from adjacent adipose cells activates adiponectin receptor in breast cancer cells, which activates the Akt-pathway and increases proliferation (243).

15. Leptin from adipocytes activates leptin receptor type B In cancer cells, which activates the JAK/STAT 3 pathway and reduces apoptosis (243).

16. Adipocytes secrete VEGF, which enhances angiogenesis (243).

17. CTCs in peripheral blood are a marker of endocrine resistance, as the treatment has not eradicated the micro metastases (244).

18. Circulating tumor DNA (ctDNA) may reveal mutations in the ERS1 gene pointing out that a change in the ER protein leads to a lack of interaction with tamoxifen metabolites and the ligand estradiol, and these issues lead to endocrine resistance (245).

19. Micro-RNAs in the tumor and circulation up- and down-regulate genes, which then alter the relationships between the ER, CoA, and CoR, which ultimately causes tamoxifen resistance (156, 246).

20. Letrozole resistance due to high expression of Let-7f in the tumor (247).

1.13 Rationale for the Present Thesis

Almost 75% of all new breast cancers in the Western World belong to the luminal breast cancer subgroup. The adjuvant drugs of choice are the anti-estrogen tamoxifen and aromatase inhibitors. However, one-third of luminal cancers belong to the luminal B subgroup, which has developed endocrine resistance with increased proliferation and gained susceptibility to chemotherapy. Luminal A cancers have preserved sensitivity for endocrine treatment. Therefore, it is important to be able to distinguish between luminal A and luminal B in order to avoid under- and over-treatment of patients. The most accurate method is the PAM-50 candidate gene method (154). However, most countries and hospitals cannot afford such advanced gene expression analysis for each luminal patient. Thus, a less expensive, reproducible, and fully reliable alternative is needed to identify the luminal B patients. We wanted to measure the prognostic power and accuracy of the MAI against Adjuvant! Online and the NBCG 2010 guidelines to determine whether the MAI may be a candidate method for identifying under- and/or over-treated subgroups of breast cancer.

During the last 50 years, the alimentary content of carbohydrates has increased. ERAS protocols have been established for many surgical procedures (200) and include high doses of oral carbohydrates pre-operatively to increase post-operative well-being. We wanted to perform a randomized controlled trial (RCT) to examine differences in the MAI and PR in an intervention group compared to a control group to examine whether pre-operative carbohydrates increase the MAI, in line with the ERAS protocol, or reduces PR, i.e. creating endocrine resistance. No prognostic or predictive factors from the metabolism of glucose have been established in breast cancer. Therefore, we also wanted to perform an explorative metabolomic study of the serum and tissues from the RCT to find putative prognostic and predictive biomarkers of endocrine resistance in luminal breast cancer patients.

2.0 Objectives, Aims, and Hypotheses of the Present Study/Thesis

2.1 Overall Objectives

2.1.1 Compare the prognostic power of tumor proliferation and classical prognostic factors in a treatment-naïve patient population (Paper I).

2.1.2 Study the effect of pre-operative carbohydrate load in operable breast cancer patients (Paper II).

2.1.3 Explore the metabolic profile in the tumor and liquid biopsies in operable breast cancer patients administered pre-operative carbohydrate loading (Paper III).

2.2 Specific Aims & Hypotheses

2.2.1 Paper I

Aim: To compare the prognostic power of MAI in relation to Adjuvant! and NBCG guidelines (2010) in a treatment-naïve population of lymph node negative (pN0) breast cancer patients <55 years old with a long-term follow-up.

Null Hypothesis (H_0): MAI does neither add prognostic information to Adjuvant! nor the NBCG 2010 guidelines.

2.2.2 Paper II

Aim 1: To study the influence of pre-operative carbohydrate load on tumor proliferation in operable breast cancer patients.

Null Hypothesis (H_0): There is no correlation between proliferation (MAI-10) and pre-operative carbohydrate load.

Aim 2: To study the influence of pre-operative carbohydrate load on insulin characteristics in operable breast cancer patients.

Null Hypothesis (H_0): There is no correlation between insulin characteristics and

pre-operative carbohydrate load in operable breast cancer patients.

Aim 3: To study the influence of pre-operative carbohydrate load on general well-being in operable breast cancer patients.
Null Hypothesis (H_0): There is no correlation between pre-operative carbohydrate load and patient well-being in operable breast cancer patients.

Aim 4: To study the influence of pre-operative carbohydrate load on survival in operable breast cancer patients.
Null Hypothesis (H_0): There is no correlation between pre-operative carbohydrate load and breast cancer survival in operable breast cancer patients.

2.2.3 Paper III

Aim 1: To explore the metabolic profile in liquid biopsies (serum) from patients who received pre-operative carbohydrates and correlate it to proliferation and survival in operable breast cancer patients. In addition, we sought to identify putative circulating exposure variables that add to understanding differences in clinical outcome between the two study groups.
Null Hypothesis (H_0): There is no correlation between pre-operative carbohydrate load and the metabolic profile in serum in estrogen receptor positive patients.

Aim 2: To explore the metabolic profile in tumor tissues from patients who received pre-operative carbohydrates, serving as a proxy for clinical outcome in operable breast cancer patients.
Null Hypothesis (H_0): There is no correlation between pre-operative carbohydrate load and the metabolic profile in tumor tissue in estrogen receptor positive patients.

3.0 Synopsis of the Studies

This thesis comprises three studies. One observational study, one randomized controlled trial and one study with an explorative design. The latter study is a continuation of the second study, where the same patients were used. An overview of the papers is presented in Fig. 35 below.

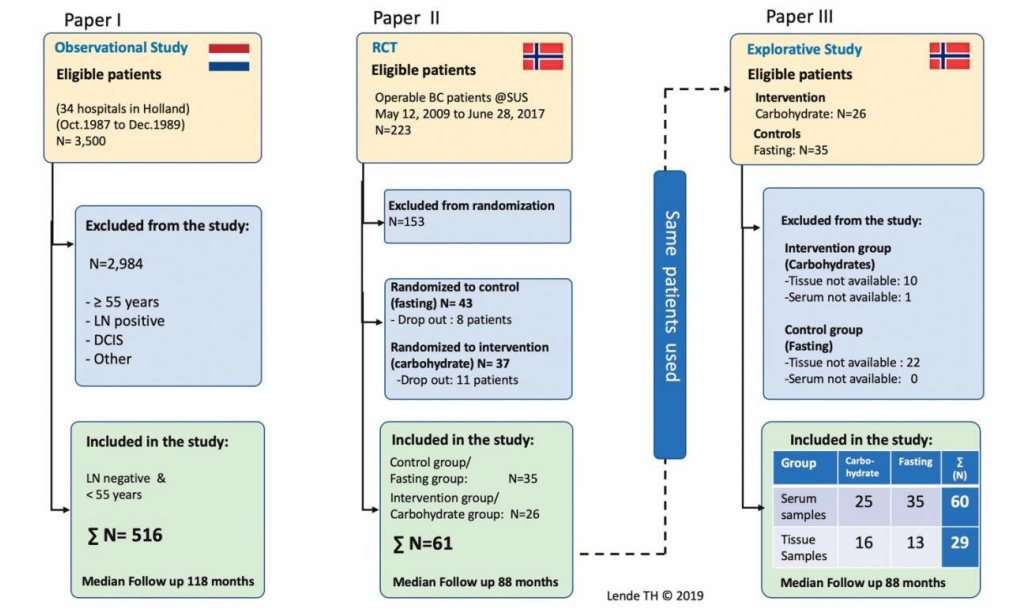


Figure 35. Overview of the three studies in the thesis

Overview of the three papers comprising the present thesis. Emphasis is made on patient selection.

The presentation of the papers in the synopsis includes key tables and figures in order to give the reader a more comprehensive overview of the papers at this point. All details are found in the individual PDF-files in the end of the thesis.

3.1 Paper I

In Patients Younger Than Age 55 Years with Lymph Node–Negative Breast Cancer, Proliferation by Mitotic Activity Index Is Prognostically Superior to Adjuvant!
Journal of Clinical Oncology 2010; 29(7): 852-8.

Background: Guidelines based on multimodal prognosticators result in under- and over-treated patients. We wanted to test whether MAI could improve clinical categorization.
Design: We applied a historical prospective observational design.
Patients: We used an anonymized dataset comprising 516 systemic treatment-naïve patients with LN-negative status and age less than 55 years, with a median follow-up of 118 months.
Controls: No controls were included.

Methods: 10-year breast cancer specific survival (BCSS) was obtained from Adjuvant! v. 8.0. Using the NBCG guidelines at the time (January 2010), the patients were re-characterized into either an NBCG no adjuvant systemic therapy (NBCG–No-AST) group or an NBCG adjuvant systemic therapy (NBCG+AST) group and the outcome evaluated in Adjuvant! Online. MAI was assessed according to the standardized protocol (see 1.7.7.3). The threshold for Adjuvant!, MAI, and NBCG was the cut-off value that distinguished when the patients were supposed to benefit from systemic adjuvant therapy (i.e., chemotherapy; see Table 4).

Table 4. Overview of variables representing low and high risk BC (Paper I).

Variable	Low risk (No AST)	High Risk (+ AST)
Adjuvant!	≥ 95% BCSS	< 95% BCSS
MAI	< 3 mitoses/1.59 mm^2	≥ 3 mitoses/1.59 mm^2
NBCG -2010	pT1+G1, all ages or PT1a-b, Grade 2-3, ≥ 35 years All pN0 and ER+ or PR+ (NBCG No-AST group)	All other (NBCG +AST group)

We used Adjuvant 95% (Adjuvant!-95%) and MAI 3 (MAI-3) as thresholds for benefit from systemic adjuvant therapy.

Results: To further investigate the prognostic interaction of Adjuvant! -95% and MAI-3, we created a new interaction variable consisting of the product of MAI-3 and Adjuvant! -95%. MAI-3 identified ~40% of patients as under-treated and ~20% of the patients as over-treated, which holds for both Adjuvant! and NBCG-2010 (Fig. 36A, table 4 and 5). This has a direct impact on survival (Fig. 36B).

A

Table 1. Comparison of Prognostic Subgroups Regarding Breast Cancer–Specific Survival of the Patients With Lymph Node–Negative Breast Cancer According to Adjuvant!, NBCG, and MAI

	MAI < 3		MAI ≥ 3		
Adjuvant! and NBCG	No. of Patients	%	No. of Patients	%	Total No. of Patients
Adjuvant!					
≥ 95%	74	61	48	39	122
< 95%	86	22	308	78	394
Subtotal	160		356		516
NBCG					
No AST	60	60	40	40	100
AST	100	24	316	76	416
Subtotal	160		356		516

NOTE. Please note that there are many discrepancies, and consensus is fair only (Adjuvant! *v* MAI: $\kappa = 0.35$; NBCG *v* MAI: $\kappa = 0.29$).

Abbreviations: NBCG, Norwegian Breast Cancer Group; MAI, mitotic activity index; No AST, NBCG guidelines indicate that patients should not be offered adjuvant systemic therapy; AST, NBCG guidelines indicate that patients should usually be offered adjuvant systemic therapy.

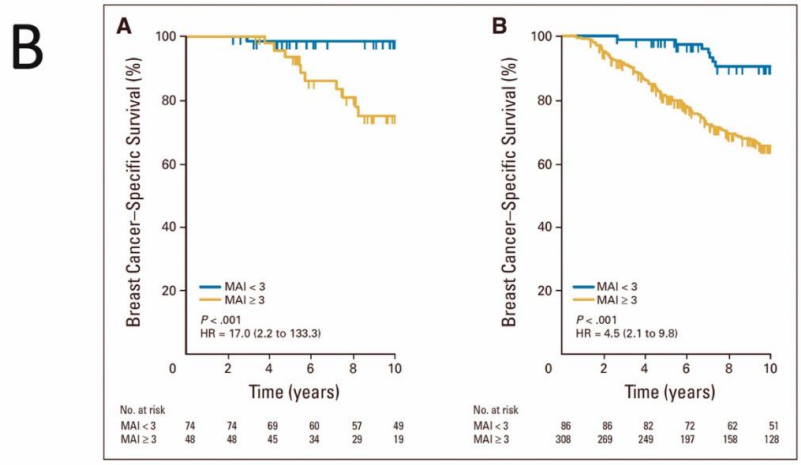


Figure 36. Prognostic information of MAI-3

A. This table is equivalent to Table 1 in Paper I, which shows that MAI-3 identifies prognostic subgroups among Adjuvant! and NBCG guidelines.

B. This figure is equivalent to Figure 2 in Paper I, which shows that MAI-3 overrules the prognostic information on BCSS for Adjuvant! ≥ 95% (A) and Adjuvant! < 95 % (B).

Table 5. Influence of Adjuvant! and MAI-3 on NBCG

Patient subgroup defined by the following interaction variables	10 y BCSS	Number /Total patients	AST Recom-mended	% Patients Under-treated	% Patients Over-treated
[Adjuvant! ≥ 95% * MAI ≥ 3]	79%	48/122	No	39%	–
[Adjuvant! < 95% * MAI < 3]	92%	86/394	Yes	–	22%
[NBCG – No-AST * MAI ≥ 3]	82%	40/100	No	40%	-
[NBCG + AST * MAI < 3]	93%	100/416	Yes	-	24%

Conclusion: These data strongly suggest that MAI adds valuable prognostic information otherwise not captured by Adjuvant! or the NBCG guidelines. MAI or another reliable proliferation marker should be added to both guidelines for LN-negative patients aged < 55 years.

3.2 Paper II

Influence of pre-operative oral carbohydrate loading vs. standard fasting procedure on tumor proliferation and clinical outcome in breast cancer patients — a randomized trial. BMC Cancer 2019 Nov 8;19(1):1076

Background: Knowledge of carbohydrates in breast cancer is conflicting. We wanted to study the effect of carbohydrates on proliferation in breast cancer tumors.

Design: Randomized control trial (RCT).

Patients: We included 26 patients who received a dose of pre-operative carbohydrates 12 h and 2 h before surgery in the intervention arm (carbohydrate group).

Controls: The 35 patients in the control group (fasting group) followed a standard fasting procedure with free drinking water per os.

Methods: Insulin characteristics, MAI, and other conventional prognosticators were assessed in all patients. Patients' post-operative well-being was assessed by a questionnaire developed by the Department of Anesthesiology in our hospital. The median follow-up was 88 months.

Results: Among luminal patients (ER+), the carbohydrate group included more patients with MAI≥ 10 (p=0.035; r= 0.301, Kendall Tau-b). In addition, the carbohydrate group comprised more PR-negative tumors than the control group (p=0.014). Moreover, pre-operative insulin and c-peptide levels were significantly higher in the carbohydrate group, whereas IGFBP3 was reduced in ER-negative patients. IGF was unchanged in all patients. In the luminal patients, the RFS was 71% in the carbohydrate group and 97% in the fasting group (p=0.012; HR=9.1, 95% CI 1.1-77.7) (Fig. 37A). This survival difference was confined to luminal patients with T2 tumors (RFS of 33% in T2 tumors and 100% in T1 tumors; p=0.031; HR=inf)(Fig. 37B). The same pattern was seen for BCSS in luminal breast cancer patients with a BCSS of 30% in ER+/T2 tumors and 100% in ER+/T1 tumors The effect on well-being was scarce, with only some reduced pain on day 5 after surgery. No adverse reactions were noted.

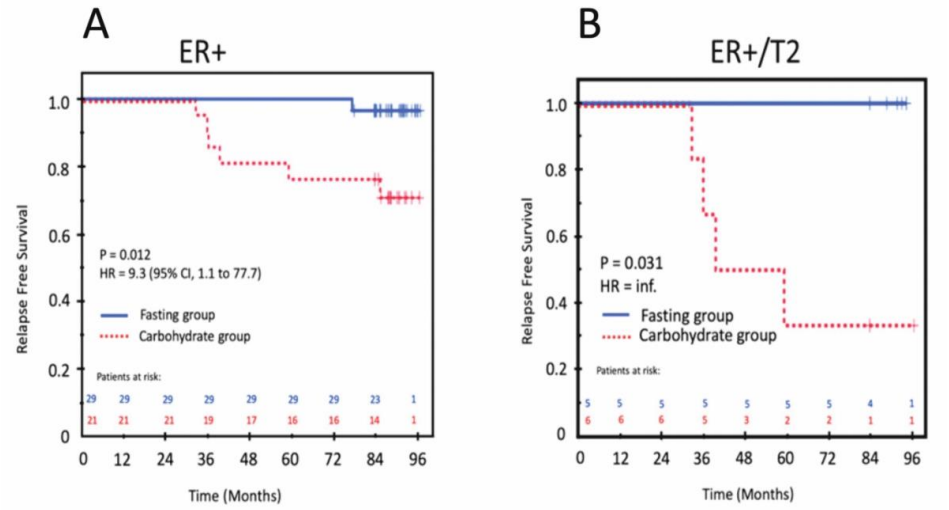


Figure 37. Relapse free survival in ER+ and ER+/T2 tumors.

This figure is equivalent to Figure 3a and 3d in Paper II.

Conclusion: These data indicate that pre-operative high carbohydrate loading correlates with increased proliferation, reduced PR, and reduced RFS and BCSS. The time from diagnosis to adjuvant systemic therapy may be more important than we think today, especially peri-operatively when CTCs are liberated from the tumor. More studies are needed to further elucidate on this hypothesis.

3.3 Paper III

Metabolic consequences of peri-operative oral carbohydrates in breast cancer patients — an explorative study Submitted BMC Cancer September 2019

Background: Systemic and local metabolic patterns in breast tumors following an oral carbohydrate load are not well elucidated.

Design: Explorative study. Based on paper II, our hypothesis was that metabolic changes were most likely to be found in the luminal (ER+) patients.

Intervention patients: Serum samples were available from all 26 patients in study II, and tumor tissue from 16 patients in the carbohydrate group in study II.

Controls: Serum samples were available from all 35 patients and tumor tissue from 13 patients in the control (fasting) group in study II.
Methods: HR-MRS analysis was performed in the serum samples, followed by PLSD analysis. HR-MAS-MRS analysis was performed in the tumors.
Results: Scores plot showed that the carbohydrate and fasting groups had significantly different metabolic profiles (Fig. 38). Fourteen of 28 metabolites explained the differences between the carbohydrate and fasting groups (Fig. 38). S-lactate and S-pyruvate were increased in the carbohydrate group, while ketone bodies were increased in the fasting group compared to the carbohydrate group.

In the carbohydrate group, there was a positive linear correlation between proliferation (Ki-67) and tumor size ($r=0.782$, $p=0.038$). When Ki-67, PPH3 and MAI were included in a forward and backward stepwise linear regression MAI was the only independent factor explaining increment in tumor size with a Beta=0.530 (95%CI, 0.201 to 0.875) $P = 0.009$. In the fasting group, there was no correlation between tumor size and proliferation.

In ER+ tumors, we measured a higher glutathione content in the tumors from the carbohydrate group, and increased glutamate in tumors with high proliferation. In ER+ tumors, the choline content was significantly higher in T2 tumors than T1 tumors. Ingenuity Pathway Analysis (IPA) and metabolite set enrichment analysis (MSEA) showed that the main functions of the involved metabolites were connected to cellular growth and proliferation, lipid metabolism, small molecule biochemistry, carbohydrate metabolism, and amino acid metabolism. Moreover, four out of seven microRNAs involved in endocrine resistance regulated the same metabolic pathways.

In ROC analysis, the optimal thresholds for RFS as outcome were S-lactate = 54.6, S-pyruvate = 12.5, and T-glutathione = 1.09. The results from the univariable analyses are given in Table 6 and Fig. 39.

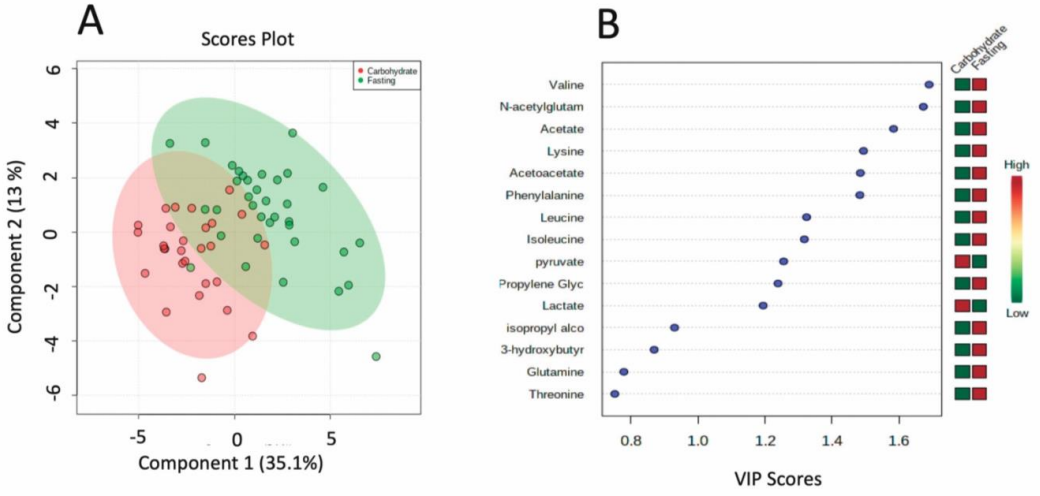


Figure 38. PCA and VIP sore from the serum samples

This figure is equivalent to Figure 2 in Paper III.

Table 6. Overview of Relapse free survival and Breast cancer specific survival

Exposure variable	Outcome variables			
	RFS (%)	P	BCSS (%)	P
Lactate <56.9	93	0.002	98	0.002
Lactate ≥56.9	56		67	
Pyruvate <12.5	95	<0.0001	100	<0.0001
Pyruvate ≥12.5	50		60	
Glutathione <1.09	100	0.038	100	0.038
Glutathione ≥1.09	63		63	

In the multivariable analysis for RFS, S-pyruvate was the only factor left in the final model (HR=13.6; 95% CI 2.61 to 70.6), and only S-lactate remained in the final multivariable model for BCSS (HR=10.4; 95% CI 1.04 to 103).

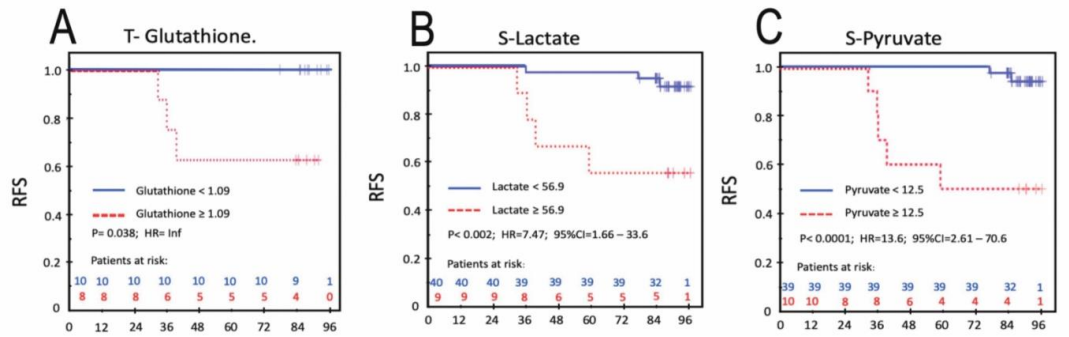


Figure 39. Relapse free survival in ER+ patients

Relapse free survival (RFS) in ER+ patients with the following explanatory variables:

A. Tissue Glutathione. B. Serum Lactate and C. Serum Pyruvate.

These figures are equivalent to Figure 5 a, b and c in Paper III.

Conclusion: Carbohydrates seem to increase two important factors from which cancer cells may benefit:

A. Better 'fuel supply': increased lactate, pyruvate, and glutamate in the systemic circulation.

B. Better cellular protection: increased glutathione content in the tumor.

4.0 Discussion of the main findings

4.1 MAI Predicts Under- and Over-treatment in Breast Cancer

Introducing MAI as a prognosticator and predictor of outcome seems to add valuable information. Alarmingly, MAI-3 revealed that as many as 40% of the lymph node negative patients as under-treated according to both Adjuvant! Online and NBCG-2010 guidelines (Paper I). Adjuvant! Online is based on SEER (248-250), which uses information from the treatment and observation of American breast cancer patients with long-term follow-up. As SEER regions in the US recruit patients from areas with a low proportion of ethnic white people, the population includes greater economic disadvantages and greater minority diversity (251). In Asian breast cancer patients, Adjuvant! online seems to be overoptimistic in predicting survival (252). Thus, Adjuvant! online has a reduced external validity and limitation regarding world-wide clinical use. Also, SEER patients may be more under-treated than Norwegian patients. However, MAI-3 identified the same proportion of under-treated patients when the NBCG 2010 guidelines were applied to MMMCP patients (Paper I). Both Adjuvant! and the NBCG guidelines are multivariable, utilizing conventional prognosticators (age, pT, pN, grade, ER) comprising overlapping prognostic information; for example, histological grade has modest interobserver agreement (kappa = 0.4-0.5). Thus, the various conventional factors may neutralize each other and lose prognostic power. In contrast, the MAI captures cells that have survived the cell cycle and entered M-phase (Fig. 23). Thus, MAI is regarded as a functional readout of cell signaling pathways belonging to all hallmarks of cancer, which change the dynamics in the cell cycle and lead to increased mitotic activity (140). Moreover, MAI-10 has been shown to be a reliable and robust prognostic factor in lymph node negative patients < 55 years old (25), and later < 70 years (253).

Many of the under- and over-treatment questions are raised in the luminal breast cancer subgroup, which comprises 75% of all breast cancers in the Western World (23). Luminal B patients have tumors with increased proliferation and will probably benefit from

chemotherapy. Thus, much of the discussion regarding gene profiling in luminal breast cancers is to identify luminal B patients. At San Antonio Breast Cancer Symposium 2012, Focke et al. presented a poster (PD06-04) (142) showing that MAI-10 is superior to various Ki-67 thresholds to differentiate luminal A patients (2/3) from luminal B patients (1/3) (132). Thus, MAI could be a reliable biomarker to identify the Luminal B patients.

Several gene expression studies have used a different approach. MammaPrint™, PAM-50 (Prosigna™), and Oncotype DX™ have based their analyses on 70, 50, and 21 genes, respectively. PAM-50 is based on the 456 genes from the original publication by Chuck Perou and Therese Sørlie in 2000 (24). Interestingly, these gene expression tests all showed that genes related to cell cycle and proliferation are prognostically the most important genes (24, 111, 254, 255). Various consensus meetings have recognized Oncotype DX (90) and Prosigna (151) as reliable tests for identifying patients among lymph node negative patients using a risk of recurrence score. Therefore, these tests give clinicians enough support in decision-making regarding the addition of chemotherapy to minimize both under- and over-treatment as shown in the TailorX study (256, 257) and MINDACT study (258).

However, many hospitals and national health care services cannot afford these expensive tests. Therefore, surrogate variables have been established. Ki-67 is the one that has gained the most attention, and various thresholds for luminal A and luminal B (15%, 30%, median ±10%) have been suggested over the years (90, 125, 126). Interestingly, MAI has not gained enough support in consensus meetings to become a proxy for proliferation. The strict guidelines for its measurement (140) and the fact that mitotic figures may be difficult to distinguish from apoptotic figures leads to pathologists having to do the job. In contrast, Ki-67 is an immunohistochemical method with better contrast between negative and positive cells, making it feasible for lab technicians to score. Another issue contributing to the hesitant introduction of MAI as a proxy for luminal A vs. luminal B, and high recurrence vs. low recurrence is the fact that there are two clinical thresholds for MAI: MAI-10 as the prognostic cut-off point and MAI-3 as the predictive threshold. MAI-3 was

superior to MAI-5 and MAI-10 in predicting the effect of chemotherapy (26). Classification and regression tree analysis in MMMCP patients showed that MAI-3 was the most important threshold and MAI-10 the second most important threshold, which was confirmed in the present thesis (Paper I). Therefore, there is a need for well-designed prospective studies to establish a more robust platform for the use of MAI (see chapter 6).

In Paper I, MAI was the strongest prognosticator, whereas in Papers II and III, it did not reach significance. The reason for this difference is likely due to MAI being tested in a pure treatment-naïve cohort of only lymph node negative patients in Paper I, whereas the patients in Papers II/III had received the maximum systemic treatment according to 2009-2010 guidelines. Furthermore, the patients in Papers II and III were a mixture of lymph node negative and positive. In Paper I we also had a much longer observation time, which gains more endpoints to increase the power in the survival analysis. Finally, the explanatory variables in Papers II and III were all optimized through ROC analysis and minimal p-values in Cox analysis.

Interestingly, only a few hallmarks of cancer translate into prognostic and/or predictive factors. Self-sufficiency in the growth signal provides only four factors: ER, PR, HER-2, and Ki-67 (91) (Appendix 3 &4). One reason for this is that a putative factor cannot be tested in a treatment-naïve population due to a lack of material. Thus, new factors are tested in patient cohorts that have already received treatment based on other prognostic/predictive factors. In addition, most of the factors are related to other factors, creating prognostic information in the univariate analysis, but are removed from the multivariable model. Thus, the existing prognostic and predictive markers are robust and reproducible (89).

4.2 Increased MAI in the carbohydrate group.

In Paper II, we found the increased MAI after only 18 hours of the carbohydrate loading commenced. This may be regarded as a too short time. However, our observation is supported by the preclinical study of MCF-7 cells which have been given glucose (259) In that study, an increase of proliferation was seen already after 12 hours. Increased signaling was also compatible with the metastatic process. Both these cellular responses were driven by impairment of the angiotensinogen expression.

Moreover, others also found increment in proliferation after glucose exposure to three different cancer cells lines (MCF-7, SKBR3 and MDAMB231). High glucose regulated EGFR activity through GTPase signaling (260) Phosphorylation of the EGFR increased the longevity of the receptor and thus sustained EGFR signaling with an increased proliferation and growth as a consequence (260).

In preclinical mouse models (261) increased carbohydrate and calorie diet showed increased EMT progression in the breast tumors mediated through insulin related signaling (262). Also, animal models simulating metabolic syndrome demonstrate increased proliferation in breast cancer tumors when the mice are fed with high carbohydrate diets (263).

Thus, there is a body of evidence that support our observation in paper II that increased proliferation follows a carbohydrate load. The question is more how we interpret this finding in the clinical setting and how we proceed and what do next (see chapter 6.0, Future Perspectives).

4.3 Progesterone Receptor as a Concomitant biomarker of Increased Proliferation, Increased IGF Signaling, and Increased Endocrine Resistance in Luminal Breast Cancer

In Paper II, we observed a concomitant reduction in PR with increased proliferation. The reason for this observation is probably the shift in cell signaling pathways away from the ER-directed systems, which stimulates PR towards cell membrane-localized RTKs such as IR and IGFR (Fig. 19, pt. 14 and Fig. 34 pt. 8) (237). These RKTs stimulate growth and protein synthesis, depriving the transcription of PR to avoid redundancy. Thus, a lack of PR expression is a sensitive marker of endocrine resistance, indicating increased signaling through cell membrane receptors with reduced survival (237). In contrast, PR positivity in Ki-67-positive cells was associated with ER signaling and ER-regulating genes, a low Oncotype DX recurrence score, and better survival.

The high carbohydrate content of the Western diet leads to increased insulin-dependent cellular signaling, which may lead to increased breast cancer mortality. In ER-positive cancers, increased signaling through the IGF signaling pathways (IGFs and IGFBPs) is associated with a worse clinical outcome (264, 265). In a recent Danish study, such a relationship could not be established for short-term prognosis but were more probable for long-term prognosis (266).

4.4 Insulin-related Pathways and Metformin in Breast Cancer

In Paper II, we observed no increased proliferation in ER-negative cancers, only in ER-positive breast cancers. Interestingly, in triple-negative breast cancer, high expression of IGFBP-3 in the nucleus was associated with poor survival in both preclinical models (267) and a clinical setting (268). Thus, the reduced IGFBP-3 in the carbohydrate group (Paper II) may explain the lack of proliferation response in ER-negative patients. In triple-negative breast cancer, IGFBP-3 may potentiate the action of IGFs (269). Thus, targeting both the

EGFR and IGFBP-3 (i.e., through sphingosine kinase) pathways in a preclinical setting has been promising (268).

The prognosis is poorer in breast cancer patients with type 2 diabetes than non-diabetic population cohorts (270, 271), probably due to insulin resistance and the hyperinsulinemic state (272). Interestingly, the drug metformin plays an important role as both an oral anti-diabetic drug and an anti-cancer drug. In type 2 diabetes, metformin reduces gluconeogenesis in the liver and increases cell sensitivity for blood glucose, reducing the absorption of glucose in the intestines (273, 274). In cancer, metformin suppresses oncogenic cell signaling pathways, such as RTKs, PI3K, Akt, and mTOR (275). There is also a positive correlation between COX-2 and IGF-1R in both ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC), which implies cross-talk between these signaling pathways in breast carcinogenesis (276). Moreover, metformin is thought to attenuate cancer stem cells by targeting pathways involved in cell differentiation, renewal, metastasis, and metabolism (275).

In breast cancer, metformin has been shown to reduce the risk of acquiring breast cancer, and the cancers that arise in metformin users are both ER and PR-positive (277), meaning that the RTK and Akt/mTOR pathways are suppressed, allowing PR transcription. Thus, breast cancers in metformin users maintain or regain their endocrine sensitivity. In non-diabetic breast cancer patients, there is contradicting epidemiological evidence regarding whether metformin increases survival (278). However, in breast cancer patients with type 2 diabetes, here is an association between metformin use and improved clinical outcome (278). Moreover, adjuvant metformin appears to improve the prognosis in diabetic ER+ breast cancer patients (279, 280). In breast cancer patients with diabetes metformin had a salutary effect on prognosis (281). This clinical effect may be due to metformin diminishing the unfavorable impact of Nrf2 (282). Notably, in triple-negative breast cancer, metformin has not demonstrated improved outcomes (283).

4.5 Increased Tumor Size in the Carbohydrate Group

In the carbohydrate group, the mean tumor size was 19.4 mm, compared to 15.0 mm in the fasting group, but this difference was only borderline significant (Paper II). In Paper III, we found a positive correlation between tumor size and proliferation in the carbohydrate group only, and not in the fasting group. The explanation for this could be that the increment of proliferation due to carbohydrate loading (Paper II) will increase the size of the tumor cells, as Ki67-positive cells are on average larger than Ki67-negative cells (284). Thus, the carbohydrate load may have increased the tumor size despite the short time span between carbohydrate exposure and measurement of the tumor size (approx. 18 hours) (Paper II and Paper III). This hypothesis should be tested by quantitative pathology methods (QPRODIT) by measuring the size of the nuclei in both study groups.

4.6 Metabolomic Changes after Carbohydrates and Fasting

The increased systemic levels of lactate and pyruvate we observed in Paper III could come from peripheral metabolism, or from the primary tumor via Warburg effect. This is a metabolic shift to aerobic glycolysis and lactate production despite the presence of oxygen (216). However, lactate and pyruvate are excreted from the cells through MCT membrane transporters (Fig. 31, pt. 6 and 7). This may be why we could not detect increased lactate/pyruvate in the tumor tissue (Paper III). However, the increased systemic levels of lactate and pyruvate serve as supportive energy for CTCs exfoliated from the tumor during surgery (285)

The metabolic switch to the Warburg effect and lactate excretion, with acidic conversion of the cell and extracellular environment, has another important effect. Tumor acidosis was recently detected as a spatial phenomenon. Cellular regions with acidic environments may alter gene expression in a way that makes the cancer cells more

aggressive (e.g., invasion and migration of malignant cells) (286). Interestingly, tumor acidosis may also communicate with the microenvironment outside the cell, which also turns acidic. Moreover, the Warburg effect increases the intracellular glutathione content (Fig. 31, pt. 13), which we observed in the ER+ tumors of the carbohydrate group (Paper III). Thus, CTCs from these tumors are loaded with a cellular protection system necessary for cell survival (209), including cancer stem cells (287).

Another consequence of the Warburg effect is increased uptake of glucose in cancer cells (Fig. 31, pt. 1 and 15). Importantly, this cellular feature may be visualized by positron emission tomography (PET) with increased uptake of the tracer 18-FDG-glucose (288) one hour after injection. Importantly, there is a relationship between PET, tumor biology features, and the prediction of distant metastases (289). Thus, metabolic coupling and the reverse Warburg effect in cancer has become a novel strategy for treating cancer (290).

In Paper III, we found another important difference between the carbohydrate and fasting groups (i.e., increased ketone bodies in the fasting group). Ketone bodies are cytotoxic (291) and will contribute to eradicate CTCs in patients. Thus, Paper III demonstrates the differential effect between a ketogenic/cytotoxic fasting context and a carbohydrate/cell supportive context. This is supported in a recent RCT of using ketogenic diet as adjuvant treatment in one of the study arms. They observed a better overall survival in the group that received ketogenic diet (292). Also, in a mouse model with xenografted breast cancer tissue they found a profound effect of ketogenic diet with increased ketone bodies and also increased amino acids (293). The latter is also in line with our observations in Paper III. Intermittent fasting (i.e. caloric restriction for 16-48 hours) (294) in animal studies have demonstrated reduction of tumor size (295). As tumor heterogeneity increases with size, the development of more therapy-resistant cell clones with greater metastatic potential may explain the poor survival in patients with ER+/T2 tumors who received carbohydrates (Paper III). Therefore, ketogenic diet/intermittent fasting may counteract tumor growth and also shed of CTCs out from the tumor. In addition, intermittent fasting improves insulin sensitivity and thus reduces insulin and IGF-1 related signaling in over weighted

individuals (295, 296). Thus, the ketones derived from intermittent fasting decreases cancer cell viability by attacking several hallmarks of cancer (297).

An important question is whether the 4 to 6-week time period between diagnosis and the introduction of systemic treatment is enough time for the primary tumor to seed CTCs to establish micro metastases. The window for the effect of carbohydrates and fasting is even shorter, only 2 days. One explanation for how carbohydrate loading creates differences in survival is that the carbohydrate load stimulates cancer cells to excrete exosomes with miRNA that regulate both systemic metabolism (Paper III) and the metabolism in micro metastases (i.e., glutathione formation) that are already formed before the operation. The effect of the microRNA is longer than 1-2 days (246). Thus, micro RNAs may be the missing link between our metabolic and clinical observations.

5.0 Validity and Methodological Considerations

The validity of a study can be divided into internal and external validity, which are like two sides of a coin; they reflect whether the results of a study can be relied upon and are meaningful. Internal validity relates to how well a study is constructed and conducted and depends largely on the methodological procedures of a study and how rigorously it is performed (298). Internal validity is the extent to which a study establishes a trustworthy cause-and-effect relationship between an exposure variable and an outcome variable. Thus, internal validity focuses on accuracy and strong research methods, and the extent to which it is possible to eliminate alternative explanations for a finding. Internal validity focuses on showing a difference that is due to the independent variable alone, whereas external validity indicate that the results can be translated to the world at large (298). Notably, better internal validity often comes at the expense of external validity and vice versa. The type of study we choose reflects the priorities of the research (299).

5.1 Factors Contributing to Internal Validity

5.1.1 Research designs

In general, data from prospective studies may give an answer as to whether there is an association between risk factors and disease outcomes (300). In Paper I, we use the term 'historical prospective', or retrospective, because the exposure (i.e., independent) variables applied in the present study were not available when the study protocol was written (300). Instead, we introduced the new exposure variable (Adjuvant! and NBCG-2010) after the follow-up had ended and the data set was anonymized. The advantage of this design is to 're-use' the original MMMCP data set (Paper I). Thus, we 'bypass' the 12-15 years of follow-up and get answers to our research questions straight away. In addition, treatment-naïve cohorts are very difficult to obtain. Therefore, this design is appropriate to the research questions in the study.

In Paper II, we performed a randomized controlled trial. The participants were randomly allocated to each treatment group and then compared with respect to a measured response (i.e., MAI, well-being, and clinical outcome) (301). A well blinded RCT is regarded as the gold standard for clinical trials (302). Our trial was blinded for the pathology lab, meaning that the patient's ID was not known to those who performed the various analyses on the patient material. However, it was impossible to blind the trial for the patients as the pre-operative carbohydrate is very sweet and water is not. This design reduces selection bias, confounders, and allocation bias and balances both known and unknown prognostic factors between the intervention group and control group (303). Admittedly, adding a pre-operative needle biopsy to the design would have made it possible to let the patients be their own control and we could use paired statistics. In such a case, more confounders would have been sorted out. However, proper determination of the MAI, Ki-67, and PPH3 requires a whole section through the tumor. Thus, this design solves one problem but creates others.

The exploratory study in Paper III sought to explore the research questions and does not intend to offer final solutions to the problem. This type of research is usually conducted to determine the nature of the problem more than provide conclusive evidence (304). Notably, exploratory research may be regarded as the initial research, forming the basis of future research. Thus, exploratory research tends to tackle new problems on which little or no previous research has been done (304). Despite being explorative, our lessons learned from paper II turned the focus towards the luminal breast cancer patients. Thus, we had a working hypothesis also for this study; the metabolomic changes were most likely to be found in this subgroup.

5.1.2 Sample size and statistical power

In Paper I, we included 516 of the 3500 patients from the MMMCP study. This created subgroups with 48, 74, 86, 308, 160, 356, 122, 394, 100, and 416 patients, combined with a 10-year follow-up time that created a minimum of 10 events in the subgroup with 48 patients and many more in the other subgroups. Ten events per exposure variable is an optimal number in the survival analyses (305). Thus, the number of patients in Paper I seems to be sufficient.

In Paper II, we had a slightly different situation. Here, a power calculation was performed based on the primary endpoint. We anticipated a 20% increase in MAI in the intervention group compared to the control group. Based on the mean MAI value in patients belonging to the catchment area of Stavanger University Hospital and the reproducibility of the method to assess MAI, a total of 30 patients in each study group (i.e. 60 patients) was necessary to achieve 80% power. We decided to randomize 80 patients to allow for a 10-15% drop-out rate. Statistical analyses were performed using SPSS statistical software v.22 (SPSS, Inc., Chicago, II, USA). T-tests, Fisher's exact test, or chi-squared tests were used, as appropriate, to test for differences between the intervention groups. In the survival analyses, we did not fulfill the criteria with 10 events per exposure variable in the Cox regression (306). In a Cox regression analysis, we should include all known variables deemed clinically relevant, and new variables with a p-value < 0.15 are reasonable to include (307). Especially, when the number of endpoints is less than 10 per exposure variable one should be careful to include too many variables in Cox analyses to avoid both type I and type II errors.

5.1.3 Representativeness

Representativeness regarding internal validity refers to the extent to which the differences identified between the two randomized arms are a result of the intervention being tested (e.g. Carbohydrate load in Paper II). Thus, this type of representativeness seeks to make

the trial results valid for the original study population, and depends on good design, conduct and analysis of the trial with minimal bias.

In Paper I, out of 3500 patients, we included 516 patients with lymph node negative breast cancer aged < 55 years, which is 15% of all cancers. This is about the same proportion as we have in Norway (91). Importantly, these patients are not randomly selected, rather selected on a biological hypothesis. Thus, the relatively high number of endpoints (approximately 30%) allows for subgroup analysis without increasing the risk of introducing bias.

In paper II, 80 patients were included for randomization, which is approx. 1/3 of a 'year-cohort' of breast cancer patients at Stavanger University Hospital. The most important issue for internal validity is that the fasting group and carbohydrate group were comparable to each other, thus reducing the risk for selection bias and confounders. Other factors that influence the external validity are discussed in chapter 5.2.

In paper III, the analysis in serum comprised all patients from paper II. Together with highly precise and accurate methods, the internal validity is also good for this part of the study. The analysis methods of the tumors are also precise, accurate and highly reproducible. The tumors are, however, skewed towards the larger tumor sizes due to the difficultness of sample tissue for metabolomic analyses when the tumors are less than 10-15 mm in diameter.

5.1.4 Random and systematic errors

A random error is due the variability in the measured data that arise purely by chance. A random error produce uncertainty whether the results obtained in one trial are real or arose by chance i.e. it is possible for the play of chance alone to have led to an inaccurate estimate of the treatment effect. Systematic errors result from flaws in either selection of study participants or gathering of information (i.e. data collection). Both these errors may hamper

the internal validity. To avoid both random and systematic errors, we need to have precise, accurate and reliable methods in the data collection phase (i.e. MAI assessment, immunohistochemistry, insulin measurements, quality of life assessment and metabolomics) (see 5.1.5).

5.1.5 Randomization

The main purpose of randomization is to eliminate selection bias / systematic errors and create a control group that is similar as possible to the treatment group . In Paper II, we applied an in-house randomization method and performed 1:1 block randomization of patients aged < 55 years and ≥ 55 years. We made two boxes, and in each of box was an equal number of folded paper notes concealing 'fasting' or 'carbohydrate'. The patients were examined completely before they draw a note and read the allocation group. Thus, the study was not blinded for the patients. However, it was blinded for the surgeon who operated on the patient and the various laboratories that received the various specimens for analysis. A better randomization method would have been to arrange an out-house method, e.g., the randomization schedule could be generated by a computer by an independent party at the Department of Clinical Science, University of Bergen, Norway. Here, the patients could be included with 1:1 randomization in blocks of 8 patients. From the randomization schedule, sequential numbers would be assigned and kept in sealed envelopes. When one of the surgeons wanted to include a patient at SUS, he/she would make a telephone call to the Department of Clinical Science in Bergen to receive the allocation group. Baseline data should be collected before randomization takes place.

5.1.6 Data collection

5.1.6.1 Immunohistochemistry

ER, PR, HER-2, Ki-67, and PPH3 are determined by IHC, giving the method a central place in this thesis. This method has both advantages and weaknesses. The key steps in the IHC process are highlighted and explained in Table 7 and Fig. 40.

Table 7. Key steps in the IHC process

Key Step/Issue	Explained	Comment	Refs
Fixation of tissue	Penetration distance (d) in mm: $d=k*\sqrt{t}$ k (liver, formalin) =0.78 5 mm tissue takes 3.7 h. 10 mm tissue takes 41.1 h. Formalin fixation is a gelating process and is slow.	Too short a fixation time leads to incomplete preservation of the central parts of the tissue and the proteins are fixed by the alcohols in the tissue dehydration process (denaturizing fixation). Antigenicity disappears and tissue morphology is poor.	
Antigen (epitope) retrieval	Optimal antigen retrieval dependent on detergent, temperature, and pressure. Antigen retrieval breaks the crosslinks, rehydrates the tissue for better penetration of the antibody, chelates Ca++ (EDTA), and restores the "original" conformation of the epitope	Computer surveillance of temperature and pressure to ensure the optimal retrieval important in IHC used for quantitative determination.	311 312
Antibody (Ab) specificity and affinity	The optimal dilution of the antibody must be titrated in the lab to ensure clear and crisp staining. Non-specific mAb binding will disappear with higher dilutions.	Monoclonal antibodies (mAbs) tend to be the most specific. Mouse mAb: high specificity, low to high affinity. Rabbit mAb: high specificity and high affinity. Polyclonal antibody: low specificity.	
pH	The antigenicity depends on the pH of the buffer used.	pH 9 will retrieve most antigens.	313
Detection system	2-layer polymer technique.	Polymers do not react with endogenous biotin. Polymers can be too large to penetrate tissue. Requires an optimal retrieval method.	
False-positive staining	Biotin (liver, kidney, breast, thyroid, intestine). Endogen peroxidase (EP)	Biotin and EP are removed/neutralized in the process to avoid false-positive staining.	
Scoring systems	Many different systems exist: Percentage positive, Allred Score, H-score.	Challenging issue. Cut-off values. Random selection in quantitation. Observer variation	

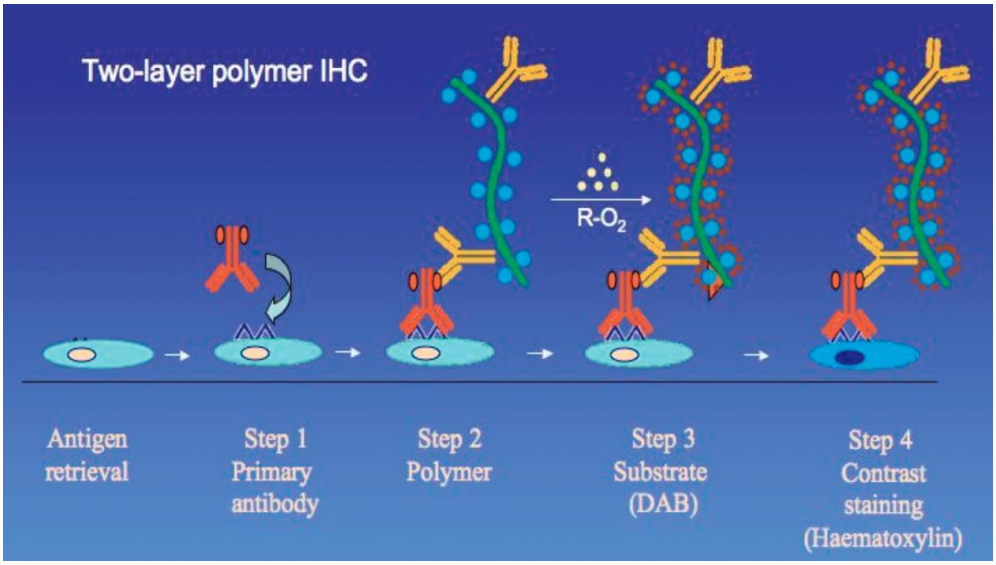


Figure 40. Overview of the main steps in the IHC method

The key steps in modern immunohistochemistry methodology. Step 0. The antigen is retrieved from the formalin fixed tissue sections by water heating. Step 1. The primary antibody directed against the target protein is applied. Step 2. A secondary antibody against the primary antibody has a coupling to a polymer with horse raddish peroxidase (HRP) units (blue circles) bound to it. Step 3. The substrate 'reduced colorless DAB'(Diaminobenzoate) will be oxidized around the HRP units into a crispy deep brown color. Step 4. The rest of the tissue is contrast stained by hematoxylin (light blue color). (Courtesy: Dr. Ivar Skaland)

Modern IHC uses sensitive and specific antibodies and makes crispy-clear sections which are quite easy to score. This translates into a quite good inter observer agreement e.g. HER-2 scoring has a kappa of 0.82 to 0.86 (308), which is comparable to the inter observer variability for ER (kappa = 0.84) (309). For PR, however, the concordance is lower with a kappa value between 0.56 and 0.71 (310).

5.1.6.2 Proliferation

Proliferation assessment is standardized (140) but dependent on correct fixation of the tissue to optimize counting. In contrast, Ki-67 and PPH3 are proteins that can be retrieved in the antigen retrieval step of IHC. Superheating (311, 312) and optimizing the pH (313) enhance antigen retrieval, improving the sensitivity of the IHC method. Consequently, the IHC method has become very sensitive and can detect small amounts of the protein of

interest. Therefore, in poorly fixed tissue, IHC may be a better method than tissue morphology (i.e., MAI). This could have led to a type II error for the MAI assessment with only borderline significance (P=0.05), whereas the IHC methods for PPH3 and Ki-67 were significant. In the MMMCP project in Paper I, the mean correlation of the reproducibility of MAI was very good with an r= 0.91 (0.81-0.96) (314). The reproducibility of the Ki-67 index is dependent on the range (class) of the positivity i.e. the intra-class interobserver correlation is perfect in sections with a Ki-67 index < 10% (kappa =1.00; CI95%=1.00 to 1.00), only modest in sections with a Ki-67 index 10-30% (kappa = 0.415; CI95%=0.300 to 0.541) and very good correlations in sections ≥ 30% (kappa=0.800; CI95%=0.730 to 0.861 (315).

5.1.6.3. Insulin characteristics

Insulin is measured in serum by well-established lab methods using built-in standards. The pre-analytical conditions were optimized by putting the blood samples in ice water (i.e., 0°C) to preserve the hormones. Furthermore, the samples were centrifuged at 4°C and frozen at –80°C before sending them to the Hormone Laboratory in Bergen on dry ice (-70°C) in batches for analysis. The method for insulin, c-peptide, IGF-1, and IGFBP3 is based on chemiluminescent immunoassay (CLIA) methodology. The intra-assay coefficient of variation (CV) for insulin was 1.8 – 2.4% and inter-assay CV 3-7.1% (316), which is good reproducibility.

5.1.6.4 QoL / PROM data

Our questionnaire was not validated. It was used as an in-house questionnaire developed by the Department of Anesthesiology to score patients after general anesthesia in day surgery. We found it appropriate to use, as it addressed the same items that we wanted to explore (Appendix 7).

However, by using a non-validated patient-reported outcome measure (PROM) instrument, there is an increased chance of type II errors (Paper II). Thus, using a validated

instrument, we could have a better chance of detecting changes in well-being between the carbohydrate group and the fasting group (Paper II). Regarding PROMs, there are two main types of validity: content validity and construct or convergent validity (317). Content validity deals with the ability of the PROM to measure what we intend to measure. Construct or convergent validity focuses on whether the PROM instrument provides scores based on existing knowledge about the construct.

5.1.6.5 Metabolomics

Pre-analytical conditions of both serum and tissue metabolomics were optimized by freezing both the serum and tissue as soon as possible after sampling. Metabolites were analyzed in serum and tissue after carefully thawing frozen samples to 4°C. HR MRS analysis of serum samples has a CV between 0.26 and 1.19% (318), whereas HR MAS MRS of tissue has a CV of 2.0% for alanine, 9.2% for choline, and 9.8% for lactate (319).

5.1.7 Survival analysis

The Kaplan-Meier approach to survival analyses was first published in 1958 (320). The advantage of this method is its ability to compare incomplete observations (321). The Cox model allows for multiple regression for time-dependent variables (322). In Paper II, survival analysis with the Kaplan-Meier method could have gained more events with a longer follow-up time. However, we had 10% events (n=8), which is what we can expect with the current treatment schedules and a follow-up of only 8 years in luminal breast cancer patients (Fig. 37). If we wanted survival as the primary endpoint, and the proportion of exposed and unexposed patients were equal (q1=q0=0.5), the number of events needed would have been 191, 65, 26, and 16 for an expected HR of 1.5, 2.0, 3.0, and 4.0, respectively. For example, if we aim for a HR of 2.0, we would need 65 events and 199 patients in each group, assuming a median survival time of 17 years in the unexposed group and a 15-year follow-up (323).

However, the Kaplan-Meier method overestimates the survival difference between two groups. In a recent meta-analysis, the Kaplan-Meier survival estimates were compared to the cumulative incidence function (CIF) approach, and the Kaplan-Meier method

overestimated survival with a HR of 1.41 (1.36 to 1.47) (324). Thus, the survival estimates in the Cox regression must be regarded as more reliable. In Paper III, S-lactate and S-pyruvate were significant as both continuous and categorical variables in the Cox regression. Therefore, these observations in Paper III strengthen the finding of a dichotomous carbohydrate/fasting variable in Paper II.

5.1.8 Bias

Bias results in non-random errors in the data and calculations (325). Bias is not evenly distributed between study subgroups, as it unintentionally favors one of the groups (326). Typically, bias may lead to type I errors, meaning that the H_0 is erroneously rejected. There are several types of bias (327) , and the main types relevant to this thesis are discussed below. Bias may produce differences that may be attributable to factors other than treatment being investigated and lead to an over- or underestimation of the true beneficial or harmful effect of an intervention.

5.1.8.1 Selection bias

In Paper II, the randomization groups were not evenly distributed between the 61 patients (26 in carbohydrate group and 35 in the fasting group). This may introduce selection bias. However, all other factors were evenly distributed, which reduces the chance of bias.

5.1.8.2 Recall bias

When patients were completing the questionnaires, they may have not been able to remember all the various symptoms well enough. In particular, it may have been difficult to remember changes in the various symptoms and scoring them if they filled them out at the end of the observation period. Typically, this bias would have affected our in-house questionnaire in Paper II. This bias may contribute to the type II error; we could not report many differences except pain. On the other hand, the time frame was only daily for 7 days. Thus, recall bias might not be a big issue in Paper II.

5.1.8.3 Confounding/spurious factors

In Paper I, the information on HER-2 status was not available; thus, HER-2 may act as a confounder and correlate with the outcome and another factor (i.e., ER negativity, which also correlates with the outcome). Moreover, in Paper II, we found a correlation between carbohydrate/fasting as the exposure variable and MAI-10 as the outcome variable. However, we could not demonstrate the same correlation using MAI as a continuous variable in a Mann Whitney U test or student t-test. Thus, there is a possibility that the correlation between carbohydrate/fasting and MAI is spurious, and a type I error may exist between MAI-10 and the exposure to carbohydrate and fasting. Even if all the various variables of interest were evenly distributed among the carbohydrate and fasting groups in Paper II, other factors may contribute to the difference between the exposure variables and outcome. Thus, is it impossible to have a situation that is 100% free of confounding factors. Furthermore, in Paper II there may be other confounders than we should correct for in the analytical phase. Here, multivariable regression, stratification, standardization, and propensity score are possible methods (328). In Papers I, II, III, we applied multivariable regression. In addition, we used stratification in Paper II.

5.1.8.4 Attrition

This challenge did not affect the papers equally. In Paper I, the number of participants included in the analysis was already decided at the beginning of the study In the RCT, we lost eight patients in the carbohydrate group at the beginning of study (Consort flow diagram, Paper II), as well as three patients during the 8 years of follow-up. In Paper III, the number of patients included in the study were those who had serum (n=61) and tumor tissue (n=29) available. Thus, attrition was not the case in this study.

5.1.9 Handling of data and statistical analyses

5.1.9.1 Missing values

In Paper II, the various variables were complete, so the multivariable analyses contained most of the patients. However, if we had replaced the missing variables, we could have increased the power in the multivariable analysis. A recognized method of correcting for

missing data is the multiple imputation approach (329). Here, a computer program replaces a missing value with the weighted mean values of the variable. Regarding missing data in Paper III, see 5.2.

5.1.9.2 Treatment of variables and assumptions for regression

We applied parametric methods for normally distributed variables and non-parametric tests when the distribution deviated from the normal. In Paper I, we used the well-recognized MAI-3, MAI-5, and MAI-10 thresholds in the analyses. In Papers II, III, we first used the continuous variable in the various correlation tests. In addition, we applied well-recognized thresholds (e.g., MAI=10, Ki-67=30). In the survival analyses, the continuous variables were analyzed directly in the Cox analysis. We then applied the ROC-detected thresholds to the Cox models. A rule of thumb was that we needed 10 events per exposure variable included in the Cox model (305, 306). This was achieved in Paper I, but not in Paper II and III. Thus, the validity of the Cox regression is lower in the latter two papers.

5.1.9.3 Biomarkers: C-statistics and the search for optimal thresholds

The C-statistic, or 'concordance statistic', is a measure of goodness of fit for binary outcomes. A value < 0.5 is a poor model, $= 0.5$ not better than random chance, > 0.7 indicates a good model, >0.8 is a strong model, and $= 1.0$ is a perfect model (330). One limitation of C-statistics is that it only measures discrimination and not calibration (331). Furthermore, several challenges have been recognized in the development of new and useful cancer biomarkers (332). Biological factors, including heterogeneity in the expression of a biomarker in various cells at a given time and during tumor growth, and development within a certain time period, are important background factors that may influence threshold values. Possible age variations, associations with other diseases, and understanding of the pathology or biology of the marker being evaluated are of great importance. Moreover, the standardization of determination methods, including thresholds or cut-off points, is important. The ROC method is a useful C-statistic tool for evaluating cut-off points for continuous variables, as it offers more than simple discrimination.

Moreover, the ROC method measures the balance between the sensitivity and specificity of each observed test result (333). Six stepping-stones to understanding the ROC analysis are presented in the table below:

Table 8. Components of the ROC statistics

	Topic	Interpretation	Comment
1	Sensitivity	The ability of a test to detect a person with the studied condition.	True positive rate
2	Specificity	The ability of a test to exclude the condition studied. Also denoted as (1- false positive rate).	True negative rate
3	Positive predictive value	The probability of having the condition if you have a positive test result. Depends on the prevalence of the condition.	PPV
4	Negative predictive value	The probability of not having the condition if you have a negative test result. Depends on the prevalence of the condition.	NPV
5	Likelihood ratio	An estimate of the relative predictive value of a test (true positive/false positives). The LR of a test indicates the increase from pre-test probability (prevalence) to post-test probability (having the condition).	A high LR (>10) indicates that a test can discriminate between "sick" and "healthy" persons. The LR very much depends on the chosen cut-off point but does not depend on the prevalence of the condition under study.
6	Accuracy	The proportion of all tests that give the correct result (true positive and true negative) divided by all results.	

In the ROC analysis, sensitivity and specificity are compared. Typically, the test variable is a continuous variable and the endpoint a dichotomous variable (healthy/sick, no cancer/cancer, etc.). The cut-off value for the continuous variable with the highest sensitivity and specificity regarding the endpoint studied is identified. To obtain this, a plot is constructed with the false-positive rate (1- specificity) on the X-axis and the true positive (sensitivity) on the Y-axis (Fig. 42) (334). There is a relationship between the ROC curve and the likelihood ratio (LR). The slope of the curve at any point is dY/dX=true positive/false positive= LR. The rationale for the optimal ROC curve is that it captures the trade-off between sensitivity and specificity over a continuous range. This trade-off is achieved at the upper left corner of the curve (333).

In addition, the area under the ROC curve (AUC; plot of false-positives, x-axis, against true positives, y-axis) is a measure of the diagnostic accuracy of the test independent of disease prevalence. An AUC > 0.5 indicates that the test has the ability to discriminate between positive and negative test results according to a chosen classification variable (335). The odds ratio (OR) can be directly derived from the AUC of the actual classifier: OR = AUC/(1-AUC). This last observation is the support for using the AUC as a singular indicator of a classifier's performance; it represents the classifier's ability to move the bias away from 0.5 and closer to 1.0 (334).

Use of ROC analysis is helpful for evaluating thresholds for biomarkers (i.e., dichotomous variables) that are not time dependent. For time-dependent endpoints, including “relapse from breast cancer” or “death from breast cancer” (Paper III), which are commonly evaluated by the Kaplan-Meier method (univariate) or a multivariate Cox regression analysis, a time-dependent ROC analysis (survival ROC) is also available (336). However, as reported in a recent study, this time-dependent ROC analysis did not provide any further information than traditional ROC analysis (337). Calculations made with Survival ROC software changed neither the cut-off values nor the AUC. Therefore, the traditional ROC analysis seems to be feasible in the evaluation of time-dependent variables, but confirmation and additional calculations with other methods is recommended (338). For time-dependent variables, the minimal p-value or maximal likelihood ratio in either the univariate setting (log-rank) or multivariate setting (Wald values) can be used to obtain optimal cut-offs (Paper III). Pitfalls exist, including variations in the underlying patient distribution under study (339). Therefore, internal and external validation is necessary to ensure that a reasonable cut-off value has been identified (340).

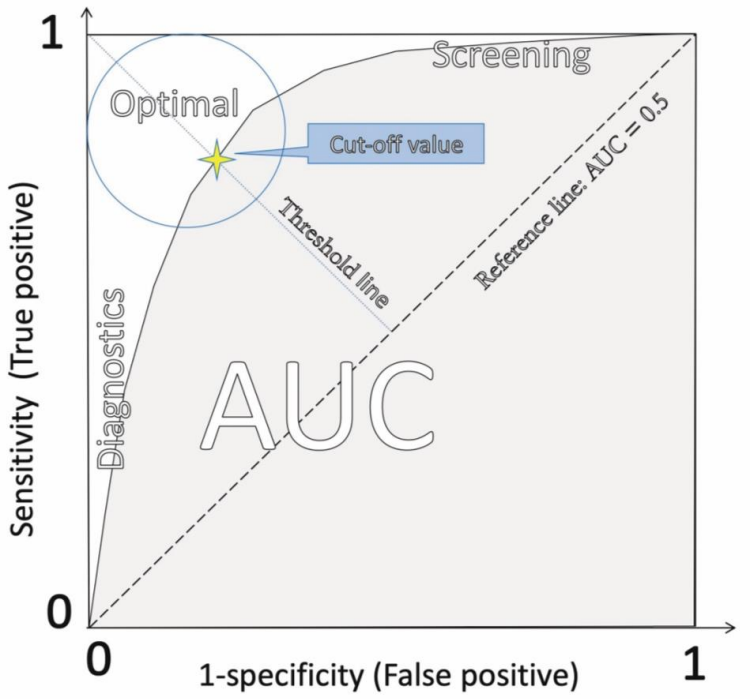


Figure 41. ROC curve

The ROC curve. Optimal threshold (star) is achieved by balance between a low false-positive rate and a high true positive rate = upper left corner of the curve (circle) When the number of observations and endpoints are sufficient the optimal threshold value will be locatd on or close to the threshold line. Situations with few patients and endpoints the ROC-curve will not be smoothed as in the figure, but more crude, and the optimal cut off value will often be located outside the threshold line. In diagnostics, a very low false-positive rate is desirable, whereas a very high true positive rate is of value in a screening setting. AUC = area under the curve = the accuracy of the cut-off value for discriminating between the two states of the dichotomous variable (healthy /sick). If AUC > 0.5(i.e., above the reference line), the accuracy is significant. Notably, the ROC curve also has a 95% confidence interval. The slope of the ROC curve is (True positive/False positive) = likelihood ratio (LR).

5.1.9.4 Correction for multiple significance testing

In Paper I, we did not apply correction for multiple testing, as there were only eight variables in the Cox-regression model (MAI, pT, grade, nuclear atypia, tubule formation, ER, Adjuvant!, and NBCG-2010). We regarded Paper II as small explorative studies with a higher risk of type II than type I error. In addition, the number of variables was less than 20 in the various analyses. Therefore, we did not apply p-value correction. However, in Paper III, we tested numerous metabolites. Therefore, we applied the Benjamini-Hochberg method for correction of p-values for multiple testing with a false discovery rate of 0.05.

5.2. External Validity

External validity relates to how applicable the findings are to the real world; from clinical trials to practice i.e. how generalizable the findings are. The outcomes can also apply to practical situations and be translated into another context. Ecological validity, an aspect of external validity, refers to whether a study's findings can be generalized to the real world (298). External validity refers to the degree to which within-study interferences generalize or can be generalized to a target population. Also, it refers to the extent to which study results can be applied to other individual or setting (i.e. generalizability). The following moments influence the external validity: 1. The setting of the trial; 2. Study population; 3. Types of intervention used; 4. Duration of follow up; and 5. Types of outcome (341).

5.2.1. Relations between external and internal validity

Rigorous research methods can ensure internal validity, which can be very high, but the external validity may be hampered by several factors and be as low as 34% (342) or lower (343). However, external validity may only be improved by a few methods. Pre-study considerations to standardize time of day, location, researcher characteristics, and how many measures are used can affect the external validity. Moreover, the inclusion and exclusion criteria used should ensure a clearly defined study population. Also, the study should be replicable (i.e., with different samples or in different settings you should get the same results). Furthermore, reprocessing and calibration are statistical methods for adjusting to problems related to external validity. For example, if a study has uneven groups for some characteristic (such as age), reweighting might be used (298).

In Paper I, out of 3500 patients, we included 516 patients with lymph node negative breast cancer aged < 55 years, which is 15% of all cancers. This is about the same proportion as we have in Norway (91). Thus, the selected patients into the study are probably representative for the Norwegian population. Therefore, we believe that the external validity is good in this study and can be applied into Norwegian context.

In Paper II, we randomized 80 patients during a year. The groups were evenly distributed regarding the various variables. The internal validity is therefore good. In the catchment area for Stavanger University Hospital approximately 250 patients are yearly diagnosed with breast cancer. Thus, we have included 1/3 of the patients possible to include. As the selection of patients were at random, we believe that also the patients are representative for the population they are recruited from. Thus, the external validity is most probably acceptable.

In Paper III, the serum analyses were available from nearly all the patients from study II and share thus the same external validity. However, the selection towards larger tumors introduces a selection bias in this part of the analyses. Due to technical challenges and the fact that the clinico-pathological examinations have priority, sampling of tissue for metabolomic analysis is not possible for tumors less than 10-15 mm depending on the growth pattern of the tumor. Thus, this part of the study may only be applicable for ER-positive T2 tumors.

5.2.2 Participation bias

Participation bias is a type of selection bias that hampers the representativeness and external validity because the participants disproportionately possess certain traits different from the target population, affecting the outcome in the subgroups differently (299). These traits mean the sample is systematically different from the target population, potentially resulting in biased estimates (344).

In Paper II, patients who declined to participate in the study may represent a different cohort with, for example, higher sugar content in the diet and lower level of physical activity than those who accepted invitations to the study. They might be afraid that the study would reveal their ‘unhealthy’ lifestyle. Thus, we may have selected towards healthier women.

Moreover, in Paper III, we miss 32 tumors in the explorative study, which must be regarded as a substantial number. Moreover, this high number of missing tumors introduced bias towards the larger tumors because the smallest tumors were impossible to harvest from the samples without jeopardizing the diagnostic process. Thus, the smallest tumors are not included, reducing the external validity of the study. As this study is purely explorative, this bias must be considered, but it does not ruin the study.

5.3 Ethical Considerations

Paper I is based on anonymized data and is therefore outside the legal requirement for a REK approval. The reason for this, is that information from studies on this data set can never be used back on the patients. The patients have already received all the treatments according to the guidelines at the time they were enrolled in the study. However, the connection between the single patients and the study variables is forever cut. Now, we find that 40% of the patients were under-treated. Patients that may read this publication may feel it unpleasant to receive this information 20 years after. Those who were undertreated according to our results are already dead. Thus, the results in Paper I are not applicable to those patients who are still alive after all these years. These patients are true breast cancer survivors. Thus, the results from paper I cannot benefit the patients in the study but is extremely important for the women with breast cancer today. From this perspective, it was both ethical and necessary to do the study.

In Papers II and III, we informed the REK that the knowledge on carbohydrates in breast cancer is conflicting. In addition, pre-operative carbohydrates are in regularly use according to the ERAS protocol (200). The results, however, are concerning, with an increased number of relapses and deaths in the carbohydrate group. These studies raise the question of whether the 4-6 week time period from diagnosis to commencement of systemic treatment is not as innocuous as we think. Therefore, it may be difficult to repeat this finding in a larger cohort. However, Paper II and Paper III may become the background for unique

intervention studies targeting the insulin signaling pathways (see Chapter 6.0, Future Perspectives)

6.0 Future Perspectives

As MAI identified subgroups of patients that, according to the NBCG 2010 guidelines, would be over- (20%) or under-treated (40%) (Paper I), it is a promising tool for discriminating between high- and low-risk patients. However, since 2010, several gene expression tests have been introduced in a clinical setting, with analytical validation (345) and clinical verification (346) of the PAM-50 (Prosigna ™) test (Appendix 3).

The Prosigna ™ test classifies luminal patients into luminal A and luminal B using many of the same genes as in the original publication by Peroue & Sørlie in 2000 (24). Moreover, Prosigna ™ calculates a ROR score on a continuous scale, but may also classify the patients into low, intermediate, and high-risk categories for recurrence. This test may be regarded as the 'gold standard' for the detection of luminal breast cancer patients that are at high risk of recurrence and s should receive chemotherapy. One of the downsides of Prosigna™ is the high cost, which is approximately 20 000 NOK per test.

Recently, NBCG supported the launch of a nationwide project, the EMIT (Establishment of Molecular profiling for Individual clinical routine Treatment decision in Early Breast cancer) study (347) in which Prosigna™ is being tested in the clinical Norwegian setting as a classifier of luminal A and luminal B patients. Thus, the NBCG guidelines in 2020 will include Prosigna™ as an alternative for distinguishing luminal A and luminal B cancers (Appendix 4). However, for those who cannot afford the Prosigna test, a version of the NBCG-2020 guidelines with Ki-67 as the proliferation biomarker (Appendix 5) is still available. Notably, the St Gallen consensus conference of 2015 implied that not all luminal B patients should receive chemotherapy (97). Thus, Prosigna meets this implication as it can also calculate the ROR score for patients and group the patients into low, intermediate, and high risk (Fig. 29). The ROR is implemented in the NBCG 2020 guidelines (Appendix 3) (28). Thus, the EMIT study serves as an excellent opportunity to evaluate MAI as a proxy for PAM-50/Prosigna™ and verify the most relevant clinical cut-

off value. Calibration of MAI against a gold standard molecular expression platform like PAM-50 has not been done previously. As MAI is a functional readout of all cellular signal pathways that drive the cell cycle towards mitosis, and Prosignatm is based on a platform of gene expression related to the cell cycle, validation of MAI against Prosignatm will become very useful. As such within the EMIT study, measurements of MAI in substudy 3 is included. As both ROR score and MAI are continuous variables, there is an opportunity to calibrate MAI against ROR and establish two thresholds for MAI: low/intermediate risk and intermediate/high risk thresholds. This may lead to more robust thresholds of the calibrated MAI to predict whether a patient would benefit from adjuvant chemotherapy. If so, MAI will have its renaissance as a predictor in decision-making in the clinical setting. Notably, as the analytical costs for MAI are extremely low (~20 NOK per test – salary costs are not included), this is an affordable option. Therefore, in low-income countries, the calibrated MAI will serve as a reliable proxy for a gene expression test like Prosigna test. Moreover, introduction of digital pathology in Norway (348) will ease consensus in difficult cases. To achieve the highest reproducibility possible, a national center in Norway should determine the calibrated MAI in all new luminal breast cancers, approximately 2500 cases per year. This will minimize under- and over-treatment. Alternatively, learning and test sets can be made available online for those who want to do the analysis of MAI themselves.

The results from Paper II and Paper III imply that pre-operative high carbohydrate loading is associated with increased proliferation, reduced PR, increased lactate, pyruvate, which may translate into the reduced RFS and BCSS. As we observed the abovementioned changes in only the ER+/T2 patients, this calls for reflection. It is possible that these patients reacted the most to the carbohydrates given. Thus, ER+/T2 tumors may be more sensitive to the systemic effect of carbohydrates on the cellular signal systems we have detected in Paper II and III (i.e. insulin/insulin C-peptide/IGF-1/lactate/glutathione) (Fig. 37 and Fig. 39). These traits can be utilized by administering metformin (274). Metformin has a 'Kinder egg effect' (i.e. 3 additive effects) by acting through endocrine mechanisms (278), paracrine effects (58) and intracrine / intracellular actions (349). The latter includes reduction in

insulin and IGF-1 signaling (350), which can benefit the patients by neutralizing the endocrine resistance. Furthermore, the time from diagnosis until commencing the adjuvant systemic therapy (i.e. 4-6 weeks) may be more important than previously thought. There is a saying in breast cancer treatment: 'What you do first ─ matters the most'. On the background of the abovementioned biological effects of metformin (351) and the fact that the patients are left untreated for 6 weeks after diagnosis, we suggest to design a randomized controlled study addressing these challenges. In this study, patients with ER+/T2 tumors will be randomized just after the diagnosis into 3 study groups; 1. Metformin, 2. Metformin + endocrine therapy and 3. Placebo upfront preoperatively (= the setting of today) (Fig. 42). All patients need a pre-operative needle biopsy to decide whether they are ER+ or not. Thus, effect of metformin on PR expression in the post-operative tumor specimen can be evaluated in comparison to the preoperative biopsy. This would allow the patients to be their own controls. Also, a better panel of circulating biomarkers must also be included: CTCs, ctDNA, exosomes, and microRNA.

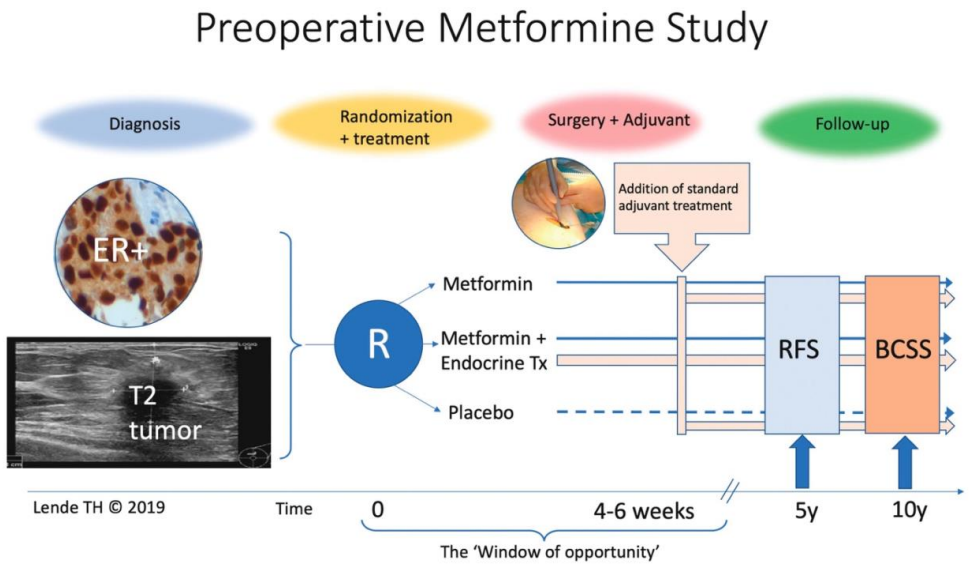


Figure 42. Overview of the Preoperative Metformin Study in ER+/T2

Suggested study: Overview of the Preoperative Metformin Study in ER+/T2 tumors are randomized ® at the point of diagnosis and given treatment in the 'window of opportunity' as shown. Then, they will be followed for 10 years. RFS, relapse free survival; BCSS, breast cancer specific survival; y, years

All patients will of cause receive additional standard adjuvant treatment according to the treatment guidelines. The patients will be followed for 10 years. Relapse free survival may be analyzed at 5 years of follow-up and Breast Cancer Specific Survival after 10 years. Of course, the patients should also be followed for 20 years to assess the effect of Metformin on long-term survival. Of good scientific reasons, such a study should be sufficiently powered and be a multicenter study.

To come to a close, of the more than 100 prognostic factors being discovered the last decades (87), only a handful are in daily use in the prognostication and prediction of the effect of treatment (Appendix 3 and Appendix 4). The main objective with the present thesis was to detect novel reliable prognostic and predictive biomarkers that can be used in breast

cancer. MAI-3 seems to be a promising predictive factor for adjuvant chemotherapy (Paper I). Systemic metabolic changes (i.e. insulin (Paper II) and lactate/pyruvate (Paper III) and local metabolic effects in tumor (i.e. glutathione: Paper III)) after preoperative carbohydrate are putative indicators for increased endocrine resistance and relapse in ER+/T2 tumors.

Therefore, a novel future project will be to examine the use of metabolic profiling for early detection of breast cancer recurrence (352). This is also the topic for the Stavanger Breast Cancer Research Group, which includes several sub-studies (353). Thus, in our research group, metabolic biomarkers will be elaborated on in the years to come. The above-mentioned strategies seem to be only a small step for biomarker research but will undoubtedly provide a giant leap for use of metabolic networks in the clinical setting.

7.0 Conclusions

7.1 Individual papers

7.1.1. Paper I

We rejected our null hypothesis as MAI-3 contributes with significant independent prognostic information to LN-neg breast cancer patients aged < 55 years. MAI-3 identified 40% of patients as under-treated and 20% of patients as over-treated compared to the prognostic grouping by Adjuvant! Online and the NBCG 2010 guidelines. Clearly, there is a need to include a proliferation biomarker in both guidelines to improve adjuvant systemic treatment in these patients. After this paper was published in 2010, Ki-67 was added to the NBCG guidelines. Luminal patients with a high Ki-67 level were offered chemotherapy.

7.1.2 Paper II

In this RCT, the four null hypotheses derived from our aims (2.2.2, page 80) were all discarded in the statistical analyses. Firstly, we observed an increase in luminal breast cancer patients with MAI≥10 among patients who received pre-operative oral carbohydrate load. In addition, the proportion of PR-negative patients increased in the carbohydrate group compared to the fasting group. The RFS and BCSS were inferior in the carbohydrate group. This paper shows that a high pre-operative oral carbohydrate load influences proliferation, PR expression, and survival. Thus, the 4-week timespan from diagnosis to commencement of systemic adjuvant treatment may be more important than currently known.

7.1.3 Paper III

This study utilized the patient material from Paper II. Thus, the null-hypotheses in this paper were generated on the basis of paper II (2.2.3, page 81). We found that oral

carbohydrate load increased the systemic lactate and pyruvate content, the tumor glutathione content, and glutamate in patients with high proliferation. Moreover, the recruited metabolic pathways are seen in the Warburg effect. Four out of seven microRNAs involved in endocrine resistance were recruited after carbohydrate influence. High levels of lactate, pyruvate, and glutathione were associated with decreased RFS, BCSS, and OS. An oral carbohydrate load seems to activate metabolic routes that favor the cancer cells and not the host.

7.2 Overall Conclusions

The following conclusions can be drawn from this thesis:

I. Proliferation (MAI) adds valuable prognostic and predictive information to lymph node-negative luminal patients. According to Adjuvant! v 8.0 and NBCG-2010, MAI-3 identified 40% of the patients as under-treated and 20% as over-treated.

II. Pre-operative oral carbohydrate loading seems to increase proliferation in luminal breast cancers and reduce the expression of progesterone receptor.

III. Explorative metabolic studies of pre-operative carbohydrate loading are probably linked to the Warburg effect with increased systemic levels of lactate and increased intratumoral protection factors (e.g., glutathione) in luminal cancers.

IV. All the above-mentioned changes contribute to inferior breast cancer survival in ER+ tumors.

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9.0 ERRATA

10.0 Appendix

APPENDIX

1-8

Appendix 1. Simplified TNM-Classification in Breast Cancer

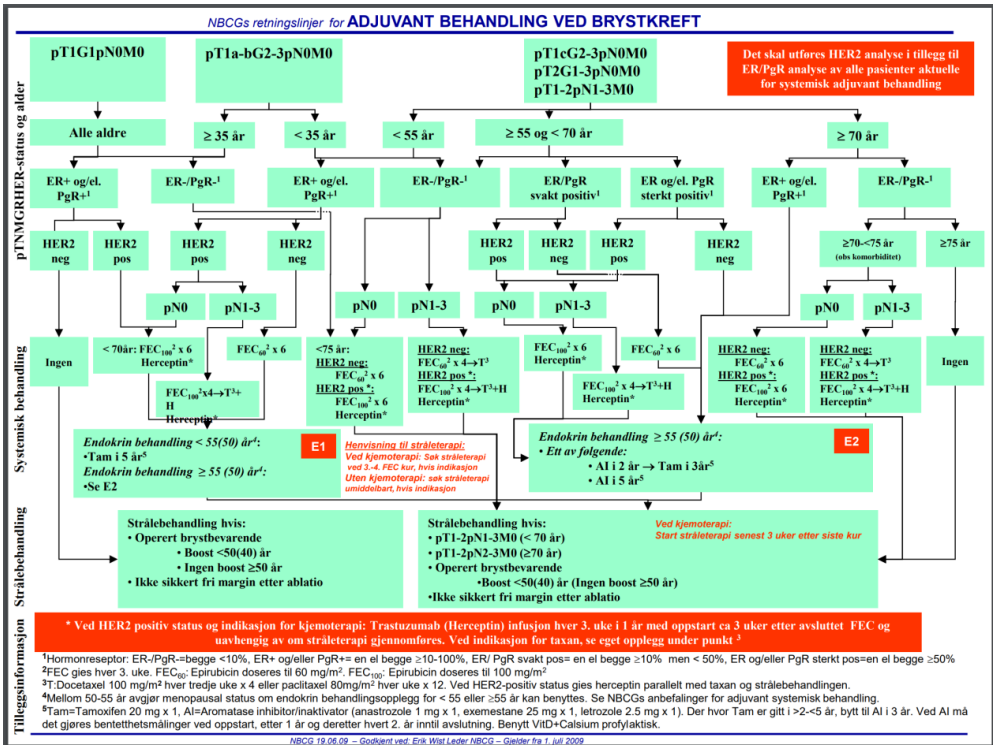
Reference: (92)

T/pT	Tumor size (clinical and pathological)	Nodes (clinical assessment)		pN (number nodes affected)	
T0	No tumor evident				
Tis	DCIS/LCIS				
T1/pT1	≤ 20 mm	N0	No mets. in regional LNs	pN0	0 positive LNs
T2/pT2	21 to 50 mm	N1	Mets. to ipsilateral movable axillary LNs	pN1	1-3 positive LNs
T3/pT3	> 50 mm	N2	Mets. to ipsilateral fixed axillary LNs	pN2	4-9 positive LNs
T4/pT4	Any T/pT involving skin/chest wall	N3	Mets. to ipsilateral movable axillary LNs + supraclav LNs	pN3	≥ 10 positive LNs
M0	No distant mets.	LN= lymph node, p= pathological, pos=positive, mets. = metastases.			
M1	+Distant mets.				

Appendix 2. Primary Treatment of Operable (early) Breast Cancer

Primary treatment	Main level	Category	Method	Objective/ Rationale	Duration	Ref (94)
Central	Local	Surgery 	BCT	Remove primary tumor	1 h	
			Mastectomy		2 h	
	Regional		Sentinel node biopsy	Staging of LN in axilla	½ h	Table1, pt. 23
			Axillary lymph node dissection (ALND)	Remove metastatic LNs	1 h	
Adjuvant	Local	Radiotherapy 	Whole breast	Destroy additional premalignant lesions and small tumors in the breast	3 weeks	
			Boost to tumor bed		8 days	
	Regional		Axilla, levels I, II, and III	Destroy regional LN metastasis	3weeks	
			Axillary + supra axillary fossa + intrathoracic LN		3 weeks	
	Systemic	Endocrine 	Tamoxifen	Block ER signaling in micro metastasis → apoptosis	5-10 y	(43)
			AIs	Block peripheral estradiol synthesis → apoptosis	5 y	(43)
		Chemotherapy 	Epirubicin	Cytotoxic antibiotics Anthracycline; binds to DNA, blocks topo-isomerase II. Induces apoptosis in micro metastatic cancer cells	4 courses 3 weekly (12 weeks)	
			Cyclo-phosphamide	Alkylating agent DNA cross-binding Induces apoptosis in micro metastatic cancer cells	4 courses 3 weekly (12 weeks)	Table1, pt. 11.
			Taxanes	Blocks microtubule function in micro metastatic cancer cells → apoptosis	4 courses 3 weekly (12 weeks)	(43)
		Biological 	Trastuzumab	Monoclonal antibody blocks the HER2HER-2 receptor in micro metastatic cancer cells → apoptosis	17 courses 3 weekly (1 year)	(354)
			Zoledronic acid	Block osteoclast activity in bone and block formation of metastatic niche ('soil') to avoid harboring of micro metastases in bones	1 injection every 6 months for 5 years	Table1, pt. 7

Appendix 3: NBCG guidelines June 2009 to February 2012



Reference: (93)

Appendix 4. NBCG recommendations for AST with Gene profiling test

NBCG Recommendations for Adjuvant Systemic Treatment (AST) Nov. 14, 2018				
+ WITH GENE PROFILING TEST (Prosigna =PAM-50)				
Main group	Prosigna™ test	Subgroup	Treatment	Ref
HR+ HER-2 —	Lum A ROR low (0-40)	pT1a-b	No AST	
		pT1c	Endocrine + zoledronic acid if postmenopausal	
		pT2	Endocrine + zoledronic acid if postmenopausal	
	Lum A ROR intermediate (41-60)	pT1a-b	No AST	
		pT1c	Endocrine + zoledronic acid if postmenopausal	
		pT2	Endocrine + zoledronic acid if postmenopausal	
	LumB ROR intermediate (41-60)	pT1a-b	ER≥50%: Endocrine ER<50%: EC90x4 + zoledronic acid if postmenopausal	
		pT1c	ER≥50%: Endocrine ER<50%: EC90x4 + zoledronic acid if postmenopausal	
		pT2	ER≥50%: EC90x4 → Endocrine ER<50%: EC90x4→Taxan→Endocrine + zoledronic acid if postmenopausal	
	ROR high (>60) (independent of luminal status)	pT1a-b	ER≥50%: Endocrine ER<50%: EC90x4 + zoledronic acid if postmenopausal	
		pT1c	EC90x4→Taxan→Endocrine + zoledronic acid if postmenopausal	
		pT2	EC90x4→Taxan→Endocrine + zoledronic acid if postmenopausal	

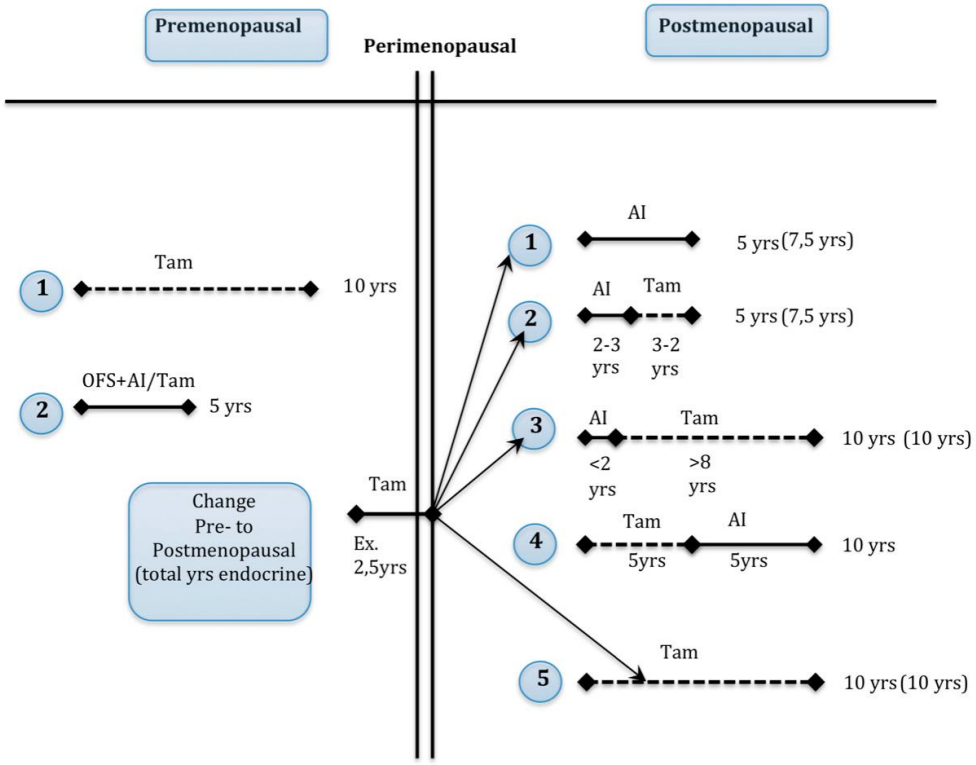
Reference: (91)

Appendix 5. NBCG recommendations for AST without gene test.

Recommendations for Adjuvant Systemic Treatment (AST) Nov. 14, 2018				
—— WITHOUT GENE PROFILING TEST				
Main group	Surrogate markers	Subgroup	Treatment	Ref
HR+ HER-2 —	LumA like: Low proliferation*, Grade 1-2, ER>50%	pT1a-b, pN0	No AST	
		pT1c, pN0, Grade1	No AST	
		pT1c, Grade 2, pN0 pT2, pN0 pT1-2, pN1	Endocrine + zoledronic acid if postmenopausal	
		pN2-3	EC90x4 → Endocrine + zoledronic acid if postmenopausal	
	LumB like: High proliferation ** AND Grade 2 -3 OR HR< 50%	pT1a-b, pN0	Endocrine + zoledronic acid if postmenopausal	
		pT1c-pT2, pN0 pT1-2, pN1-3	EC90x4 → Endocrine Zoledronic acid if postmenopausal	
HR+ HER-2+		pT1, pN0	Taxan/Trastuzumab →Trastuzumab+endocrine Zoledronic acid if postmenopausal	
		All others	EC90x4→ Taxan/Trastuzumab →Trastuzumab+endocrine Zoledronic acid if postmenopausal	
HR— HER-2+		pT1,pN0	Taxan/Trastuzumab →Trastuzumab Zoledronic acid if postmenopausal	
		All others		
HR — HER-2 —			EC90→ Taxan Zoledronic acid if postmenopausal	
	*Ki-67 low: lab median value - 10%	When Ki-67 has an intermediate value: Use the other prognostic and predictive factors to decide e.g., Grade 3 = high proliferation (LumB) Grade 1 = low proliferation (LumA)		
	**Ki-67 high: lab median value + 10%			

Reference: (93)

Appendix 6. Endocrine Treatment of Breast Cancer Patients



Norwegian Breast Cancer Group (NBCG) 2015 [93] and based on international recommendations (St. Gallen, 2017). Premenopausal patients have two options (1 and 2 on the left side) and postmenopausal patients five options (1–5 on the right side) comprising aromatase inhibitor (AI), tamoxifen (TAM), and ovarian function suppression (OFS) alone or in combination. Total duration of endocrine treatment for a premenopausal patient that becomes postmenopausal after 2 or 5 years on TAM (example) is illustrated in brackets. The choice between alternatives 1–5 is made individually based on tumor biology, side effects, and preferences among clinicians and patients. Peri: perimenopausal; Yrs.: years; Dotted line: years on tamoxifen; Solid line: years on AI.

Reference: (246)

Appendix 7. PROM of Well-being

TILBAKEMELDING OM PLAGER ETTER OPERASJON VED FASTING VERSUS SUKKER-DRIKK

Pas initialer :

Studienr:

Takk for at du med dette er med og gir oss tilbakemelding!

Det er viktig at dette skjemaet blir nøyaktig utfylt, så noter deg tall hver dag og ikke vent med oppsummering etter noen dager.

Kryss av: Faste ☐ Sukkerdrikk ☐

Tallet du velger å sette beskriver din opplevelse av situasjonen hver dag:

1 ingen (smerte, kvalme, blødning….etc)

2 lett

3 moderat

4 veldig sterk

	1.dag	2.dag	3.dag	4.dag	5.dag	6.dag	7.dag
Smerte							
Kvalme							
Svimmelhet							
Blødning							
Usikkerhet							

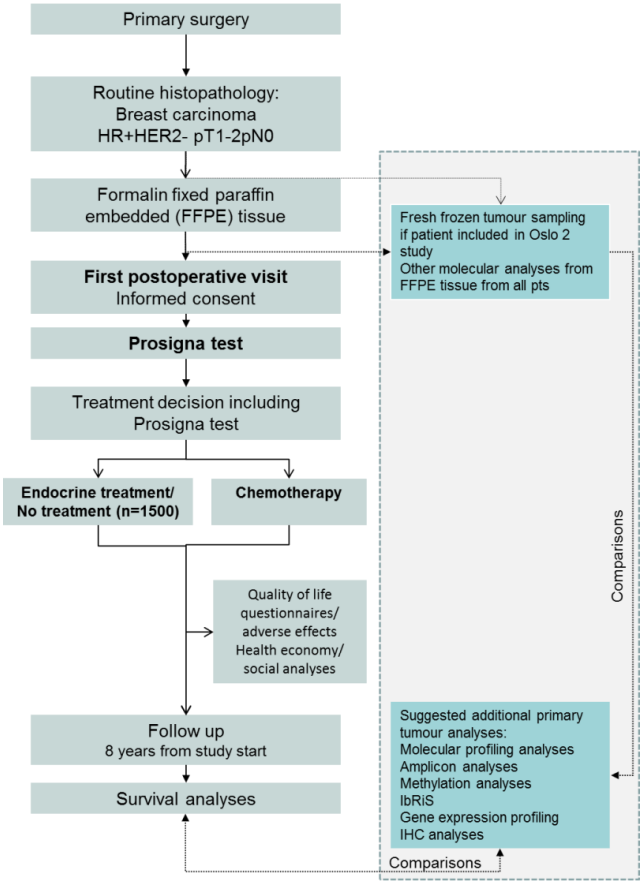
På de neste spørsmål sett kryss i ruta ved det som stemmer for hver dag:

	1.dag	2.dag	3.dag	4.dag	5.dag	6.dag	7.dag
Sengeliggende							
Sitter oppe							
Normalt funksjonsnivå							

Kommentar:

(For legen: type anestesi gitt, pre-/postop medik., operasjonsvarighet, lett bilsyk)

Appendix 8. EMIT Protocol

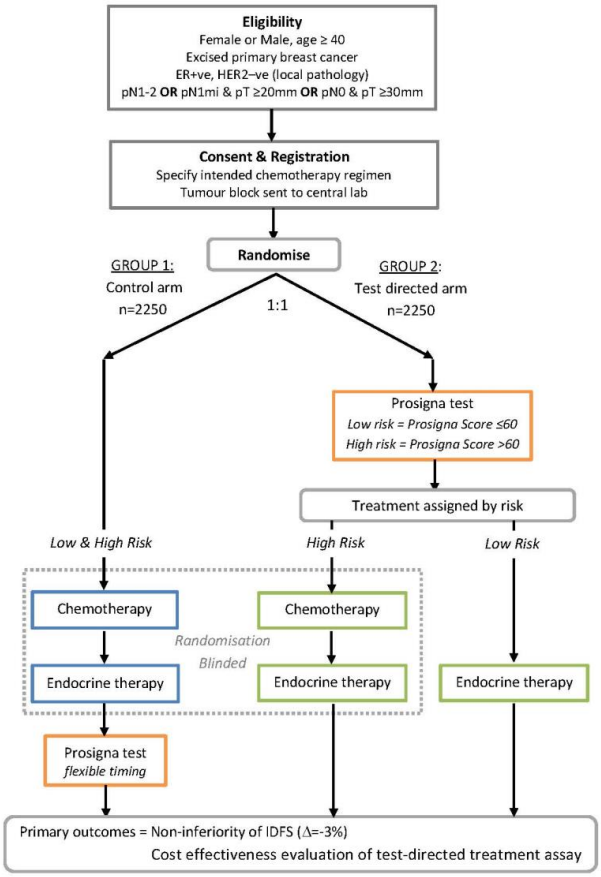


Patients with node-negative early breast cancer who have completed surgery and are classified as ER and/or PgR positive (HR-positive) with ≥1% receptor expression are candidates for this study. Patients can be included after written informed consent is obtained and eligibility has been established and approved. An overview of the study is illustrated in Figure 3. It will be organized as a multi-center study and run as a one-armed trial. Patients with appropriate primary tumor characteristics will be informed at the first postoperative visit.
Treatment recommendations will be based on the Prosigna test results, in addition to conventional clinicopathological parameters. The Prosigna test will be performed after study inclusion.

The study will recruit a total of 2150 patients, including approximately 1500 who will not be recommended for chemotherapy. After inclusion, the patients will be followed for breast cancer-related events for at least 5 years.

References: (28, 347)

2. Trial Schema



References: (28, 347)

11.0 Paper I-III

Paper I

Paper II

Paper III

11.0 List of publications

Paper I

Lende TH, Janssen EAM, Gudlaugsson E, Voorhorst F, Smaaland R, Van Diest P, Søiland H, Baak JPA. (2010) In Patients Younger Than Age 55 Years With Lymph Node–Negative Breast Cancer, Proliferation by Mitotic Activity Index Is Prognostically Superior to Adjuvant! Journal of Clinical Oncology 2010: 29(7), 852-8.

Paper II

Lende TH, Austdal M, Varhaugvik A, Skaland I, Gudlaugsson E, Kvaløy JT, Akslen LA, Søiland H*, Janssen EAM*, Baak JPA*. (2019). Influence of pre-operative oral carbohydrate loading vs. standard fasting procedure on tumor proliferation and clinical outcome in breast cancer patients — a randomized trial. BMC Cancer 2019 Nov 8; 19:1076

Paper III

Lende TH§, Austdal M§, Bahten TF, Varhaugvik AE, Skaaland I, Gudlaugsson E, Egeland NG, Lunde S, Akslen LA, Jonsdottir KI, Janssen EAM*, Søiland H* and Baak JPA*. Metabolic consequences of perioperative oral carbohydrate in breast cancer patients — an explorative study. BMC Cancer 2019, Dec 7;19:1183

Paper II

Lende et al. BMC Cancer (2019) 19:1076
https://doi.org/10.1186/s12885-019-6275-z

BMC Cancer

RESEARCH ARTICLE Open Access

Influence of pre-operative oral carbohydrate loading vs. standard fasting on tumor proliferation and clinical outcome in breast cancer patients – a randomized trial

Tone Hoel Lende[1,2*], Marie Austdal[3,4], Anne Elin Varhaugvik[4,5], Ivar Skaland[4], Einar Gudlaugsson[4], Jan Terje Kvaløy[3,6], Lars A. Akslen[2,7], Håvard Søiland[1,8†], Emiel A. M. Janssen[4,6†] and Jan P. A. Baak[4,9,10†]

Abstract

Background: Conflicting results have been reported on the influence of carbohydrates in breast cancer.

Objective: To determine the influence of pre-operative per-oral carbohydrate load on proliferation in breast tumors.

Design: Randomized controlled trial.

Setting: University hospital with primary and secondary care functions in South-West Norway.

Patients: Sixty-one patients with operable breast cancer from a population-based cohort.

Intervention: Per-oral carbohydrate load (preOp™) 18 and 2–4 h before surgery ($n = 26$) or standard pre-operative fasting with free consumption of tap water ($n = 35$).

Measurements: The primary outcome was post-operative tumor proliferation measured by the mitotic activity index (MAI). The secondary outcomes were changes in the levels of serum insulin, insulin-c-peptide, glucose, IGF-1, and IGFBP3; patients' well-being, and clinical outcome over a median follow-up of 88 months (range 33–97 months).

(Continued on next page)

* Correspondence: leth@sus.no
Håvard Søiland, Emiel A.M. Janssen and Jan PA Baak are Equal senior contribution.
[1]Department of Breast & Endocrine Surgery, Stavanger University Hospital, Helse Stavanger HF, P.O. Box 8100, N-4068 Stavanger, Norway
[2]Centre for Cancer Biomarkers CCBIO, Department of Clinical Medicine, Faculty of Medicine and Dentistry, University of Bergen, Jonas Lies vei 87, N-5012 Bergen, Norway
Full list of author information is available at the end of the article

(Continued from previous page)

Results: In the estrogen receptor (ER) positive subgroup ($n = 50$), high proliferation (MAI ≥ 10) occurred more often in the carbohydrate group (CH) than in the fasting group ($p = 0.038$). The CH group was more frequently progesterone receptor (PR) negative ($p = 0.014$). The CH group had a significant increase in insulin (+ 24.31 mIE/L, 95% CI 15.34 mIE/L to 33.27 mIE/L) and insulin c-peptide (+ 1.39 nM, 95% CI 1.03 nM to 1.77 nM), but reduced IGFBP3 levels (− 0.26 nM; 95% CI − 0.46 nM to − 0.051 nM) compared to the fasting group. CH-intervention ER-positive patients had poorer relapse-free survival (73%) than the fasting group (100%; $p = 0.012$; HR = 9.3, 95% CI, 1.1 to 77.7). In the ER-positive patients, only tumor size ($p = 0.021$; HR = 6.07, 95% CI 1.31 to 28.03) and the CH/fasting subgrouping ($p = 0.040$; HR = 9.30, 95% CI 1.11 to 77.82) had independent prognostic value. The adverse clinical outcome of carbohydrate loading occurred only in T2 patients with relapse-free survival of 100% in the fasting group vs. 33% in the CH group ($p = 0.015$; HR = inf). The CH group reported less pain on days 5 and 6 than the control group ($p < 0.001$) but otherwise exhibited no factors related to well-being.

Limitation: Only applicable to T2 tumors in patients with ER-positive breast cancer.

Conclusions: Pre-operative carbohydrate load increases proliferation and PR-negativity in ER-positive patients and worsens clinical outcome in ER-positive T2 patients.

Trial registration: CliniTrials.gov; NCT03886389. Retrospectively registered March 22, 2019.

Keywords: Breast cancer, Carbohydrate load, Proliferation, Insulin, Insulin c-peptide, IGF-1, IGFBP3, Tumor size, Relapse-free survival, Breast cancer-specific survival

Background

Breast cancer is the most frequent malignancy among women [1], representing 12% of all new cancer cases and 25% of all cancers in women worldwide [2, 3]. In Norway, the incidence of breast cancer has doubled during the last 50 years. The lifetime risk for a Norwegian woman developing the disease is 10–12% [4]. A total of 570,000 women across the globe died of breast cancer in 2015, comprising 15% of cancer deaths among women [3]. Approximately 75% of all new breast cancers are luminal breast cancer subtypes, which express estrogen receptor (ER) and/or progesterone receptor (PR) [5]. The etiological factors of breast cancer comprise genetic, hormonal, environmental, and lifestyle-related elements [6]. Risk factors relating to the Western lifestyle, including lack of physical exercise, being overweight, certain hormonal and dietary factors, and diabetes mellitus type 2, have recently gained increased attention [2].

The effect of carbohydrate consumption on breast cancer incidence and outcome is probably mediated through three parallel routes. One route is through stimulation of the insulin/ insulin-like growth factor-1 (IGF-1) axis in epithelial breast cells, which comprises the insulin receptor (IR) [7] and IGF1 signaling pathways [8]. This results in crosstalk between cellular signaling systems and endocrine resistance in luminal breast cancers (i.e., ER-positive tumors) [9, 10]. Secondly, a substantial part of the insulin effect is mediated by paracrine signaling in the tumor micro-environment between adjacent adipocytes, fibroblasts, and the epithelial cancer cell. Signaling factors, such as ER, IR, IGF1-R, adiponectin, and leptin are involved [11]. Thirdly, alimentary glucose may affect cancer cells directly through the Warburg effect, which is an expedient switch that changes cellular energy metabolism from oxidative mitochondrial ATP production to cytoplasmic aerobic glycolysis [12]. This transition enables the proliferative cancer cells to produce both ATP for energy and ribose for DNA synthesis [13].

In human breast cancer patients, studies on the relationship between carbohydrate/glucose content in food and quantitative insulin characteristics are lacking. Insulin is a growth factor that increases proliferation and decreases apoptosis, and elevated levels of insulin are associated with different cancers, including breast cancer [14]. In breast cancer patients without diabetes, high insulin levels have been associated with a poor prognosis [15]. Insulin receptors have been detected on breast cancer cells [16], though there is conflicting evidence on whether insulin directly regulates cancer proliferation, and how fast such an effect will occur. Also, there is a research deficit on the influence of carbohydrates on clinical outcome or prognostic endpoint biomarkers such as proliferation. Generally, proliferation is measured by the mitotic activity index (MAI), phosphohistone-H3 (PPH3), and Ki-67 [17, 18]. The MAI and PPH3 estimate the number of cells in M phase (mitosis) and G_2M phase, respectively, whereas Ki-67 detects all cells outside the G_0phase. Notably, insulin influences cell cycle kinetics by more rapid transit through the G_1 phase in ER-positive cells [7].

A meta-analysis has shown that, in patients undergoing abdominal surgery, administration of two per-oral carbohydrate loads administered 12–18 h, and again 2–4 h, before elective surgery reduces postoperative insulin resistance and leads to enhanced recovery after surgery

(ERAS) [19]. During surgery, however, breast cancer cells are pushed into the circulation [20]. Moreover, due to the pre-operative oral carbohydrate load used in ERAS protocols, these cells may have a much better chance of survival and of forming viable metastatic foci [21, 22]. Pre-operative oral hyperglycemic loading may bring breast cancer cells into a favorable state to escape, divide, thrive, and survive during surgery, which may then lead to an inferior long-term prognosis for breast cancer patients [23]. Therefore, it is of great importance to gain more insight into the effects of pre-operative carbohydrate administration in breast cancer regarding insulin-related characteristics, proliferation, and clinical outcomes.

The cell cycle in breast cancer is fast enough to be influenced by the two pre-operative oral carbohydrate loads in ERAS protocols [24, 25]. We chose to use the MAI as our primary endpoint for proliferation. Our hypotheses were that an ERAS protocol comprising two oral carbohydrate loads will improve post-surgical recovery in breast cancer patients, the oral carbohydrate load will stimulate cellular signaling and increase proliferation as measured by the MAI, and pre-operative carbohydrate loading will lead to an adverse prognosis in breast cancer patients. A subgroup analysis of ER-positive patients was planned before the study was started.

Thus, the aim of this study was to investigate whether a pre-operative carbohydrate load according to a standard ERAS protocol influences tumor proliferation, post-surgical recovery, and/or clinical outcome.

Methods

This population-based cohort of operable breast cancer patients was randomized into an intervention group receiving pre-operative per-oral carbohydrate loading or a control group comprising the standard fasting pre-operative protocol with unlimited access to drinking water. The investigation was an open-labeled study for the patient and breast surgeon. However, all researchers at the Department of Pathology and hormone laboratory were blinded to the intervention.

Patients

A total of 253 patients were assessed for eligibility between May 12, 2009, and June 23, 2010, in the catchment area of Stavanger University Hospital in South-West Norway. The exclusion criteria were clinical or radiological T3–4 tumors at clinical examination, overt systemic metastases, ductal carcinoma in situ (DCIS), micro-invasive cancer < 2 mm, or comorbidity, including diabetes mellitus type I and II, Cushing syndrome, previously diagnosed cancer, or being unable to co-operate in the study (e.g., dementia, other serious psychiatric illnesses, language barriers, or unwillingness to sign the informed consent papers). A total of 80 patients with unequivocal operable breast cancers (Stage I and II) diagnosed by fine needle aspiration cytology (FNAC) agreed to participate in the study and were randomized (Fig. 1). The last follow-up date was June 28, 2017. A larger proportion of dropouts in the intervention group for various random reasons created an imbalance in the numbers of patients between allocation groups (Fig. 1).

Randomization and intervention

Randomization was performed after the patients provided written consent to participate in the study. The randomization procedure was organized as an in-house procedure with concealed envelopes generated and distributed in two boxes by the study nurse. The allocation sequence was performed by the trial administration committee. The sequence was balanced according to age by choosing between two boxes, one for age < 55 years (i.e., possible and certain premenopausal) and one for age ≥ 55 years (i.e., most probably postmenopausal), each with 1:1 block randomization regarding the carbohydrate (intervention) and fasting (control) groups in each box. The surgeon in the out-patient clinic enrolled consecutively operable breast cancer patients who agreed to participate in the trial.

Intervention

Patients who were randomized to pre-operative carbohydrates drank 400 ml pre-Op™ (Nutricia, Netherlands) containing 12% carbohydrates, 2% glucose, and 10% polysaccharides the evening before (i.e., 18 h before surgery) and in the morning on the day of the operation (i.e., 2–4 h before surgery). Each patient was asked before surgery if they had been able to finish the carbohydrate drink or if they were fasting according to the randomization. The control group followed the standard fasting procedure with free intake of tap water.

Blinding

The study was not blinded for the patients due to use of the carbohydrates and tap water by the participants. The information on the grouping was known only to THL, who was head of the clinical part of the trial, and this information was kept in a locked safe. Others involved in the study had no access to this information. Thus, the investigation was blinded for the laboratory personnel performing various assessments (MAI, PPH3, Ki67, histological grading, insulin, C-peptide etc.).

Primary treatment

The primary surgery was performed according to the recommendations of the Norwegian Breast Cancer Group (NBCG) [4]. The surgery was either breast conserving treatment (BCT) or mastectomy, and sentinel

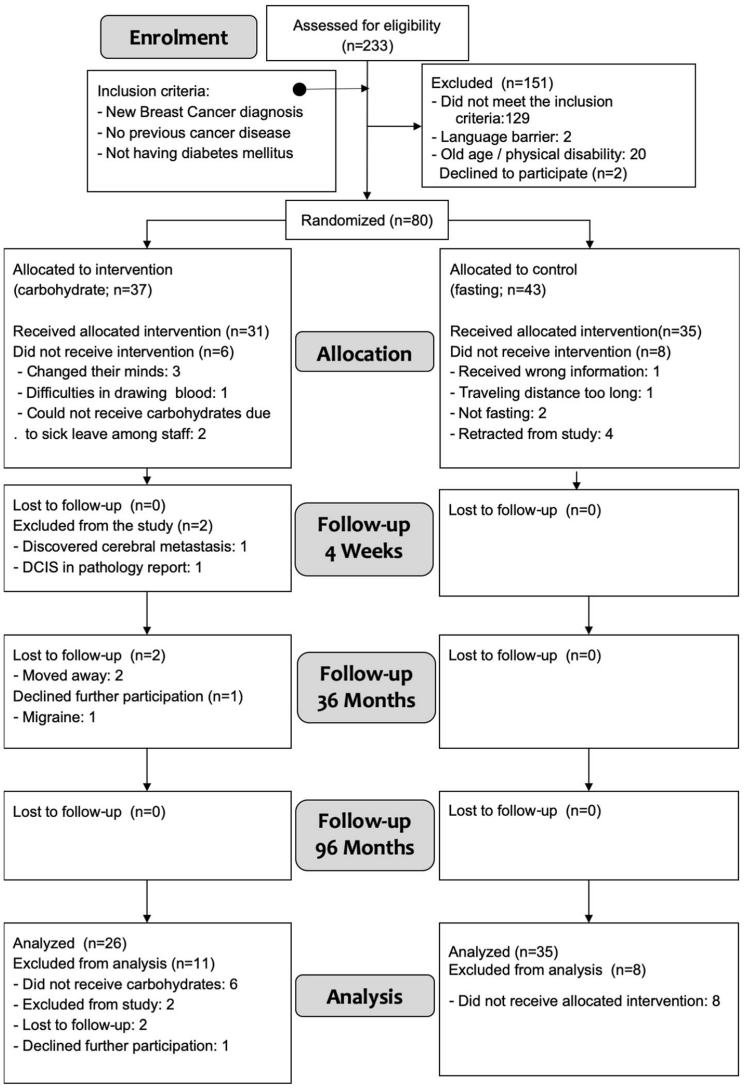


Fig. 1 Study flow diagram

node (SN) diagnostic or axillary lymph node clearance of level I and II. Adjuvant chemotherapy was also given based on the NBCG guidelines [4]. Notably, we found no differences between the two allocation groups regarding the type of primary treatment received (Table 1).

Safety issues

The patients were hospitalized for 1–2 days after surgery. Any complications, such as hemorrhage, infection, or others, were recorded on the Case Report Forms. No patients died or experienced any serious complications from the pre-operative treatment.

Blood sampling for serum analysis

Five blood samples were obtained from the participants: 1) at the time of diagnosis, 2) at admission (the day before surgery), 3) pre-operatively before surgery, after the second pre-Op™ carbohydrate dose, 4) the day after

Table 1 Baseline characteristics of patients in the two study groups

Variable	Carbohydrate group ($n = 26$)	Missing data (Intervention group)	Fasting group ($n = 35$)	Missing data (Control group)	*P*
	n (%)		*n* (%)		
Age					
< 55	12 (46)	0	16 (46)	0	0.973
> 55	14 (54)	0	19 (54)	0	
BMI (kg/m^2)	25.0 (3.9)	4	25.1 (3.0)	3	0.868
BMI < 25[a]	14 (64)	4	17 (53)	3	0.443
BMI ≥ 25	8 (36)		15 (47)		
BMI < 75 percentile[b]	18 (82)	4	23 (76)	3	
BMI ≥ 75 percentile	4 (18)		13 (24)		0.401
Menopausal status					
Premenopausal	4 (17)	1	7 (22)	1	0.627
Postmenopausal	20 (83)	1	25 (78)	2	
HRT - yes	8 (35)	3	10 (32)	4	0.937
HRT – no	14 (61)		19 (61)		
HRT- not relevant	1 (4)		2 (7)		
HRT use (years)	4.7 (4.3)	16	7.9 (5.8)	25	0.176
Tumor size (mm)	19.4	0	15.0	0	0.094
Tumor category					
T1	16 (62)	0	29 (83)	0	
T2	10 (38)	0	6 (17)	0	0.061
Histological Grade[c]					0.157
1	4 (15)	0	7 (20)	0	
2	10 (39)	0	20 (57)	0	
3	12 (46)	0	8 (23)	0	
pN negative	18 (69)	0	25 (71)	0	0.852
pN positive	8 (31)	0	10 (29)	0	
Number LNs removed	5.5	2	5.8	0	0.843
Number positive LNs	0.38	2	0.86	0	0.191
Estrogen receptor					
Positive (≥1%)	21 (81)	0	29 (83)	0	0.834
Negative (< 1%)	5 (19)	0	6 (17)	0	
Progesterone receptor					
Positive (≥ 10%)	13 (50)	0	28 (80)	0	0.014
Negative (< 10%)	13 (50)	0	7 (20)	0	
HER2					
Positive	3 (12)	0	1 (3)	0	0.176
Negative	23 (88)	0	34 (97)	0	
MAI (median, IQR)	7 (2–9)	1	5 (2–9)	0	0.647
MAI < 10	14 (56)	1	27 (77)	0	
MAI ≥ 10	11 (44)		8 (23)	0	0.083
Ki67 (mean, SD)	30.4 (28.2)	0	28.0 (26.5)	1	0.747
Ki67 < 15%	9 (35)	0	17 (50)	1	
Ki67 ≥ 15%	17 (65)	0	17 (50)	0	0.233

Table 1 Baseline characteristics of patients in the two study groups *(Continued)*

Variable	Carbohydrate group ($n = 26$)	Missing data (Intervention group)	Fasting group ($n = 35$)	Missing data (Control group)	*P*
	n (%)		*n* (%)		
Ki67 < 30%	14 (54)	0	24 (71)	1	0.182
Ki67 ≥ 30%	12 (46)	0	10 (29)	0	
PPH3 (mean, SD)	20.2 (24.7)	0	20.5 (26.9)	0	0.966
PPH3 < 13	14 (54)	0	21 (60)	0	0.631
PPH3 ≥ 13	12 (46)	0	14 (40)	0	
TILs (mean %, SD)	4.7 (10.7)	0	4.3 (7.3)	1	0.137
TILs					
Positive (> 10%)	2 (8)	0	4 (11)	0	0.663
Negative (< 10%)	24 (92)	0	31 (89)	0	
Luminal type[d]					
Luminal A	16 (62)	0	23 (66)	0	
Luminal B	10 (38)	0	12 (34)	0	0.737
Glucose					
Admission[e]|	5.4 (1.1)	0	5.3 (0.6)	0	0.864
Pre-operative[f]	5.2 (1.8)	0	5.1 (0.6)	0	0.739
S-Insulin					
Admission[e]	9.4 (8.5)	0	9.1 (6.6)	0	0.886
Pre-operative[f]	33.7 (20.2)	0	9.1 (5.9)	0	< 0.0001
S-insulin-c-peptide					
Admission[e]	0.69 (0.32)	0	0.75 (0.32)	0	0.517
Pre-operative[f]	2.10 (1.05)	0	0.75 (0.27)	0	< 0.0001
Surgery					
BCT	15 (58)	0	23 (66)	0	
Mastectomy	11 (42)	0	12 (34)	0	0.523
Axillary staging					
SN	21 (81)	0	28 (80)	0	
ALND	5 (19)	0	7 (20)	0	0.940
Reoperation - 1					
-Breast	1 (20)	0	1 (50)	0	
-Axilla	4 (80)	0	1 (50)	0	0.427
Chemo therapy					
Yes	12 (46)	0	17 (47)	0	
No	14 (53)	0	18 (51)	0	0.852
Radiation therapy					
Yes	17 (68)	0	26 (74)	0	
No	8 (32)	1	9 (26)	0	0.594
Endocrine therapy					
Yes	17 (65)	0	22 (63)	0	
No	9 (35)	0	13 (37)	0	0.839
Smoking status		5		4	
-Never smoked	5 (24)		10 (32)		0.650
-Former smoker	9 (43)		14 (45)		

Table 1 Baseline characteristics of patients in the two study groups *(Continued)*

Variable	Carbohydrate group ($n = 26$)	Missing data (Intervention group)	Fasting group ($n = 35$)	Missing data (Control group)	*P*
	n (%)		*n* (%)		
-Ongoing smoking	7 (33)		7 (23)		

Tumor size category analyzed as T1 vs. T2
[a]BMI-25 represents a dichotomized BMI < 25 or ≥ 25 on the BMI scale
[b]BMI-75p represents a dichotomized BMI with cut off < /≥ 75 percentile, i.e., </≥ 26.8 on the BMI scale
[c]Histological grading was performed according to the Nottingham algorithm
[d]Luminal A = ER+/HER2−/Ki67 < 15% and Luminal B = ER+/HER2−/Ki67 ≥ 15%
[e]Blood samples taken in the fasting state at the time patients were admitted in the hospital approx. 24–30 h before surgery
[f]Pre-operative blood samples taken 1–2 h before the surgical procedure commenced
BMI Body mass index, *HRT* Hormonal replacement therapy, *pT* Pathological tumor size in mm or category, *pN* Pathological lymph node status, *LN* Lymph node, *HER-2* Human epidermal growth factor receptor 2, *MAI* Mitotic activity index, *TILs* Tumor infiltrating leucocytes, *PPH3* Phosphorylated phospho-histone 3, *SN* Sentinel node, *ALND* Axillary lymph node dissection

surgery, and 5) 4 weeks post-surgery. Immediately after being drawn, the blood samples were put in ice water for transport to the in-house medical laboratory. The samples were spun and the serum frozen for transport to the Hormone Laboratory, Haukeland University Hospital, Bergen, Norway, where insulin, insulin c-peptide, IGF-1, and IGFBP-3 were measured by the IMMULITE 2000 two-site chemiluminescent immunometric assay (Siemens Medical Solutions Diagnostics).

Histology

Tumor size was measured macroscopically in fresh specimens following excision. The tissues were cut into 0.5-cm slices. The axillary lymph nodes from sentinel node biopsy, or axillary fat from axillary dissection were examined macroscopically by a pathologist. All detectable lymph nodes (median 3 per patients, range 1–21) were prepared for histological examination. No lymph nodes were detected in two patients. For hematoxylin–eosin–saffron (HES) staining, the tissues were fixed in buffered 4% formaldehyde, embedded in paraffin, and sectioned (4 μm). The histological type and grade were assessed according to World Health Organization criteria (by two pathologists, EG and JPAB) [26].

Immunohistochemistry

Immunohistochemistry (IHC) was performed to identify ER, PR, PPH3, Ki-67, and human epidermal growth factor receptor 2 (HER2) in whole sections. The antigen retrieval and IHC techniques were based on DAKO technology [27]. Formalin-fixed paraffin-embedded (FFPE) sections (4-μm thick) were serially sectioned after the preparation of HES sections and mounted onto siliconized slides (#S3002, DAKO, Glostrup, Denmark). A highly stabilized retrieval system (ImmunoPrep; Instrumec, Oslo, Norway) was used for antigen retrieval with the retrieval buffer (10 mM Tris/1 mM EDTA, pH 9.0). Sections were heated for 3 min at 110 °C, and then 10 min at 95 °C, before cooling to 20 °C. The following antibodies and dilutions were used: ER (clone SP1, Neomarkers/LabVision, Fremont, CA, USA), 1:400; PR (clone SP2, Neomarkers/LabVision), 1:1000; rabbit polyclonal anti-PPH3 (ser 10) (Upstate #06–570; Lake Placid, NY), 1:1500; and Ki-67 (clone MIB-1, DAKO, Glostrup, Denmark), 1:100. All antibodies were incubated for 30 min at 22 °C. Visualization was achieved using the EnVision™ FLEX detection system (DAKO, K8000). Sections were incubated with the peroxidase-blocking reagent (SM801) for 5 min, followed by the primary antibody for 30 min, EnVision™ FLEX/HRP Detection Reagent (SM802) for 20 min, EnVision™ FLEX DAB+ Chromogen (DM827)/EnVision™ FLEX Substrate Buffer (SM803) mix for 10 min, and EnVision™ FLEX Hematoxylin (K8008) for 5 min. Next, the slides were dehydrated, mounted, and stained using a Dako Autostainer Link 48 instrument and EnVision™ FLEX Wash Buffer (DM831). To assess HER2, the DAKO HercepTest™ was used according to the manufacturer's protocol.

Quantitative measures

MAI was assessed as the total number of mitotic figures in 10 consecutive fields of vision at 400× magnification (objective 40, specimen level field diameter 450 μm) in the most poorly differentiated periphery of the tumor, representing a total area of 1.59 mm^2. Areas with necrosis or inflammation were avoided. This was performed as a routine diagnostic procedure, but controlled by EJ as described elsewhere [28]. We assessed the PPH3 index as described previously [29] and evaluated PPH3 expression using the fully automated VIS analysis system (Visiopharm, Hørsholm, Denmark) and previously described image processing principles [27]. The semi-automatic interactive computerized QPRODIT system (Leica, Cambridge) was used to measure the percentage of Ki-67-positive cells as described elsewhere [30]. A total of 250–350 fields of vision were systematically selected at random for each measurement. The Ki-67 percentage was defined as [(Ki-67 positive)/ (Ki-67 positive + Ki-67 negative)] × 100. ER-positivity was the presence of nuclear staining in > 1% of the cancer cells and ER-

negative when < 1% of the cells were stained. For PR, positive was defined as nuclear staining present in > 10% of the cancer cells, borderline as 1–10% of the cancer cells exhibiting nuclear staining, and negative as < 1% of the epithelial breast cancer cells exhibiting nuclear staining. The DAKO Hercep-Test scoring protocol was used to score HER2, with 2+ and 3+ cases considered to be positive. Two of the authors (BH and EJ) scored all sections independently.

The relative number of stromal tumor-infiltrating lymphocytes (TILs) was assessed according to Salgado et al. [31]. HE-stained tissue sections were scored semi-quantitatively according to the presence or absence of stromal TILs. The degree of infiltration was scored from 0 to 100%, with positive TILs defined as ≥10%. Tumors were also classified as luminal A (ER+/HER2−/Ki67 < 15%) or luminal B (ER+/HER2−/Ki67 ≥ 15% or ER+/HER2+ regardless of Ki67) cancers according to the St. Gallen 2013 recommendations [32].

Main outcome measures

The main primary outcome measure was the difference in proliferation (measured by MAI) in the primary tumor between the study groups. The secondary outcome measures were differences in insulin-related characteristics (i.e., insulin/c-peptide, IGF1, and IGFBP3) between the intervention and control groups. Patient-reported outcome measures (PROMs) on the following complaints and symptoms were also regarded as secondary outcomes: nausea, pain, mobilization, dizziness, insecurity, and bleeding. We applied an in-house questionnaire with which the patients were asked to score the six variables on a 4-step Likert scale (1 = 'no', 2 = 'little', 3 = 'moderate', and 4 = 'very much') on days 1, 2, 3, 4, 5, 6, and 7 after the operation.

For long-term outcome measures, we looked at relapse-free survival (RFS), defined as the time from surgery until the time the patient was diagnosed with a relapse in any location (i.e., locoregional, systemic, or contralateral). The time from surgery until death due to breast cancer was the breast cancer-specific survival (BCSS). The time from surgery until death from any cause constituted overall survival (OS). For both the primary and secondary outcomes, a subgroup analysis was planned for the ER-positive (luminal) breast cancer subtype.

Statistical analysis

Power calculations were performed on the basis of the primary endpoint. We anticipated a 20% increase in MAI in the intervention group compared to the control group. Based on the mean value of MAI in patients belonging to the catchment area of Stavanger University Hospital [33, 34] and the reproducibility of the method to assess MAI, a total of 30 patients was needed in each study group (i.e., 60 patients) to achieve 80% power. We decided to randomize 80 patients to allow for a 10–15% drop-out rate.

As ER- positive breast cancer comprises approximately 75% of all breast cancer cases, there should be a reasonable number of patients to perform a subgroup analysis of luminal breast cancers. Statistical analyses were performed using SPSS statistical software v.22 (SPSS, Inc., Chicago, IL, USA). Differences in the clinical variables between the intervention groups were determined using T-tests, Fishers exact test, or chi-squared tests as appropriate. Kaplan-Meier survival curves were constructed, and the log-rank test was used to evaluate survival differences between groups. Cox proportional hazard analysis was used to test the relative importance of potential prognostic variables. In multivariable Cox regression, a backward stepwise model selection procedure was used, in which all covariates deemed clinically relevant were included in the initial model.

The proportion of patients reporting at least mild problems on each of the items on the PROM questionnaire each day for the first 7 postoperative days was analyzed using a mixed effects logistic regression model. Using this model, we tested for differences between the intervention and control groups. If a significant difference was found, a post-hoc analysis was performed using chi-squared tests for each of the days. We did not apply any correction for multiple testing due to the pilot and exploratory nature of the study. A two-tailed *P*-value of 0.05 was considered the threshold for significance.

Manuscript reporting

We ensure that the manuscript reporting adheres to CONSORT guidelines for reporting clinical trials, including sticking to the CONSORT check list.

Results

The various characteristics of the two allocation groups are shown in Table 1. Fifty patients had ER-positive tumors and 11 ER-negative tumors. Of the latter, 8 were HER2-negative (ER-, HER2-) and 4 were triple-negative (ER-, PR-, HER2-) based on IHC profiling. Notably, we found no differences in the distribution of the basic covariates between the carbohydrate-intervention group and the fasting group (Table 1).

Proliferation markers

In the total study cohort, none of the continuous variables (MAI, Ki67, or PPH3) were different between the carbohydrate and fasting groups. However, when applying the robust and well-established prognostic threshold for MAI (< 10/≥10), among the ER-positive patients (n = 50) significantly more patients in the carbohydrate

intervention (70%) had high proliferation (MAI ≥ 10) than in the fasting group (30%; $p = 0.038$; Table 2). The same trend was found when all tumors were considered (58% vs. 42%, carbohydrate vs. fasting; $p = 0.083$). In lymph node-negative luminal patients, the same correlation was stronger with a Kendall's tau-b r = 0.488 ($p = 0.017$), Gamma r = 1.000 ($p = 0.017$), and Pearson chi-squared = 7.62 ($p = 0.006$; Fischer exact = 0.014 (two-sided); Table 3).

Progesterone receptor

Significantly more patients in the carbohydrate group had PR-negative tumors (50%) compared to the fasting group (20%; $p = 0.014$), independent of luminal A/B status.

Serum glucose and insulin responses

The response to pre-operative carbohydrate loading was assessed by the difference between the pre-operative serum values and the values obtained at admission (i.e., serum levels after carbohydrate loading minus fasting baseline values in both groups; Table 4). As expected, the intervention group had a significant increase in both S-insulin (+ 24.31 mIE/L, $p < 0.0001$, 95% CI 15.34 mIE/L to 33.27 mIE/L) and S-insulin c-peptide (+ 1.39 nM, $p < 0.0001$; 95% CI 0.21 nM to 0.97 nM). The upper quartile (Q_4) border value of 2.40 nM was equal to the upper value of the normal range of insulin c-peptide (Table 4), indicating that 25% of the patients had c-peptide values compatible with insulin resistance. Regarding IGFBP3, a significant reduction of − 0.43 nM was measured after carbohydrate loading ($p < 0.0001$, 95% CI − 0.56 nM to − 0.27 nM) and − 0.26 nM compared to the control group ($p = 0.015$, 95% CI − 0.46 nM to − 0.051 nM). We found no changes in S-glucose or S-IGF-1 values within or between the two study groups (Table 4, Fig. 2a-f).

Quality of life data

In the carbohydrate intervention group, fewer patients reported mild and moderate pain during the first 7 post-operative days than in the fasting group ($p < 0.001$), which in post-hoc analysis was significant on postoperative day 5 (28% vs. 47%; $p = 0.038$) and day 6 (28% vs. 50%; $p < 0.001$). Otherwise, there were no significant differences between the two groups regarding the other items from the PROM questionnaire (nausea, mobilization, dizziness, insecurity, and bleeding) (data not shown).

Table 2 Cross table MAI and allocation groups in ER+ patients

			Carbohydrate	Fasting	Total
	MAI < 10	Count	13	26	39
		%	65.0%	89.7%	79.6%
	MAI ≥ 10	Count	7	3	10
		%	35.0%	10.3%	21.4%
Total		Count	20	29	49
		%	100.0%	100.0%	100,0%

Pearson chi-squared: 4.430, df = 1, $p = 0.035$
Fischer exact: 0.041 (one-sided) and 0.068 (two-sided)
r (gamma) = 0.647 ($p = 0.042$)
r (Kendall's tau-b) = 0.301 ($p = 0.042$)

Table 3 Cross table MAI and allocation groups in ER+ /LN negative patients

			Carbohydrate	Fasting	Total
	MAI < 10	Count	8	20	28
		%	66.7%	100.0%	87.5%
	MAI ≥ 10	Count	4	0	4
		%	33.3%	0.0%	12.5%
Total		Count	12	20	49
		%	100.0%	100.0%	100.0%

Pearson chi-squared: 7.619, df = 1, $p = 0.006$
Fischer exact: 0.014 (one-sided) and 0.014 (two-sided)
r (gamma) = 1.000 ($p = 0.017$)
r (Kendall's tau-b) = 0.488 ($p = 0.017$)

Long-term clinical outcome

The median follow-up for RFS was 88 months (range 33 to 97 months) and for BCSS 88 months (range 45 to 97 months). Eight patients experienced a relapse: one loco-regional, six systemic, and one contralateral. Five of these patients died of breast cancer.

Relapse-free survival

Randomization to intervention with pre-operative carbohydrates had a weak and borderline influence on RFS when analyzed in the whole study cohort (Table 5). However, in the ER-positive patients who received carbohydrates pre-operatively, a reduced RFS of 71% compared to 97% in the control group ($p = 0.012$, HR = 9.3, 95% CI 1.1 to 77.7; Table 5 and Fig. 3a) was observed. The covariates tumor diameter between 2 and 5 cm (T2) and the proliferation marker Ki67 (both ≥15% and ≥ 30%) had a significant negative influence on RFS in both the whole cohort and in the ER-positive cohort (Table 5). In the ER-negative subgroup, there was no influence of the carbohydrate/fasting grouping on RFS (Fig. 3b). The following co-variates were deemed clinically relevant: tumor size, nodal status, histological grade, PR and HER2 status, Ki67–15%, Ki67–30%, PPH3–13, MAI-10, TILs, luminal A/B status, carbohydrate/fasting grouping, chemotherapy, radiotherapy and endocrine therapy, BMI-75p, BMI-25, and smoking status. In the multivariable analysis, tumor size (T1/T2; $p = 0.021$, HR = 6.07, 95% CI = 1.31 to 28.03) and carbohydrate/fasting grouping ($p = 0.040$; HR = 9.30, 95% CI 1.11 to 77.82) were the only two variables left in the final Cox model. As T2 tumors were more frequent in the intervention group, we performed a Kaplan Meier analysis of the influence of

Table 4 Changes in glucose and insulin-related characteristics in the study groups

	Carbohydrate group (CH)				Fasting group (F)				Between groups		Normal range
	Admission values (A)	Pre-operative Values (Pop)	Difference within group (A-Pop)	P-diff within group	Admission values (A)	Pre-operative Values (Pop)	Difference within group (A-Pop)	P-diff within group	Difference between group CH vs. F	P-diff between groups	
GLUCOSE (mmol/L)											4.0 to 6.0
Median	5.05	4.70			5.30	5.10					
Mean	5.37	5.22	− 0.15	0.625	5.34	5.11	− 0.23	0.009	+ 0.072	0.824	
IQR	4.88 to 5.05	4.25 to 5.50			4.80 to5.80	4.70 to 5.40					
Range	4.40 to 10.00	3.2 to 12.1			4.20 to 6.40	3.30 to 6.90					
95% CI	4.90 to 5.80	4.48 to 5.96	− 0.79 to 0.49		5.15 to 5.53	4.89 to 5.33	−0.39 to −0.061		−0.59 to 0.73		
INSULIN (mIE/L))											< 29.1
Median	6.80	26.65			6.90	8.60					
Mean	9.43	33.68	+ 24.25	< 0.0001	9.14	9.09	− 0.58	0.940	+ 24.31	< 0.0001	
IQR	3.60 to 10.33	20.90 to 45.28			5.00 to 12.1	3.30 to 8.60					
Range	2.00 to 32.50	6.00 to 86.60			2.00 to 24.80	2.00 to 22.00					
95% CI	6.00 to 12.90	25.52 to 41.85	15.39 to 33.11		6.88 to 11.04	7.06 to 11.12	−1.58 to 1.47		15.34 to 33.27		
C-PEPTIDE (nM)											< 2.4
Median	0.61				0.66	0.68					
Mean	0.70	2.10	+ 1.40	< 0.0001	0.75	0.76	+ 0.004	0.926	+ 1.39	< 0.0001	
IQR	0.50 to 0.83	1.50 to 2.41			0.53 to 0.66	0.53 to 0.68					
Range	0.34 to 1.91	0.71 to 5.10			0.38 to 1.92	0.40 to 1.45					
95% CI	0.57 to 0.83	1.67 to 2.53	0.98 to 1.83		0.64 to 0.86	0.66 to 0.85	− 0.083 to 0.091		1.03 to 1.77		
IGF-1 (nM)											5 to 28
Median	18.60	18.45			18.10	17.90					
Mean	18.67	18.98	+ 0.31	0.541	18.30	18.88	+ 0.58	0.145	+ 0.62	0.672	
IQR	14.2 to 23.0	14.15 to 18.45			15.70 to 21.60	18.80 to 23.30					
Range	8.5 to 30.6	10.20 to 33.50			8.60 to 32.80	9.80 to 32.80					
95% CI	16.36 to 20.98	16.59 to 21.37	−0.72 to 1.35		16.34 to 20.25	16.89 to 20.86	− 0.21 to 1.36		−1.51 to 0.98		
IGFBP-3 (mg/L)											2.9 to 5.1
Median	4.55	4.05			4.20	4.40					

Table 4 Changes in glucose and insulin-related characteristics in the study groups *(Continued)*

	Carbohydrate group (CH)				Fasting group (F)				Between groups		Normal range
	Admission values (A)	Pre-operative Values (Pop)	Difference within group (A-Pop)	P-diff within group	Admission values (A)	Pre-operative Values (Pop)	Difference within group (A-Pop)	P-diff within group	Difference between group CH vs. F	P-diff between groups	
Mean	4.43	4.02	−0.42	< 0.0001	4.53	4.37	− 0.16	0.042	− 0.26	0.015	
IQR	3.95 to 5.05	3.50 to 4.58			4.00 to 5.30	3.80					
Range	3.00 to 5.60	2.70 to 5.20			2.80 to 6.50	2.80 to 5.60					
95% CI	4.11 to 4.75	3.74 to 4.30	−0.56 to −0.27		4.24 to 4.83	4.11 to 4.64	−0.31 to − 0.0061		−0.46 to − 0.051		

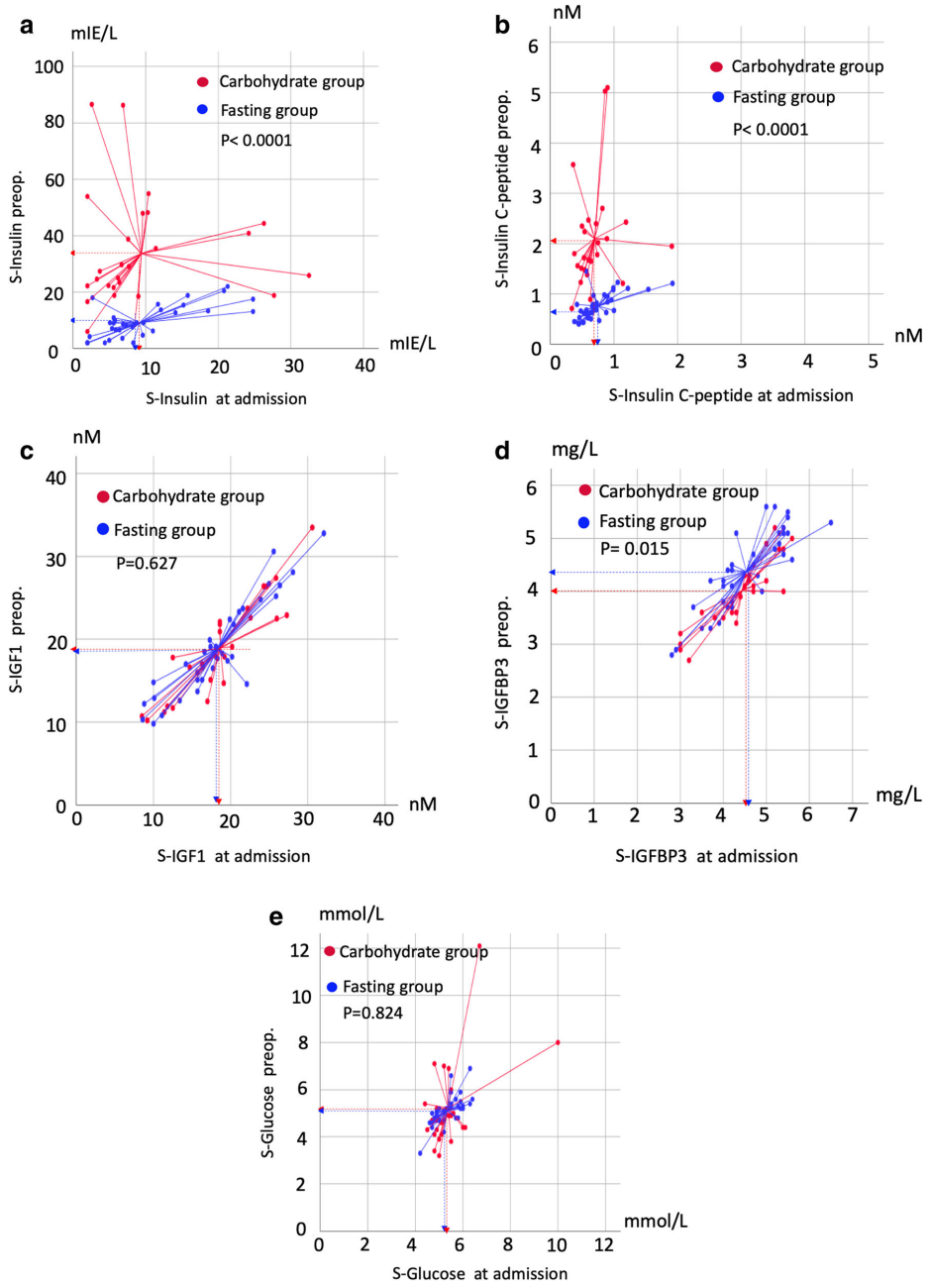


Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Scatterplot of the various insulin-related measures in serum in the two study groups. **a** S-insulin. **b** S-insulin c-peptide. **c** S-IGF. **d** S-IGFBP3. **e** S-glucose. The center of the centroid reference lines represents the mean value in each group (dotted lines). *P*-values were determined using t-tests. Units are given by the x-axis and y-axis. All values on the x-axis are at admission, and the y-axis values represent pre-operative measurements. Red, carbohydrate group; blue, fasting group; S, serum; Preop., pre-operatively; IGF, insulin-like growth factor; IGFBP3, IGF-binding protein 3

the carbohydrate intervention on RFS stratified for T1 vs. T2. This analysis showed that the unfavorable prognostic effect of carbohydrate loading was not present in the T1 (≤ 2 cm) patients, but was strongly prognostic in the T2 patients (Fig. 3c and d). In the T2 group, the carbohydrate-loaded and fasting patients had an RFS of 33 and 100%, respectively ($p = 0.031$; HR = inf). In the T2 subgroup, there was a significantly higher mean serum level of pre-operative insulin c-peptide among patients who experienced a relapse versus those who were relapse-free (2.02 nM vs. 0.838 nM, $p = 0.025$). Notably, there was an even distribution of luminal A and luminal B tumors among the patients with T2 tumors who experienced a relapse versus those who did not ($p = 0.47$).

Breast cancer-specific survival

In the unadjusted analysis of BCSS, intervention with carbohydrates resulted in a significantly inferior BCSS in ER-positive patients compared to the control group (Table 6; Fig. 4a). In ER-positive T2 tumors, the carbohydrate intervention group had the worst BCSS (30%), compared to 100% in the control fasting group (p = 0.031, HR = infinite, due to zero relapses in one of the two groups; Fig. 4b). In addition, tumor size, nodal status, and Ki67–30% provided significant prognostic information in the unadjusted analysis (Table 6). In the multivariable analysis, only Ki67–30 remained in the final model. In general, the small number of patients and endpoints hampered a robust multivariable analysis.

Overall survival

The univariate analysis of OS in ER+ patients showed only a borderline significance of OS for the carbohydrate group (81%) compared to the fasting group (99%; $p = 0.068$; HR = 6.02; 95% CI 0.672–53.8; Fig. 5a). Only tumor size remained as an explanatory factor in the final Cox model (HR = 17.1; 95% CI 17.1–153). In the ER+/T2 patients, the corresponding OS was 33% vs. 100%, respectively ($p = 0.031$; HR = inf; Fig. 5b). In the Cox model, carbohydrate/fasting status was entered in the last step, but the model was considered too unstable for a reliable report.

Adverse events

No adverse events were recorded in either of the two study arms. No signs of pathologically elevated fasting blood sugar levels (i.e., > 6 mmol/L) was noted. Furthermore, in the carbohydrate arm, no signs of occult diabetes mellitus were seen (i.e., blood sugar levels > 10 mmol/L) after carbohydrate loading.

Discussion

Glucose has been correlated with cancer for nearly a century. Warburg (1925) was the first to describe the phenomenon that cancer cells have a much stronger tendency to take up glucose [35], for which (amongst other findings) he received the Nobel prize in 1932 [36]. However, to the best of our knowledge, the current study is the first prospective randomized trial to evaluate the effects of pre-operative carbohydrate loading on tumor proliferation and outcome (short-term vs. long-term) in operable breast cancer patients. In patients with ER-positive tumors (i.e., luminal tumors), significantly more patients with MAI ≥ 10 were observed in the carbohydrate group than the fasting group. Luminal cancers have, on average, a lower proliferation rate than ER-negative and triple-negative cancers [37]. As such, the proliferation-increasing effect of carbohydrate loading in luminal cancers understandably leads to a higher percentage increase in patients crossing the prognostically essential MAI-10 threshold. Most ER−/triple-negative breast cancer patients already have an MAI greatly exceeding 10. Therefore, carbohydrate loading will probably not increase proliferation in a clinically significant manner, as they have an a priori high risk of distant metastases [38]. In addition, the luminal A patients exposed to excess carbohydrates may turn into luminal B tumors, thereby statistically increasing their risk for recurrence. This is in agreement with luminal breast cancers responding directly to an increase in circulating insulin through altered transmembrane IRs [39]. Thus, in the present study, the observation of an increase in insulin/c-peptide in the intervention group could explain the increased MAI and Ki67 in the ER-positive group. Similarly, as triple-negative cancers better utilize the IGFBP3 pathway in EGF1-signaling [40], the observed reduction in IGFBP3 after carbohydrate loading may account for the lack of response to proliferation in the ER-negative group. This could suggest that the differential responses to the insulin/IGF1 axis between luminal and triple-negative cancers [41] explain our observed differences in response to per-oral carbohydrate loading and mitotic activity between the ER-positive and ER-negative groups.

Table 5 Univariable analysis of relapse-free survival

	Whole cohort ($n = 61$)				ER positive patients ($n = 50$)			
Characteristics	Event/at risk (% survival)	Log rank P	HR	95% CI	Event/at risk (% survival)	Log rank P	HR	95% CI
Pre-operative randomization								
Fasting	2/35 (94)	0.049	1		1/29 (97)		1	
Carbohydrates	6/26 (77)		4.4	0.9 to 21.7	6/21 (71)	0.012	9.3	1.1 to 77.7
Nodal status								
N0	3/43 (93)		1		3/33 (91)		1	
N+	5/18 (13)	0.03	9.8	1.10 to 88.1	4/17 (77)	0.16	2.8	0.63 to 12.6
Tumor size								
T1	3/45 (93)		1		3/39 (92)		1	
T2	5/16 (69)	0.009	5.5	1.3 to 23.2	4/11 (64)	0.008	6.0	1.3 to 27.0
Nottingham grade[b]		0.33				0.31		
Grade 1	0/11 (100)		1		0/11 (100)		1	
Grade 2	5/30 (83)	–	Inf.	Inf.	5/30 (83)		Inf.	Inf.
Grade 3	3/20 (85)	–	inf.	Inf.	2/9 (78)		inf.	Inf.
Estrogen receptor					–	–	–	–
Positive (≥ 1%)	7/50 (86)		1		–	–	–	–
Negative (< 1%)	1/11 (91)	0.67	1.6	0.2 to 12.7	–	–	–	–
Progesterone receptor								
Positive (≥10%)	4/41 (37)		1		3/37 (92)		1	
Negative (< 10%)	4/20 (80)	0.27	2.1	0.5 to 8.6	4/13 (69)	0.048	4.0	0.90 to 18.1
HER2								
Negative (0 to 1+)	7/57 (88)		1		6/49 (88)		1	
Positive (2+ to 3+)	1/4 (75)	0.46	2.1	0.3 to 17.5	1/1 (0)	0.005	11.7	1.3 to 105.1
MAI								
< 10	5/41 (88)		1		4/39 (90)		1	
≥ 10	3/19 (66)	0.66	1.4	0.3 to 5.8	3/10 (70)	0.09	3.4	0.8 to15.2
MAI								
< 3	2/16 (88)		1		2/16 (88)		1	
≥ 3	6/44 (86)	0.89	1.1	0.2 to 5.5	5/33 (85)	0.80	1.2	0.2 to 6.4
PPH3								
< 13	3/35 (91)		1		3/35 (91)		1	
≥ 13	5/26 (81)	0.26	2.2	0.5 to 9.4	4/15 (73)	0.12	3.1	0.7 to 14.0
Ki67								
< 15	0/26 (100)				0/25 (100)		1	
≥ 15	8/34 (77)	0.008	–	–	7/24 (71)	0.003	[a]	[a]
Ki67								
< 30	3/38 (92)		1		3/37 (92)		1	
≥ 30	5/22 (77)	0.093	3.2	0.8 to 13.4	4/12 (67)	0.023	4.8	1.1 to 21.8
TILs								
Negative (< 10%)	2/13 (85)				7/55 (87)		1	
Positive (≥10%)	6/48 (88)	0.77	1.4	0.2 to 3.9	1/6 (83)	0.75	2.2	0.24
Luminal status[c]								
Luminal A	3/39 (92)				2/28 (93)			
Luminal B	5/22 (77)	0.091	3.2	0.77 to 13.5	5/22 (77)	0.11	3.5	0.68 to 18.1

Table 5 Univariable analysis of relapse-free survival *(Continued)*

	Whole cohort ($n = 61$)				ER positive patients ($n = 50$)			
Characteristics	Event/at risk (% survival)	Log rank P	HR	95% CI	Event/at risk (% survival)	Log rank P	HR	95% CI
Chemotherapy								
Yes	6/29 (79)		1		5/20 (75)		1	
No	2/32 (94)	0.096	0.28	0.06 to 1.4	2/30 (93)	0.069	0.25	0.05 to 1.3
Radiotherapy								
Yes	6/43 (86)		1		5/38 (87)		1	
No	2/17 (88)	0.90	0.91	0.18 to 4.5	2/12 (83)	0.72	1.4	0.26 to 7.0
Endocrine Therapy								
Yes	7/39 (82)		1		6/36 (83)		1	
No	1/22 (96)	0.15	0.24	0.03 to 2.0	1/14 (93)	0.38	0.40	0.05 to 3.3
BMI-25[d]								
< 25	3/31 (90)		1		3/26 (89)		1	
≥ 25	4/23 (83)	0.40	1.9	0.42 to 8.4	3/20 (85)	0.70	1.4	0.28 to 6.8
BMI-75p[e]								
< 75p	4/41 (90)		1		4/36 (89)		1	
≥ 75p	3/13 (77)	0.201	2.57	0.57 to 11.5	2/10 (80)	0.417	1.99	0.36 to 10.9
Smoking								
-Never smoked	4/15 (73)		1		3/12 (87)		1	
-Former smoker	1/23 (96)		0.22	0.025 to 2.00	1/20 (95)		0.26	0.027 to 2.5
-Ongoing smoking	1/14 (93)	0.065	0.14	0.015 to 1.22	1/12 (92)	0.15	0.17	0.017 to 1.6

BMI Body mass index, *HRT* Hormonal replacement therapy, *T* Tumor size in mm or category, *N* Pathological lymph node status, *LN* Lymph node, *N0* Node negative, *N+* Node positive (assessed by pathologists), *HER-2* Human epidermal growth factor receptor 2, *MAI* Mitotic activity index, *TILs* Tumor infiltrating leucocytes, *PPH3* Phosphorylated phospho-histone 3

[a]HR (95% CI) was not computed, as the equation did not converge, and no events occurred in one or more categories

[b]Histological grading was performed according to the Nottingham algorithm

[c]Luminal A = ER+/HER2−/Ki67 < 15% and Luminal B = ER+/HER2−/Ki67 ≥ 15% or ER+/HER2 +

[d]BMI-25 represents a dichotomized BMI < 25 or ≥ 25 on the BMI scale

[e]BMI-75p represents a dichotomized BMI with cut off < /≥ 75 percentile, i.e., </≥ 26.8 on the BMI scale

The observed inferior RFS for ER-positive T2 tumors suggests that larger tumor size may influence the extent to which cancer cells activate all necessary features to promote the epithelial-mesenchymal transition (EMT) [42] and seed out micro-metastases. These processes turn into clinically overt relapses after some years [43]. This is in line with other research that has found a positive correlation between tumor size and relapse [44], and between tumor size and the development of endocrine resistance [45]. A crucial question is to what extent the pre-operative carbohydrate load to patients in the present study promoted the EMT process in the T2-T3 tumors and created more micro-metastases [46, 47]. Importantly, increased signaling through the insulin/IGF axis is known to promote both the EMT process [48] and chemotaxis [49], which increases the risk for minimal residual disease to occur. Furthermore, the pre-operative carbohydrates may have been administered in a critical window of the cancer's life cycle. The number of liberated circulating tumor cells (CTCs) from the primary tumor sharply increases during surgery [50]. Thus, the administered carbohydrates may have given the CTCs systemic biological support with a triple survival benefit through the Warburg effect [12], the insulin/IGF-1 axis [51], and paracrine signaling with distant adipocytes [11]. Furthermore, increased IR/IGF-signaling promotes protein synthesis in the same way the PR pathway does. Consequently, the upregulation of IR/IGF-signaling will suppress the transcription of PR in the cell [52], which is considered to be part of the endocrine switch. Moreover, dietary carbohydrates may down-regulate the gene expression of PR through epigenetic mechanisms [53]. These mechanisms support our finding of less PR-positivity in the carbohydrate arm. Taken together, these components of the endocrine switch make CTCs more resilient to the adjuvant endocrine treatment following surgery [9, 54]. The present study seems to support the novel principle of manipulating the perioperative nutrient status for adjuvant treatment purposes. Recently, the complete opposite situation with a postoperative low carbohydrate/ketogenic diet was advocated in pancreatobiliary cancer surgery as an option for adjuvant anticancer therapy [55].

As the distribution of larger tumor sizes was skewed to the carbohydrate group, there may be another

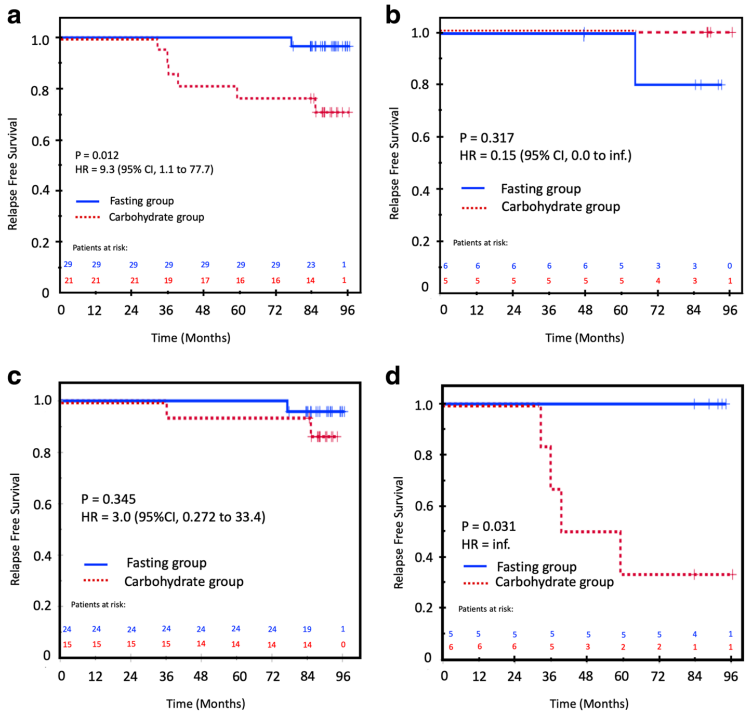


Fig. 3 Relapse-free survival (RFS) in the carbohydrate and fasting groups. **a** In all ER-positive patients. **b** In all ER-negative patients. **c** In ER-positive, T1 patients. **d** In ER-positive, T2 patients. Fasting group, blue solid line; carbohydrate group, red dotted line. Patients at risk are above the X-axis with the same color coding as the treatment groups. Censored patients are marked with a + sign on the survival curves

explanation than statistical chance. As the carbohydrates affected proliferation, they may have also affected the growth of tumor cells in the tumor periphery, where the MAI is measured. This may have resulted in more blurry demarcations of the tumor, which then interferes with the accuracy of measuring the tumor size. Thus, the increased tumor size in the carbohydrate group may have a biological basis.

The inferior prognosis of patients who received the carbohydrate load and had T2 tumors requires some reflection. Patients with higher levels of insulin c-peptide may be more responsive not only to the carbohydrate loading they received in the present study, but also to carbohydrates in every meal they consume during the period in which they receive adjuvant therapies and thereafter. These patients may have a subclinical insulin-resistant state, which is known to be a risk factor for relapse from breast cancer in non-diabetic women [56]. Therefore, tumor size combined with insulin c-peptide status may predict an increased effect of adjuvant metformin or other insulin-lowering drugs in the treatment of breast cancer patients. Metformin attenuates the systemic biological effect of IR/IGF on tumor-promoting signaling by improving insulin sensitivity and suppressing liver glucose output, which leads to reduced levels of systemic circulating insulin [14]. This further mitigates paracrine signaling, overcoming endocrine resistance [51, 57] and improving prognosis in breast cancer [58–61]. The present study supports the hypothesis that adjuvant metformin or other insulin-lowering therapeutic interactions may have their greatest effect in breast cancer patients with ER-positive T2 tumors. In addition, the greatly increased glucose consumption by cancer cells as measured by positron emission tomography (PET) with the tracer ^{18}F-deoxy-glucose (FDG) [62] identifies patients with an inferior clinical outcome [63]. This may also serve as a promising proxy for insulin/metformin responders.

The effect of carbohydrate loading on well-being had a very limited clinical subjective effect in the present study (i.e., only reduced pain on the 5th and 6th day after surgery). Notably, no difference in mobilization or hospitalization was found. This is probably due to the short duration of the operation and the extraperitoneal nature of the surgical procedure in breast cancer

Table 6 Univariable analysis of breast cancer-specific survival

Variable	Whole study cohort ($n = 61$)				ER-positive patients ($n = 50$)			
	Event/at risk (% survival)	Log rank *P*	HR[a]	95% CI	Event/at risk (% survival)	Log rank *P*	HR[a]	95% CI
Pre-operative randomization								
Fasting	1/35 (97)		1		0/29 (100)		1	
Carbohydrates	4/26 (85)	0.086	4.4	0.88 to 21.7	4/21 (81)	0.015	[a]	[a]
Nodal status								
N0	1/43 (98)				1/33 (82)		1	
N+	4/18 (78)	0.012	4.4	1.05 to 18.5	3/17 (82)	0.080	2.80	0.63 to 12.6
Tumor size			–	–				
T1	0/45 (100)		1		0/40 (100)		1	
T2	5/16 (69)	< 0.0001	5.5	1.32 to 23.1	4/10 (60)	< 0.0001	[a]	[a]
Nottingham grade [b]		0.556				0.352		
Grade 1	0/11 (100)		1		0/11 (100)		1	
Grade 2	3/30 (90)		[a]	[a]	3/30 (90)		[a]	[a]
Grade 3	2/20 (90)		[a]	[a]	1/9 (89)		[a]	[a]
Estrogen receptor					–	–	–	–
Positive (≥ 1%)	4/50 (92)		1		–	–	–	–
Negative (< 1%)	1/11 (91)	0.852	0.64	0.079 to 5.21	–	–	–	–
Progesterone receptor								
Positive (≥10%)	4/41 (90)		1		3/37 (92)		1	
Negative (< 10%)	1/20 (95)	0.543	0.51	0.057 to 4.59	1/13 (92)	0.94	0.93	0.1 to 8.9
HER2								
Negative (0 to 1+)	4/57 (93)		1		3/49 (94)		1	
Positive (2+ to 3+)	1/4 (75)	0.248	3.37	0.38 to 30.2	1/1 (0)	0.001	11.7	1.31 to 105.1
MAI								
< 10	3/41 (93)		1		3/39 (92)		1	
≥ 10	2/19 (90)	0.645	1.5	0.25 to 9.1	2/10 (80)	0.23	4.1	0.6 to 29.3
MAI								
< 3	1/16 (94)		1		1/16 (88)		1	
≥ 3	4/44 (91)	0.735	1.46	0.16 to 13.0	3/33 (85)	0.76	1.4	0.15 to 13.8
PPH3								
< 13	2/35 (94)		1		2/35 (94)		1	
> 13	3/26 (89)	0.426	2.0	0.34 to 12.2	2/15 (87)	0.40	2.3	0.32 to 16.1
Ki67								
< 15	0/26 (100)		1		0/25 (100)		1	
≥ 15	5/34 (82)	0.040	–	–	4/24 (83)	0.014	[a]	[a]
Ki67								
< 30	1/38 (97)		1		1/37 (95)		1	
≥ 30	4/22 (82)	0.033	7.5	0.84 to 67.5	3/12 (75)	0.023	9.9	1.03 to 95.3
TILs								
Negative	4/55 (93)		1		3/45 (93)		1	
Positive	1/6 (83)	0.479	2.16	0.24 to 19.4	1/4 (75)	0.24	3.6	0.37 to 34.6
Luminal status[c]								
Luminal A	3/39 (92)		1		2/28 (93)		1	
Luminal B	2/22 (91)	0.847	1.2	0.20 to 7.41	2/22 (91)	0.777	1.33	0.19 to 9.42

Table 6 Univariable analysis of breast cancer-specific survival *(Continued)*

Variable	Whole study cohort ($n = 61$)				ER-positive patients ($n = 50$)			
	Event/at risk (% survival)	Log rank *P*	HR[a]	95% CI	Event/at risk (% survival)	Log rank *P*	HR[a]	95% CI
Chemo therapy								
Yes	5/39 (87)		1		3/20 (85)		1	
No	0/22 (100)	0.089	0.22	0.024 to 1.95	1/30 (97)	0.15	0.22	0.023 to 2.10
Radiation therapy								
Yes	3/43 (93)		1		2/38 (95)		1	
No	2/17 (88)	0.499	1.84	0.33 to 11.0	2/12 (83)	0.19	3.9	0.48 to 24.1
Endocrine therapy								
Yes	5/39 (87)		1		4/36 (89)		1	
No	0/22 (100)	0.089	0.024	0 to 46.4	0/14 (100)	0.20	0.03	0 to 262
BMI-25[d]								
< 25	1/31 (97)		1		1/26 (96)		1	
≥ 25	3/23 (87)	0.177	4.19	0.44 to 40.3	2/20 (90)	0.398	2.70	0.25 to 29.8
BMI-75p[e]								
< 75p	2/41 (95)		1		2/36 (94)		1	
≥ 75p	2/13 (85)	0.218	3.20	0.45 to 22.8	1/10 (90)	0.622	1.81	0.16 to 20.0
Smoking								
-Never smoked	3/15 (80)		1		2/12 (83)		1	
-Former smoker	0/23 (100)		0.003	Inf.	0/20 (100)		0.003	Inf.
-Ongoing smoking	0/14 (100)	0.020	0.003	Inf	0/12 (100)	0.052	0.003	Inf.

BMI Body mass index, *HRT* Hormonal replacement therapy, *T* Pathological tumor size in mm or category, *LN* Lymph node, *N0* Node negative, *N+* Node positive (assessed by pathologists), *HER-2* Human epidermal growth factor receptor 2, *MAI* Mitotic activity index, *TILs* Tumor infiltrating leucocytes, *PPH3* Phosphorylated phospho-histone 3

[a]HR (95% CI) was not computed, as the equation did not converge and no events occurred in one or more categories

[b]Histological grading was performed according to the Nottingham algorithm

[c]Luminal A = ER+/HER2−/Ki67 < 15% and Luminal B = ER+/HER2−/Ki67 ≥ 15%

[d]BMI-25 represents a dichotomized BMI < 25 or ≥ 25 on the BMI scale

[e]BMI-75p represents a dichotomized BMI with cut off < /≥ 75 percentile, i.e., </≥ 26.8 on the BMI scale

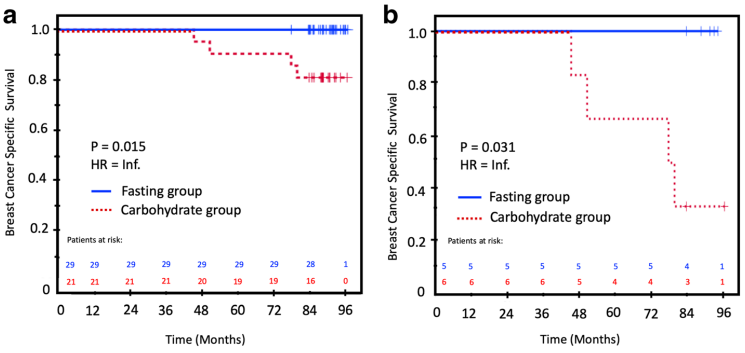


Fig. 4 Breast cancer-specific survival (BCSS) in the intervention and control groups. **a** In all ER-positive patients. **b** In ER-positive, T2 patients Fasting group, blue solid line; carbohydrate group, red dotted line. Patients at risk are above the X-axis with the same color coding as the treatment groups. Censored patients are marked with a + sign on the survival curves

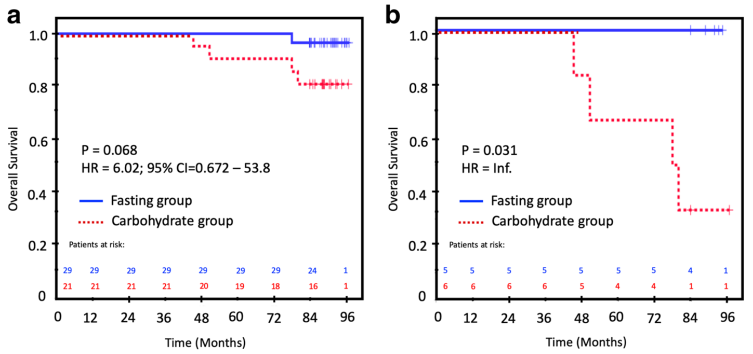


Fig. 5 Overall survival (OS) in the intervention and control groups. **a** In all ER-positive patients. **b** In ER-positive, T2 patients. Fasting group, blue solid line; carbohydrate group, red dotted line. Patients at risk are above the X-axis with the same color coding as the treatment groups. Censored patients are marked with a + sign on the survival curves

patients. The health authorities in Norway recently introduced new national guidelines for a more standardized trajectory in breast cancer, without preoperative carbohydrate loading included [64]. Day-care surgery comprising anesthesiology medication with a short half-life, leading to fewer side effects for the patients and an optimized pain relief regimen, has been introduced since this trial was performed. Thus, the present study does not support introducing carbohydrate loading in this patient group, especially due to the worrying inferior RFS observed in the carbohydrate group.

The strengths of the biological model described above are that it allows changes in the breast tumor to be assessed after manipulating the metabolic environment pre-operatively; thus, it combines the assessment of primary tumor characteristics in concert with systemic metabolic changes. The stable nature of insulin c-peptide also compensated for the more short-lived insulin and IGF. This may explain the more robust nature of insulin c-peptide in the various analyses.

The present study has some weaknesses. First, the number of patients in the intervention arm turned out to be lower than calculated in the power analysis. This may have introduced a type II error in the various statistical analyses. Furthermore, the low number of events and patients at risk in the various survival analyses requires caution in interpreting the results. Moreover, the unbalanced number of participants in the carbohydrate group and fasting group may have introduced confounders. However, as all basic characteristics were evenly distributed between the two study arms, the risk for such confounders is probably quite low. In addition, the proportion of missing data was very low, which contributes to strengthening the study. Regarding tumor markers, a pre-operative biopsy of the tumor would have turned the patients into their own controls. Thus, we could have addressed several questions raised in the discussion, such as the increased PR-negativity in the carbohydrate group. In future studies, pre-operative biopsy must be included to improve the internal validity of the trial.

Finally, the external validity of the present study is limited to luminal breast cancers with T2 tumors. Thus, the present study should be expanded in a multicenter manner, but only in luminal type breast cancers without the PROM quality of life questionnaire. Moreover, a high insulin c-peptide response to a carbohydrate load may predict high risk for relapse. Future research should pursue this clue by adding metabolomic studies to future research on predictive/prognostic circulating biomarkers for systemic relapse in the minutest state possible [65].

Conclusion

The goal of this study was to investigate the influence of carbohydrates on the biological characteristics of breast cancer. Our working hypothesis was that pre-operative carbohydrate loading affects proliferation and clinical outcome. In the carbohydrate-loading group, the levels of insulin and insulin-c-peptide were increased, whereas those of IGFB3 were decreased. We found that there were more ER+ patients with MAI ≥ 10 among patients who received pre-operative carbohydrate loading than among those who fasted. In addition, the proportion of PR- patients was higher in the carbohydrate group. In ER+ patients with tumors larger than 2 cm (T2), carbohydrate loading seemed to affect clinical outcome with significantly decreased RFS, BCSS, and OS. Only RFS had enough events to enter into a Cox regression model, in which carbohydrate/fasting status and tumor size were the only independent explanatory factors. However,

because this study was not powered for survival outcomes, these analyses must be regarded as suggestive. In addition, caution is needed when interpreting the results due to the small sample size and relatively short follow-up. Intriguingly, the decreased expression of PR in the carbohydrate-loaded group suggests the development of endocrine resistance through signaling via membrane-bound receptors, opening up another possibility for the change in clinical outcome than increased proliferation. Taken together, the results of this study indicate that per-oral carbohydrates given pre-operatively may influence both systemic and tumor biology to the benefit of breast cancer cells. Thus, explorative metabolic investigations that focus on identifying novel biomarkers associated with the observed impairment in clinical outcome are warranted.

Abbreviations

ATP: Adenosine triphosphate; BCSS: Breast cancer-specific survival; BCT: Breast conservative therapy; CI: Confidence interval; CTC: Circulating tumor cell; DCIS: Ductal carcinoma in situ; EGFR: Epidermal growth factor receptor; EMT: Epithelial mesenchymal transition; ER: Estrogen receptor; ERAS: Enhanced recovery after surgery; FNAC: Fine needle aspiration cytology; HER2: Human epithelial growth factor receptor 2; HES: Hematoxylin-eosin saffron staining; HR: Hazard ratio; IGF1: Insulin-like growth factor 1; IGF1R: Insulin-like growth factor 1 receptor; IGF-R: Insulin-like growth factor receptor; IHC: Immunohistochemistry; IR: Insulin receptor; MAI: Mitotic activity index; MRI: Magnetic resonance imaging; NBCG: Norwegian Breast Cancer Group; NSD: Norwegian Center for Research Data; PPH3: Phosphorylated phosphohistone 3; PR: Progesterone receptor; PROM: Patient-reported outcome measure; RFS: Relapse-free survival; SN: Sentinel node; TIL: Tumor-infiltrating lymphocytes; WHO: World Health Organization

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Manuscript reporting

We ensure that the manuscript reporting adheres to CONSORT guidelines for reporting clinical trials, including filling out the CONSORT check list.

Authors' contributions

THL included and operated on all of the patients, built the database, and contributed to statistical analyses and interpretation of data. MA contributed to quality assurance for the database. AEV drew all of the blood samples and performed the laboratory analyses. IS contributed to the laboratory analyses. EG performed the surgical pathological analysis with histological grading and morphological analysis of the tumor. JTK provided expert advice on the statistical analyses. LAA contributed scientific support and advice. HS contributed to the concept of the study, statistical analyses, interpretation of data, and funding of the study. EAMJ contributed to the concept of the study, performed the assessment of the pathological parameters and scorings, and interpreted the data. JPAB contributed to the concept of the study, and the analysis and interpretation of the data. All co-authors contributed to writing the manuscript and gave their final approval of the last version to be published. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from Stavanger Breast Cancer Research Group, but restrictions apply to the availability of these data, which were used under license for the current study and are not publicly available. However, data are available from the authors upon reasonable request and with permission from Stavanger Breast Cancer Research Group.

Ethics approval and consent to participate

The randomized trial was approved by the Regional Ethics Committee (Accession number 2015/1445), NSD (Norwegian Centre for Research data, #20984), and The Norwegian Biobank registry (#2239). An informed consent form was signed by each patient. The trial was retrospectively registered at Clinicaltrials.gov (NCT03886389). The reason for delayed registration was that we were not aware of the obligation to register prospectively at the time.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Breast & Endocrine Surgery, Stavanger University Hospital, Helse Stavanger HF, P.O. Box 8100, N-4068 Stavanger, Norway. [2]Centre for Cancer Biomarkers CCBIO, Department of Clinical Medicine, Faculty of Medicine and Dentistry, University of Bergen, Jonas Lies vei 87, N-5012 Bergen, Norway. [3]Department of Research, Stavanger University Hospital, Helse Stavanger HF, P.O. Box 8100, N-4068 Stavanger, Norway. [4]Department of Pathology, Stavanger University Hospital, Helse Stavanger HF, P.O. Box 8100, N-4068 Stavanger, Norway. [5]Department of Pathology, Helse Møre og Romsdal HF, P.O. Box 1600, N-6026 Ålesund, Norway. [6]Department of Mathematics and Physics, University of Stavanger, P.O. Box 8600 Forus, N-4036 Stavanger, Norway. [7]Gades Institute, Laboratory Medicine Pathology, University of Bergen, Jonas Lies vei 87, N-5012 Bergen, Norway. [8]Department of Clinical Science, University of Bergen, Jonas Lies vei 87, N-5012 Bergen, Norway. [9]Risavegen 66, N-4056 Tananger, Norway. [10]Vierhuysen 6, 1921 SB Akersloot, Netherlands.

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Publisher's Note

Paper III

Metabolic consequences of perioperative oral carbohydrates in breast cancer patients — An explorative study

Tone Hoel Lende, MD † [1,2] (leth@sus.no), Marie Austdal, PhD † [3,4] (marie.austdal@sus.no), Tone Frost Bathen, PhD [5] (tone.f.bathen@ntnu.no), Anne Elin Varhaugvik, MSc [4,6] (anne.elin.varhaugvik@helse-mr.no) , Ivar Skaland, PhD [4] (skiv@sus.no), Einar Gudlaugsson, MD, PhD [4] (guei@sus.no), Nina G. Egeland, MSc [4,7] (nina.gran.egeland@sus.no) , Siri Lunde, MSc [1] (siri.lunde@sus.no), Lars A. Akslen, MD, PhD) [2] (lars.akslen@uib.no), Kristin Jonsdottir, PhD [3] (kristin.jonsdottir@sus.no), Emiel A. M. Janssen, PhD § [4,7] (jaem@sus.no), Håvard Søiland, M.D, PhD § (hsoiland@gmail.com)[1,8] and Jan PA Baak, MD, PhD§ [4,9] (info@drjanbaak.com)

1. Department of Breast & Endocrine Surgery, Stavanger University Hospital, Helse Stavanger HF, P.O. Box 8100, N-4068 Stavanger, Norway
2. Centre for Cancer Biomarkers CCBIO, Department of Clinical Medicine, Faculty of Medicine and Dentistry, University of Bergen, Jonas Lies vei 87, N-5012 Bergen, Norway
3. Department of Research, Stavanger University Hospital, Helse Stavanger HF, P.O. Box 8100, N-4068 Stavanger, Norway
4. Department of Pathology, Stavanger University Hospital, Helse Stavanger HF, P.O. Box 8100, N-4068 Stavanger, Norway
5. Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trondheim, Norway
6. Department of Pathology, Helse Møre og Romsdal, Ålesund, Norway,

7. Department of Chemistry, Bioscience and Environmental Technology, University of Stavanger, P.O. Box 8600 Forus, N-4036 Stavanger, Norway
8. Department of Clinical Science, University of Bergen, Jonas Lies vei 87, N-5012 Bergen, Norway
9. Dr. Med. Jan Baak AS, Risavegen 66, N-4056 Tananger, Norway

† Shared 1st authorship.

§ Equal senior contribution

*Corresponding author:

Tone Hoel Lende

Department of Breast & Endocrine Surgery, Stavanger University Hospital,

Helse Stavanger HF, P.O. Box 8100, N-4068 Stavanger, Norway

Phone: +47 478 61 295

Abstract

Background: The metabolic consequences of preoperative carbohydrate load in breast cancer patients are not known. The present study investigated the systemic and tumor metabolic changes after preoperative per-oral carbohydrate load and their influence on tumor characteristics and survival.

Design: Explorative study.

Setting: University hospital with primary and secondary care functions in south-west Norway.

Interventions and Outcome Measures: Serum and tumor tissue were sampled from a population-based cohort of 60 patients with operable breast cancer who were randomized to either per-oral carbohydrate load (preOp™; n=25) or standard pre-operative fasting (n=35) before surgery. Magnetic resonance (MR) metabolomics was performed on serum samples from all patients and high-resolution magic angle spinning (HR-MAS) MR analysis on 13 tumor samples available from the fasting group and 16 tumor samples from the carbohydrate group.

Results: Fourteen of 28 metabolites were differently expressed between fasting and carbohydrate groups. Partial least squares discriminant analysis showed a significant difference in the metabolic profile between the fasting and carbohydrate groups, compatible with the endocrine effects of insulin (i.e., increased serum-lactate and pyruvate and decreased ketone bodies and amino acids in the carbohydrate group). Among ER-positive tumors (n=18), glutathione was significantly elevated in the carbohydrate group compared to the fasting group (p=0.002), with a positive correlation between preoperative S-insulin levels and the glutathione content in tumors (r=0.680; p=0.002). In all tumors (n=29), glutamate was increased in tumors with high proliferation (t-test; p=0.009), independent of intervention group. Moreover, there was a positive correlation between tumor size and proliferation markers in the carbohydrate group only. Patients with ER-positive / T2 tumors and high tumor glutathione (≥1.09), high S-

lactate (≥56.9), and high S-pyruvate (≥12.5) had inferior clinical outcomes regarding relapse-free survival, breast cancer-specific survival, and overall survival. Moreover, Integrated Pathway Analysis (IPA) in serum revealed activation of five major anabolic metabolic networks contributing to proliferation and growth.

Conclusions: Preoperative carbohydrate load increases systemic levels of lactate and pyruvate and tumor levels of glutathione and glutamate in ER-positive patients. These biological changes may contribute to the inferior clinical outcomes observed in luminal T2 breast cancer patients.

Keywords: breast cancer, carbohydrate load, proliferation, insulin, insulin c-peptide, S-lactate, S-pyruvate, tumor glutamate, tumor glutathione, fasting state, ketonic bodies, clinical outcome

Registration of trial: CliniTrials.gov; NCT03886389. Retrospectively registered March 22, 2019. Available at: https://clinicaltrials.gov/ct2/show/NCT03886389?cond=Breast+cancer+diet&rank=1

Full project title: 'The Effects of Insulin and Insulin-related Characteristics, and Short-Term Low-glycemic and High-glycemic Carbohydrate Intervention on Breast Cancer Biomarkers and Survival.'

Introduction

Breast cancer is the most common female malignancy and one of the most frequent causes of death among women in the Western world [1]. Breast cancer incidence has more than doubled in the last 50 years, probably due to increased estrogen exposure and a change towards high levels of alimentary carbohydrates and fat [2, 3]. Even though breast cancer originates locally in the breast, circulating tumor cells (CTCs) may spread to the systemic circulation before and during surgery [4] and establish distant micrometastases [5]. These CTCs must thrive and survive attacks from the innate and adaptive immune system. Thus, tumor cells have to establish a favorable metabolism that can produce energy, protection mechanisms, and the necessary biomass to survive the journey from the breast tumor to remote locations, including transformation into dormancy [6]. The luminal breast cancer subtype, which express estrogen receptor (ER) and/or progesterone receptor (PR) in the tumor cells, comprise the largest subgroup, accounting for approximately 75% of all breast cancers. Endocrine resistance in this subtype can creates micrometastases that escape anti-estrogen therapy and can hibernate for many years before they become clinically overt [7]. The molecular features underlying these cellular characteristics are driven by hallmarks of cancer [8], including changes in cellular energetics and metabolism, followed by a vast number of necessary metabolic modifications to strengthen the metabolic needs of breast cancer cells [9]. A well-known cellular characteristic of tumor cells is increased glucose consumption and glycolysis towards lactate despite the presence of oxygen, a feature called ‘the Warburg effect’ [10, 11]. This metabolic switch includes the production of ribose for DNA synthesis and allowing amino acids to be a source for ATP production [12]. Furthermore, the Warburg effect extends to increased choline

metabolism for cell membrane synthesis and increased amino acid turnover for protein synthesis [10, 13].

Even though much is known about metabolism in breast cancer cells [14], little is known about the influence of carbohydrate loading in the *early recovery after surgery* (ERAS) program [15] on peri-operative metabolism in the systemic circulation and locally in the breast tumor. We recently conducted a randomized controlled trial (RCT) in which operable breast cancer patients were treated with either two oral loads of enriched carbohydrate solution or a standard fasting procedure comprising free drinking of tap water before surgery [16]. In this study, luminal breast cancer patients, who received oral pre-operative carbohydrates, had a higher tumor proliferation and an adverse survival. The goal of the present paper, using the same patients, was to further explore the metabolic differences in serum and the tumor. Based on our previous findings, we hypothesize that the metabolic changes after carbohydrate loading will correlate with proliferation and outcome in patients with ER positive tumors. Also, we also wanted to study whether such metabolic alterations correlate with other tumor characteristics or translate into differences in clinical outcome.

Methods

Ethics statement

This paper is an explorative study based upon a recently published randomized controlled trial (RCT) approved by the Regional Ethics Committee in Western Norway (#2015/1445) and was retrospectively registered at Clinicaltrials.gov (NCT03886389).

Patients

Details on these patients have been described previously [16]. In short, between 12 May 2009 and 23 June 2010 a population-based cohort of 61 operable breast cancer patients (Stage I and II) were randomized into an intervention group receiving preoperative per-oral carbohydrate loading (n=26) or a control group (n=35) receiving the standard preoperative fasting protocol.

The patients in the carbohydrate group drank 200 mL pre-Op™ (Nutricia, the Netherlands). This non-carbonated carbohydrate enriched drink contained 100 kCal per bottle containing 4.2 g (2.1 %) glucose and 20g (10%) polysaccharides. A loading dose of two bottles pre-Op™ were given 18 hours before surgery (i.e., the evening before surgery) and another 2 bottles were administered 2-4 hours before surgery (i.e., the morning of the operation day). In contrast, the control group practiced the standard fasting procedure with free intake of tap water 12-14 hours before surgery. From this cohort, patients with available fresh frozen tissue and serum samples were included in the present study (Figure 1). The patient characteristics are given in Table 1.

Blood sampling

Blood samples were drawn immediately before surgery. In total three serum gel tubes and one EDTA plasma tube were drawn in this study. One serum gel tube and one EDTA plasma tube were delivered within an hour to the department of medical biochemistry for standard analysis. For metabolomics analyses, two serum gel tubes were centrifuged within one hour at 4°C, 2500 x g in 10 minutes. After centrifugation, the serum of the two tubes were mixed and a minimum of 1.1mL serum were sent for analyses in Haukeland University Hospital, Bergen, Norway, the rest of the serum were stored in 1mL cryotubes at -80°C in the biobank at Stavanger University Hospital, Stavanger, Norway.

Tumor tissue sampling

Immediately after removal of the surgical specimen from the systemic circulation, it was transported to the Department of Pathology for further sampling. To avoid necrotic areas, cancerous tissue from the invasive front of the tumor (i.e. tumor periphery) was immediately snap-frozen in liquid nitrogen and stored at -80ºC until assayed for tissue metabolomics. Before HR-MAS analysis, tissues from all of the patients were analyzed consecutively for histopathology and immunohistochemistry as described preciously [16].

Serum hormone and protein analyses

Serum was transported to the Hormone Laboratory, Haukeland University Hospital, Bergen, Norway. Insulin, insulin c-peptide, insulin growth factor 1 (IGF-1), and insulin growth factor binding protein 3 (IGFBP-3) were measured by the IMMULITE 2000 two-site chemiluminescent immunometric assay (Siemens Medical Solutions Diagnostics).

Serum metabolomics analyses

A separate aliquot of serum was transported to the MR Core Facility at NTNU, Trondheim, Norway for metabolomics analyses. Thawed samples (100 µL) were mixed with bacteriostatic buffer (100 µL; pH 7.4, 0.075 mM Na_2HPO_4, 5 mM NaN_3, 5 mM TSP), transferred to 3-mm NMR tubes, and stored at 5°C until analysis (<15 hours). The MR analysis was performed using a Bruker Avance III Ultrashielded Plus 600 MHz spectrometer (Bruker Biospin GmbH, Germany) equipped with a 5 mm QCI Cryoprobe with integrated, cooled pre-amplifiers for ^{1}H, ^{2}H, and ^{13}C. Experiments were fully automated using the SampleJet™ in combination with Icon-NMR in TopSpin 3.1 software (Bruker Biospin). One-dimensional ^{1}H Nuclear Overhauser effect spectroscopy (NOESY) and Carr–Purcell–Meiboom–Gill (CPMG) spectra with water

presaturation were acquired at 310.15 K. The spectra were Fourier transformed to 128 K after 0.3 Hz exponential line broadening and automatically phased and baseline-corrected.

Spectra were further processed in Matlab 2013b (The Mathworks Inc., Natick, MA, USA). The CPMG spectral region between 0.1 and 4.2 ppm was selected for further processing. Chemical shifts were referenced to the left alanine peak at 1.47 ppm. Metabolites were identified based on previous assignment. [17, 18] Twenty-eight metabolites were identified as measurable and their areas calculated by integrating the area under the signal curve.

Breast tumor tissue metabolomics analyses

In the 29 patients with available tissue, the tumors were larger (45% vs. 9% pT2/3/4, p=0.003), had a higher histological grade (52% vs. 18% grade 3, p=0.022), were more often ER-negative (35% vs. 3%, p=0.002), and had higher proliferation (59% vs. 27% PPH3-positive, p=0.002) than those without tissue. Thus, we had a selection bias of larger, non-luminal and a more proliferative tumors into the present study compared to the original study[16]. Tissue was transported on dry ice to the MR Core Facility at NTNU, Trondheim, Norway, for metabolomics analyses. Tissue samples were prepared frozen on a metal plate bathed in liquid nitrogen to minimize tissue degradation. Biopsies (11.0 ± 2.3 mg) were cut to fit 30 µL disposable inserts (Bruker Biospin Corp, USA) filled with 3 µL D_2O containing 25 mM formate. The insert containing the frozen sample was placed in a 4-mm diameter zirconium rotor (Bruker, Biospin GmbH, Germany) and kept at -20°C until analysis (<8 hours). Spin-echo spectra were acquired on a Bruker Avance DRX600 spectrometer with a $^{1}H/^{13}C$ magic angle spinning (MAS) probe with gradient (Bruker Biospin GmbH, Germany) using the following parameters: 5 KHz spin rate, 5°C probe temperature, 5-minute temperature acclimatization before shimming and spectral acquisition, CPMG pulse sequence (cpmgpr1d; Bruker) with 4s

water suppression prior to a 90° excitation pulse, total echo time 77 ms, 256 scans, and spectral width 20 ppm. Spectra were Fourier transformed into 64 K following 0.3 Hz line broadening. Phase correction was performed automatically for each spectrum using TopSpin 3.1.

Spectra were preprocessed in Matlab 2013b as follows [19]. The spectral region between 1.4-4.70 ppm, which contained the majority of the metabolite signals, was selected for further processing. Chemical shifts were referenced to the creatine peak at 3.03 ppm. The spectra were baseline-corrected using asymmetric least squares [20] with parameters λ = 1e7 and p=0.0001, setting the lowest point in each spectrum to zero. Lipid peaks at 4.34-4.27, 4.19-4.14, 2.90-2.7, 2.31-2.18, 2.11-1.92, and 1.68-1.5, and ethanol at 3.67-3.62, were excluded. The resulting spectra were normalized to the total area to correct for differences in sample size and tumor cell content. Metabolite peak assignment was based on previous identification [21]. Twenty metabolites were identified as measurable, and the area under the signal curve in the preprocessed spectra was used to calculate their relative intensities. The metabolite integrals were log10 transformed to satisfy prerequisite assumptions of normality.

Endpoints

Proliferation differences between the carbohydrate and fasting groups were evaluated by Ki67 (<15% or ≥15% and <30% or ≥30%), mitotic activity index (MAI; <10 or ≥10), and PPH3 (<13 or ≥13). The metabolic response to preoperative oral carbohydrate loading was evaluated in serum (preoperative) by ^{1}H NMR and in tumor tissue by HR-MAS MRS.

Univariate analysis

Metabolite differences between groups were assessed by student T-tests. Correlations between continuous variables were assessed by Pearson correlation. Categorical variables were

compared by Chi square tests. P-values were considered significant when $p<0.05$. When multiple variables were compared, the resulting p-value tables were corrected for multiple testing by the Benjamini-Hochberg method [22].

Multivariate analyses (serum and tissue)

Multivariate analyses were performed in R V.3.5 [23] using the package PLS [24] and MetaboAnalyst [25]. Metabolite values were auto-scaled (mean-centered and divided by variance) before multivariate analysis. Principal component analysis (PCA) was performed to evaluate the data sets for outliers. Partial least squares discriminant analysis (PLS-DA) was performed to explore differences in serum and tissue metabolic profiles between categories: carbohydrate loading vs fasting. Partial least squares (PLS) was used to find correlations between the tissue metabolic profile and variables (MAI, PPH3, Ki67, serum (S)-glucose, S-insulin, S-insulin c-peptide, S-IGFR, S-IGFPB3, S-estradiol). Metabolites were evaluated by Variable Importance in Projection (VIP) score. The VIP score is a measure of how important each variable was for creating the discrimination model. It is calculated as a weighted sum of squares of the PLS loadings, where the weights are based on the amount of y-variance explained in each dimension [26]. PLS and PLS-DA classification parameters were evaluated by 'leave-one-out' cross validation due to the limited sample numbers. Permutation testing was carried out as an additional model validation; sample classes or responses were shuffled, and the model rebuilt with the same numbers of latent variables as the original model. One thousand permutations were performed, and models were considered significant if the final accuracy (of classification models) or R^2 (of regression models) were >95% of the permuted accuracy values ($p<0.05$).

Thresholds in survival analyses

Relapse-free survival (RFS) was defined as the time from surgery until a relapse from any site. Breast cancer-specific survival (BCSS) was defined as the time from surgery until death from breast cancer, whereas overall survival (OS) was until death from any cause. Receiver-operator characteristic (ROC) analysis identified optimal thresholds for the various continuous metabolite variables using relapse 'Yes/No' as the categorical variable (Appendix Table 1). The cut-off values obtained in RFS analysis were also used in the BCSS and OS analyses. In ER-negative patients, none of the explanatory variables with ROC-derived thresholds were significant for analysis of RFS, BCSS, or OS. Therefore, further analyses were limited to ER-positive patients. The ROC-obtained thresholds were confirmed with the minimal p-value/maximal Wald-value in a Cox model. In the multivariabel Cox analyses the 'Forward Wald' method was primarily used. In cases of an unstable model, a stepwise backward analysis was performed.

Metabolite Set Enrichment Analysis and Ingenuity pathway analysis (IPA)

Serum metabolite levels were uploaded to the Enrichment module of MetaboAnalyst to explore the pathways affected by the carbohydrate intervention. Pathway-associated metabolite sets with sets containing at least two metabolites were used. Pathways with p-values ≤ 0.05 (after FDR correction) were interpreted as significant.

Serum metabolites with significantly different expression ($p=0.05$) and their corresponding fold changes were imported into the Ingenuity Pathway Analysis (IPA) software (Ingenuity, Redwood City, USA) to explore which biological and molecular functions these metabolites were involved in and how these and their direct and indirect target molecules were connected, using the network function in IPA. Additionally, we examined if there were a direct or indirect

connection between the top network and seven microRNAs related to tamoxifen resistance from our previous paper [27], using the grow function with a moderate or experimentally observed confidence level.

Results

Systemic metabolism

The results of the quantification of serum metabolites in the carbohydrate and fasting groups are given in Table 2. Fourteen out of 28 metabolites were significantly altered between the groups. PLS-DA revealed a significant difference in metabolic profiles between the two groups.; (one component, classification accuracy = 0.85; $p<0.001$; Figure 2A). The main increased markers were increased serum (S) lactate and S-pyruvate in the carbohydrate group ($p<0.0001$; Figure 2A and 2B). Among the patients in the fasting group, the levels of ketone bodies, such as S-acetate, S-acetoacetate, and S-3-hydroxybutyrate, were increased (Table 2). In addition, we observed increased S-N-acetylated groups, S-leucine, S-valine and S-isoleucine in the fasting group (all $p<0.05$; Figure 2B). We found positive correlations between tumor size and S-lactate ($r=0.344$; $p=0.016$) and tumor size and S-pyruvate ($r=0.370$; $p=0.009$).

In the carbohydrate group, there was a positive linear correlation between proliferation (Ki-67) and tumor size ($r=0.782$, $p=0.038$). When Ki-67, PPH3 and MAI were included in a forward and backward stepwise linear regression MAI was the only independent factor explaining increment in tumor size with a Beta=0.530 (95%CI, 0.201 to 0.875) $P = 0.009$. In the fasting group, there was no correlation between tumor size and proliferation.'

Serum glucose and insulin responses

The mean fasting glucose and insulin values at admission were 5.4 mmol/L (95% CI 5.1 to 10.0) and 9.4 mIU (95% CI 6.8 to 32.5), respectively (normal ranges: glucose, 4.0 to 6.0 mmol/L; insulin, 6.0 to 27.0 mIU; c-peptide, 0.3 to 2.4 nmol/L). In the carbohydrate group, the mean preoperative insulin value was 35.6 mIU (26.7 to 106 mIU), compared to 9.1 (8.6 to 22 mIU) in the fasting group (student`s t-test $p<0.001$). For C-peptide, the mean values in the carbohydrate and fasting groups were 2.10 nmol/L and 0.76 nmol/L, respectively ($p<0.001$).

We found significant univariate correlations between the serum concentrations of preoperative insulin (Table 3), Insulin C peptide (Appendix Table 2) IGFBP3 (Appendix Table 3), but not to IGF1 (Appendix Table 4). Multivariate analysis with leave-one-out cross-validation showed significant correlations between the serum metabolic profile and insulin (Cross-validated (CV) (R^2=0.33, p<0.001), Insulin C-peptide (CV R^2 = 0.35, p <0.001), IGFBP3 (CV R^2=0.11, p < 0.001), but not IGF-1. (Figure 3) For both insulin and insulin C-peptide, the most important metabolites for predictions were increased S-glucose, S-lactate and decreased S-Leucine. For IGFBP3, the most important metabolites were increased S-Acetone, S-Glycoprotein, and S-Leucine. We also found positive correlations between S-lactate and the preoperative increase in S-insulin and S-insulin / c-peptide (r=0.57; p<0.001 and r=0.61; p<0.0001), and between S-pyruvate and the increase in preoperative S-insulin and S-insulin c-peptide (r=0.54; p<0.001 and r=0.60; p<0.001).

Tumor metabolism

Metabolites included in the analysis are presented in Table 4. PLS-DA did not result in a significant model discriminating between fasting and carbohydrate-fed patients, and no metabolites were significantly different in univariate testing when all tumors were analyzed (Figure 4A). However, for ER-positive tumors (n=18), glutathione was significantly elevated in the carbohydrate group compared to the fasting group (p=0.002; Figure 4 B), even after adjusting for tumor size. In the ROC analysis, we found an area under the curve (AUC) of 0.894 (95%CI=0.687-1.000, p=0.0015) for glutathione in discriminating between fasting and carbohydrate-fed patients with ER-positive tumors (Figure 4 C). The difference was also significant in the ER-positive tumors with low proliferation (MAI<10; n=7). Moreover, we found a positive correlation between preoperative S-insulin levels and the glutathione content in tumor tissue (r=0.680; p=0.002). Furthermore, we observed a higher level of tissue

glutamate in tumors with a high proliferation as measured by Ki67</≥ 15% (p=0.004). This association remained significant when adjusted for intervention group using a general linear model with intervention status as fixed factor, Ki67</≥ 15% as random factor, and tissue Glutamate as dependent variable (p=0.009). Also, choline (p=0.002) and phosphoethanolamine (p=0.019) were increased in T2 tumors compared to T1 tumors.

Survival analysis

First, we used S-lactate, S-pyruvate, and tissue (T) glutathione as continuous variables in a univariate Cox model for RFS, BCSS and OS. Both S-pyruvate and S-lactate, but not T-glutathione reached significance with a hazard ratio (HR) for RFS of 1.53 (95% CI, 1.11 to 2.11; p=0.009) and 1.08 (95% CI, 1.01 to 1.17; p=0.029), respectively. For BCSS the HR for the continuous variables of S-pyruvate and S-Lactate were 1.85 (95%CI, 1.15 to 2.97; p=0.011) and 1.13 (95%CI, 1.01 1.26; p=0.028) respectively. The corresponding observations for OS were 1.63 (95%CI, 1.11 to 2.40; p=0.014) for lactate and 1.10 (95%CI, 1.002 to 1.20; p=0.045) for pyruvate. Thereafter, the following independent variables were dichotomized according to the optimal ROC-derived thresholds: S-lactate, S-pyruvate, preoperative S-insulin, preoperative S-insulin-c-peptide, and tissue glutathione. In addition, the well-established prognostic factors tumor size, nodal status, histological grade, MAI 10, Ki-67-30 and PPH3-13 were deemed clinically relevant and included as explanatory variables in the multivariable analyses. The results of the univariate RFS, BCSS, and OS analyses are given in table 5 ,6 and 7, respectively.

Patients with a high glutathione content in the tumor (≥1.09) had a 37% risk of experiencing a relapse and 37% risk of dying of breast cancer compared to no relapses and no deaths in patients with a low glutathione content in the tumor (both comparisons: p=0.038; HR=Inf.; Figure 5A

and 5D). Patients with high S-lactate (≥56.9) had RFS of 71% compared to 97% for those with lower S-lactate (p=0.002, HR=7.47; 95% CI 1.66-33.6; Figure 5B). Patients with S-pyruvate ≥12.5 had an adverse RFS of 50% compared to 95% for the patients with S-pyruvate <12.5 (p<0.0001; HR=13.6; 95% CI 2.61-70.6; Figure 5C). The same pattern was observed in the BCSS and OS analyses for these three prognostic variables (Figure 5E-I). Notably, only one contralateral relapse occurred in the fasting group – all others were in the carbohydrate group. Even though the relapses were restricted to patients with T2 tumors, tumor category was not an independent prognostic factor in the multivariable analyses. In the multivariable analysis for RFS, S-pyruvate was the only factor left in the final model (HR=12.8; 95% CI, 2.47 to 66.8), and only S-lactate remained in the final multivariable model for BCSS (HR=14.8; 95% CI 1.54 to 142). Furthermore, S-pyruvate was the sole factor to reach significance in the multivariable model of the OS analysis (HR=18.2; 95% CI 2.03 to 164).

Pathway analyses

In the Pathway analyses, MetaboAnalyst and IPA showed complimentary information. Quantitative metabolite set enrichment analysis (MSEA) identified biologically meaningful patterns in serum metabolite concentration changes (Figure 6A and Appendix Table 5). Significantly enriched pathways included energy associated metabolic pathways (amino sugar metabolism and pyruvate metabolism which links to glutamate metabolism, the citric acid cycle, gluconeogenesis and the Warburg effect). IPA showed the main functions of the involved metabolites as cellular growth and proliferation, molecular transport, small molecule biochemistry, carbohydrate metabolism and amino acid metabolism (Figure 6B). Interestingly, the metabolites showed a pattern congruent with growth of organism (Figure 6C) with metabolites increased in carbohydrate-fed patients activating growth pathways, and

downregulation of metabolites acting as inhibitors of growth. Finally, four (miR-26a-5p, miR-30c-5p, miR126-3p and miR-210-3p) out of the seven microRNAs found to be involved in resistance to tamoxifen in our previous review [27] could indirectly be associated with the metabolic network through insulin signaling pathways (Figure 6D). The same metabolic pathways were evident when only ER positive patients were considered.

Discussion

We present the first study to examine the effect of per-oral preoperative carbohydrate load on perioperative metabolism in operable breast cancer patients. Among the 15 different serum metabolites that distinguished fasting from the per-oral carbohydrate load, we observed increased systemic lactate and pyruvate, decreased ketone bodies, increased glycerol, and reduced amino acids in the patients who received the carbohydrate load. Moreover, we found highly significant positive correlations between S-insulin and S-lactate and S-pyruvate. Thus, changes in these 15 key metabolites are consistent with increased glycolysis, increased ketolytic activity, reduced lipolysis, and reduced proteolysis, which are exactly the same metabolic modifications seen after carbohydrate challenge in healthy persons [28]. Being able to capture these well-known metabolic effects of insulin increases the reliability of our model to detect other changes that may follow a carbohydrate load.

It may be considered that 18 hours is too short to expect effect of the carbohydrate load on tumor cell proliferation and metabolism. However, in vitro studies show that glucose fed MCF-7 cells increase their proliferation after 12-24 hours [3] .Others found the same pattern in three different breast cancer cell lines [29]. As the cell lines lack the in vivo endocrine response to glucose the increased proliferation was based on GTP-ase driven phosphorylation of EGFR with increased activity and longevity of this receptor as a consequence. Also, animals fed with

a diet containing increased glucose show an increased epithelial mesenchymal transition (EMT) [30]

’ The increased S-lactate and S-pyruvate in the carbohydrate patients stems primarily from two sources. Firstly, lactate is the product of glycolysis, especially in muscle cells, and is transported to the liver for conversion back to glucose, known as the Cori cycle [31]. The intended effect of preOP is to contra act and reduce insulin resistance that follows surgical stress[32]. This stressor leads to reduced mitochondrial ATP production and lactate formation[33, 34]. In healthy individuals, an oral glucose tolerance test (OGTT) showed a negative correlation between differences in S-glucose concentrations and differences in S-lactate levels (i.e. a rise in S-glucose leads to a reduction in S-lactate) [35]. Moreover, during 180 minutes after a OGTT among non-insulin dependent diabetic mellitus (NIDDM) patients there was no significant alteration in S-lactate levels [36]. Thus, it is unlikely that preOp itself creates a systemic lactate production. Therefore, S-lactate in our patients may come from excretion of intracellular lactate and pyruvate produced in the breast cancer cells. Consequently, lactate and pyruvate in the present study are probably translocated into the systemic circulation via mono carboxylate transporter type 4 (MCT-4), which is a known part of the Warburg effect [13]. Despite the fact that systemic metabolite concentrations are functional read outs of the numerous homeostatic reactions in the body, which will blur the contribution from the cancer cell metabolism to the serum levels[18], our present observation of positive correlation between larger tumor size and increasing S-lactate is supported by Hui S et al. [37].. Also, the positive correlation between proliferation and tumor size solely occurs in the carbohydrate group this suggests that carbohydrate exposure to larger tumors (i.e.T2 tumors) increases both proliferation and S-lactate. Thus, this indicates that lactate from the Warburg effect in the tumor cells may have a substantial contribution to the systemic lactate and pyruvate levels. This observation also adheres to the lack of correlation between intra tumor lactate/pyruvate and fasting/carbohydrate

status in the present study, as the former are probably excreted from the cells into the systemic environment.

Moreover, tumor cells not only produce lactate for excretion through MCT-4, they are also able to take up systemic circulating lactate and pyruvate via the MCT-1 transporters [38]. Regardless of the source, systemic lactate and pyruvate will certainly benefit the free CTCs shed from the tumor during surgery that are on their way to distant tissue to form micrometastases [39], but may also benefit the preoperatively established occult micrometastases [40, 41]. Lactate and pyruvate are the most preferred substrates for lactate/pyruvate dehydrogenase (LDH/PDH), ensuring a 1:1 ratio between lactate and pyruvate when equilibrium is reached. Thus, LDH provides substrate for both the production of ATP via the tricarboxylic acid (TCA) cycle [37] and also increased gluconeogenesis for the production of ribose for nucleotide synthesis via the pentose phosphate pathway (PPP) [38]. Notably, increased levels of serum LDH [42] and increased expression of LDH in breast cancer tissue [43] and lung tumors [44] are associated with an inferior prognosis.

In line with other studies [45], we observed a positive correlation between higher proliferation and increased glutamate content in tumor tissue. Glutamate is a metabolic product of glutaminolysis, which drives membrane trafficking to promote breast cancer cell invasiveness [46]. In addition, the expression of glutaminase genes *GLS* and *GLS2* correlates with increased tumor growth rates [47]. Many tumors become glutamine-dependent, as it serves as a direct route into the TCA cycle at the alpha-ketoglutaric acid level with consequential ATP production. Together with glycine and cysteine, glutamate is a precursor to the tripeptide glutathione, which is an antioxidant molecule that serves to 'buffer' superoxide insults encountered in the tumor microenvironment [45]. Glutathione is the major thiol-containing endogenous antioxidant and serves as a redox buffer against various sources of oxidative stress. In tumors, maintaining a supply of glutathione is critical for cellular survival because it allows

cells to resist the oxidative stress associated with rapid metabolism, DNA-damaging agents, and inflammation, among others [48, 49]. Glucose metabolism and biosynthesis of glutathione are often modulated by the PI3K/Akt pathway, which is often dysregulated in breast cancer tumors [50, 51]. Importantly, one of the effects of targeting the PI3K/Akt-pathway upstream [52] and downstream [19] is reduced glutathione content in tumor cells. In the PPP-pathway, NAD+ and NADP are converted into NADH and NADPH, respectively, which contribute to maintaining glutathione (GSSG) in the reduced state (GSH) [53]. Thus, the PPP-pathway in the Warburg effect secures a high intracellular level of glutathione, which is regarded as the most important cellular protection system against attack from reactive oxygen species (ROS) in both dividing and hibernating luminal cells [10], and also in cancer stem cells [54]. Thus, preoperative carbohydrate loading seems to create a doubly favorable environment that will probably serve the CTCs liberated during surgery [39] more than the already established micrometastases [55]. First, CTCs have a surplus of cellular fuel via lactate and pyruvate available systemically. Second, they benefit from an increased level of intracellular protection systems against ROS via increased tumor glutathione. Both effects will increase the probability of CTCs thriving and surviving as micrometastases, which then may erupt as clinical relapse years later, compatible with the tumor biology of luminal breast cancers. However, our observed clinical endpoint between 3 to 7 years must be regarded as 'early relapses' when coming to luminal cancers [56]. Thus, we need a much longer follow up to capture the late recurrences in order to get the correct picture of the clinical outcome of the present study.

Several attempts have been made to reverse the above-mentioned metabolic pathways for treatment purposes. The first attempt was to reverse the Warburg effect with the polyphenol resveratrol, which blocks PDH/LDH. In colon cancer cells, resveratrol inhibits proliferation, gluconeogenesis, and PPP [57]. By blocking PDH, resveratrol promotes mitochondrial electron transport chain overload with increased ROS production, ultimately resulting in apoptosis [58].

Secondly, a ketogenic diet has been shown to be effective in preclinical studies [59]. A ketogenic diet produces a large amount of intracellular ketone bodies that have a direct cytotoxic effect. Furthermore, the ketogenic state inhibits insulin/IGF signaling and downstream signaling pathways, such as PI3K/Akt/mTOR [60]. Interestingly, in the present study, the patients in the fasting group reached a ketogenic state with increased ketone bodies, which may have created an unfavorable environment for the cancer cells in the tumor and for the liberated CTCs. This is in line with a recent RCT of using ketogenic diet as adjuvant treatment in one of the study arms. They observed a better overall survival in the group that received ketogenic diet[61]. Others have recently shown a profound effect of ketogenic diet in a xenografted breast cancer mouse model with increased ketone bodies and increased aminoacidic [62], which is in line with our observations. The authors hypothesize that the anti-cancer effect may be mediated through immunological mechanisms[62]. Thus, use of a ketogenic diet as adjuvants to conventional therapy is rooted in several studies [63].

Likewise, physical activity is known to prevent and improve survival in several cancer forms and is thus recommended as a measure to both prevent and treat breast cancer [64] [65].One of the mechanisms behind these observation is a change in the estrogen metabolism after 180 minutes exercise pr. week. They found an increased 2 hydroxy-estrone level known to antagonize the estradiol action [66] This observation is important for both in the preventive setting as breast cancer risk is correlated to total life exposure of estrogens [67]. Also, changes in diet affect the cancer incidence [68], and also prognosis in breast cancer patients [69].

A combination of calorie restriction and physical exercise in postmenopausal women did also reduce insulin levels [70]. In our patients, we found that metabolic changes after the carbohydrate load affected the ER-positive breast cancer patients. Thus, ketogenic diet combined with physical exercise would probably be beneficial for our patients as this approach will affect both the ER and insulin signaling pathways.

Interestingly, intermittent fasting (i.e. caloric restriction for 16-48 hours [71] has been proven to affect the metabolism and disease process in a beneficial manner. Notably, intermittent fasting in animal studies have demonstrated reduction of tumor size [72]. In humans, intermittent fasting improves insulin sensitivity and thus reduces insulin and IGF-1 related signaling in over weighted individuals.[72, 73] Preclinical studies show that intermittent fasting more than 2 days is as effective as chemotherapy to reduce cancer load [74]. Thus, the ketones derived from intermittent fasting decreases cancer cell viability by attacking several hallmarks of cancer [75]

The IPA-analyses confirmed that the systemic response to the carbohydrate load converge towards pathways involved in proliferation and growth of the organism. Moreover, other pathways related to the Warburg effect were also involved. Thus, peroral preoperative carbohydrate load shifts the systemic metabolism towards a very fortunate and beneficial environment for CTCs liberated from the tumor under the operation. Interestingly, four out of seven microRNAs related to endocrine resistance [27] also regulate the same metabolic pathways through insulin signaling pathways, which are known to be involved in endocrine resistance with reduced effect of tamoxifen and aromatase inhibitors. Thus, it seems plausible to introduce metformin early on as adjuvant treatment to regain the endocrine sensitivity. Intriguingly, circulating microRNAs from the tumor in exosomes [76] can perform cell-independent microRNA biogenesis and promote tumorigenesis away from the primary tumor[77]. Thus, we may speculate that one of the steps in the metastatic process is to control the systemic metabolic pathways to ensure a beneficial environment and survival of the liberated cancer cells. [54] Moreover, increased cellular uptake of glucose via the Warburg effect [10] favor differentiating glycosylation of intracellular proteins included paucimannosylation [78]. Intriguingly, the metastatic Epithelial-Mesenchymal-Transition (EMT) process is regulated through glycosylation of key regulator proteins, that are frequently

modulated via the insulin /IGF signaling [79]. Thus, glycosylation opens up a connection between the glucose/insulin signaling and increased survival of CTCs trough enhancement of the EMT-processes.

Taken together, this explorative study indicates that the carbohydrate loading state and fasting state have opposite systemic and micro-environmental effects, which may explain why the relapses in the present study were skewed towards the carbohydrate group, with an inferior RFS, BCSS, and OS in patients with high tissue glutathione, high S-lactate, and high S-pyruvate. The favorable macro- and micro-environmental changes for the tumor that come from carbohydrate loading reflect the Warburg effect, which serves the CTCs and micrometastases more than the patient [80]. In luminal cancers, the Warburg pathway enzyme PFKFB4 acts as a molecular fulcrum that couples sugar metabolism to transcriptional activation by stimulating the ER co-activator SRC-3 to promote aggressive metastatic tumors [81].

The present study has several weak points. First, it is a post hoc explorative analysis of an RCT. Therefore, the various analyses are not sufficiently powered regarding the various endpoints. In addition, tissue samples were not available for all patients, which reduces the number of patients in the various analyses. Thus, this creates a greater risk of a type II error than a type I error. Furthermore, the tissue analyses were skewed towards patients with larger tumors. This could introduce systematic error in the analysis. However, tumor size was not included in the final Cox models in any survival analysis, indicating that this error was not strong enough to blur the effects of the metabolites. Also, including diet recalls and demographic data of the patients would have strengthened the study. Detecting the well-known endocrine metabolic fingerprint of insulin strengthens the method and the reliability of the various findings in this study. However, the study is too small to conclude on preoperative preparation guidelines; fasting or carbohydrate loading. Moreover, the pilot nature of the present study calls for validation in a larger study with a long-term follow-up. Introducing a ketogenic diet as a third

study arm may test out whether ketone bodies could wipe out the liberated CTCs and thus improve survival.

Conclusion

Preoperative oral glucose loading increases systemic levels of lactate and pyruvate, and tumor levels of glutathione and glutamate in luminal breast cancer patients. In fasting patients, the proapoptotic ketone bodies are increased. These biological changes may contribute to the survival differences observed between these two study groups. Integrated Pathway Analysis (IPA) in serum revealed activation of five major anabolic metabolic networks contributing to proliferation and growth mainly through insulin signaling pathways.

Abbreviations

ATP: adenosine triphosphate

AUC: area under the curve

BCSS: breast cancer specific survival

CI: confidence interval

CPMG: Carr–Purcell–Meiboom–Gill spectra

CTC: circulating tumor cell

ER: estrogen receptor

EMT: epithelial mesenchymal transition

ERAS: enhanced recovery after surgery

GSSG: glutathione, oxidized form

GSH: glutathione, reduced form

HER2: human epithelial growth factor receptor 2

HR: hazard ratio

HR-MAS-MR: high-resolution magic angle spinning - magnetic resonance

IGF1: insulin-like growth factor 1

IGF1R: insulin-like growth factor 1 receptor

IGFBP3: Insulin-like growth factor 1 binding protein 3

IHC: immunohistochemistry

IPA: Ingenuity Pathway Analysis

IR: insulin receptor

LDH: lactate dehydrogenase

MAI: mitotic activity index

MCT-1: monocarboxylate transporter 1

MCT-4: monocarboxylate transporter 4

MR: magnetic resonance

MRI: magnetic resonance imaging

MSEA: Metabolite Set Enrichment Analysis

NAD: nicotinamide adenine dinucleotide

NADP: nicotinamide adenine dinucleotide phosphate

NOESY: One-dimensional ^{1}H Nuclear Overhauser effect spectroscopy

NSD: Norwegian Center for Research Data

OS: overall survival

PHD: pyruvate dehydrogenase

PLS: partial least square

PLS-DA: partial least square discriminant analysis

PPH3: phosphorylated phosphohistone 3

PPP: pentose phosphate pathway

PR: progesterone receptor

RCT: randomized controlled trial

ROS: reactive oxygen species

RFS: relapse free survival

S: Serum

SRC-3: steroid receptor co-activator 3

TCA-cycle: tri carboxyl acid cycle

Declarations

Ethics approval and consent to participate

The RCT, which this study is based upon, was approved by the Regional Ethics Committee (Accession number 2015/1445), Norwegian Centre for Research Data (#20984), and The Norwegian Biobank Registry (#2239). An informed consent form was signed by each patient. The trial was retrospectively registered at Clinicaltrials.gov (NCT03886389).

Consent for publication

Not applicable

Availability of data and material

The data that support the findings of this study are available from Stavanger Breast Cancer Research Group, but restrictions apply to the availability of these data, which were used under license for the current study and as such are not publicly available. However, data are available from the authors upon reasonable request and with permission from Stavanger Breast Cancer Research Group.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

THL included and operated on all of the patients, built the database, and contributed to statistical analyses and interpretation of data. MA contributed to analyses of the metabolite spectra of all serum and tissue samples and also to the IPA analyses. TFB provided all the metabolomic analyses in her MR-imaging lab and interpreted the results of the metabolic spectra. AV obtained all of the blood samples and performed the laboratory analyses. IS contributed to the laboratory analyses. EG performed surgical pathological analysis with histological grading and morphological analysis of the tumor. NGE and SL provided detailed information of the micro RNA in the IPA analyses. LAA contributed with scientific support and advice. KJ performed the IPA analyses. EAM contributed to the concept of the study, assessed the pathological parameters and scorings, and participated in interpreting the data. HS contributed to the concept of the study, statistical analyses and interpretation of data. JPAB contributed to the concept of the study and the analysis and interpretation of the data.

All co-authors contributed to writing the manuscript and gave their final approval of the last version to be published.

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Tables

Table 1

Clinical variables in the randomized groups

	Carbohydrate group (N=26)	Fasting group (N=35)	Carbohydrate group with tissue (n=16)	Fasting group with tissue (n=13)
Age				
<55	12 (46%)	16 (46%)	9 (56%)	7 (53%)
≥ 55	14 (54%)	19 (54%)	7 (44%)	6 (46%)
Lymph Node status				
Negative	19 (70%)	25 (71%)	11 (69%)	9 (69%)
Positive	8 (30%)	10 (29%)	5 (31%)	4 (31%)
Tumor size (pT)				
pT1 (<2cm)	16 (61%)	30 (85%)*	7 (44%)	9 (69%)
pT2 (≥2cm)	10 (39%)	5 (14%)	9 (57%)	4 (31%)
Grade				
1	4 (15%)	7 (20%)	2 (13%)	2 (15%)
2	10 (37%)	20 (57%)	4 (25%)	7 (53%)
3	13 (48%)	8 (23%)	10 (63%)	4 (31%)
ER status				
Positive	21 (81%)	29 (83%)	11 (69%)	9 (69%)
Negative	5 (19%)	6 (17%)	5 (31%)	4 (31%)
PR status*				
Positive	13 (50%)	28 (80%)**	7 (44%)	11 (85%)
Negative	13 (50%)	7 (20%)	9 (56%)	2 (15%)
HER2 status				
Negative	23 (88%)	34 (97%)	13 (81%)	12 (92%)
Positive	3 (12%)	1 (3%)	3 (19%)	1 (8%)
MAI				
<10	14 (56%)	27 (77%)	6 (38%)	10 (77%)
≥ 10	11 (44%)	8 (23%)	10 (62%)	3 (23%)
PPH3				
<13	14 (56%)	21 (60%)	7 (44%)	6 (46%)
≥ 13	12 (44%)	14 (40%)	9 (56%)	7 (54%)
Ki67				
≥ 15	17 (65%)	17 (50%)	3 (19%)	5 (42%)
<15	9 (35%)	17 (50%)	13 (81%)	7 (58%)
≥ 30	12 (46%)	10 (29%)	6 (38%)	8 (67%)
<30	14 (54%)	24 (71%)	10 (62%)	4 (33%)
TILs				
<10%	24 (92%)	31 (89%)	15 (94%)	13 (100%)
≥10%	2 (8%)	4 (11%)	1 (6%)	0 (0%)
End of follow-up status				
No distant metastasis	22 (85%)	33 (94%)	11 (67%)	11 (85%)
Distant metastasis	4 (15%)	2 (6%)	3 (20%)	1 (7%)

*Significantly different between fasting and carbohydrate group (Fisher's exact test)

** p=0.052 in tissue subset

Table 2

Serum metabolites with p-values from t-tests, fasting group versus carbohydrate (CH), for all patients and for the ER positive subset.

Metabolite	p-value*	Fold change	p-value* ER+	Fold change ER+
3-Hydroxybutyrate	**0.010**	**-1.06**	**0.010**	**-1.07**
Acetate	**<0.001**	**-1.22**	**<0.001**	**-1.21**
Acetoacetate	**<0.001**	**-1.25**	**<0.001**	**-1.20**
Acetone	0.250	-1.18	0.508	-1.11
Alanine	0.692	1.01	0.544	-1.02
Asparagine	0.237	-1.05	0.376	-1.04
Citrate	0.503	1.03	0.726	1.01
Creatine	0.905	-1.01	0.704	-1.02
Creatinine	0.066	-1.06	0.039	-1.07
Dimethylsulfone	0.319	-1.09	0.154	-1.15
Glucose	0.969	1.00	0.972	1.00
Glutamine	**0.005**	**-1.06**	**0.013**	**-1.07**
Glycerol	0.065	-1.05	0.054	-1.06
Glycoprotein	0.243	-1.06	0.408	-1.05
Isoleucine	**<0.001**	**-1.26**	**0.001**	**-1.22**
Isopropyl alcohol	**0.009**	**-1.12**	0.038	-1.10
Lactate	**<0.001**	**1.36**	**<0.001**	**1.26**
Leucine	**<0.001**	**-1.20**	**0.002**	**-1.17**
Lysine	**<0.001**	**-1.12**	**<0.001**	**-1.11**
Methanol	0.495	-1.04	0.511	-1.04
Methionine	0.052	-1.11	0.062	-1.11
N-acetylated groups	**<0.001**	**-1.15**	**<0.001**	**-1.15**
Phenylalanine	**<0.001**	**-1.12**	**<0.001**	**-1.13**
Proline	0.298	-1.03	0.236	-1.03
Propylene Glycol	**<0.001**	**-1.13**	**0.004**	**-1.10**
Pyruvate	**<0.001**	**1.27**	**<0.001**	**1.23**
Threonine	**0.035**	-1.07	**0.016**	**-1.08**
Valine	**<0.001**	**-1.31**	**<0.001**	**-1.29**

Abbreviations: ER+, Estrogen Receptor positive.

*Significant at $p \leq 0.016$ after Benjamini-Hochberg correction for multiple testing

Table 3

Serum metabolite values correlated to insulin (Pearson's correlation) for the total study population, and the carbohydrate and fasting groups separately.

Metabolite	R (All)	P (All)*	R (CH)	P (CH)	R (F)	P (F)
Lactate	**0.57**	**<0.001**	0.31	0.136	**0.70**	**<0.001**
pyruvate	**0.54**	**<0.001**	0.26	0.203	**0.54**	**0.001**
Acetate	**-0.53**	**<0.001**	-0.40	0.046	-0.22	0.212
N.acetylgroups	**-0.41**	**0.001**	-0.06	0.788	0.10	0.576
Acetoacetate	**-0.34**	**0.008**	-0.04	0.847	0.21	0.221
Valine	-0.31	**0.016**	0.31	0.137	0.28	0.105
Lysine	-0.29	**0.027**	0.01	0.947	**0.43**	**0.010**
Citrate	0.28	**0.029**	0.27	0.192	**0.50**	**0.002**
Isoleucine	-0.28	**0.030**	-0.03	0.881	0.36	**0.035**
Glucose	0.26	**0.043**	0.40	0.047	-0.09	0.622
Propylene_Glycol	-0.24	0.062	0.07	0.748	0.21	0.219
Creatine	-0.23	0.075	-0.39	0.054	-0.18	0.292
Leucine	-0.23	0.079	0.17	0.425	0.25	0.149
Phenylalanine	-0.19	0.149	0.43	0.033	0.24	0.163
Glycerol	-0.19	0.152	-0.12	0.555	0.11	0.541
Alanine	0.15	0.262	0.18	0.386	0.30	0.076
Isopropyl alcohol	-0.12	0.344	0.20	0.331	0.12	0.500
3-Hydroxybutyrate	-0.10	0.442	0.12	0.562	0.36	**0.035**
Methanol	-0.10	0.457	-0.06	0.778	-0.05	0.761
Glutamine	-0.09	0.506	0.30	0.150	0.06	0.737
Creatinine	-0.08	0.543	0.23	0.263	-0.05	0.755
Threonine	-0.08	0.567	0.10	0.627	0.32	0.062
Acetone	0.04	0.780	0.15	0.489	0.39	**0.019**
Proline	-0.04	0.789	0.11	0.593	-0.03	0.880
Glycoproteins	0.02	0.873	0.06	0.787	**0.49**	**0.003**
Asparagine	-0.01	0.923	-0.05	0.818	**0.47**	**0.005**
Methionine	0.01	0.941	0.37	0.067	0.08	0.653
Dimethylsulfone	0.00	0.997	0.17	0.404	0.05	0.777

Abbreviations: CH, carbohydrate group; F, Fasting group; P, Pearson's correlation p-value; R, Pearson's correlation R value.

*Significant at $p \leq 0.043$ after Benjamini-Hochberg correction

** Significant at $p \leq 0.03$ after Benjamini-Hochberg correction

*** Significant at $p \leq 0.035$ after Benjamini-Hochberg correction

Table 4

Tumor metabolites with fold changes and t-test p-values in carbohydrate vs fasting groups

Tumor metabolite	P (All)*	FC (All)	P (ER+) **	FC (ER+)
Acetate	0.844	-1.030	0.620	-1.095
Alanine	0.322	1.038	0.163	1.067
Ascorbate	0.300	-1.099	0.991	-1.001
Aspartate	0.385	1.100	0.545	1.088
Choline	0.136	1.056	0.547	1.027
Creatine	0.418	-1.062	0.558	-1.051
Glucose	0.495	-1.151	0.500	-1.201
Glutamate	0.172	1.047	0.146	1.055
Glutamine	0.955	1.003	0.816	-1.015
Glutathione	**0.006**	**1.082**	**0.002**	**1.103**
Glycerophosphocholine	0.712	-1.018	0.762	-1.018
Glycine	0.186	1.063	0.162	1.090
Lactate	0.862	1.006	0.922	1.004
Leucine	1.000	1.000	0.947	-1.004
Myoinositol	0.445	-1.038	0.768	-1.018
Phosphocholine	0.517	1.027	0.291	1.051
Phosphoethanolamine	0.211	1.050	0.544	1.031
Scylloinositol	0.926	-1.007	0.565	1.060
Succinate	0.788	1.022	0.503	1.067
Taurine	0.982	1.001	0.902	1.004

Abbreviations:, ER+, Estrogen receptor positive; FC, fold change, P, t-test p-value.

***** Significant at $p \leq 0.001$ after Benjamini-Hochberg correction

** Significant at $p \leq 0.030$ after Benjamini-Hochberg correction

Table 5.

Univariate analysis of Relapse Free Survival in ER+ patients

Variable	Events / At risk	% Survival	P	HR	95% CI
Fasting /Carbohydrate					
Fasting	1/29	97			
Carbohydrate	6/21	71	0.012	9.34	1.12 –77.7
S-Pyruvate *					
< 12.5	2/39	95			
≥ 12.5	5/10	50	<0.0001	13.59	2.61– 70.6
S-Lactate*					
< 56.9	3/40	93			
≥ 56.9	4/9	56	0.002	7.47	1.66 – 33.6
S-Preoperative Insulin					
< 18.3 I.U.	1/29	97			
≥ 18.3 I.U.	6/21	71	0.012	9.34	1.12 – 77.7
S-Preoperative					
C-peptide					
< 1.22 nM	1/29	97			
≥1.22 nM	6/21	71	0.011	9.51	1.14-79.0
Tumor					
Glutathione					
< 1.09	0/10	100			
≥ 1.09	3/8	63	0.038	Inf.	
Tumor size					
T1	3/40	93			
T2	4/10	60	0.003	7.09	1.57-31.9
Nodal status					

N0	3/33	91			
N+	4/17	73	0.160	2.80	0.625-12.6
Grade					
1	0/11	100			
2+3	7/39	82	0.136	31.1	0.019 – 50547
MAI*					
<10	4/39	90			
≥10	3/10	70	0.092	3.38	0.751–15.2
Ki67*					
<30%	3/37	92			
≥30%	4/12	67	0.023	4.84	1.08 – 21.8
PPH3					
<13	3/35	91			
≥13	4/15	73	0.116	3.13	0.699-14.0

*Missing information on one patient in the ER+ group leading to n=49 patients analyzed for this variable

Table 6.

Univariate analysis of Breast Cancer Specific Survival in ER+ patients

Variable	Events / At risk	% Survival	P	HR	95% CI
Fasting /Carbohydrate					
Fasting	0/29	100			
Carbohydrate	4/21	81	0.015	Inf.	
S-Pyruvate					
< 12.5	0/40	100			
≥ 12.5	4/10	60	<0. 0001	Inf.	
S-Lactate*					
< 56.9	1/40	98			
≥ 56.9	3/9	67	0.002	14.8	1.53-142
S-Preoperative Insulin					
< 18.3 I.U.	0/29	100			
≥ 18.3 I.U.	4/21	81	0.015	Inf.	
S-Preoperative					
C-peptide					
< 1.22 nM	0/29	100			
≥1.22 nM	4/21	81	0.015	103	0.025-429676
Tumor					
Glutathione					
< 1.09	0/10	100			
≥ 1.09	3/8	63	0.038	Inf.	
Tumor size					
T1	0/40	100			
T2	4/10	60	<0.0001	Inf.	
Nodal status					

N0	1/33	97			
N+	3/17	82	0.080	5.92	0.615 – 56.9
Grade					
1	0/11	100			
2+3	4/39	90	0.277	30.1	Inf.
MAI*					
<10	2/39	95			
≥10	2/10	80	0.124	4.12	0.580- 29.3
Ki67*					
<30%	1/37	97			
≥30%	3/12	75	0.014	9.91	1.03-95.3
PPH3					
<13	2/35	94			
≥13	2/15	87	0.399	2.27	0.320 – 16.1

*Missing information on one patient in the ER+ group leading to n=49 patients analyzed for this variable

Table 7.

Univariate analysis of Overall Survival in ER+ patients

Variable	Events /At risk	% survival	P	HR	95% CI
Carbo/Faste					
Faste	1/29	97			
Carbohydrate	4/21	81	0.068	6.02	0.675–53.8
S-Pyruvat*					
< 12.5	1/39	97			
≥ 12.5	4/10	60	<0.0001	19.2	2.14–172
S-Lactate*					
< 56.9	2/40	95			
≥ 56.9	3/9	67	0.009	7.58	1.26–45.4
S-Preop Insulin					
< 18.3 I.U.	1/29	97			
≥ 18.3 I.U.	4/21	81	0.068	6.016	0.672–53.9
S-Preoperative C-peptide					
< 1.22 nM	1/29	97			
≥1.22 nM	4/21	81	0.068	6.02	0.672–53.9
Tissue Glutathione					
≤1.0855	1/10	90			
>1.0855	3/8	63	0.140	4.72	0.488–45.7
Tumor size					
T1	1/40	98			
T2	4/10	60	< 0.0001	19.2	2.20 –176
Nodal status					
N0	2/33	94			

N+	3/17	82	0.205	3.01	0.502–18.0
Grade					
1	0/11	100			
2+3	5/39	87	0.222	30.2	0.004–223736
MAI*					
<10	3/39	92			
≥10	2/10	80	0.235	2.83	0.471–16.9
Ki67*					
<30%	2/37	95			
≥30%	3/12	75	0.049	5.040	0.842–30.2
PPH3					
<13	3/35	91			
≥13	2/15	87	0.641	1.53	0.255–9.13

*Missing information on one patient in the ER+ group leading to n=49 patients analyzed for this variable

Appendix Table 1

ROC – analysis with 'Relapse / No relapse' as dichotomous variable in ER+ patients.

Test variable	AUC	95% CI	Sens	Spec	P	Threshold
S-lactate	0.769	0.609 – 0.929	57	88	0.024	56.9
S-pyruvate	0.765	0.541 – 0.989	71	86	0.026	12.5
Tumor-Glutathione	0.711	0.485 – 0.938	100	66	0.260	1.09
S-preoperative Insulin	0.724	0.554 – 0.896	86	67	0.059	18.3 I.U./L
S-preoperative insulin c-peptide	0.735	0.566 – 0.903	86	67	0.049	1.22 nM

Appendix Table 2

Metabolites correlated to serum insulin C peptide (Pearson's correlation) for all patients, carbohydrate group, and fasting groups.

Metabolite	R All	P All*	R CH	P CH*	R F	P F**
Lactate	**0.611**	**<0.001**	0.401	0.047	**0.577**	**<0.001**
Pyruvate	**0.596**	**<0.001**	0.395	0.051	**0.431**	**0.010**
Acetate	**-0.513**	**<0.001**	-0.344	0.092	-0.092	0.598
N-acetylgroups	**-0.398**	**0.002**	-0.092	0.663	**0.397**	**0.018**
Valine	**-0.366**	**0.004**	0.189	0.366	**0.385**	**0.023**
Acetoacetate	**-0.352**	**0.006**	-0.058	0.781	0.348	**0.041**
Isoleucine	**-0.333**	**0.009**	-0.166	0.428	**0.488**	**0.003**
Lysine	-0.301	**0.020**	-0.064	0.763	**0.646**	**<0.001**
Propylene Glycol	-0.260	**0.045**	0.044	0.833	0.305	0.075
Citrate	0.244	0.060	0.262	0.205	0.348	**0.041**
Leucine	-0.242	0.063	0.125	0.551	**0.416**	**0.013**
Glucose	0.233	0.074	0.345	0.092	0.187	0.282
Creatine	-0.209	0.110	-0.444	0.026	0.005	0.978
Phenylalanine	-0.206	0.115	0.357	0.080	**0.503**	**0.002**
Methanol	-0.185	0.157	-0.162	0.438	-0.269	0.118
Glycerol	-0.152	0.246	-0.111	0.596	0.323	0.059
Glutamine	-0.152	0.248	0.216	0.300	0.058	0.740
Alanine	0.150	0.252	0.207	0.320	0.303	0.077
Isopropyl alcohol	-0.134	0.307	0.185	0.375	0.222	0.199
Threonine	-0.108	0.409	0.000	1.000	**0.462**	**0.005**
3-Hydroxybutyrate	-0.098	0.455	0.153	0.464	**0.439**	**0.008**
Creatinine	-0.077	0.557	0.176	0.399	0.158	0.365
Dimethylsulfone	0.076	0.565	0.264	0.202	0.332	0.051
Acetone	0.027	0.835	0.078	0.710	**0.530**	**0.001**
Proline	-0.014	0.913	0.154	0.462	0.049	0.778
Asparagine	-0.012	0.927	-0.126	0.547	**0.624**	**<0.001**
Glycoproteins	0.012	0.928	0.003	0.989	**0.606**	**<0.001**
Methionine	0.010	0.938	0.412	0.041	0.138	0.430

***Significant at p≤ 0.045 after Benjamini-Hochberg correction**

****Significant at p≤0.041 after Benjamini-Hochberg correction**

Appendix Table 3

Metabolites correlated to serum Insulin Growth Factor Binding Protein 3 (IGFBP3) (Pearson's correlation) for all patients, carbohydrate group, and fasting groups.

Metabolite	R (All)	P (All)*	R (CH)	P (CH)*	R (F)	P (F)*
Isoleucine	**0.424**	**0.001**	0.414	0.040	0.351	**0.039**
Glycoproteins	**0.410**	**0.001**	0.224	0.282	0.478	**0.004**
Asparagine	**0.401**	**0.001**	0.364	0.073	0.399	**0.018**
Leucine	**0.393**	**0.002**	0.107	0.612	0.440	**0.008**
Acetone	**0.383**	**0.003**	0.298	0.148	0.397	**0.018**
Lysine	**0.378**	**0.003**	-0.017	0.937	0.459	**0.006**
N.acetylgroups	**0.342**	**0.007**	-0.241	0.247	0.484	**0.003**
Phenylalanine	**0.322**	**0.012**	0.114	0.586	0.314	0.066
Propylene-Glycol	**0.321**	**0.012**	0.044	0.833	0.349	**0.040**
Isopropyl-alcohol	**0.319**	**0.013**	-0.079	0.706	0.434	**0.009**
Alanine	**0.310**	**0.016**	0.100	0.635	0.413	**0.014**
Acetoacetate	0.243	**0.062**	0.036	0.866	0.192	0.268
Threonine	0.206	0.114	-0.250	0.228	0.395	**0.019**
Valine	0.196	0.134	0.050	0.814	0.082	0.640
Acetate	0.180	0.169	0.079	0.707	0.066	0.707
Lactate	-0.166	0.205	-0.347	0.090	0.207	0.232
pyruvate	-0.164	0.211	-0.287	0.164	0.131	0.453
Methionine	-0.158	0.228	-0.484	0.014	-0.066	0.704
Glycerol	0.137	0.296	-0.384	0.058	0.360	**0.034**
Proline	-0.124	0.345	-0.358	0.079	0.004	0.982
Creatine	0.103	0.435	0.175	0.401	0.051	0.773
Creatinine	0.084	0.522	-0.189	0.365	0.146	0.403
Methanol	0.079	0.549	0.288	0.162	-0.110	0.528
Glutamine	0.075	0.569	-0.176	0.399	0.123	0.481
Glucose	0.055	0.678	0.007	0.972	0.186	0.285
Citrate	0.031	0.812	-0.052	0.804	0.134	0.442
Dimethylsulfone	-0.005	0.967	-0.255	0.219	0.096	0.584
3-Hydroxybutyrate	-0.001	0.993	-0.320	0.119	0.046	0.794

Abbreviations: CH, carbohydrate group; F, Fasting group; P, Pearson's correlation p-value; R, Pearson's correlation R value.

*Significant at $p \leq 0.01$ after Benjamini-Hochberg correction for multiple testing

** Significant at $p \leq 0.037$ after Benjamini-Hochberg correction for multiple testing

*** Significant at $p \leq 0.04$ after Benjamini-Hochberg correction for multiple testing

Appendix Table 4

Metabolites correlated to serum Insulin Growth Factor 1 (IGF1) (Pearson's correlation) for all patients, carbohydrate group, and fasting groups.

Metabolite	R (All)	P (All)*	R (CH)	P (CH)*	R (F)	P (F)*
Methionine	-0.318	0.013	-0.591	**0.002**	-0.135	0.438
Isopropyl_alcohol	-0.314	0.015	-0.318	0.121	-0.330	0.052
Creatinine	-0.302	0.019	-0.381	0.061	-0.265	0.124
Proline	-0.274	0.034	-0.465	0.019	-0.095	0.585
Valine	-0.251	0.053	-0.122	0.563	-0.406	0.015
Propylene_Glycol	-0.230	0.077	-0.159	0.446	-0.291	0.089
Acetoacetate	-0.230	0.077	-0.168	0.421	-0.308	0.072
Methanol	-0.218	0.094	-0.001	0.996	-0.400	0.017
Acetone	-0.184	0.159	-0.061	0.772	-0.247	0.152
pyruvate	-0.173	0.186	-0.271	0.191	-0.179	0.303
Leucine	-0.160	0.222	-0.090	0.670	-0.208	0.231
3.Hydroxybutyrate	-0.157	0.230	-0.231	0.266	-0.114	0.515
Dimethylsulfone	0.155	0.238	0.032	0.880	0.245	0.155
Threonine	-0.152	0.248	-0.397	0.050	0.013	0.941
Lactate	-0.139	0.289	-0.323	0.115	-0.026	0.882
N.acetylgroups	-0.131	0.320	-0.385	0.057	-0.038	0.830
Glycerol	-0.125	0.342	-0.430	0.032	0.075	0.668
Lysine	-0.118	0.370	-0.169	0.418	-0.104	0.551
Glutamine	0.100	0.447	-0.053	0.801	0.257	0.136
Isoleucine	-0.096	0.468	0.116	0.582	-0.225	0.193
Acetate	-0.092	0.483	0.031	0.883	-0.148	0.395
Creatine	-0.087	0.507	-0.002	0.994	-0.154	0.378
Glycoproteins	-0.081	0.539	-0.059	0.779	-0.088	0.614
Citrate	0.063	0.631	0.115	0.584	0.014	0.936
Alanine	-0.048	0.717	-0.070	0.738	-0.048	0.783
Glucose	0.017	0.899	-0.008	0.969	0.079	0.652
Phenylalanine	0.011	0.931	0.253	0.222	-0.080	0.648
Asparagine	-0.006	0.962	0.393	0.052	-0.163	0.349

* Significant at $p \leq 0.002$ after Benjamini-Hochberg correction for multiple testing.

Abbreviations: CH, carbohydrate group; F, Fasting group; P, Pearson's correlation p-value; R, Pearson's correlation R value.

Appendix Table 5

Results from Quantitative Metabolite Set Enrichment Analysis

Metabolic pathway	Total Cmpd	Hits	Statistic Q (Expected 1.613)	Raw p	FDR
Amino Sugar Metabolism	33	3	24.50	0.000	0.000
Propanoate Metabolism	42	1	44.09	0.000	0.000
Valine, Leucine and Isoleucine Degradation	60	4	31.60	0.000	0.000
Pyruvate Metabolism	48	3	28.66	0.000	0.000
Phenylalanine and Tyrosine Metabolism	28	2	31.54	0.000	0.000
Fatty Acid Biosynthesis	35	3	25.58	0.000	0.000
Aspartate Metabolism	35	3	16.23	0.000	0.000
Ethanol Degradation	19	1	33.64	0.000	0.000
Tyrosine Metabolism	72	1	32.22	0.000	0.000
Butyrate Metabolism	19	1	32.22	0.000	0.000
Lysine Degradation	30	1	31.52	0.000	0.000
Biotin Metabolism	8	1	31.52	0.000	0.000
Carnitine Synthesis	22	1	31.52	0.000	0.000
Ammonia Recycling	32	3	14.09	0.000	0.000
Warburg Effect	58	5	13.16	0.000	0.000
Cysteine Metabolism	26	1	27.23	0.000	0.000
Pyruvaldehyde Degradation	10	1	27.23	0.000	0.000
Urea Cycle	29	3	13.38	0.000	0.000
Glutamate Metabolism	49	3	13.38	0.000	0.000
Gluconeogenesis	35	3	17.45	0.000	0.000
Ketone Body Metabolism	13	2	17.24	0.000	0.000
Glycolysis	25	2	13.61	0.000	0.000
Citric Acid Cycle	32	2	14.00	0.000	0.001
Glycine and Serine Metabolism	59	5	8.26	0.000	0.001
Alanine Metabolism	17	2	13.75	0.000	0.001
Transfer of Acetyl Groups into Mitochondria	22	3	9.34	0.001	0.001
Glucose-Alanine Cycle	13	3	9.17	0.001	0.002
Pyrimidine Metabolism	59	1	12.64	0.005	0.008
Nicotinate and Nicotinamide Metabolism	37	1	12.64	0.005	0.008
Purine Metabolism	74	1	12.64	0.005	0.008
Phenylacetate Metabolism	9	1	12.64	0.005	0.008
Threonine and 2-Oxobutanoate Degradation	20	1	7.42	0.035	0.048
Methionine Metabolism	43	1	6.38	0.052	0.065
Betaine Metabolism	21	1	6.38	0.052	0.065
Spermidine and Spermine Biosynthesis	18	1	6.38	0.052	0.065
Glycerolipid Metabolism	25	1	5.74	0.065	0.080
Galactose Metabolism	38	2	2.87	0.184	0.219
Arginine and Proline Metabolism	53	2	0.94	0.575	0.665
Glutathione Metabolism	21	1	0.27	0.692	0.743

Selenoamino Acid Metabolism	28	1	0.27	0.692	0.743
Tryptophan Metabolism	60	1	0.27	0.692	0.743
Sphingolipid Metabolism	40	1	0.00	0.969	0.969
Lactose Synthesis	20	1	0.00	0.969	0.969
Lactose Degradation	9	1	0.00	0.969	0.969

Legends to figures

Figure 1

Flowchart of study participants

Figure 2

Partial Least Square Discriminant Analysis (PLS-DA) in serum.

A) Scores plot showing serum samples from the fasting group (green) and carbohydrate group (red). The carbohydrate and fasting groups have significantly different metabolic profiles as evidenced by permutation testing.

B) Variable Importance in Projection (VIP) scores showing the top ~~15~~ 14 metabolites contributing to differences between the groups. The right column indicates increased (red) or decreased (green) metabolite in the indicated group.

Figure 3

Correlation between serum metabolic profile and serum insulin, insulin C-peptide, and IGFBP3. Samples from carbohydrate-fed patients are shown in red, while samples from fasting patients are shown in blue. Metabolites are colored according to their variable importance in projection (VIP) score and labeled when VIP≥1.

A) Measured insulin vs. predicted insulin levels based on metabolic profile (cross-validated measurements).

B) Metabolites versus regression coefficient for insulin. Increased S-glucose, S-lactate, and decreased S-Leucine are important to prediction of serum insulin from the metabolic profile.

C) Measured insulin C peptide vs. predicted insulin C-peptide levels.

D) Regression weight plot showing metabolites versus the regression coefficient for insulin C-peptide. Increased S-Glucose, S-Lactate, and decreased S-Leucine are important to prediction of serum insulin C-peptide from the metabolic profile.

E) Measured Insulin Growth Factor Binding Protein 3 (IGFBP3) vs. predicted IGFBP3 based on metabolic profile.

F) Regression weight plot showing metabolites versus the regression coefficient for IGFBP3. Increased S-Acetone, S-Glycoproteins, and S-Leucine are important to prediction of serum IGFBP3 from the metabolic profile.

Figure 4

A) Principal Component Analysis (PCA) of tumor metabolites. No grouping of fasting vs carbohydrate groups observed.

B) Glutathione levels in ER positive tumors.

C) ROC curve for classification into carbohydrate or fasting group by glutathione concentration in ER-positive tumors. AUC= 0.894; 95%CI=0.0.687-1.000, P=0.002.

Figure 5

Survival analyses for Tumor-Glutathione, Serum-lactate and Serum-pyruvate.

A-C: Relapse Free Survival (RFS); D-F: Breast Cancer Survival (BCSS);

G-I: Overall Survival (OS).

Figure 6

Pathway analyses in serum metabolites

A) Metabolite Set Enrichment Analysis of serum metabolism. Significantly enriched pathways are annotated in the pathway network. The circle size denotes significance of the pathway, and lines denote at least 25% shared metabolites in the pathways.

B) Ingenuity pathway analysis (IPA) bar chart showing the top 5 functions enriched in the dataset.

C) IPA pathway network showing the metabolites connected to four microRNAs found to be involved in tamoxifen resistance. Metabolites in green are downregulated in carbohydrate-fed patients, while metabolites in red are upregulated. MicroRNAs are colored purple.

D) IPA Function plot showing metabolites involved in organismal growth. Orange arrows indicate activation, while blue arrows indicate inhibition.

Appendix Figure 1

Receiver operating characteristics (ROC) analysis with ‘fasting / carbohydrate load’ as the dichotomous variable and T-glutathione (A), S-lactate (B) and S-Pyruvate (C) as the continuous variables. The area under the curve (AUC), sensitivity, specificity, p-values and criterion (threshold) value are listed under the reference line in each figure. The criterion is visualized with a red asterix on the ROC-curve.

Appendix Figure 2

Receiver operating characteristics (ROC) analysis with ‘relapse/no relapse’ as the dichotomous variable and T-glutathione (A), S-lactate (B) and S-Pyruvate (C) as the continuous variables. The area under the curve (AUC), sensitivity, specificity, p-values and criterion (threshold) value are listed under the reference line in each figure. The criterion is visualized with a red asterisk on the ROC-curve.

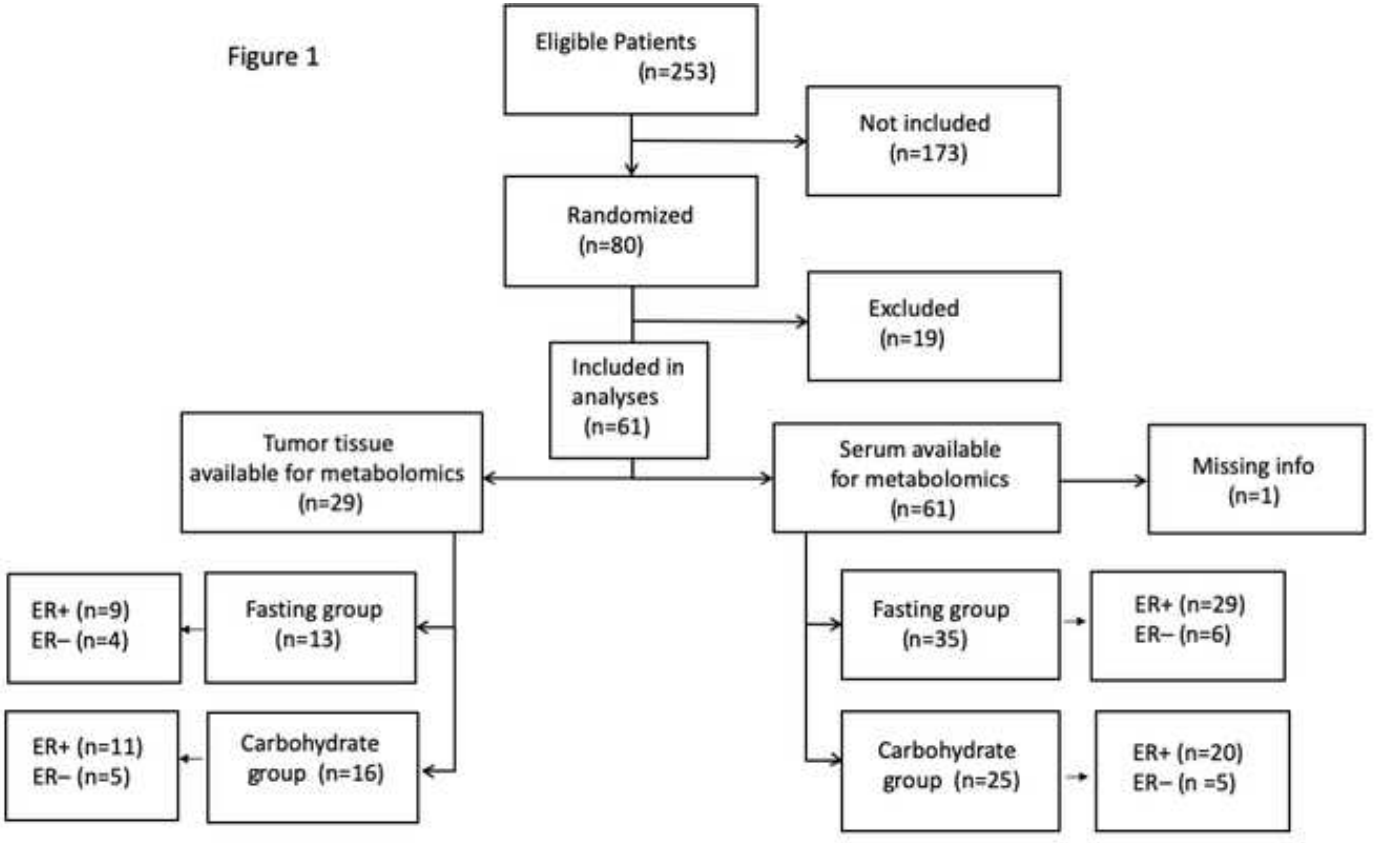
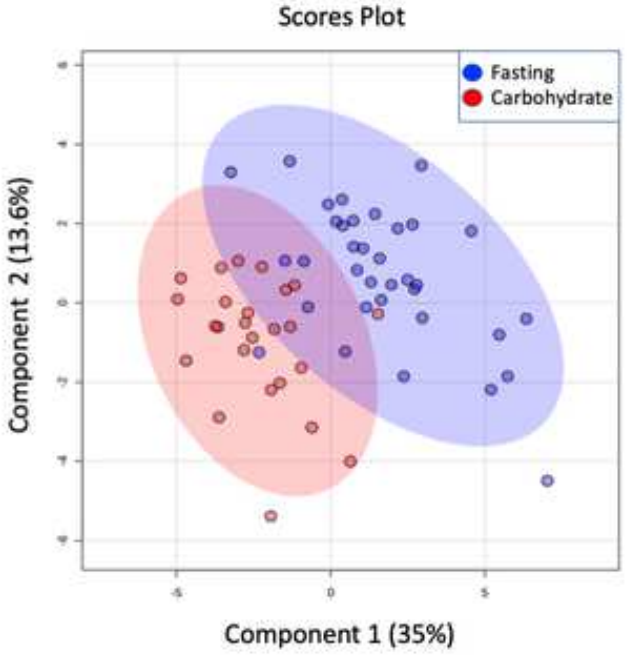


Figure 2A



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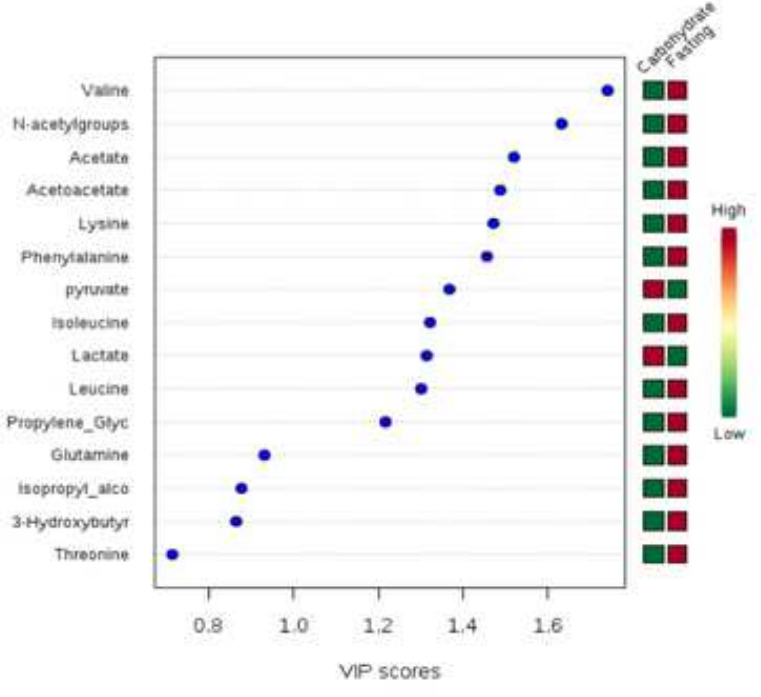


Figure 3

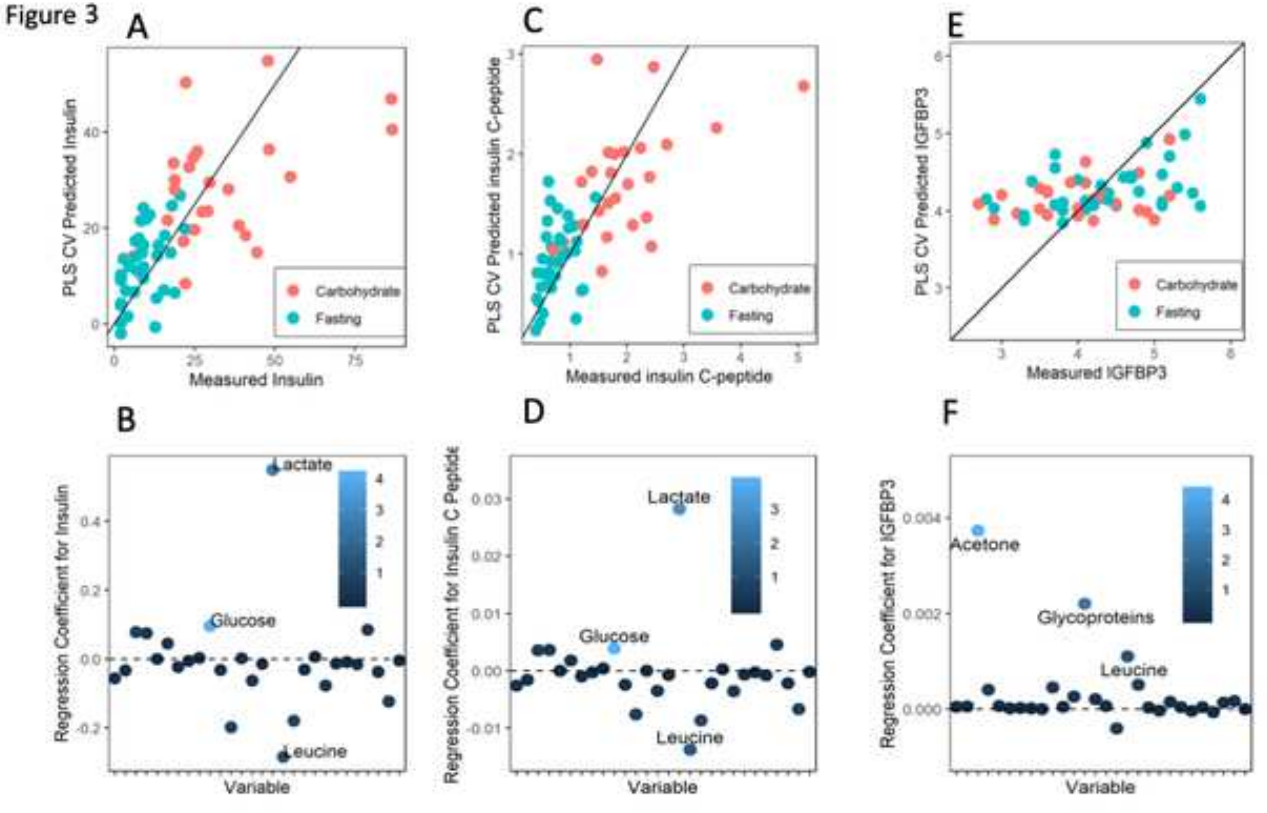
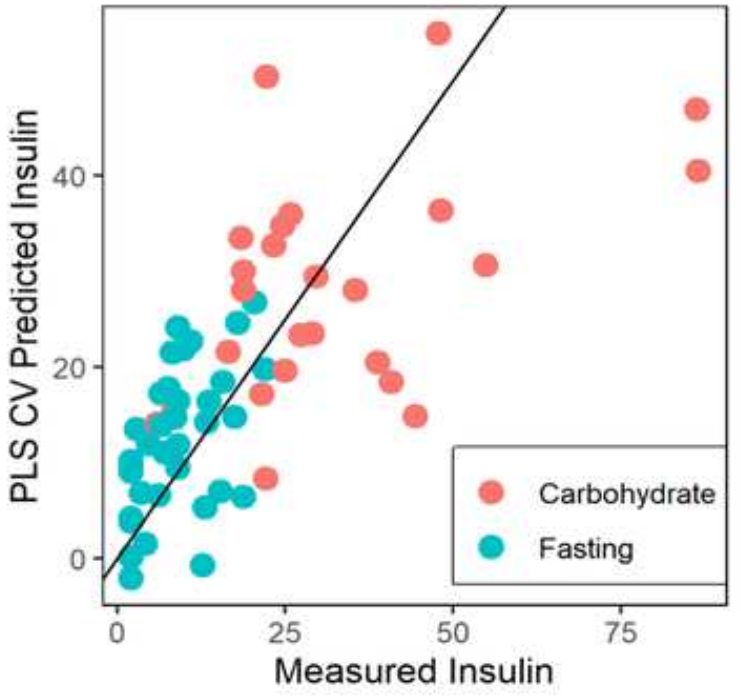


Figure 3A



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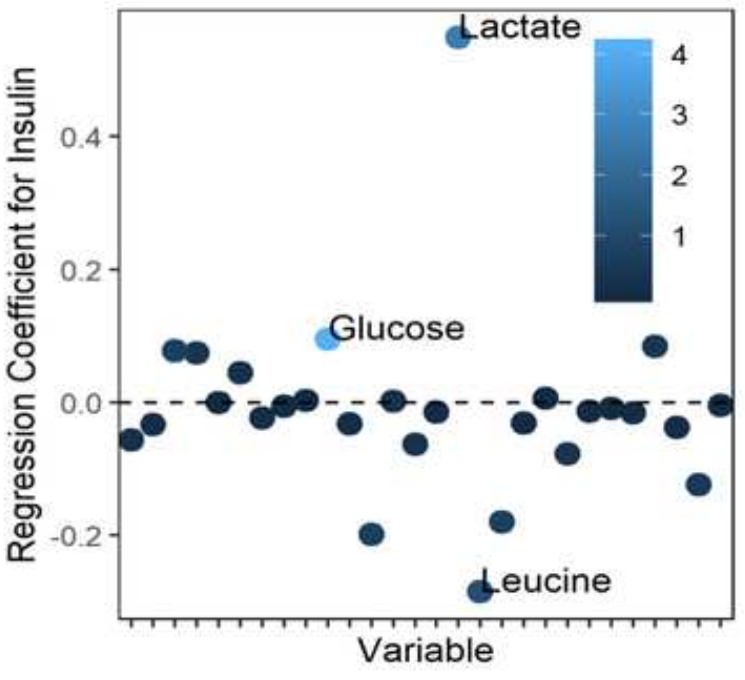

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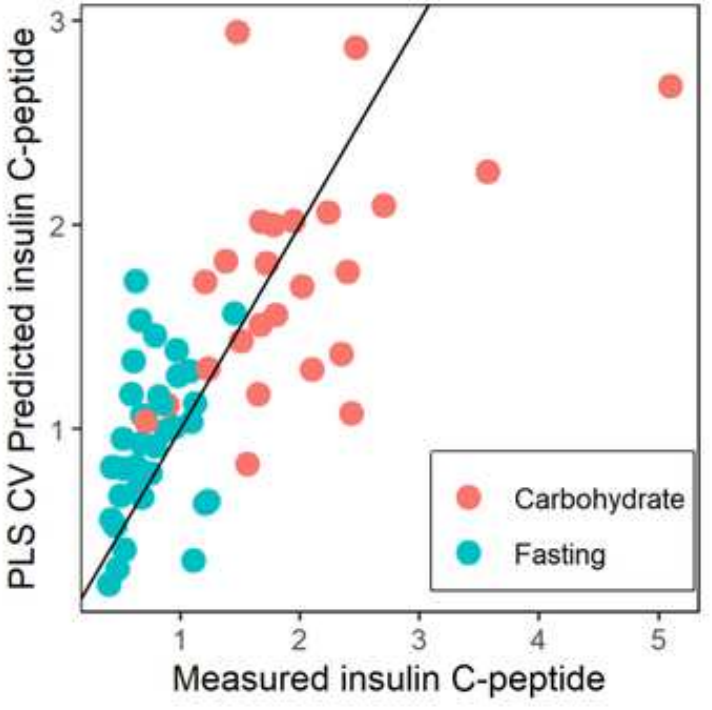

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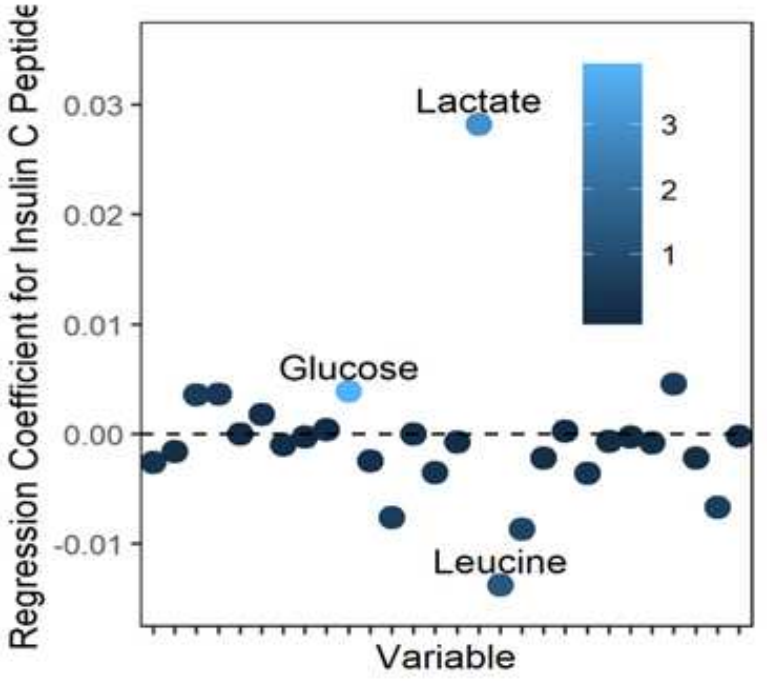

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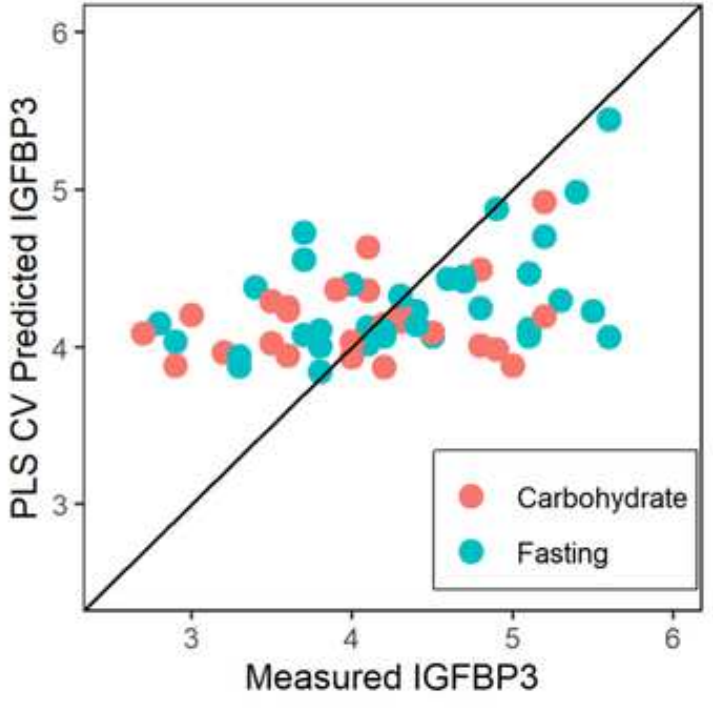

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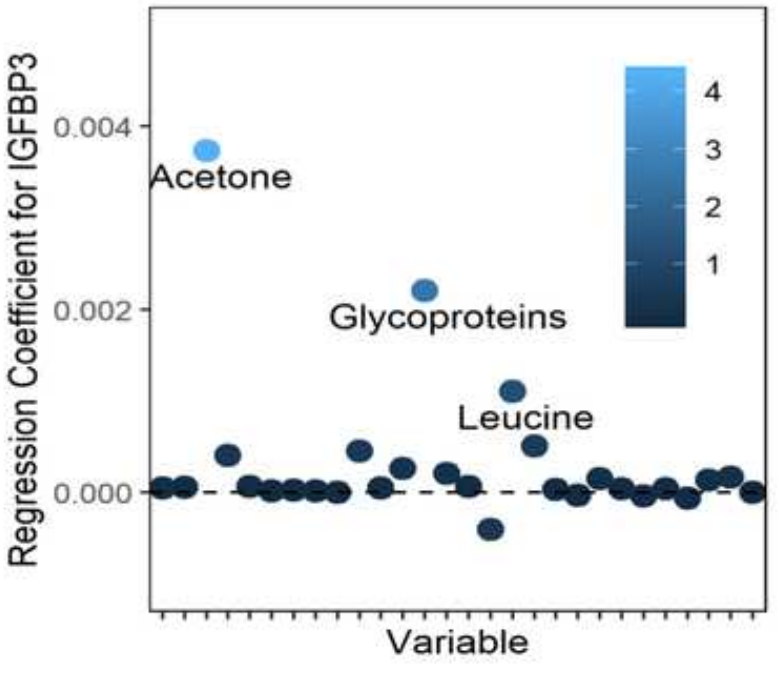

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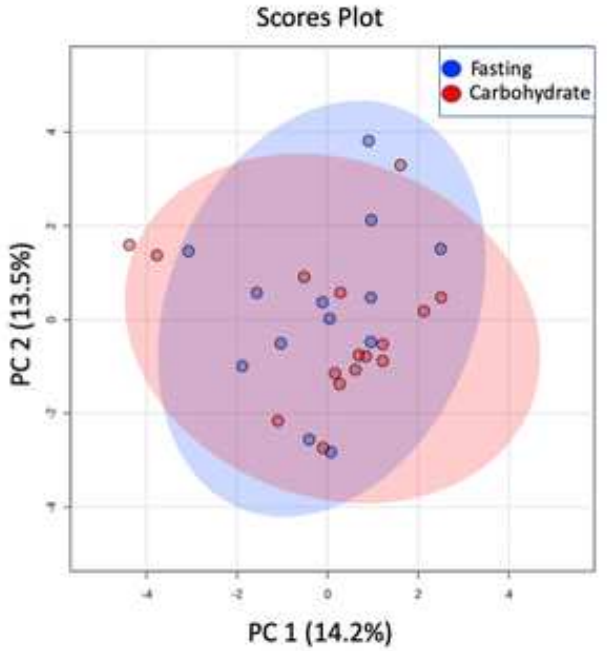

Figure 4B

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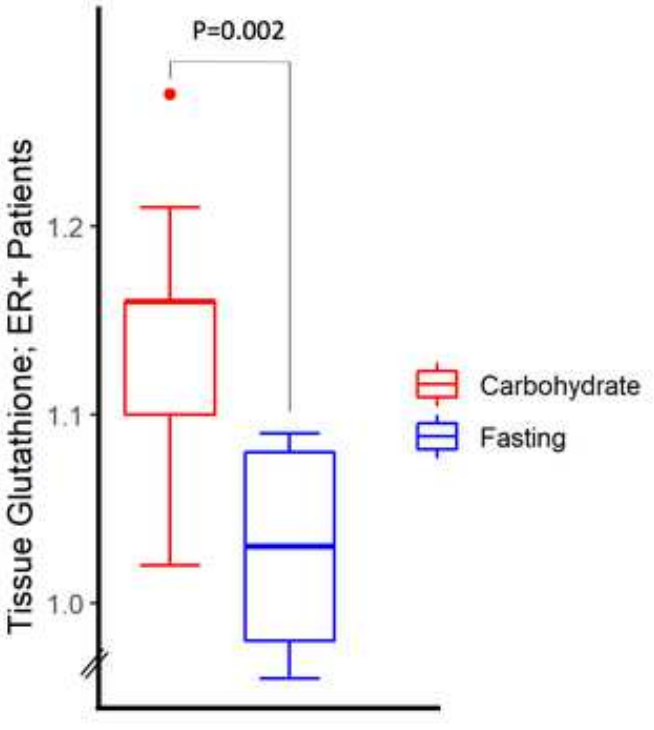

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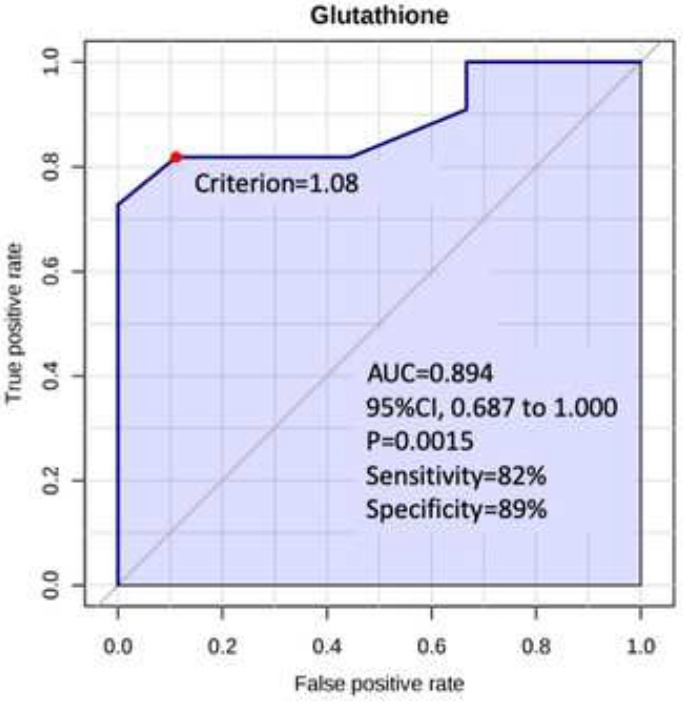
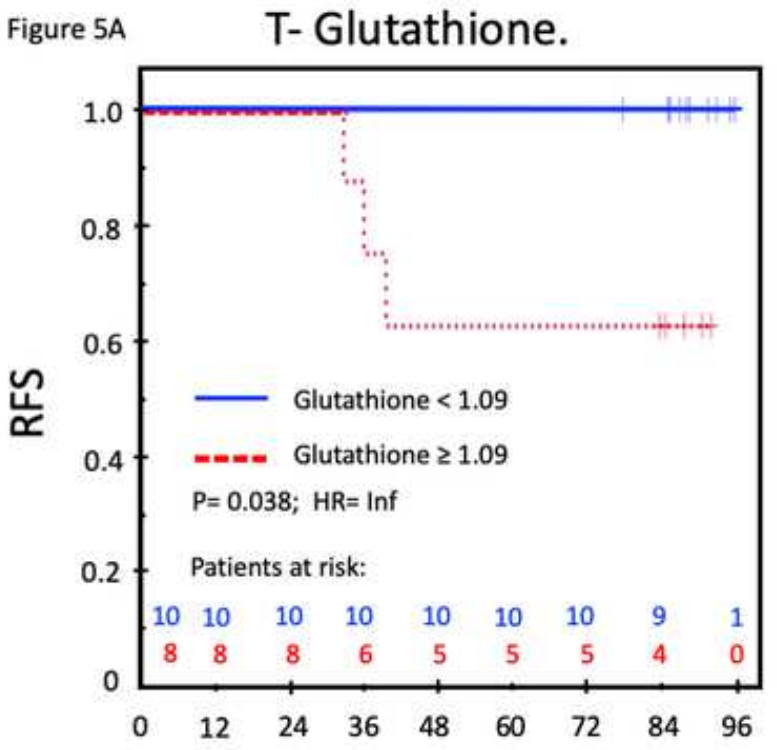
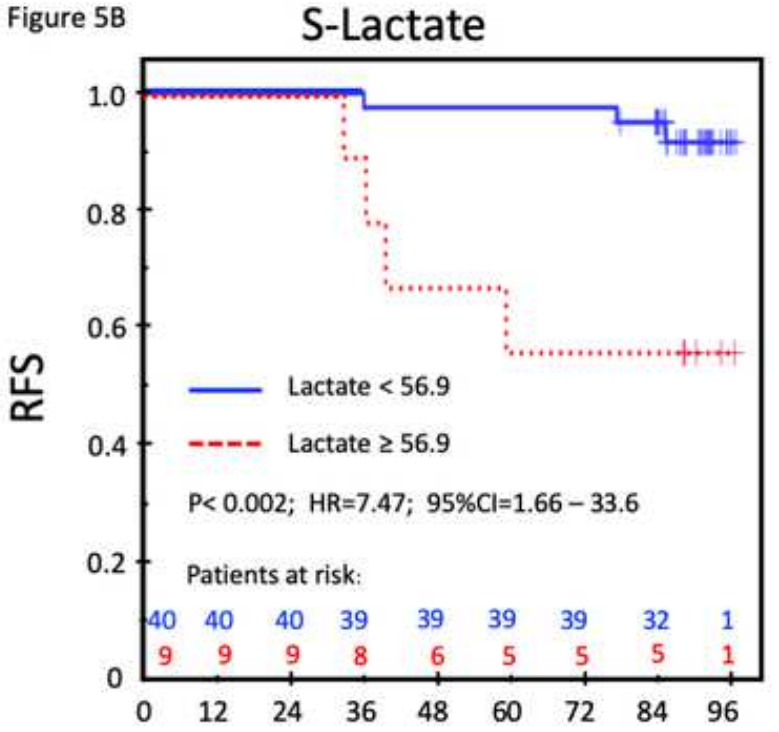
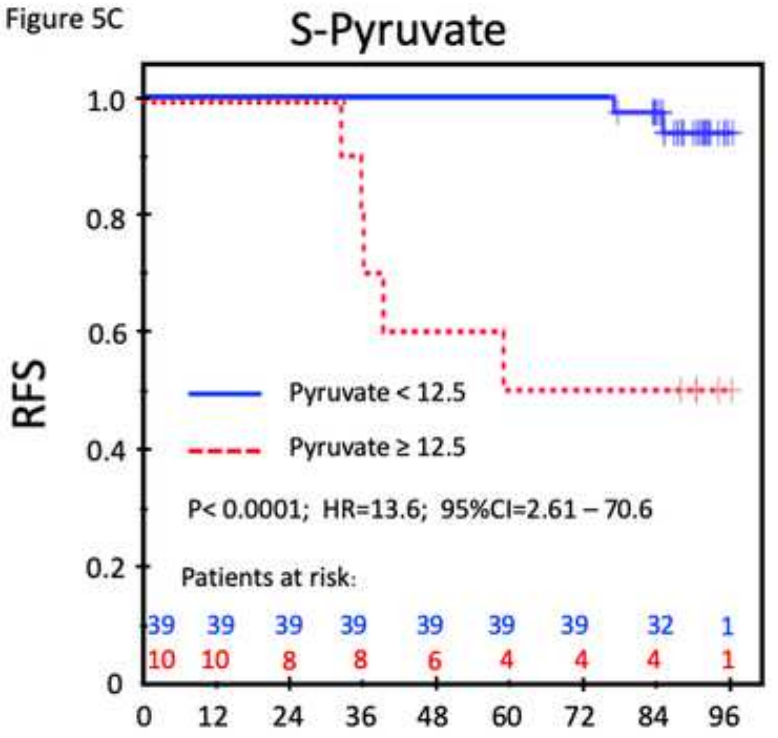
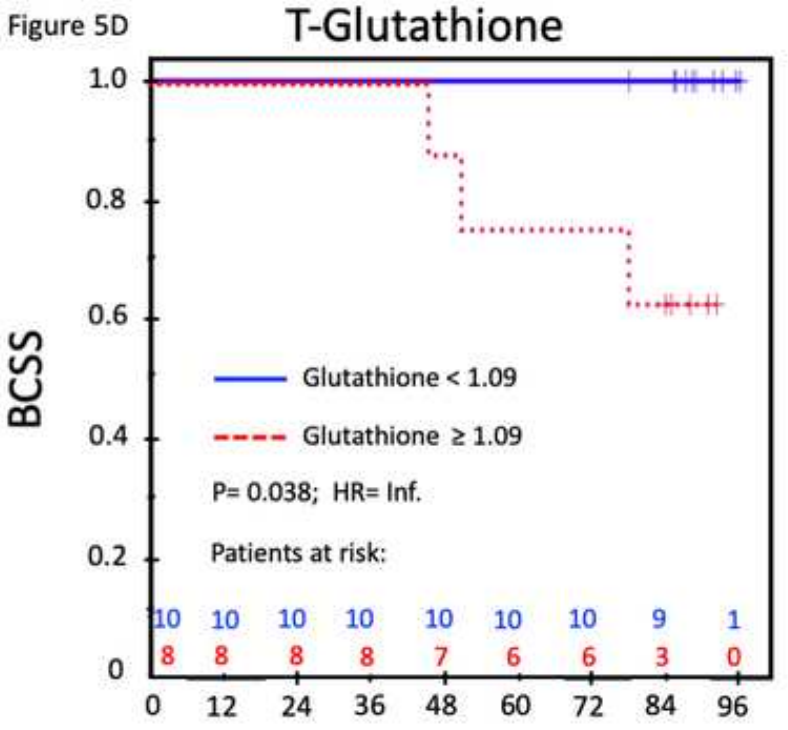
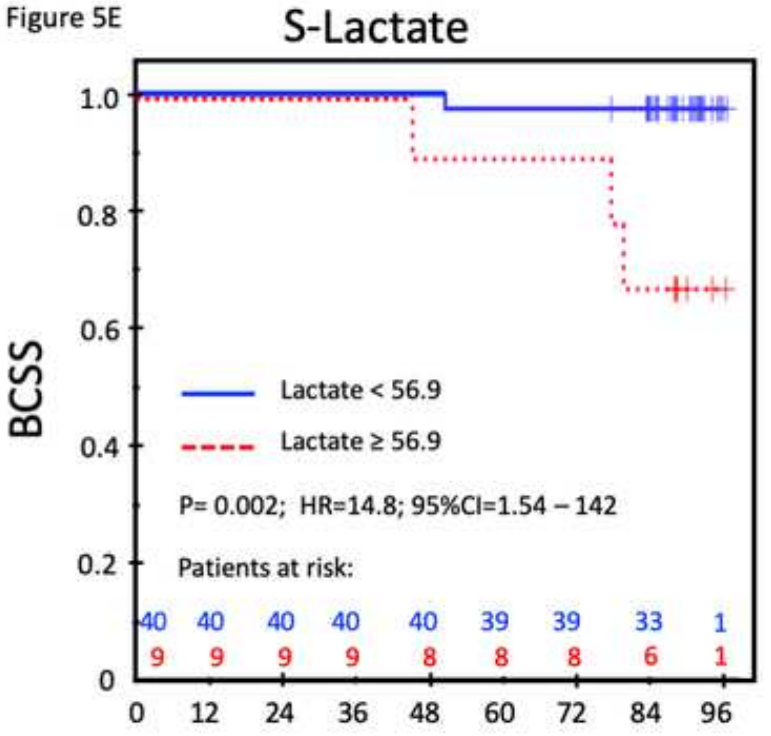


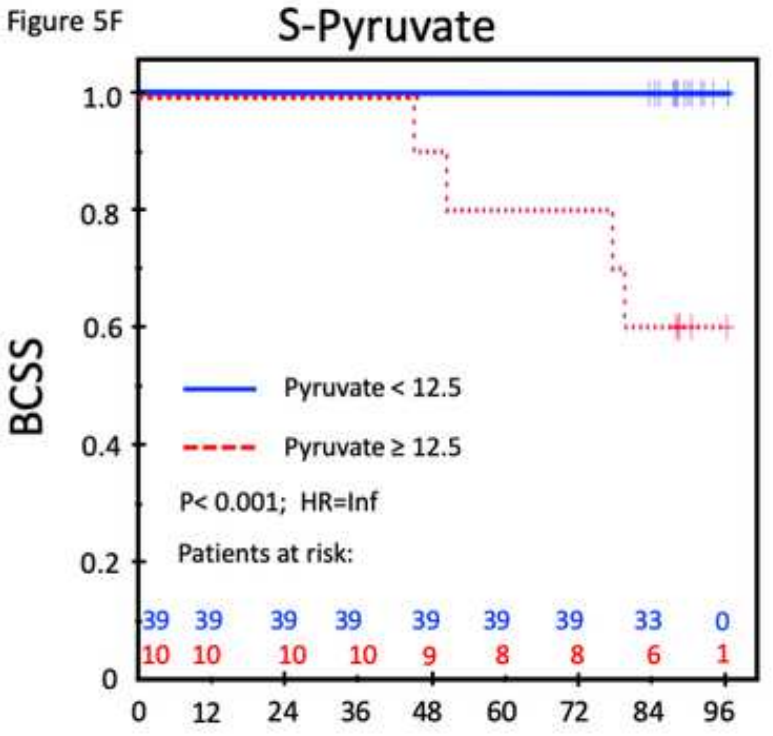












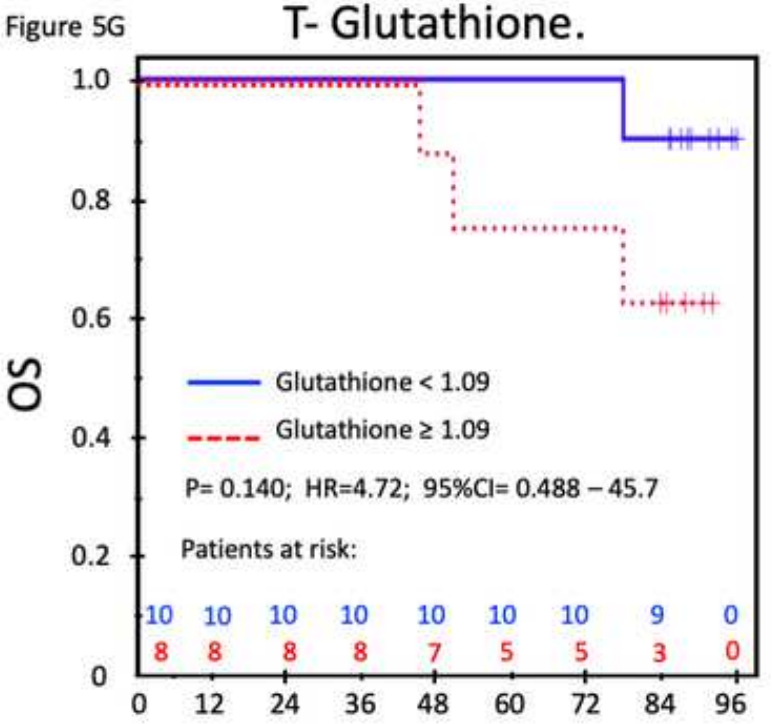
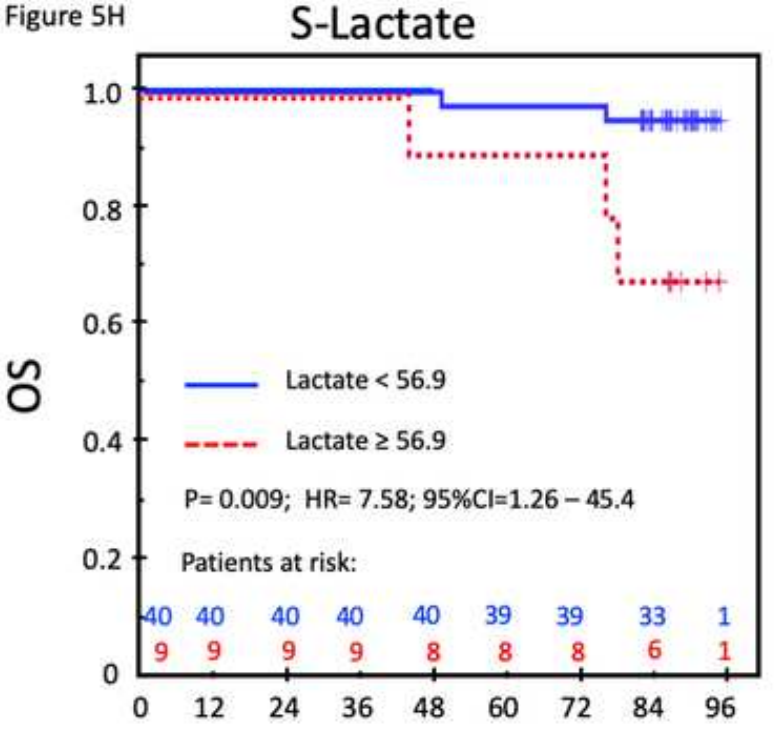


Figure 5H



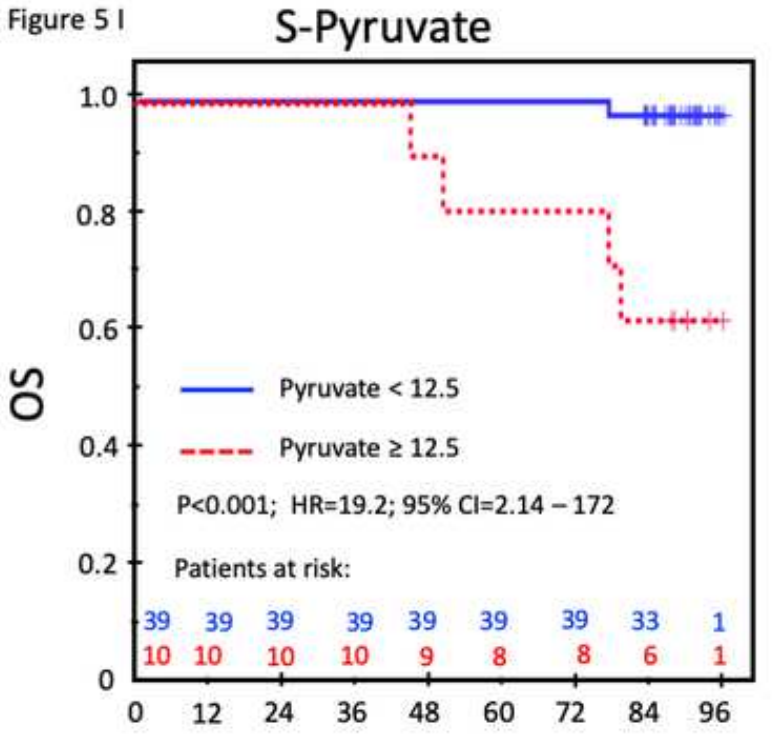


Figure 6A

Figure 6B

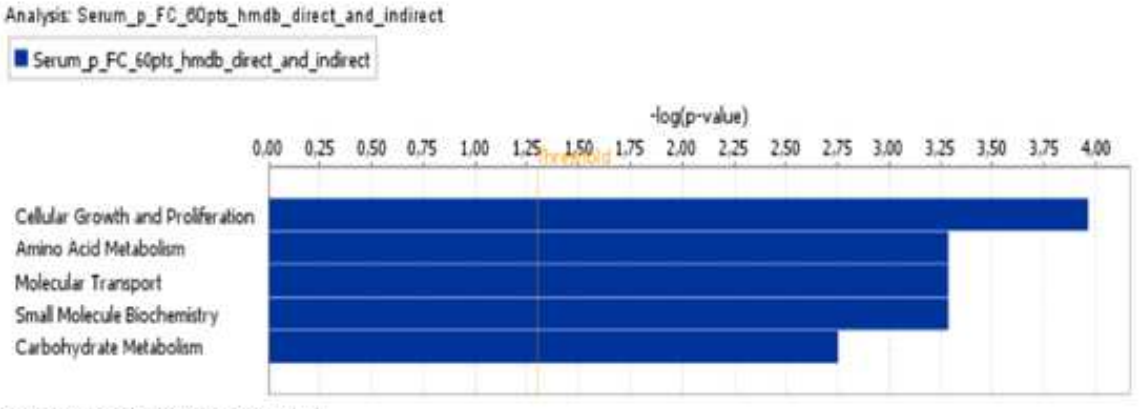
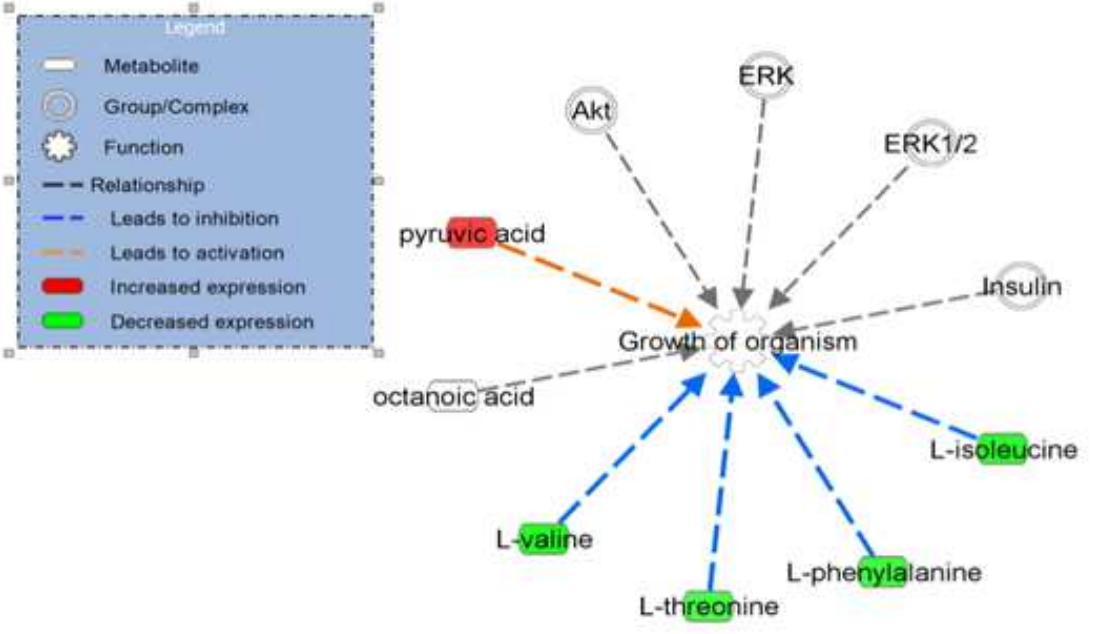
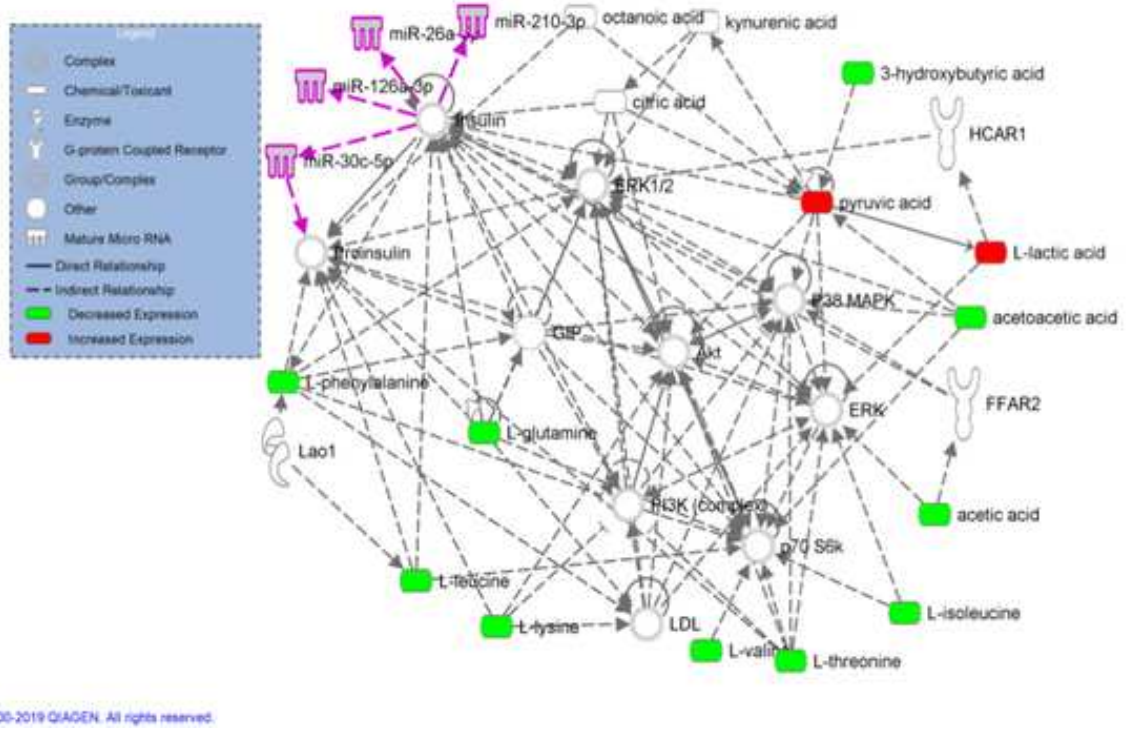


Figure 6C



Click here to access/download;Figure;Figure 6D.tiff

Figure 6D



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