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Characterization of breast cancer using MR metabolomics and gene expression analysis

Thesis for the degree of Philosophiae Doctor

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Sammendrag

Brystkreft er den vanligste kreftformen blant kvinner i Norge. Prognose og overlevelse avhenger av type kreft, tumorstørrelse, lymfeknutemetastaser og reseptorstatus (østrogen, progesteron og HER2). Basert på genekspresjonanalyser av tumorvev kan brystkreft deles inn i fem grupper; luminal A, luminal B, basal-like, HER2 positive og normal-like. Prognosene til pasientene i de ulike gruppene varierer der pasienter med basal-like brystkreft har den dårligste prognosen, mens pasienter med luminal A brystkreft har de beste prognosene.

Metabolismen i kreftceller og normale celler er svært forskjellig. Et kjennetegn ved kreftceller er endret glykolytisk aktivitet. Kreftcellene kan forbruke glukose og omdanne dette til laktat til tross for at det er rikelig med oksygen til stede. Denne effekten er ofte omtalt som Warburgeffekten. En annen karakteristisk egenskap ved kreftceller er endringer i kolinmetabolismen.

High resolution magic angle spinning MR spektroskopi (HR-MAS MRS) er en metode som er egnet for å studere biokjemiske forbindelser, kalt metabolitter, i vev. Glykolyse- og kolinmetabolitter i kreftvev kan derfor studeres med denne teknikken. Proton (^{1}H) MRS gir et spekter med informasjon om hvilke metabolitter som finnes i vev. ^{13}C MRS er velegnet til å studere metabolsk omsetning i celler, dyr eller mennesker. Ved administrering av ^{13}C-merkede metabolitter, kan man derfor kartlegge metabolske reaksjonsveier. Siden MRS er en kvantitativ metode kan den brukes til å beregne metabolittkonsentrasjoner i vev.

Ved bruk av multivariate dataanalyser kan flere metabolitter i HR-MAS MR-spektrene analyseres samtidig. Denne metoden er derfor egnet til å studere metabolske forskjeller mellom ulike brystkreftgrupper. Siden vevet er intakt etter HR-MAS MRS kan det brukes til andre analyser etterpå, som for eksempel histopatologi eller genekspresjonsanalyser. Genekspresjonsanalyse er en egnet metode for å kartlegge hele

eller deler av genomet. Med denne metoden kan man undersøke de genetiske forandringene som oppstår i kreftceller.

Denne doktorgraden består av tre studier. Målet med det første studiet var å kartlegge prognostiske faktorer i brystkreftvev ved bruk HR-MAS MRS og multivariate dataanalyser. Tre ulike typer multivariate metoder ble benyttet for å undersøke om HR-MAS MR spektrene inneholder informasjon som kan brukes til å prediktere østrogenreseptorstatus, progesteronreseptorstatus og lymfeknutestatus. Resultatene viste at det finnes metabolske forskjeller mellom tumorer som har positiv og negativ hormonreseptorstatus.

I det andre studiet ble ^{13}C HR-MAS MRS og genekspresjonsanalyser brukt til å kartlegge den glykolytiske aktiviteten i to ulike brystkreft musemodeller som representerer luminal-like og basal-like brystkreft. ^{13}C-merket glukose ble injisert i de to modellene og tumorvev samlet 10 eller 15 minutter etter injeksjon. HR-MAS MRS-analysene av tumorvevet viste at glukose/laktat (Glc/Lac) og glukose/alanin (Glc/Ala)-ratioene var større i de raskt voksende basal-like svulstene sammenlignet med den luminal-like modellen. De fleste glykolytiske genene var dessuten oppregulert i den luminal-like modellen. Disse resultatene indikerer at den luminal-like modellen har større glykolytisk aktivitet enn den basal-like modellen, og at tumorvekst ikke nødvendigvis er en avgjørende faktor for glykolytisk aktivitet.

Hensikten med det tredje studiet var å beskrive den metabolske profilen til et større utvalg av brystkreft musemodeller som representerer både luminal A, luminal B, basal-like og HER2 positiv brystkreft. Resultatene viste at luminal B-svulstene hadde en større fosfokolin/glyserofosfokoline (PCho/GPC)-ratio enn de fleste basal-like svulstene. I tillegg var kolin, PCho og GPC korrelert til andre gener i kolinmetabolismen i luminal B- svulstene enn i de basal-like svulstene. Dette kan bety at reguleringen av kolinmetabolismen er ulik i de to undergruppene av brystkreft. Det var i tillegg god overensstemmelse mellom både metabolitt- og genekspresjonsprofiler mellom xenograftprøvene og brystkreftprøver fra pasienter i de to undergruppene. Resultatene fra studiet viser at dette panelet av xenograftmodeller er representativt for

brystkreft hos mennesker, og betyr at modellene kan brukes til å identifisere nye behandlingsregimer ved bruk av HR-MAS MRS og genekspresjonsanalyser.

Studiene beskrevet i denne avhandlingen har vist at HR-MAS MRS og genekspresjonsanalyser reflekterer ulike karakteristikker i brystkreft og at disse metodene derfor kan brukes til å utvikle prognostiske verktøy for brystkreftpasienter.

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Summary

Breast cancer is a heterogeneous disease characterized with dysregulation of multiple, complex molecular events. Prognosis and survival depends on tumor size, lymph node involvement, metastatic state, histological grade, estrogen receptor (ER) status, progesterone receptor (PgR) status, and human epidermal growth factor receptor 2 (HER2) status. Five subtypes of breast cancer named luminal A, luminal B, basal-like, HER2 enriched, and normal breast-like have been identified on the basis of gene expression patterns. The prognosis for patients in the different subtypes varies where basal-like breast cancer patients have poorest prognosis and patients with luminal A breast cancer have the best prognosis.

Malignant and normal cells are known to have a different metabolism. Tumor cells have increased glycolytic activity, and glucose is mainly used to form lactate and alanine, even when high concentrations of oxygen are present (Warburg effect). Another known characteristic of tumor cells is modifications in the choline metabolism.

High resolution magic angle spinning MR spectroscopy (HR-MAS MRS) is a suitable tool to study metabolic changes occurring in intact cancer tissue. Glucose and choline compounds in the cancer cells can therefore be studied with this technique. ^{13}C MRS can map intracellular metabolic pathways. By injection of stable isotopic ^{13}C traces into animal models, the metabolic end products can be detected in the ^{13}C MR spectra. MRS is a quantitative technique and can therefore be used to measure metabolite concentrations in the tissue.

Multivariate analyses are powerful statistical techniques for analyzing data with many variables simultaneously, in order to identify patterns and relationships in data sets. Since MR spectra contain several thousand variables, this technique is suitable for metabolic analyses of MR spectra from breast cancer tissue samples. The tissue is intact after HR-MAS MRS analysis and can therefore be used for histopathological or gene expression analysis. Gene expression microarray analysis is suitable for studying the expression of the entire or parts of the genome and is therefore an appropriate tool for understanding genetic alterations that occur in cancer tissue.

The aim of this thesis was to investigate the relationship between prognostic factors and metabolic profiles, and to characterize the glycolytic activity and choline metabolism in breast cancer tissue samples from xenografts and patients using HR-MAS MRS in combination with gene expression analyses.

In paper I, three different multivariate methods were used to examine if HR-MAS MR spectra of tissue from breast cancer patients contain information that can be used to predict the important breast cancer prognostic factors estrogen and progesterone receptor status, and axillary lymph node status. The results showed a relation between metabolic profiles, and estrogen and progesterone receptor status which indicates that MR spectra contain prognostic information about breast cancer tissue.

In paper II, the glucose metabolism in two xenograft models representing basal-like and luminal-like breast cancer was studied using ^{13}C HR-MAS MRS and gene expression analysis. A significant lower glucose/alanine (Glc/Ala) and glucose/lactate (Glc/Lac) ratio was detected from tissue samples in the luminal-like compared to the basal-like subgroup. A higher expression of the glycolytic genes was also detected in the luminal-like model. The results demonstrate that the transformation from glucose to lactate and alanine occurs faster in the luminal-like compared to the faster growing basal-like model.

A larger panel of xenograft models was studied in paper III. A higher phosphocholine/glycerophosphocholine (PCho/GPC) ratio was found in the luminal B compared to most basal-like breast cancer xenograft tissue samples. Choline, PCho and GPC were correlated to different choline genes in the luminal B compared to the basal-like xenograft samples, suggesting that the regulations of choline metabolism may vary between the different breast cancer subgroups. These findings were in agreement with findings in tissue samples from patients which indicate that these xenografts are representative models of human breast cancer.

In conclusion, this thesis demonstrates how MR metabolomics reflect various molecular characteristics of breast cancer, and this method may therefore be used to develop prognostic and predictive tools for breast cancer patients.

Abbreviations

1D:	One-dimensional
2D:	Two-dimensional
Ala:	Alanine
ALNS:	Axillary lymph node status
Asp:	Aspartate
BBN:	Bayesian belief networks
cDNA:	Complementary DNA
CHKA:	Choline kinase alpha
CPMG:	Carr-Purcell-Meiboom-Gill
cRNA:	Complementary RNA
ECM:	Extracellular matrix
ER:	Estrogen receptor
ER+/-:	Estrogen receptor positive/negative
ERBB2:	Epidermal growth factor receptor 2
FDG-PET:	18F-deoxy-glucose positron emission tomography
FID:	Free-induction decay
GDPD:	Glycerophosphodiester phosphodiesterase
Glc:	Glucose
Glc-6P:	Glucose-6-phosphate
GLUT:	Glucose transporter
GPC:	Glycerophosphocholine
HER2:	Human Epidermal growth factor receptor 2
HES:	Hematoxylin, eosin and saffron
HIF:	Hypoxia-inducible factor
HK:	Hexokinase
HR-MAS:	High Resolution Magic Angle Spinning
HSQC:	Heteronuclear single quantum coherence
IDC:	Invasive ductal carcinoma
ILC:	Invasive lobular carcinoma
KEGG:	Kyoto Encyclopedia of Genes and Genomes
Lac:	Lactate

LDH:	Lactate dehydrogenase
LNS:	Lymph node status
LV:	Latent variables
mRNA:	Messenger RNA
MRS:	Magnetic Resonance Spectroscopy
NOE:	Nuclear Overhauser effect
PC:	Principal component
PCho:	Phosphocholine
PCA:	Principal component analysis
PI3K:	Phosphatidylinositol-3-kinase
PgR:	Progesterone receptor
PgR+/-:	Progesterone receptor positive/negative
PLA2:	Phospholipase A2
PLC:	Phospholipase C
PLD:	Phospholipase D
PFK:	Phosphofructokinase
PFKFB:	Fructose-2,6-bisphosphatase
PLS:	Partial least square
PLS-DA:	Partial least square discriminant analysis
PNN:	Probabilistic neural networks
ppm:	Parts per million
Ptd-Cho:	Phosphatidylcholine
PULCON:	Pulse length based concentration determination
RF:	Radio frequency
RIN:	RNA Integrity Number
SCID:	Severe combined immune deficient
SNR:	Signal-to-noise ratio
SLNS:	Sentinel lymph node status
SPXY:	Sample set partitioning based on joint x-y distances
TCA:	Tricarboxylic acid
tCho:	Total choline
TOCSY:	Total Correlation Spectroscopy
TNM:	Tumor Node Metastasis

TSP:	Trimetylsilyl-3-propionic acid sodium salt d4
VAST:	Variable stability
VEGF:	Vascular endothelial growth factor

List of papers

Paper I

Multivariate modeling and prediction of breast cancer prognostic factors using MR metabolomics

Giskeødegård G.F., Grinde M.T., Sitter B., Axelson D.E., Lundgren S., Fjøsne H.E., Dahl S., Gribbestad I.S., Bathen T.F.

Journal of Proteome Research, 2010; 9(2): 972-9

Paper II

^{13}C High-resolution-Magic Angle Spinning MRS reveals differences in glucose metabolism between two breast cancer xenograft models with different gene expression patterns

Grinde, M.T., Moestue S.A., Borgan E., Risa Ø., Engebraaten O., Gribbestad I.S.

NMR in Biomedicine, 2011; 24(10): 1243-52

Paper III

Interplay of choline metabolites and genes in patient-derived breast cancer xenografts

Grinde M.T., Skrbo N., Moestue S.A., Rødland E.A., Borgan E., Kristian A., Sitter B., Bathen T.F., Børresen-Dale A.L., Mælandsmo G.M., Engebraaten O., Sørlie T., Marangoni E., Gribbestad I.S.

Manuscript

Contents

1 Introduction

1.1 Cancer

While normal cells are controlled by regulatory signals, cancer cells have the ability to proliferate uncontrolled, invade surrounding tissue, and metastasize to distant organs. Cancer starts with mutations in the DNA which can be caused by radiation, chemicals, virus, or errors during DNA replication [1, 2]. It is a heterogeneous disease characterized by a dysregulation of multiple and complex molecular events. Hanahan and Weinberg described that the complexity of cancer can be reduced to a small number of underlying principles related to self-sufficiency in growth signals, insensitivity to anti-growth signals, invasion and metastasis, limitless reproductive potential, sustained angiogenesis, and evading apoptosis [1]. Conceptual progress in the last decade has added two new hallmarks to this list; evading immune destruction, and reprogramming of energy metabolism, in addition to two enabling characteristics; genome instability, and tumor promoting inflammation [2].

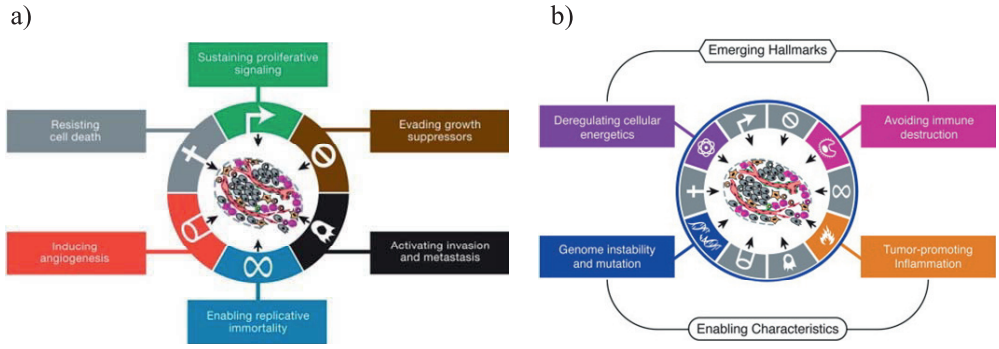

Figure 1.1: Hallmarks and enabling characteristics of cancer. a) The six hallmark of cancer originally proposed in the 2000 perspective. b) The two emerging, and two enabling hallmarks of cancer, proposed in the 2011 perspective. Adapted from Hanahan and Weinberg 2011 [2], with permission.

Diagnosis of cancer is mainly based on histopathological examinations of biopsies, often in combination with medical imaging methods. Treatment of cancer varies based on the type and stage of the cancer. Usually a combination of local treatment (surgery and radiation) and systemic treatment (chemotherapy, hormone treatment, and immunotherapy) is used. Optimization of treatment is important both for the patient and socio-economic reasons. Due to the heterogeneous nature of cancers, today's diagnostic tools are not sensitive enough to identify patients who will benefit from particular systemic treatments, and markers that will enable clinicians to provide more personalized treatment is requested.

1.2 Breast cancer

Breast cancer is the most common type of cancer affecting women, with more than 2700 new cases every year in Norway [3]. It is a heterogeneous disease, where patients with similar diagnosis can have very different prognosis. Some tumors develop metastasis to distant organs at an early stage, while others grow slowly and remain *in situ* in the mammary gland. The breast consists of fatty tissue, connective tissue, and mammary glands as shown in Figure 1.2. The most common breast cancers are developed from epithelial cells in the ducts (70-80%) and lobules (10%), known as invasive ductal carcinoma (IDC), and invasive lobular carcinoma (ILC), respectively [4].

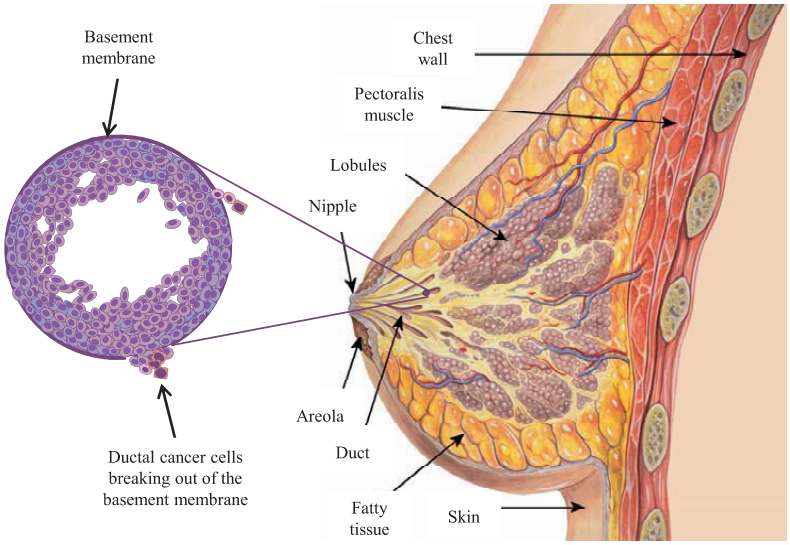

Figure 1.2: Breast with invasive ductal carcinoma (IDC) in an enlarged cross-section of the duct. The cancer cells have broken through the wall of the milk duct and begun to invade the surrounding tissue of the breast. Over time, IDC can spread to the lymph nodes and possibly to other organs in the body. Adapted from Wikimedia Commons with credits to Patrick J. Lynch and C. Carl Jaffe, with permission.

1.2.1 Breast cancer diagnostics

Breast cancer is diagnosed based on clinical examination, mammography often in combination with ultrasound, and pathologic examinations of fine-needle aspiration biopsies of the tumor. Breast cancers are commonly staged by the TNM (T: tumor size, N: lymph node involvement, and M: presence of distant metastases) classification system. Combination of these three factors defines the tumor stage and provides a strategy for grouping patients with respect to prognosis [7]. Patients with small primary tumors (<2cm, Stage I) have best prognosis while patients with distant metastasis (stage IV) have the poorest prognosis. Tumor grade is determined according to guidelines recommended by the Norwegian Breast Cancer Group (NBCG), which is based on the Bloom and Richardson classification system [5, 6]. The grade is found by microscopic examinations of the tumor biopsy. Scores from three criteria; tubule formation, nuclear

pleomorphism, and mitotic count give the final grade counting from 1 to 3. Grade 1 tumors are characterized with well-differentiated tumors and patients in this group have good prognosis, while grade 3 tumors are poorly differentiated and patients in this group have worst prognosis [5, 6]. Important prognostic factors are tumor size, grade, lymph node metastasis, and estrogen (ER) and progesterone (PgR) receptor status. The overall survival rate in Norway in 2009 was 89% [3]. However, for the breast cancers with advanced breast cancer, the survival rate is much lower. The relative survival for breast cancer patients in general has increased over the last decades. Better treatment and a mammography screening program may be possible explanations for the improved survival.

In normal mammary glands there are usually low expressions of ER and PgR. Breast cancer cells are often hormone sensitive, since growth and regression of tumors are frequently modulated by endocrine manipulations. The general trend in breast cancer is an overexpression of ER and PgR where about 70-80% of the breast cancer tumors are ER positive (ER+) and/or PgR positive (PgR+) [7]. Patients with ER+ and PgR+ breast cancer have better prognosis [7], and ER and PgR status is used to identify patients who may respond to endocrine agents like Tamoxifen. A growth factor receptor gene, human epidermal growth factor receptor 2, *ERBB2* (commonly referred to as *HER2*), is amplified in about 20% of breast cancers and in these cases the encoded protein is present in abnormally high levels in the malignant cells [8]. HER2 status predicts tumors that may be suitable for trastuzumab treatment. Trastuzumab inhibits the growth of breast cancer cells having high expression of HER2. Triple-negative cancers have a negative expression for ER, PgR and HER2. These cancers are usually highly proliferative, the patients have poor prognosis, and chemotherapy is an appropriate treatment for these patients.

1.2.2 Breast cancer subclassification

Five subtypes of breast cancer (luminal A, luminal B, basal-like, HER2 enriched, and normal-like) have been identified on the basis of expression patterns of so called intrinsic genes [9, 10]. Patients with luminal-like breast cancers have a better prognosis

(particularly luminal A) compared to patients with basal-like breast cancers and HER2 enriched cancers, who have fairly poor prognosis, as shown in Figure 1.3 [11].

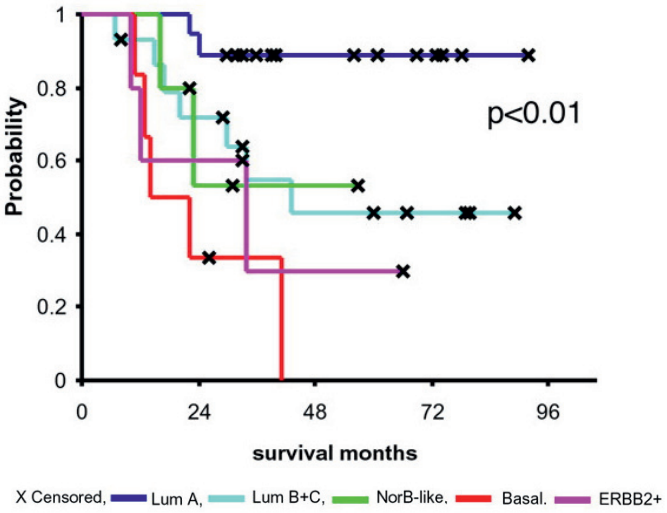

Figure 1.3: Overall survival for luminal A, luminal B, basal-like, normal breast-like, and HER2 enriched breast cancer patients. Adapted from Sørlie et al 2001 [11], with permission from the National Academy of Sciences, USA (Copyright 2001).

Other molecular subgroups of breast cancer have also been suggested. The Claudin-low subtype has been recently identified in human breast tumors [12, 13]. Clinically, the majority of Claudin-low tumors are triple-negative cancers, but distinct in low expression of cell-cell junction proteins. Furthermore, metabolic data from breast cancers have been used to refine the genetic subclassification of luminal A by combining transcriptomic and metabolic data. Three subgroups within the luminal A subgroup were identified using multivariate analyses of High Resolution Magic Angle Spinning Magnetic Resonance Spectroscopy (HR-MAS MRS) spectra [14]. The samples in one of the groups showed significantly lower glucose and higher alanine levels than the other luminal A samples, suggesting a higher glycolytic activity in these tumors. This group was also enriched for genes related to cell cycle and DNA repair. Recent research has focused on creating effective customized treatments for individual patients based on the biological characterization of the tumors [15, 16]. Refinement of

cancer subgroups can help in selecting patient who most likely will to respond to specific therapies and recognize those at risk of serious toxicity.

1.2.3 Breast cancer metabolism

Glycolysis

Cancer cells have a different metabolism compared to normal cells. A common property of invasive cancers is altered glucose metabolism. Normal cells would metabolize glucose to pyruvate via glycolysis and then completely oxidize most of that pyruvate during the process of oxidative phosphorylation which provides up to 36 molecules of ATP per glucose molecule. Conversion of glucose to lactic acid in the presence of oxygen is known as aerobic glycolysis or the Warburg effect, and is uniquely observed in cancers [17, 18]. Tumors display aerobic glycolysis through activation of oncogenes or loss of tumor suppressor genes [19]. This can further be enhanced by stabilization of the hypoxia-inducible factor (HIF) as a response to hypoxic microenvironment, and this leads to an enhanced aerobic glycolysis through coordinated upregulation of glycolytic enzymes and downregulation of mitochondrial oxidative metabolism [20-22]. Aerobic glycolysis is an inefficient way to generate ATP since it produces a net of two ATPs from each glucose molecule. It has been suggested that aerobic glycolysis gives malignant cells a selective advantage [23], and can be a supply of metabolic intermediates essential for macromolecular biosynthesis necessary for cell growth and proliferation [18, 24, 25]. Some studies have shown that high levels of lactate are correlated with the ability to metastasize in several types of cancers [26, 27]. The metabolic products of glycolysis can cause an acidification of the extracellular space, which might result in cellular toxicity [28]. The transformation from glucose to lactate and alanine is regulated by several enzymes as shown in Figure 1.4. Glucose transporters (GLUT), hexokinase (HK), and lactate dehydrogrenase (LDH) are known to be important regulators of the glycolysis [23, 29-31]. Another rate limiting control step is catalyzed by phosphofructokinase 1 (PFK1) and fructose-2,6-bisphosphatase (PFKFB) is considered to be a major regulator of PFK1 [23, 29, 32]. It has recently been increased interest in the development of treatments that target glycolysis [28-30].

Several studies have reported normalization of glucose metabolism as an indication of response to targeted treatment in various cancers [33, 34].

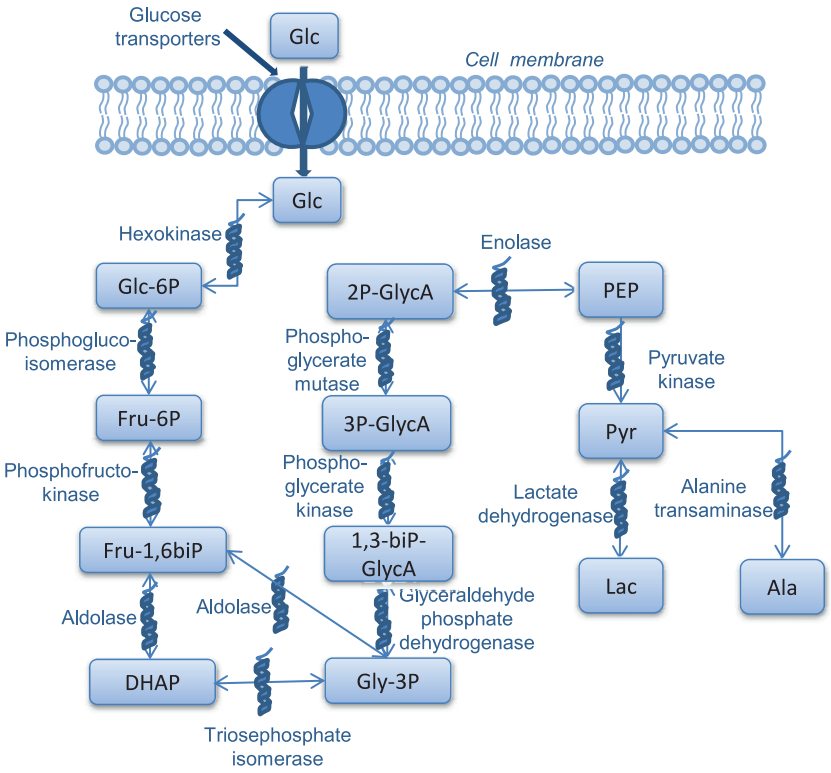

Figure 1.4: The different steps in glycolysis. Glucose enters the cells and is converted to pyruvate during several steps in glycolysis. Cancer cells drive pyruvate conversion to lactate even in the presence of oxygen (Warburg effect). Pyruvate can also be converted to alanine. The following abbreviations are used: Glc: glucose, Glc-6P: glucose 6-phosphate, Fru-6P: fructose 6-phosphate, Fru-1,6biP: fructose 1,6-biphosphate, DHAP: dihydroxyacetone phosphate, Gly-3P: glyceraldehyde 3-phosphate, 1,3-biP-GlycA: 1,3-bisphosphoglycerate, 3P-GlycA: 3-phosphoglycerate, 2P-GlycA: 2-phosphoglycerate, PEP: phosphoenolpyruvate, Pyr: pyruvate, Lac: lactate, Ala: alanine.

Choline metabolism

Phosphatidylcholine (Ptd-Cho) is the predominant component of cellular membranes. The synthesis of Ptd-Cho is shown in Figure 1.5. Rapid proliferation of cancer cells requires a continuous reorganization of the cell membranes. Choline metabolism and choline-derived metabolites can therefore undergo extensive alterations as a result of a malignant transformation [35, 36]. Magnetic Resonance Spectroscopy (MRS) has shown to be a valuable tool in cancer studies of choline metabolism. Phosphocholine (PCho), glycerophosphocholine (GPC), and choline are visible using *in vitro* and *ex vivo* MRS. Using *in vivo* MRS, the signals from choline, PCho and GPC is detected as one peak assigned to total choline (tCho). *In vivo* tCho has been used to differentiate malignant and benign lesions in untreated tumors. A correlation between tumor response to neoadjuvant chemotherapy and a disappearance or reduction of the tCho signal has also been reported [37]. Despite the diagnostic value of tCho as a cancer biomarker, the underlying mechanisms causing these alterations are not fully understood [38]. Studies of cell cultures have suggested that increased levels of tCho are mainly caused by increased PCho [35, 39]. Choline transport and activity of choline kinase alpha (CHKA) which catalyzes the phosphorylation of free choline to PCho have been suggested as indicators of increased PCho in cancer cells. However, in breast cancer xenograft and human tissue samples, higher GPC compared to PCho have been observed [40-42]. GPC has also been correlated with malignancy in xenograft tissue samples from prostate cancer [43]. Altered expression of isoforms of phospholipase D (PLD) [44, 45], phospholipase C (PLC) [46] and phospholipase A2 (PLA2) [47] have also been identified in breast cancers. Drugs targeting choline metabolism have displayed selective cytotoxic efficacy against a variety of cancer types [48-50].

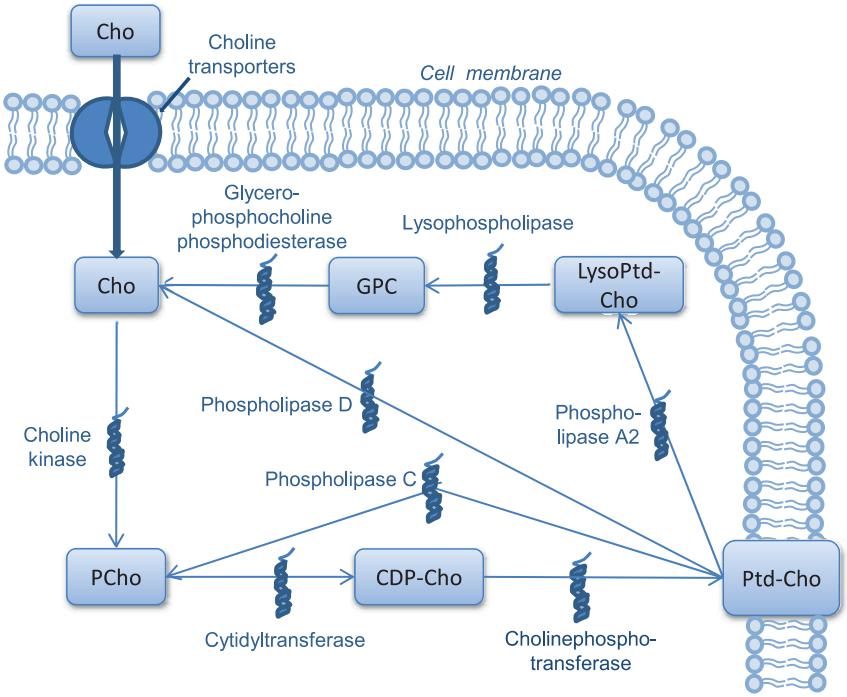

Figure 1.5: Pathway for synthesis and degradation of phosphatidylcholine. The following abbreviations are used: Cho: choline, PCho: phosphocholine, CDP-Cho: cytidine diphosphate choline, Ptd-Cho: phosphatidylcholine, GPC: glycerophosphocholine.

1.3 Tumor xenograft models

Human tumor cells or tissue can be transplanted into immunodeficient mice to form a xenograft. A defect immune system makes these mice appropriate for research since they can receive different types of tissue and tumor grafts without rejection response. Nude mice and severe combined immune deficient (SCID) mice are commonly used as hosts. The nude mice lack a functional thymus, which is essential for production of T-lymphocytes and necessary for a functional immune system [51, 52]. The SCID mice show additional defects in innate and adaptive immunity including impaired production of functional T- and B-lymphocytes [53, 54].

Most of the existing *in vivo* models for preclinical assays are based on cell lines previously isolated from human tumors and selected through culture before implantation into mice. Xenografts derived from cell lines generally show a homogeneous and undifferentiated histology [55]. This is probably an indicator of the high selection pressure to *in vitro* conditions during extensive culturing [56]. Preclinical models based on xenografts obtained by engrafting tumor tissue directly from human to mice have been established [55, 57-59]. Tumors transplanted into a host animal are subject to different stroma and selection pressures, which may impact on the tumor's molecular profile. It has been shown that these models recapitulate the features of the original tumors and maintain the cell differentiation, morphology, vasculature, and histology, even after several passages [57, 58, 60]. The fact that genomic alterations are conserved for several years suggests that genomic profiles remain relatively stable over time despite new selection pressures and loss of human stroma [61]. Human tumor models established by direct engraftment have demonstrated a high correlation of drug response compared to that observed in the clinic [57, 62].

Tumors can be established either by engraftment under the skin (subcutaneously) or in the same organ as the primary human tumor (orthotopic). An orthotopic localization can give tumors that have similar morphology because of nearby blood vessel system, and supporting tissue that closely mirror the tumor's original microenvironment [63]. An increased metastatic potential have also been found in orthotopic tumors [58, 64].

Breast cancer xenograft models are particularly hard to establish [65]. ER- tumors are easier to establish than ER+ tumors, as reported in several studies [57, 66]. This could be caused by slower growth, lower proliferation and usually a lower grade detected in the ER+ tumors. For these reasons, most of the established xenografts models usually display an aggressive phenotype, with a high tumor grade, and the majority of breast xenograft models are classified as triple-negative cancers [57].

1.4 Magnetic Resonance Spectroscopy (MRS)

1.4.1 The principles of MRS

It is possible to acquire MR spectra from all nuclei with spin angular momentum (spin quantum number $I \neq 0$). Quantum mechanics describes that nucleus of spin I will have $2I+1$ possible orientations. The most used nucleus in MRS is proton (^{1}H), but other types of nuclei, for instance ^{13}C, ^{31}P, ^{17}O, ^{19}F, and ^{23}Na have also been utilized in MRS studies. All these have a spin I =1/2 and will therefore have two possible orientations (spin up, and spin down).

When magnetic active nuclei with spin I =1/2 are placed in an external magnetic field, they will either align in a parallel or an antiparallel direction compared to the magnetic field. The relative number of parallel versus antiparallel spins is given by the Boltzmann's distribution as described by (1.1)

$$\frac{N^{+}}{N^{-}} = e^{\frac{\gamma h B_0}{kT}} \tag{1.1}$$

where N^{+} is the number of parallel spins, N^{-} is the number of antiparallel spins, γ is the gyromagnetic ratio, h is the Planck's constant, B_0 is the magnitude of the magnetic field, k is the Boltzmann's constant, and T is the temperature. The spins demonstrate at the same time a circular oscillation (precession) which is proportional to the magnetic field strength. The frequency of the oscillation (Larmor frequency) is described by (1.2)

$$\omega = -\gamma B_0 \tag{1.2}$$

where ω is the angular frequency, γ is the gyromagnetic ratio, and B_0 is the magnitude of the magnetic field. In addition, nuclei in a region of high electron density will experience a magnetic field proportionately weaker than those in a region of low electron density. This is called shielding. Consequently, nuclei from different compounds and in different parts of a molecule experience a slightly different magnetic field and create small differences in the resonance frequency. The difference between the resonance frequency of nuclei in a given molecule and a reference compound is called chemical shift and is defined by (1.3)

$$\delta_i(ppm) = \frac{\omega_i - \omega_{ref}}{\omega_{ref}} 10^6 \tag{1.3}$$

where ω_i is the frequency for the molecule of interest, and ω_{ref} is the frequency of the reference compound. *J* coupling is mediated by up to four chemical bonds and can be exploited for structural elucidation. The presence of such couplings creates multiplets (doublets, triplets, quartets etc.) in the MR spectra. The combination of chemical shift and couplings gives each metabolite a unique MR spectrum.

The precession of magnetic active nuclei is what we detect in an MRS experiment. As described by the Boltzmann's distribution (1.1), a larger fraction of the spins will be aligned in the parallel compared to antiparallel direction. The population differences of the spins will cause a net magnetization which is parallel to the magnetic field. When a radio frequency (RF) pulse with a frequency close to the Larmor frequency is applied, the magnetization is tilted away from the main magnetic field. After a spin system has been excited by an RF pulse, it initially behaves like a coherent system, i.e. the microscopic magnetic components precess in phase around the external magnetic field. However, the magnetic components are quickly becoming incoherent due to (1) B_0 inhomogeneities, and (2) static and oscillating fields locally induced by neighboring magnetic moments. This transversal relaxation is called T_2* relaxation. With adequate techniques explained in next section, it is possible to eliminate the effect of the B_0 inhomogeneities, so that the transverse magnetization decays with a time constant T_2. Longitudinal or T_1 relaxation refers to the phenomenon of macroscopic magnetization recovery along the direction of the static magnetic field. The signal that is induced in the receiver coil is denoted Free-induction decay (FID). After acquisition, the FIDs can be multiplied by windows functions in order to increase the signal-to-noise ratio (SNR) or resolution, and the FIDs are Fourier transformed for spectral analysis.

1.4.2 MRS pulse sequences

The simplest MRS experiment is a single-pulse experiment which consists of a recycling delay, an RF pulse, and the acquisition period in which the signal is recorded. Since biological tissue contains large amounts of water it is often necessary to suppress the water signal in order to enhance signals from other metabolites. Water is commonly suppressed by applying a low power continuous wave irradiation on the water signal prior to the acquisition, usually during the recycle delay.

Carr-Purcell-Meiboom-Gill (CPMG) is a commonly used MR sequence for suppression of fat and macromolecules. This type of spin echo pulse sequence consists of a 90° RF pulse followed by an echo train induced by successive 180° pulses. The train of 180° pulses will refocus the spins that are dephased according to B_0 inhomogeneities (T2*). The intensity of lines with short T_2 (broad lines from for instance fatty acids and proteins) diminishes much more quickly than that for lines with long T_2 (sharp lines). The CPMG sequence is therefore useful for enhancing sharp lines in the MR spectra.

Carbon MRS detects only the ^{13}C isotope, whose natural abundance is low, only 1.1%. The gyromagnetic ratio (γ) of ^{13}C is only one-fourth of that of the ^{1}H. These two conditions make ^{13}C MRS an insensitive technique. ^{13}C MR spectra yield a much larger spectral dispersion than ^{1}H spectra since it has a chemical shift range around 250 parts per million (ppm) compared to 10 ppm for ^{1}H spectra. The most frequently used ^{13}C sequence is ^{1}H decoupled. These sequences involve continuous irradiation of the ^{1}H with a repeated set of pulses. The coupled multiplets are thus collapsed into singlets, and an enhanced in SNR is attained.

Two or multiple dimensional MRS experiments present MR spectra in a space defined by two or more frequency axes rather than one, and utilize intramolecular nuclear interactions mediated through chemical bonds or through space. These MR spectra provide structural information about a molecule in addition to the information provided by one-dimensional (1D) MR spectra. Frequently used MR experiments are J-spectroscopy, correlation spectroscopy and Nuclear Overhauser Effect (NOE) spectroscopy. In correlation spectroscopy, cross peaks demonstrate that different nuclei

with different chemical shifts are coupled to each other, either directly or in a chain [67]. It is common to distinguish between homonuclear couplings (between nuclei of the same type, frequently used experiment is Total Correlation Spectroscopy; TOCSY), and heteronuclear couplings (between nuclei of different types, frequently used experiment is Heteronuclear Single Quantum Coherence; HSQC).

1.4.3 High Resolution Magic Angle Spinning MRS (HR-MAS MRS)

In liquids, molecules do not experience significant motion restriction since they tumble at fast rates. Spectral broadening effects due to molecular interactions are thus averaged, resulting in narrow spectral lines. In solid state, the nuclear spins experience a great number of interactions, due to the lack of isotropic molecular motion. The major line-broadening factors are dipole–dipole interactions, and chemical shift anisotropy which produce spectral broadening with an angular dependency of

$$3\cos^2\theta - 1 \qquad (1.4)$$

where θ is the angle between the static magnetic field and the spinning axis. However, if the sample is rapidly spun (typically 5kHz) at an angle of θ=54.7° to the external magnetic field (which meets the criterion of $3\cos^2(\theta)-1=0$), the effects on line widths of dipolar couplings and chemical shift anisotropy are drastically decreased [68]. The principle of MAS was described in 1958 by Andrew and Lowe [68, 69], and HR-MAS MRS was first applied on human tissue samples in 1997 [70]. The resolution of HR-MAS MR spectra is comparable to that of liquid state MRS. HR-MAS MRS of breast tumor tissue can give detailed biological information from more than 30 metabolites in intact tissue samples [71]. HR-MAS MRS analysis requires only simple sample preparations. The tissue is intact after analysis and can therefore be used for gene expression analysis or histopathological examinations.

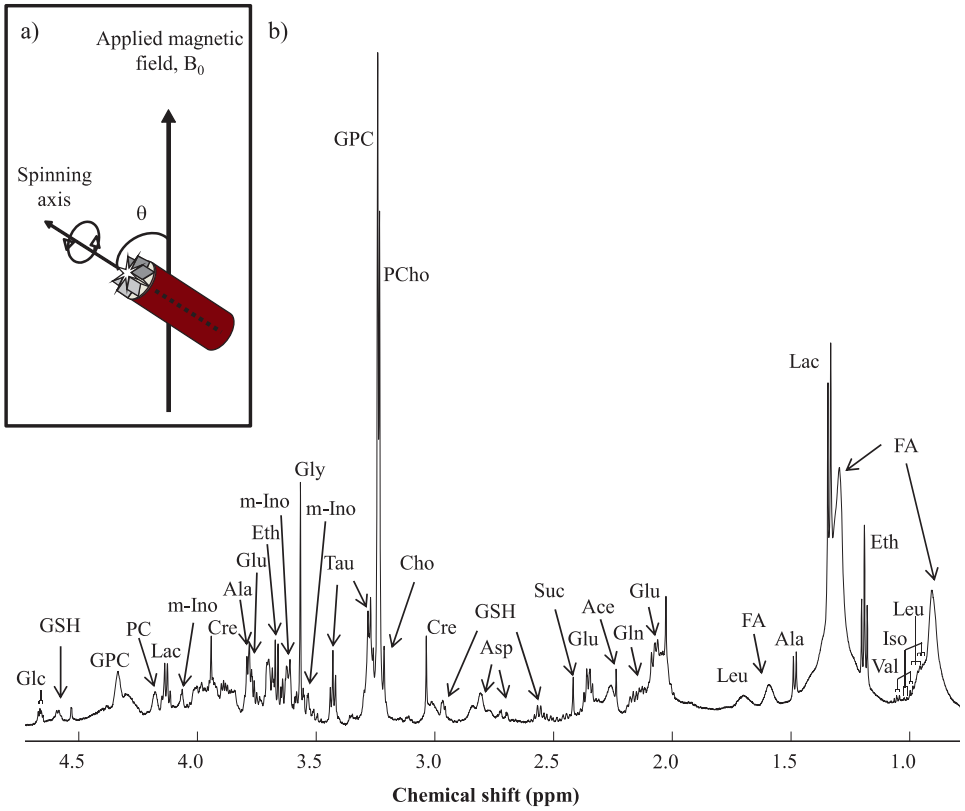

Figure 1.6: a) Schematic presentation of a magic angle spinning rotor. The rotor is inclined at an angle θ of 54.7° to the static magnetic field (B_0). b) Example of a HR-MAS MR spectrum of breast cancer xenograft tumor tissue. Assigned metabolites are: Ace: acetone (contamination), Ala: alanine, Asp: aspartate, Cho: choline, Cre: creatine, Eth: ethanol (contamination), FA: fatty acids, Glc: glucose, Gln: glutamine, Glu: glutamate, Gly: glycine, GPC: glycerophosphocholine, GSH: glutathione, Iso: isoleucine, Lac: lactate, Leu: leucine, m-Ino: myo-Inositol, PCho: phosphocholine, Tau: taurine, and Val: valine. The MAS rotor is reprinted with permission from Beathe Sitter.

1.4.4 ^{1}H HR-MAS MRS of breast cancer

MRS is a powerful technique to study metabolic changes in cancer tissue. Such analysis can be performed either by HR MRS of tissue extracts, or by using HR-MAS MRS of intact tissue samples. The most frequently used nucleus is ^{1}H, since it has the highest sensitivity and a high natural abundance in biological tissue.

Metabolic characteristics in tissue samples from various cancers including breast, prostate and brain, have been studied by ^{1}H MRS for almost 25 years [42, 72-75]. The potential use of MRS in oncology includes detection of cancer, differential diagnosis, and assessment of treatment response. HR-MAS MRS studies of breast cancer tissue samples have showed that tumor samples can be distinguished from non-involved samples with a high sensitivity, and specificity [42, 76, 77]. Histopathological grade have been correlated with metabolic markers, such as PCho, lactate, and lipids [78]. Choline compounds, glucose, and glycine have been suggested as metabolic markers in samples from patients with differential diagnosis [79], and samples from patients with different hormone status (both ER and PgR) have been distinguished with MRS [80]. MRS studies of samples from patients with different axillary lymph node involvement, which is one of the most important prognostic factor in breast cancer, have been proposed with various levels of correct classifications [80, 81]. In addition, changes in metabolic profiles as a response to treatment have been reported where HR-MAS MRS analysis revealed a significant decrease in choline compounds for tissue samples in xenograft models treated with docetaxel compared to non-treated controls [82]. The potential value of HR-MAS MRS analysis of breast tumor biopsies to predict long-term survival and evaluate response to treatment, has also been previously reported [83]. Tissue samples from patients with long-term survival (≥5 years) had higher levels of tCho, and lower levels of lactate compared to patients who died of cancer recurrence (< 5 years). A significant decrease in GPC after neoadjuvant chemotherapy was, in addition, associated with long-term survival for the same patients [83]. A different study showed that non-survivors had a significant increase in lactate levels in response to neoadjuvant chemotherapy treatment compared to survivors where no change in lactate levels were observed [84].

1.4.5 ^{13}C MRS of breast cancer

While ^{1}H MRS offers high SNR, the characterization of many metabolites in the ^{1}H MR spectra are often limited by overlapping signals due to a narrow chemical shift range. In contrast, ^{13}C MRS offers a wide chemical shift range and therefore a better separation of individual metabolite peaks. Despite an improved spectral resolution in ^{13}C MR spectra, the use of ^{13}C MRS has traditionally been limited by a low SNR due to low natural abundance (1.1% ^{13}C).

^{13}C MRS is a particularly useful technique to map biochemical reactions in cell cultures, animals, or human. After administration of ^{13}C labeled substrates the metabolic flux through different metabolic pathways can be mapped. Changes in metabolic fluxes in cancer through glycolysis, tricarboxylic acid (TCA) cycle, or fatty acid synthesis can therefore be measured with this technique. When for instance ^{13}C labeled glucose is administered, the glucose can be metabolized to ^{13}C labeled pyruvate, which is metabolized aerobically through the TCA cycle, or to ^{13}C labeled lactate. Most tumors exhibit labeling of ^{13}C lactate (Warburg effect) [85-87], which results directly from glycolysis, whereas some studies have demonstrated labeling of both ^{13}C lactate and ^{13}C glutamate, reflecting various levels of both glycolytic and oxidative metabolism [88]. Using ^{13}C labeled glucose, a reduction in the rate of glycolysis for cancer treated with antiestrogen treatment (Tamoxifen) has been observed [89, 90]. Numerous studies have been performed to study the fate of glucose in cancer cells, but only a few of these have been performed on intact tumor tissue samples [91, 92].

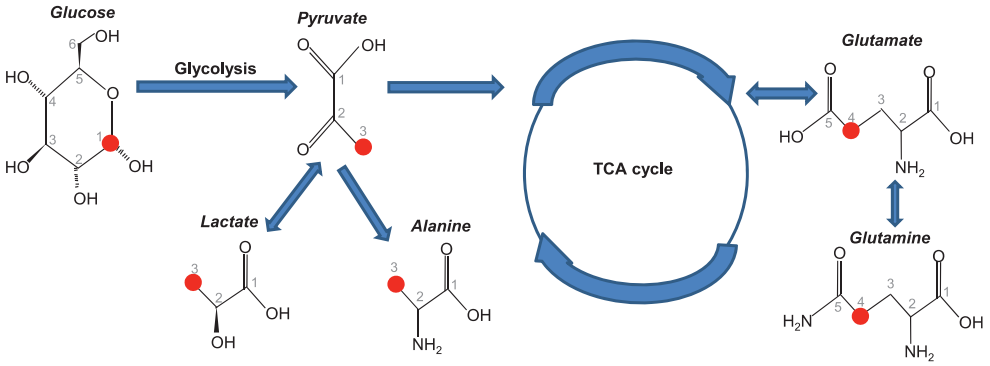

Figure 1.7: [1-^{13}C] labeled glucose enters the cell and is converted to [3-^{13}C] pyruvate via glycolysis. In the presence of oxygen, [3-^{13}C] pyruvate can be converted to acetyl coenzyme A and enter the TCA cycle and be transformed to for instance [4-^{13}C] glutamate. In absence of oxygen, and frequently in malignant tumors, pyruvate is converted to [3-^{13}C] lactate (Warburg effect). [3-^{13}C] pyruvate is also commonly transformed to [3-^{13}C] alanine in cancer cells. Red ring: ^{13}C isotope.

^{13}C labeled choline has also been used to study the metabolic flux in breast cancer cell lines. After administration of ^{13}C labeled choline, higher levels of PCho and betaine were observed in the cancer cells [47, 93, 94]. Recently, ^{13}C labeled substrates have been polarized using dynamic nuclear polarization (DNP) to achieve more than 10 000 fold signal enhancement of the ^{13}C MR signal from substrates and metabolic products [95, 96]. The sensitivity enhancement gained by hyperpolarization offers the possibility of using ^{13}C MRS and imaging *in vivo* to measure fluxes through enzyme-catalyzed reactions. Hyperpolarized [1-^{13}C] pyruvate and other ^{13}C labeled substrates have recently been widely used in metabolic studies of cancers [97-102].

1.4.6 Quantification of metabolites

In principle, the total area under a metabolite resonance in a MR spectrum is proportional to the concentration of the metabolites. Metabolites can thus be quantified by comparing peak areas to a reference with a known concentration. The reference compound has to satisfy several criteria; solubility with the sample, chemical shifts different from those of the sample, and no interactions with the sample components. Sample concentrations can also be measured by MRS without an internal reference compound using a pulse length based concentration determination (PULCON) [103]. PULCON is based on the reciprocity principle [104], which states that for a given RF coil, the length of the 90° pulse is proportional to the obtained sensitivity. If two samples are measured using the same MR probe, and one metabolite has a known concentration, the concentration can be calculated based on their integrals.

1.5 Multivariate analysis

Multivariate analysis is an appropriate tool for observation and analysis of data containing more than one statistical variable at a time. Several variables from a data set may be correlated with each other, and their statistical dependence should be taken into account during analysis. The purpose of multivariate analysis is to reduce the number of variables from the data set into a smaller set of uncorrelated variable and detect or model “hidden phenomena”. There are two categories of multivariate analysis; supervised, and unsupervised. No prior knowledge is added to the data set in unsupervised analyses while supervised analyses use prior knowledge about the sample to do statistical predictions or classifications. Multivariate analysis is an appropriate tool for analyses of MRS data since the MR spectra usually contain several thousand variables.

1.5.1 Principal component analysis (PCA)

PCA is based on a linear transition of a data set from a large number of original variables to a smaller number of uncorrelated (orthogonal) variables entitled principal components (PCs). PCA is an unsupervised method since no prior knowledge about the data set is used during analysis. The first principal component (PC1) lies along the direction of maximum variance in the data set. The second principal component (PC2) defines the direction that maximizes the remaining variability in the data set, PC3 maximize the remaining variability, and so on. All PCs are orthogonal to each other. Usually the first few PCs explain a large fraction of the total variance between the samples. It is common to present the data in score plots. The scores are the projection of the data to the new coordinate system which is spanned by the eigenvectors (Figure 1.8a). Samples with similar patterns will form clusters in a score plot. Each PC has an associated loading profile. The loading profile for each PC describe the importance of each original variable (Figure 1.8.b) [105].

1.5.2 Partial least squares (PLS)

Partial least squares (PLS) is another dimension reduction method for modeling relations between a large set of observed variables to smaller set of uncorrelated variables called latent variables (LVs). PLS is similar to PCA, but is a supervised statistical method since it uses prior knowledge about the samples during analysis. The LVs are calculated by successively extracting factors from the two matrixes X (the MR spectra) and Y (properties of the MR spectra) such that covariance between the extracted factors is maximized. PLS discriminant analysis (PLS-DA) is a special case of PLS that attempts to discriminate between distinct classes, i.e. the response variable describes which class the samples fit into [106].

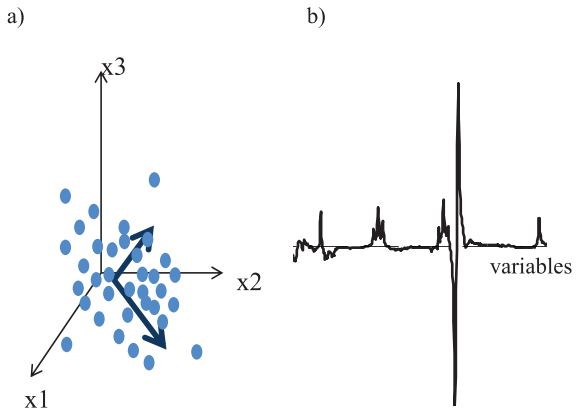

Figure 1.8: a) Schematic presentation of PCA and PLS performed on a dataset containing 35 samples with three variables x1, x2, and x3. The two vectors represent the direction in which the variance/covariance is maximized. PCs/LVs are always orthogonal. b) Representation of a loading profile for one PC/LV, describing the importance of the original variables.

1.5.3 Bayesian belief networks (BBN)

BBN is a complex non-linear statistical method that uses probability theory to classify samples into groups. The variables in BBN are represented by nodes, as showed in Figure 1.9a. Each node or variable may take one of a number of possible states or values. The certainty of each of these states is determined by the states of connected nodes. Directed links (edges) between the nodes represent the conditional dependencies between nodes, and nodes that are not connected represent variables which are conditionally independent of each other [107, 108]. If a direct link is pointing from node A to node B, then it is said that A is the parent of B, and B is the child of A. Naïve Bayesian networks consist only of one parent and several child nodes, and the variables are therefore assumed to be independent of each other.

1.5.4 Probabilistic neural networks (PNN)

PNN perform classification where the target variable is categorical. A PNN is organized into four layers, as shown in Figure 1.9b. The first layer contains nodes representing the variables. The second layer has one node for each sample in the training data set. In this layer, probability density functions (pdfs), typically of Gaussian shape, are created for each sample in the training set. The third layer is a summation of the pdfs for each subgroup in the second layer. The fourth layer compares the weighted votes for each subgroups accumulated in the summation layer and uses the largest vote to classify the unknown samples [109].

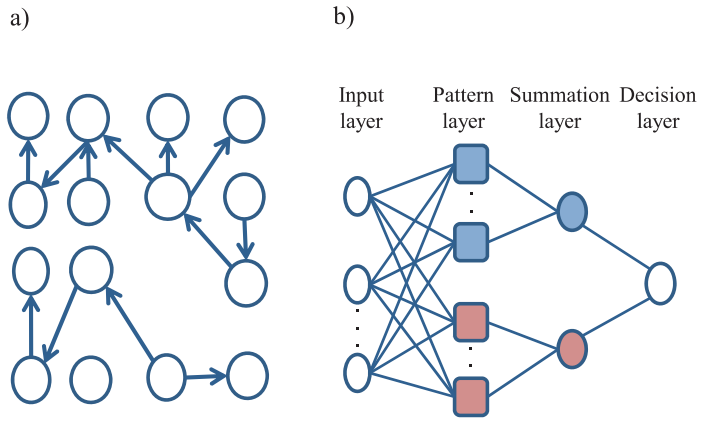

Figure 1.9: a) Schematic presentation of Bayesian belief networks. Each node represents variables, and the links between each node indicate direct dependencies between the variables. The strengths of these dependencies are quantified by conditional probabilities. b) Representation of the four layers in probabilistic neural networks. The input layer has nodes for each variable in the data set. The pattern layer contains nodes for each sample in the training data set, here categorized into two subgroups (blue, red). In the summation layer, the network sums up the information from the pattern layer. Finally, the unknown sample is classified into a subgroup in the decision layer.

1.6 Gene expression analysis

Genes encoded in the DNA of all cells can be trancribed into messenger RNA (mRNA) which are translated into proteins. The level of mRNAs is referred to as gene expression and the total number of a specific mRNA is dependent on the number of mRNA transcriptions in addition to the life time of the mRNA. Rapid decay of mRNAs in response to various stimuli can have a profound impact on the amount of protein synthesized, and the same mRNA can be translated into a protein several times [110]. In addition, post-transcriptional events such as alternative mRNA splicing increase the diversity of proteins that can be synthesized from a fixed number of genes [111].

One of the most used techniques for quantification of mRNA is gene expression microarrays. Gene expression microarrays can give a snapshot of the expression of thousands of genes. Before microarray analysis, RNA must be extracted from the biological sample. The RNA Integrity Number (RIN) is a measure of the quality of the RNA. A RIN of 7 or greater is usually recommended for a sample to be used for microarray analysis [112]. A DNA microarray slide may contain several thousands DNA probes, containing specific genomic sequences spotted at fixed locations on a support material such as a glass slide. Complementary DNA (cDNA) is synthesized from the extracted mRNA and amplified to either cDNA or cRNA and labeled with a tracer, such as a fluorescent dye. cDNA or cRNA are then hybridized (hydrogen bonded) to the probes on the microarray and the signal intensities from each spot can be measured using for example a laser scanner. The signal intensities of the individual spots can be used as a relative measure of the levels of specific transcripts in each sample compared with other samples.

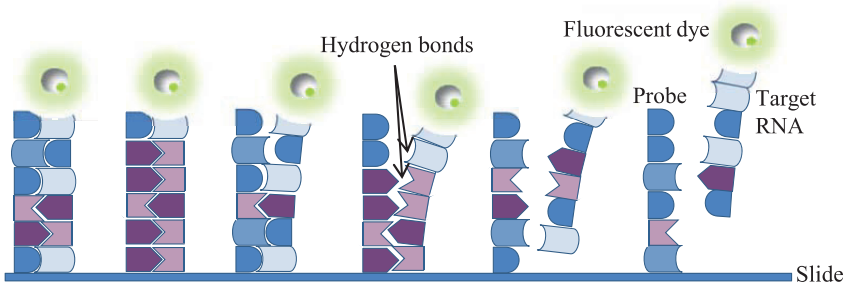

Figure 1.10: Part of a microarray slide, showing hybridization between the labeled target and the probe. The target RNAs are labeled with a fluorescent dye and the intensity of the dye in each spot represents the transcript level in the samples.

In recent years, microarrays have been extensively used to study molecular differences among different types of breast cancer. One of the common features of these studies has been the suggestions of new tumor subtypes with distinct gene expression patterns, identified using hierarchical clustering analysis [9]. Characteristic gene expression patterns can be used to classify new samples into the five intrinsic molecular subtypes [10]. Samples can be classified according to these subtypes by correlating the expression of so called intrinsic genes to centroids representing the mean expression of intrinsic genes for each of the subtypes. A new sample is generally assigned to the subtype with which it has the strongest correlation. Intrinsic subtyping is dependent on having a representative cohort that is normalized in a similar manner as the original cohort which these centroids are based on.

1.7 Histopathology

Histopathology refers to the microscopic examination of cells and tissue in order to study the indications of disease. The most commonly used staining is a combination of hematoxylin, eosin, and saffron (HES). Hematoxylin is used to stain nuclei blue, eosin stains cytoplasm and the extracellular connective tissue matrix pink, and saffron stains collagen yellow. The aim of staining is to reveal cellular components where counterstains are used to provide contrast. During surgery, breast tumor tissue, and one or more lymph nodes are removed. Tumor tissue sections are later examined under microscope to characterize the cancer and to search for cancer cells in the lymph nodes.

Immunohistochemistry (IHC) refers to the process of detecting antigens in biologic tissue by exploiting the principle of antibodies binding to specific antigens in tissue sections. Specific molecular markers are characteristic of particular cellular events such as proliferation, apoptosis, or expression of specific receptors. The presence of hormone receptors (typically ER/PgR) and growth factors receptors (typically HER2) are commonly determined by IHC in breast cancer cells. IHC is therefore used to assess which tumors are potentially responsive to targeted therapy like Tamoxifen or Herceptin.

2 Objectives

The main objectives of the research presented in this thesis were to:

- Determine prognostic and predictive factors of breast cancer by multivariate analysis of HR-MAS MRS data from human breast cancer tissue
- Measure the glycolytic activity in two breast cancer xenograft models by HR-MAS MRS in combination with gene expression analysis
- Characterize a large panel of breast cancer xenograft models and tissue from human breast cancer patients using HR-MAS in combination with gene expression analysis with a particular focus on choline metabolism

3 Materials and methods

This thesis describes data of tissue from breast cancer patients and xenograft models obtained using *ex vivo* HR-MAS MRS and gene expression analysis. An overview over samples and experiments is given in Figure 3.1.

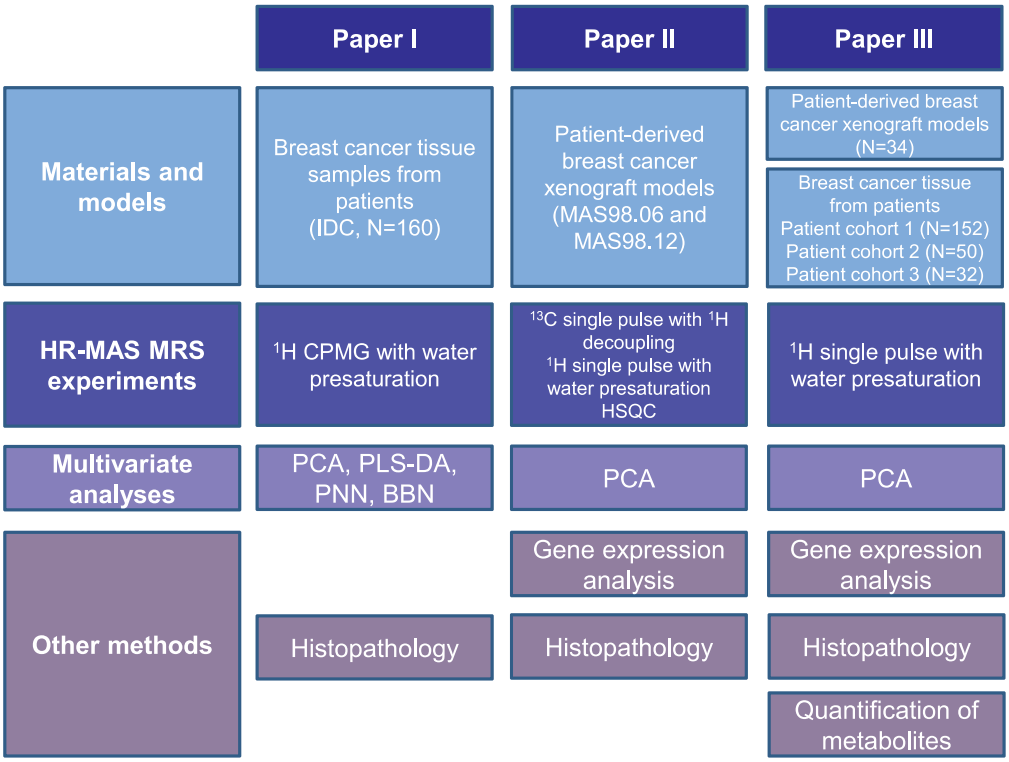

Figure 3.1: Schematic presentation of samples and methods used in the papers presented in this thesis. Abbreviations: BBN: Bayesian belief network, CPMG: Carr-Purcell-Meiboom-Gill, ER: Estrogen receptor, HSQC: Heteronuclear single quantum coherence, IDC: Invasive ductal carcinomas, PCA: Principal component analysis, PLS-DA: Partial least square discriminant analysis, PNN: Probabilistic neural networks.

3.1 Human tissue samples

Paper I and III included patients diagnosed with breast cancer. Written informed consent was obtained from all patients, and the study was approved by the Regional Committee for Medical and Health Research Ethics. After surgery, the tissue samples were immediately frozen (within minutes) and stored (~two years) in liquid nitrogen until HR-MAS MRS analysis. The MR spectra from paper I (N=160) were selected from our internal database. All patients had been diagnosed with IDC, and none of them received neoadjuvant chemotherapy (NAC) prior to surgery. Hormone receptor status (ER, and PgR) was also previously determined by IHC. In paper III, three different patient cohorts were included. Tissue samples from Patient cohort 1 (N=152) was used for breast cancer subclassification of the xenograft models. Patient cohort 2 (N=50) was used to evaluate metabolic characteristics in breast cancer tissue samples being ER+ (N=37) and ER- (N=13). The MR spectra were selected from our local spectral database based on the estrogen receptor status. Some of these samples have also been analyzed by HR-MAS MRS in paper I, and in another study [113]. Patient cohort 3 (N=32) was used for comparative analysis of gene expression between human and xenograft samples. The gene expression analysis of these samples is described in [114, 115]. Only the basal-like (N=18) and luminal B samples (N=14) were selected from the data set, and only expression of genes directly involved in choline metabolism were used in the analyses.

3.2 Tumor models

The luminal-like (MAS98.06) and basal-like (MAS98.12) xenograft models used in Paper II were established at the Oslo University Hospital, Radiumhospitalet, as described previously [114]. Briefly, primary tumor samples from breast cancer patients were implanted subcutaneously into a back pocket of SCID mice in the presence of continuous release of estradiol. The xenografts were subsequently transplanted from

mouse to mouse when reaching a diameter of 15 mm. The tumor tissue samples were later implanted orthotopically into the mammary fat pad of the mice. The animals were transported from Oslo to Trondheim prior to the HR-MAS MRS experiments. The molecular characteristics of the xenograft models are described by Bergamachi et al. [114]. Mice carrying the xenograft tumors were sacrificed by cervical dislocation during isoflurane anesthesia. Tumor tissue samples from one group of mice (N=19) were collected and frozen in liquid nitrogen for MRS analysis. Another group (N=16) received a bolus injection of [1-^{13}C]-labeled glucose via the lateral tail vein, and the mice were sacrificed 10 or 15 minutes after the injection. All tumor samples were collected and immediately stored in liquid nitrogen for MRS analysis.

The tumor models studied in Paper III were established at The Translational Research Department, Institut Curie, Paris as previously described [57, 61]. Primary tumor samples from breast cancer patients were implanted into the interscapular fat pad of female Swiss nude mice receiving estrogen. The xenografts were subsequently transplanted from mouse to mouse. The molecular characteristics of the xenograft models are described by Marangoni and Reyal et al. [57, 61]. Tumor tissue samples (N=32) were collected and sent from Paris to Norway, one part to Oslo for gene expression analysis and another part to Trondheim for metabolic analysis. The two xenograft models from paper II were also included in paper III.

a) b)

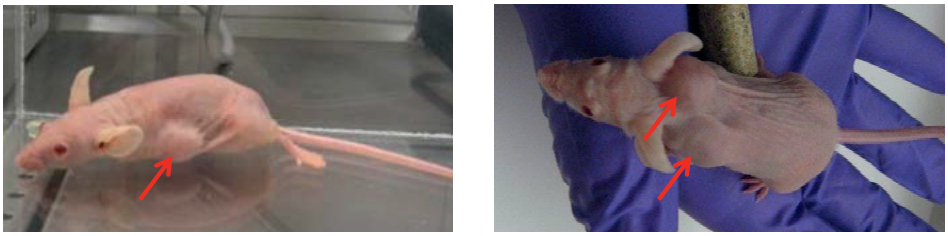

Figure 3.2: Patient-derived breast cancer xenograft models. a) Orthotopic breast cancer xenograft implanted into the mammary fat pad of SCID mouse. b) Subcutaneous breast cancer xenograft implanted into the interscapular fat pad of Swiss nude mice. Red arrows point out the tumors. The xenograft model in Figure b) is reprinted with permission from Elisabetta Marangoni.

3.3 HR-MAS MRS

Before HR-MAS MRS analysis, the tumor samples were cut to fit either a 50 µL rotor (paper I, II and III) or a 25 µL insert placed into an 80 µL rotor (paper III). Buffer containing D_2O with trimetylsilyl-3-propionic acid sodium salt d_4 (TSP) and formate was added to the samples. The HR-MAS MR spectra were acquired using a Bruker Avance DRX600 spectrometer (paper I, II and III), or a Bruker Avance III 600MHz/54 mm US (paper III), equipped with a $^{1}H/^{13}C$ HR-MAS probe with magnetic field gradients aligned with the magic angle axis (Bruker Biospin, Germany). The the spin rate for all experiments was 5 kHz and temperature in the probe fixed at 4 °C or 5 °C.

The MR spectra in paper I were acquired using a 1D water and fat suppressing CPMG sequence (cpmgpr, Bruker). The MR spectra in paper II and III were acquired using 1D ^{1}H single pulse MR spectra with water presaturation (zgpr; Bruker). The ^{13}C MR spectra in paper II were acquired using a single pulse experiment with decoupling during recycle delay and acquisition (zgpg, Bruker). Two-dimensional (2D) ^{1}H-^{13}C HSQC MR spectra with decoupling during acquisition (hsqcetgpsi2, Bruker) were acquired in paper II to assist spectral assignment. The HR-MAS MR spectra were processed using TopSpin 3.0 (Bruker). The acquisition parameters are shown in Table 3.1.

Table 3.1: HR-MAS MRS parameters. Abbreviations: cpmgpr: Carr-Purcell-Meiboom-Gill with water presaturation, NA: not applicable, ppm: parts per million, zgpg: single pulse spectra with power-gated proton decoupling, zgpr: single pulse spectra with water suppression.

	Paper I	**Paper II**		**Paper III**	
	Human tissue	Xenograft tissue		Xenograft tissue	Human tissue
MRS pulse sequence	^{1}H cpmgpr	^{1}H zgpr	^{13}C zgpg	^{1}H zgpr	^{1}H zgpr
Temperatur	4 °C	4 °C	4 °C	5 °C	4 °C
Spin rate	5 kHz	5 kHz	5 kHz	5 kHz	5 kHz
Acquisition time	1.64 s	4.6 s	0.5 s	3.4 s	2.7 s
Number of scans	128	128	26 k	32	32
Number of points	32 k	32 k	32 k	32 k	32 k
Sweep width	16.7 ppm	12 ppm	210 ppm	16 ppm	20 ppm
Echo time	285 ms	NA	NA	NA	NA

3.4 Data analyses

The metabolite ratios obtained in paper II were calculated by integration of the peaks. Peak fitting (PeakFit, SeaSolve Software, Inc. San Jose, USA) and integration (TopSpin 3.0; Bruker) was used for quantification of the metabolite concentrations and calculation of metabolite ratios. In paper III, PULCON was used for quantification of the metabolites in the xenograft tissue samples, while the metabolite concentrations in the human tissue samples were calculated from the internal standard TSP and sample wet weight.

PCA were performed using PLS toolbox for MATLAB (Eigenvector Research, USA) or Unscrambler (Camo Software AS, Norway). The relationship between the different metabolites of the MR spectra and breast cancer prognostic factors was studied with PLS-DA, BBN and PNN in paper I. PLS-DA was performed using PLS toolbox for MATLAB, BBN analyses were performed using Netica (Norsys Software Corp,

Canada), and PNN analyses were performed in NeuroShell Classifier (Ward Systems Group, USA).

3.5 Gene expression analysis

Gene expression analysis in paper II and III was performed with support from the Dept. of Genetics, Institute of Cancer Research, Oslo University Hospital, Radiumhospitalet.

A microarray-based platform was used where total RNA was isolated from frozen tumor tissue samples. The RNA was amplified, labeled with Cy3, and hybridized to Agilent Human Whole Genome Oligo 4x44k or 8x60k Microarrays, according to the manufacturer's protocol (Agilent Technologies, USA). The arrays were scanned and the data normalized and analyzed using R (v 2.9.0) and the Limma Bioconductor package [115]. The raw signals were detrended for multiplicative effects, the arrays were quantile normalized and log2 transformed. Genes reported to be relevant for glycolysis (hsa:00010) and glycerophospholipid pathway (hsa:00564) were selected from Kyoto Encyclopedia of Genes and Genomes (KEGG). The selected genes were tested for differential expression using a t-test or an Empirical Bayes' modified t-test.

In order to determine the molecular intrinsic subtype of the xenograft tumors in paper III, the gene expression data from the 30 samples were mean centered against a larger, more heterogeneous human breast cancer gene expression dataset of 152 tumors. The molecular subclassification of the xenografts was determined based on distinct variation in gene expression pattern of 500 "intrinsic" genes, characteristic for five major molecular breast cancer subtypes.

3.6 Histopathology

Histopathological examinations of the human tissue samples in paper I, II, and III were performed at Dept. of Laboratory Medicine, Children's and Women's Health, NTNU. In addition, receptor status of ER, PgR and HER2 for the xenograft models in paper III was determined at Institut Curie, Paris, France.

After HR-MAS MRS analyses, the samples were fixed in formalin and embedded in paraffin. Paraffin sections of 3-5 μm were cut and stained with HES. Relative areas of normal, tumor, fat, necrotic, and fibrous tissue were estimated by visual examination using light microscopy. Axillary lymph nodes were removed during surgery, either by axillary lymph node dissection (ALND) or by sentinel lymph node dissection (SLND). The samples were considered lymph node positive if at least one node contained cancer cells.

Receptor status of ER, PgR and HER2 were determined with immunohistochemistry (IHC). The ER and PgR staining cut-off point was 10% where samples < 10% were considered negative. For HER2, protein positivity was defined if ≥65% of the cells were positive.

4 Summary of papers

Paper I

Multivariate modeling and prediction of breast cancer prognostic factors using MR metabolomics

The purpose of this study was to investigate if MR metabolomics can be a useful tool in the prediction of the breast cancer prognostic factors estrogen (ER), progesterone (PgR), and axillary lymph node status. Three different multivariate classification techniques; partial least-squares discriminant analysis (PLS-DA), probabilistic neural networks (PNN), and Bayesian belief networks (BBN), were tested.

High Resolution Magic Angle Spinning (HR-MAS) MR spectra from totally 160 breast cancer patients diagnosed with invasive ductal carcinoma were analyzed. The spectral data were preprocessed and variable stability (VAST) scaled, and training and test sets were generated using the Kennard-Stone and sample set partitioning based on joint x-y distances (SPXY) sample selection algorithms. Blind samples from the test sets (50 spectra) were predicted for verification. PLS-DA was the best predictor for ER and PgR receptor status, where 44, and 39 of the 50 samples were correctly classified, respectively. Higher levels of phosphocholine (PCho), taurine, creatine, and ascorbate and lower levels of glycine, choline, alanine, and glycerophosphocholine (GPC) were found in ER+ compared to ER- samples. The PgR+ samples were characterized with less glycine, ascorbate, PCho, choline, lactate, alanine, and creatine than PgR- samples. BBN was the best predictor of lymph node status, where 34 of 50 samples being correctly classified. The number of correctly classified samples was better than expected by chance.

These results show that MR spectra contain prognostic information about breast cancer patients and that MR metabolomics may be a tool that can benefit patients during cancer treatment.

Paper II

^{13}C High-resolution-Magic Angle Spinning MRS reveals differences in glucose metabolism between two breast cancer xenograft models with different gene expression patterns

The purpose of this study was to investigate glucose metabolism in two xenograft models representing basal-like and luminal-like breast cancer using ^{13}C high-resolution-magic angle spinning (HR-MAS) MRS, and gene expression analysis.

^{13}C and ^{1}H HR-MAS MR spectra were acquired from two groups for each model, untreated mice (n = 19), and a group of mice (n = 16) that received an injection of [1-^{13}C]-glucose 10 or 15 minutes before harvesting the tissue. The predominant metabolites observed after [1-^{13}C]-glucose administration were [3-^{13}C]-lactate, and [3-^{13}C]-alanine. [4-^{13}C]-glutamate was observed in most of the samples in the luminal-like model, indicating an active TCA cycle in this model. A significant lower glucose/alanine (Glc/Ala) and glucose/lactate (Glc/Lac)-ratio was detected in the luminal-like compared to the basal-like subgroup ($p<0.05$) both for the labeled and unlabeled group. In addition, most genes contributing in glycolysis were higher expressed in the luminal-like compared to the basal-like models. These results indicate an agreement between expression of glycolytic genes and amounts of glycolytic end products in the two models. The results demonstrate that the transformation from glucose to lactate and alanine occurs faster in the luminal-like than in the faster growing basal-like model.

Paper III
Interplay of choline metabolites and genes in patient-derived breast cancer xenografts

Dysregulated choline metabolism is a well-known feature of breast cancer, but the underlying mechanisms are not fully understood. In this study, the metabolomic and transcriptomic characteristics of a panel of breast cancer xenograft models were mapped, with focus on the association between gene expression and choline metabolites.

Tumor tissue specimens from 34 xenograft models were collected and divided in two pieces. One part was examined using HR-MAS MRS while the other was analyzed using gene expression microarrays. Gene expression data from genes involved in the choline metabolic pathway was analyzed. The correlation between gene expression levels and the levels of choline, phosphocholine (PCho), and glycerophosphocholine (GPC) was evaluated using Pearson's correlation analysis. Metabolic and gene expression analysis was also performed on tissue samples from breast cancer patients in corresponding molecular subgroups.

Most of the xenograft models were classified as basal-like (N=19) or luminal B (N=7) breast cancer, with significantly different choline metabolic and gene expression profiles. The luminal B xenografts were characterized with a high PCho/GPC ratio while the basal-like xenografts were characterized with highly variable PCho/GPC ratios. Choline, PCho and GPC levels were correlated to expression of several genes in the choline metabolism, including choline kinase alpha (*CHKA*) and glycerophosphodiester phosphodiesterase 5 (*GDPD5*).

The higher PCho/GPC ratio found in the luminal B compared to most basal-like breast cancer xenograft models and human tissue samples do not correspond to results from *in vitro* studies. Choline, PCho and GPC were correlated to different choline genes in the luminal B samples than in the basal-like xenograft samples. The metabolic and gene expression profiles were in concordance with findings in tissue samples from patients in corresponding breast cancer subgroups. This indicates that these xenografts are representative models of human breast cancer.

5 Discussion

The main objective of this thesis was to characterize the metabolic profiles of different breast cancer subtypes, both in patient tumor tissue and tissue from animal xenograft models. The metabolic data was combined with gene expression analysis and histopathology in order to understand how metabolic pathways are regulated and associated with tumor aggressiveness. This may be used to establish prognostic and predictive tools based on metabolomics and transcriptomics and provides possibilities for more individualized treatment for breast cancer patients. A better understanding of the correlations between aggressiveness and metabolism offers possibilities for exploiting metabolic pathways in breast cancer treatment. Therapeutic strategies aimed at reducing glycolysis or choline metabolism may successfully prevent carcinogenesis or suppress growth of established invasive cancers.

5.1 Patient tissue samples and patient-derived xenograft models

Patient tumor tissue samples were analyzed by HR-MAS MRS and gene expression analysis in paper II and III. The tissue samples contain a heterogenous tumor environment including both tumor cells, and stromal and fatty tissue. After resection, the tissue samples were immediately frozen (within minutes) and stored (~two years) in liquid nitrogen until HR-MAS MRS analysis. Long term storage of samples in liquid nitrogen has shown little or no effect on tissue metabolite degradation [42, 116]. All HR-MAS MR spectra were acquired at 4-5°C in order to reduce tissue metabolite degradation. However, some degradation may be expected during acquisition, especially for experiments performed with a long acquisition time.

Patient-derived xenograft models were used to study the metabolic profiles of different subtypes of breast cancer (paper II and III). Different from animal models based on cultured cancer cells, patient-derived xenograft models will more closely resemble the whole system of human tumors [55]. However, there are clear limitations to xenograft

models. First, immune responses, which may both protect or facilitate tumor growth [117] are impaired in the immunosuppressive mice. Second, the properties of the implanted cancer cells are retained in these models, but connective tissue and blood vessels surrounding the tumor cells are from the host. In line with this, the extracellular matrix (ECM)-related genes were found to be downregulated in the xenografts compared to the primary tumors in paper II. The downregulation of stroma-related genes was compensated by overproduction of ECM-related genes by the mouse host tissue [114]. Therefore, care has to be taken when transferring results from these models into the clinic.

The xenograft models studied in this thesis were established by direct grafting of human cancer tissue into mice. In contrast to cell line derived xenograft models which are originated from one cell clone, the patient-derived xenograft models originate from a more heterogenous tumor environment, containing both tumor cells and stromal tissue. Patient-derived xenograft models appear to retain the morphological and molecular markers of the source tumors, despite serial passing across several generations of mice [58, 62, 118]. The models we have studied maintained morphology, cell differentiation, and molecular signatures of the original patient's tumors [57, 114]. Vasculature, necrosis and metastatic potential in the tumorbearing mice were also similar to the patient's tumor.

Most human tumor xenograft experiments have employed subcutaneous injection procedures, and one of the main limitation of subcutaneous site is very often the lack of metastasis [119]. The xenograft tumors in paper II were first subcutaneously engrafted into the mice. Because of a gradually slower tumor growth at this site, an orthotopically engraftment into the mammary fat pad was later chosen, and gave better conditions for tumor growth. An orthotopic location will often give grafts that better resemble human tumors compared to subcutaneous locations [120]. The xenograft models in paper III were engrafted into the interscapular fat pad of the mice, a site which was found to be favorable for tumor take and growth. In addition, about a third of xenografts in paper III developed lung metastases, and three were consistently metastatic [57].

ER+ models are more difficult to establish than ER- xenograft models, which is reflected in high number of ER- compared to ER+ models. In addition, the ER+ models often show an aggressive phenotype, which most likely also is the case for the ER+ models in this panel. Although the panel reflects a strong selection bias toward aggressive tumors, disease heterogeneity persists in terms of histology and biological parameters. These models could therefore be valuable in predicting clinical outcome of cancer patients. MR spectroscopy is an appropriate tool to study the metabolic profiles of the models. These models also represent a variety of different breast cancer tumors and may be important tools in studies of molecular properties associated with sensitivity or resistance to chemotherapy or targeted anticancer drugs. It also allows further studies of the unique biology of the different subtypes of breast cancer, which may be important for future clinical applications based on molecular fingerprint.

5.2 HR-MAS MRS experiments and analysis

HR MRS and HR-MAS MRS have shown to be candidates technologies in breast cancer diagnosis and treatment monitoring [80, 83, 121-123]. The HR-MAS MRS technique gives high quality MR spectra of intact tissue and more than 30 different metabolites can be assigned in breast cancer tissue samples [71].

The MR spectra of the breast cancer tissue samples from patients in paper I were acquired using a CPMG presaturation MR sequence. Breast tissue consists of large fractions of fatty acids, and the CPMG MR sequence is therefore suitable for suppressing broad MR signals from fatty acids and thus enhancing signals from other metabolites. A water presaturation single-pulse MR sequence was applied on the xenograft models in paper II and III. This MR sequence gives spectra with a higher SNR compared to a CPMG sequence. The xenograft samples consist mainly of tumor and stromal tissue, and suppression of broad peaks from fatty acids is therefore less essential. The ^{13}C MRS is an insensitive technique, because of low natural abundance of ^{13}C isotopes (1.1%) in biological tissues, in addition to a relatively low gyromagnetic ratio (γ). MR sequences with numerous transients, especially for natural abundance

experiments, are therefore required in order to acquire ^{13}C MR spectra with acceptable SNR. The ^{13}C MR spectra were acquired using ^{1}H decoupling to suppress coupling between ^{1}H and ^{13}C, which would otherwise lead to signal splitting and reduced sensitivity.

Choline metabolites were quantified in the xenograft tissue samples in paper III. Several factors may potentially lead to errors during quantification. The RF field of the HR-MAS probes, which are commonly inhomogeneous, may lead to errors in the quantification, as described by Ziarelli et al. [124]. PULCON [103], which may be used to measure metabolite concentrations without the use of an internal reference, was used for quantification of metabolites in the xenografts in paper III. For quantification of the metabolites in the human tissue samples in paper III (Cohort 2), metabolite peak areas of choline, PCho, and GPC were related to the internal standard TSP and sample wet weight. Quantification using TSP as reference may cause additional errors since an internal standard can bind to molecules in the tissue causing an overestimation of the metabolite concentration. Curve fitting using PeakFit was used to find the area of the choline metabolites. Curve fitting is necessary to achieve a precise quantification from overlapping peaks in the MR spectra. This curve fitting method may be biased by subjective interpretations. However, since the fitting was performed by one user only, the inter-spectral effects were considered to be minimal. In order to validate the accuracy of the quantified data, several spectra with known creatine concentrations (~0.25-10mM) were acquired. The results showed a mean relative error of 6.8%, where the samples of low concentrations had a higher relative error compared to that of high concentrations. It can therefore be assumed that low concentration metabolites (like choline) are more difficult to quantify with a high precision compared to metabolites with higher concentrations. In paper II, integration was performed to study metabolite ratios and calculate percent enrichment. For calculation of ^{13}C enrichment, the concentration of ^{13}C-taurine was estimated based on the assumption that 1.1% of the taurine isotopes were ^{13}C-taurine. Taurine was selected because it did not overlap with other metabolites. In addition, it is very unlikely that ^{13}C from glucose would be incorporated in taurine.

The MR spectra in all papers were analyzed using multivariate methods. These methods are not quantitative, thus only relative contents and variations in metabolites can be evaluated. Absolute quantification may in some cases be beneficial over multivariate methods, since the concentrations more easily are comparable with other studies. Advantages of multivariate methods are the possibility of extracting important features from complex data sets and identify patterns from several metabolites simultaneously. Proper preprocessing including the selection of spectrum region, baseline correction, mean normalizing, and peak alignment is important in order to get meaningful results. The MR spectra in paper I were variable stability (VAST) scaled [125]. The purpose of VAST scaling is to focus on the most stable variables of the data set and downweight the least stable variables. In order to select samples that are representative for the whole data set when training the model, two different sample selection algorithms were used, Kennard Stone sample selection [126] and SPXY [127]. PCA was performed on MR spectra from tissue samples in all papers, while PLS-DA, PNN, and BBN in addition was used in paper I. PCA, and PLS-DA are both linear methods, while PNN and BBN are nonlinear methods, usually conducted in a black box manner, and interpretation of the underlying molecular biology responsible for clustering or classification of distinct groups may consequently be more challenging. In paper I, PLS-DA provided the best classification results for ER and PgR. BBN was found to be the best predictor for lymph node status using the SPXY sample collection and the number of correctly classified samples was better than by chance. This indicates that the MR spectra are different in lymph node positive and lymph node negative tissue samples.

5.3 Metabolic characterization of breast cancer

MRS is widely applied in biomedical research, with particular attention to molecular evaluations regarding diagnosis, treatment, and prognosis of human neoplasms. HR-MAS MRS gives a description of the molecular composition of intact tissue samples and represents consequently an important tool for metabolic analysis of cancer tissue. Cancer cells are commonly characterized with dysregulations in metabolic pathways. They commonly have a high glycolytic activity where cancer cells consume glucose and

convert it to lactate even when oxygen is present (Warburg effect). Cancer cells show in addition modifications in the choline metabolism. Since glycolysis and choline metabolism are dysregulated in cancer and metabolites from glycolysis and choline metabolism are MRS-visible, these pathways have been thoroughly studied in this thesis.

The objective of paper II was to use ^{13}C HR-MAS MRS to analyze the glycolytic activity in two breast cancer xenograft models. In contrast to ^{1}H MRS, ^{13}C MRS allows detection of ^{13}C labeled parent substrate and their downstream metabolites, and the metabolic flux through metabolic pathways can be mapped. The results in paper II showed that both the luminal-like, and the basal-like model had a high glycolytic activity where ^{13}C labeled glucose mainly was transformed into ^{13}C labeled lactate, and alanine. Small amounts of ^{13}C labeled glutamate were also detected in the luminal-like model indicating an active TCA cycle in these tumors. The percent enrichment of ^{13}C labeled lactate and alanine in the two models indicates that the differences in glucose/lactate (Glc/Lac) and glucose/alanine (Glc/Ala)-ratios between the two models is caused by lower levels of Glc in the luminal-like compared to the basal-like model. The lower Glc/Lac and Glc/Ala-ratio, and higher expression of glycolytic genes observed in natural abundance and ^{13}C glucose labeled samples in the luminal-like model, indicates a faster conversion from glucose to lactate, and alanine in this model compared to the basal-like model. Conversion of glucose to lactate in the presence of oxygen is known as the Warburg effect and is uniquely observed in cancer [23]. It can accordingly be assumed that the Warburg effect is higher in this model compared to the basal-like model. Hypoxia is another factor that may lead to greater production of lactate in tissue, since the cells are forced to produce energy from glycolysis rather than from oxidative phosphorylation. A significant higher hypoxic fraction was found in the luminal-like than in the basal-like model [128]. In addition, a greater angiogenic activity was detected in the basal-like compared to the luminal-like model [128]. As a result of the fewer tumor blood vessels in the luminal-like model, the glucose is probably less available for the cancer cells, and nearly all of the distributed glucose is consumed and metabolized in this model. It can be speculated that the cancer cells in the luminal-like

model have adapted to compensate for the glucose shortage by having an increased glycolytic efficiency.

The glycolytic activity in the luminal-like and the basal-like models from paper II has also been measured using 18F-deoxy-glucose positron emission tomography (FDG-PET) in a different study [129]. The findings in this study were in accordance with the findings in paper II, and showed that (i): Basal-like xenografts had higher initial FDG uptake, which may be caused by two factors; either glucose transport, and/or a higher angiogenic activity. Since the expression of glucose transport genes were upregulated in the luminal-like model, it can be assumed that the angiogenic factor plays a major role in glucose availability in the basal-like tumor tissue. (ii): the transformation from glucose to glucose-6-phosphate (Glc-6P) occurred faster in the luminal-like model. This can be caused by upregulation of hexokinase and explain a higher Warburg effect in this model. A different study performed on breast cancer patients showed that triple-negative tumors had a higher FDG uptake compared to ER+/PgR+/HER2− tumors [130]. This study was not performed dynamically, and therefore, the transformation from glucose to Glc-6P was not modeled. For the patient tissue samples in paper I, glucose was only detectable in some samples, and no significant differences in glucose levels between samples with different ER/PgR status were found. Higher levels of lactate were, however, found in ER-, and PgR-, compared to ER+ and PgR+ tissue samples. For the xenograft models in paper II, no differences in the fractional enrichment of ^{13}C-lactate between the luminal-like and the basal-like model were found, but for the panel of xenograft models in paper III, the PCA indicated higher levels of lactate in basal-like/triple-negative compared to luminal B/ER+ samples. Acidosis, which is a consequence of upregulated glycolysis and poor perfusion is a common microenvironmental feature of most solid tumors, and may promote invasion and metastasis [131]. Overall, these results indicate that lactate may be a biomarker for tumor aggressiveness.

Abnormal choline metabolism in an emerging hallmark of cancer and have been associated with oncogenesis and tumor progression. Most *in vitro* studies of breast cell cultures have demonstrated a switch from a low PCho/high GPC to a high PCho/low

GPC metabolic profile with increased malignancy [35, 39]. A similar switch from low to high PCho/GPC ratio has also been reported in cell lines from prostate cancer [132] and immortalized, oncogene-transformed rat Schwann cells [133]. These findings are not in agreement with the findings from human breast cancer tissue samples in paper I and the xenograft models in paper II and III, where a lower PCho/GPC ratio was observed in more malignant ER- compared to ER+ human, and xenograft tissue samples. Higher levels of GPC and thus lower PCho/GPC ratios have also been correlated with malignancy in xenograft tissue samples from prostate cancer [43]. The discrepancy in PCho/GPC ratios between cell lines and tissue samples can presumably arise from different interactions and a more heterogeneous environment found in solid tumors than in cultured cell lines. In a study of perfused mammalian cells, an acidic extracellular pH led to a significantly increase in GPC and a decrease in the PCho levels [134]. Since the luminal-like model from paper II is less vascularized, has a higher hypoxic fraction, [128], and probably has a higher glycolytic activity than the basal-like model, it can be speculated that the extracellular pH is lower in this model. However, significant lower GPC concentrations has been observed in this model compared to the basal-like model [113]. On the other hand, hypoxia leads to stabilization of HIF, which has shown to enhance the CHK activity and may cause increased levels of PCho [135]. This may, at least partly, explain the higher expression of *CHKA* and higher PCho/GPC level observed in the luminal-like model compared to the basal-like model from paper II [113]. Another study showed that lower content of GPC were associated with malignant tumors with high expression of ER [136], indicating that ER may play a role for contents of choline metabolites.

Prediction of prognostic factors in breast cancer by use of MRS was evaluated in paper I. ER and PgR was predicted with a high sensitivity and specificity. The metabolic regression profile of the ER+ samples appeared to be similar to that of the PgR+ samples. This can partly be caused by the fact that PgR is regulated by ER [137], and that therefore the majority of the samples are either ER+/PgR+ or ER-/PgR-. The loading profiles indicated that the PgR- samples were characterized with higher levels of ascorbate, lactate, glycine, GPC, PCho, choline, creatine, and alanine compared to the PgR+ samples. The ER- samples were characterized with higher amounts of glycine,

GPC, choline, and alanine, and lower amounts of ascorbate, creatine, taurine, and PCho than ER+ samples. In concordance with this were also the lower amounts of taurine, and creatine and higher amounts of lactate found in basal-like/triple-negative compared to the luminal B/ER+ samples from paper III. The role of taurine and creatine in cancer is still not clear. Studies of breast tissue biopsies have demonstrated that taurine is elevated in cancer compared to normal cells [77, 138]. The amino acid glycine was, on the other hand found to be higher in the luminal B/ER+ xenograft samples, in contrast to the lower glycine content in ER+ patient tissue samples in paper I. Glycine is mainly derived from glycolysis, but can also be converted from choline [139]. It has been shown that relatively large amounts of glycolytic carbon can be diverted into glycine metabolism and that this may confer several advantages for tumor growth [140]. High levels of glycine have been associated with low survival in hormone receptor positive breast cancer patients [141], and it has identified in high grade compared to low grade brain tumors [142]. The role of glycine is, however, still not understood, and more studies are therefore needed in order to understand its role in cancer progression.

Lymph node metastasis is a main prognostic factor in a number of malignancies, including breast carcinomas [143]. In paper I, lymph node status (LNS) was predicted from MR profiles of human breast cancer samples with a sensitivity and specificity of 52% and 79%, respectively. This result is better than expected by chance, but shows an error that is not reliable enough for a correct classification of LNS. It can be hypothesized that metabolic changes appear before a lymphatic spread and makes it difficult to distinguish between patients with spread and patients with risk of spread. Moreover, the lymph node status was identified both on axillary and sentinel lymph node status (ALNS, and SLNS), which could have affected the classification. The sentinel nodes are most likely to contain metastasizing cancer cells if lymphatic metastasis is present [144]. Still, several investigators have reported variable rates of a false negative result, i.e. negative SLNS in the presence of other metastatic axillary nodes [144-146]. Lymph node status from MR profiles was also predicted in another study with a 94% correct classification [81]. In this study, however, the same samples were used both in the training and testing set. This can lead to overoptimistic results since the training model cannot forecast as well on new data as on the data that has been

trained on. The underlying mechanisms leading to lymphatic spread are not fully understood. Growth of lymphatic vessels (lymphangiogenesis) near solid tumors is often associated with lymph node metastasis. This may begin before tumor cells arrive in the nodes, presumably promoted by lymphangiogenic growth factors derived from the primary tumor [147]. This process may facilitate further metastatic spread to distant organs [148]. A number of factors that may play a role in development of lymph node metastases have been identified. These include the well-known vascular endothelial growth factor (VEGF)-C, VEGF-D, and VEGFR-3 as well as chemokine-receptor interactions, integrins and downstream signaling pathways [149-154].

5.4 Regulation of metabolic pathways

Different molecular subclasses of breast cancer (luminal A, luminal B, basal-like, HER2 enriched, and normal breast-like) have been described based on gene expression profiles [9, 10]. Most ER+ tumors are classified in the luminal-like subgroup, ER- tumors often classified as basal-like, while HER2 enriched cancers are commonly characterized with a high expression of the HER2 receptors [10, 155, 156]. The association between histopathological characteristics and intrinsic subtype in paper III was in agreement with other published data, since all of the ER+ models were classified as luminal-like models and eighteen of twenty-four triple-negative models were classified as basal-like models. This strengthens the suggestion that these xenograft models are representative models for human breast cancer.

When correlating metabolites with gene expression it should be kept in mind that the gene expression not necessarily represents enzymatic activity. The expressions of specific mRNAs are dependent on the number of mRNA transcripts, and the life time of the mRNA. In addition, post-translational modifications of the enzymes may lead to differences in the protein activity. Other studies have shown various degree of correlations between protein and gene expression levels [157]. Therefore, care has to be taken when correlating gene expression levels, and not protein activity, with metabolite concentrations.

Discussion

The majority of the glycolytic genes analyzed in paper II were higher expressed in the luminal-like compared to the basal-like model. This result was consistent with the more rapid conversion of glucose to lactate in the luminal-like model. GLUT, HK and LDHA are known to be important regulators in the glycolysis, and isoenzymes of these were higher expressed in the luminal-like model. One rate limiting step in glycolysis is the control point catalyzed by PFK1 [29]. PFK1 has three known isoenzymes; PFKM, PFKL, and PFKP, where the gene transcript encoding for the isoenzyme *PFKM* was higher expressed in the basal-like model. It has been suggested that glycolytic efficiency primarily depends on the preferential expression of another isoform, *PFKL* [158] , which was not differentially expressed between the models.

The regulation of choline metabolism is not fully understood. In paper III, seven of the 54 genes contributing in choline metabolism were found to be correlated to the concentrations of either PCho or GPC. Several other studies have shown a correlation between PCho and *CHKA* [159-161], and this was also confirmed in our study. PCho was also positively correlated to glycerophosphodiester phosphodiesterase 5 (*GDPD5*) suggesting an increased Ptd-Cho-turnover in these tumors. A positive correlation between *GDPD5* and PCho concentration was also found, which is in accordance with another study [162]. The regulation of GPC is poorly understood, which is a problem since it contributes significantly to the tCho which is detected by *in vivo* MRS. In addition, several studies have demonstrated that GPC may be a potential biomarker for response to treatment [82, 83, 163]. It would therefore be valuable to clarify mechanisms causing high GPC concentration in some tumors. As GDPD5 is responsible for GPC degradation, the positive correlation between *GDPD5* and GPC concentration in paper III was not expected. Feedback mechanism leading to Ptd-Cho turnover may be a possible explanation of these findings. This interpretation is supported by the positive correlation between expression of *CHKA* and *GDPD5*, which has also been observed in another study [162]. The contribution of PLC, PLD and PLA2 in the regulation of choline metabolism is also unclear [164], but several studies have found that isoenzymes of these are overexpressed in some cancers [45, 122, 165, 166]. In our xenograft models, a higher expression of *PLA2G6* was found in samples with high GPC, suggesting that this isoform may be a catalyst in the transformation from Ptd-Cho

to GPC. PLD hydrolyse Ptd-Cho to phosphatidic acid and choline, and the *PLD3* isoform was negatively correlated with GPC. PLC contributes in the conversion from Ptd-Cho to PCho and diacylglycerol. However, no mammalian isoforms of PLC have been sequenced to date, and it is therefore difficult to study the associations between expression of PLC and the concentration of choline metabolites.

The gene expression profiles from paper III were found to be different in the luminal B compared to the basal-like xenograft samples. The luminal B samples were characterized with a higher expression of *PLCD4*, *GDPD3*, and glycerol-3-phosphate dehydrogenase 1-like *(GPD1L)*, while the basal-like samples had a higher expression of *PLCG2*, patatin-like phospholipase domain-containing protein 3 (*PNPLA3)*, and *PLCE1*. In addition, different choline metabolites were correlated to other genes in the luminal B than in the basal-like subgroup. This suggests that luminal B and basal-like breast cancer may have a different choline metabolism regulation. Similar metabolic and gene expression profiles in choline metabolism were found in the xenograft and human breast cancer tissue with corresponding molecular subtype. This suggests that the xenograft models are valuable for studies of human breast cancer, despite the presence of mouse stromal cells and potentially different tumor/host interaction than in human tumors.

Several enzymes involved in choline metabolism have been found to be affected by metabolic signaling pathways like RAS and phosphatidylinositol-3-kinase (PI3K)-AKT, and transcription factors like MYC, and HIF [167-169]. In paper III, the gene expression profiles in the xenografts were determined by a large number of genes. None of the 54 genes selected genes from choline metabolism were included in the intrinsic gene set that defines the breast cancer subtypes. As some driver mutations, with specific regulatory effects on metabolism are more frequent in some subtypes, the metabolic profiles can still be used to discriminate between the subtypes. Activation of the PI3K pathway in cancer has been associated with ER and PgR negative status, and a basal-like phenotype [170]. In addition, PI3K inhibition has shown to significantly change the levels of PCho and GPC in breast cancers [171], and a significantly higher expression of *CHKA*, *GDPD5*, and *PLD1* has been identified in ER- compared to ER+ breast cancer

[160, 162]. It is therefore possible that cancers with different estrogen receptor status have a different regulation of the choline metabolism.

6 Conclusions and future perspectives

The papers in the thesis demonstrate how MR metabolomics reflect various molecular characteristics of breast cancer. Therefore, this method may be used to develop prognostic and predictive tools. Based on metabolic profiles, patients may be individually stratified to treatment algorithms, and metabolic changes can report on response to treatment.

The work in paper I included characterization of prognostic factors in breast cancer tissue samples using HR-MAS MRS analysis and demonstrated that MR spectra contain information about ER and PgR receptor status. Since some patient don't respond well to hormonal treatment, it is possible that MR metabolomics may be a useful tool to identify these patients for a different treatment regime. A larger patient cohort could help validating these results further and help explaining why some samples are false positives or false negatives.

A high glycolytic activity was observed in the luminal-like, and basal-like breast cancer xenograft models in paper II using ^{13}C labeled glucose and ^{13}C HR-MAS MRS. By administration of ^{13}C labeled glucose in a larger panel of xenograft models, the glycolytic activity in different breast cancer subgroups could be further explored. This would allow determination of the most important factors for glycolytic activity, including growth rate, breast cancer subgroups, specific genes, metabolic pathways, or vascularization. Of special interest is assessment of the glycolytic activity and response to treatment in animals treated with drugs targeting glycolysis. In addition, ^{13}C hyperpolarized experiments using ^{13}C labeled pyruvate would allow *in vivo* assessment of glycolytic activity.

A larger panel of breast cancer xenograft models was studied in paper III and the results showed that different breast cancer subgroups had a different choline metabolism. These findings were in agreement with corresponding subgroups of human breast cancer

which indicate that this panel of xenografts is representative of human breast cancer, and may be valuable for further exploration of subtype-specific metabolic and transcriptomic traits. Several genes contributing in choline metabolism was correlated to the concentrations of choline, PCho and GPC. Future studies could help further revealing which genes are the most important regulators of choline, PCho and GPC concentrations. In addition, it would help identifying potentially treatments targeting choline metabolism. This would be of special interest for the poor prognosis basal-like subgroup where no targeted treatment is available to date.

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PAPER I

research articles Journal of proteome research

Multivariate Modeling and Prediction of Breast Cancer Prognostic Factors Using MR Metabolomics

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Axillary lymph node status together with estrogen and progesterone receptor status are important prognostic factors in breast cancer. In this study, the potential of using MR metabolomics for prediction of these prognostic factors was evaluated. Biopsies from breast cancer patients ($n = 160$) were excised during surgery and analyzed by high resolution magic angle spinning MR spectroscopy (HR MAS MRS). The spectral data were preprocessed and variable stability (VAST) scaled, and training and test sets were generated using the Kennard-Stone and SPXY sample selection algorithms. The data were analyzed by partial least-squares discriminant analysis (PLS-DA), probabilistic neural networks (PNNs) and Bayesian belief networks (BBNs), and blind samples ($n = 50$) were predicted for verification. Estrogen and progesterone receptor status was successfully predicted from the MR spectra, and were best predicted by PLS-DA with a correct classification of 44 of 50 and 39 of 50 samples, respectively. Lymph node status was best predicted by BBN with 34 of 50 samples correctly classified, indicating a relationship between metabolic profile and lymph node status. Thus, MR profiles contain prognostic information that may be of benefit in treatment planning, and MR metabolomics may become an important tool for diagnosis of breast cancer patients.

Keywords: Chemometrics • Estrogen receptor • Progesterone receptor • Lymphatic spread • PLS-DA • Bayesian network • Probabilistic neural network • HR MAS MRS

Introduction

Breast cancer has the highest incidence and mortality of all cancer diseases among women globally.[1,2] Early diagnosis is of vital importance for successful treatment results, and during the past few years, the mortality of breast cancer has decreased, probably due to earlier diagnosis and more adjuvant treatment.[1] The most common type of breast cancer is invasive ductal carcinoma (IDC), accounting for approximately 80% of invasive breast cancers.[2]

There are few predictive and prognostic markers in breast cancer, but some specific markers are routinely being used for treatment planning and evaluating prognosis.[3] Estrogen receptor (ER) and progesterone receptor (PgR) status predict a possible endocrine responsive tumor, whereas human epidermal growth factor receptor 2 (HER-2) positive tumors may be suitable for trastuzumab treatment. ER, PgR and axillary lymph node status together with tumor size and lymphovascular invasion are important for predicting the clinical outcome of breast cancer patients.[3–5]

High resolution magic angle spinning magnetic resonance spectroscopy (HR MAS MRS) can be used to describe the metabolic profile of intact tissue samples. Metabolic profiles have been shown to correlate with characteristics of several malignant diseases such as breast,[6–8] brain,[9] colon,[10] and cervical cancer.[11] More than 30 metabolites have been described by HR MAS MRS analysis of breast cancer tissue.[12] The study of the metabolic profile of certain cell or tissue types in combination with multivariate and analytical statistics is referred to as metabolomics. In a previous study, Bathen et al.[7] showed that hormone receptor and axillary lymph node status, as well as histological grade, could be predicted by MR metabolomics. The study by Bathen et al. was, however, performed using spectra from a restricted number of patients ($n = 77$), and verified on a small amount of blind samples ($n = 12$).

The purpose of this study was to further explore the potential of MR metabolomics to provide clinically useful prognostic factors for breast cancer patients. The use of HR MAS MRS and chemometrics as tools for determining prognostic and predictive factors of breast cancer was evaluated. Several multivariate classification techniques exist, and in this study, partial least-

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Table 1. Characteristics of Patients and Tumors

Mean age, years (range)	62 (33−92)
Histological grade, *n* (1/2/3/unknown)	20/72/65/3
Axillary lymph node status, *n* (positive/negative/unknown)	64/88/8
ER status, *n* (positive/negative/unknown)	118/39/3
PgR status, *n* (positive/negative/unknown)	94/60/6
Mean tumor size, cm (range)	2.3 (0.6−7.0)

squares discriminant analysis (PLS-DA), probabilistic neural networks (PNNs) and Bayesian belief networks (BBNs) were used. The relationship between the metabolic profiles of breast cancer tissue and the status of ER, PgR and axillary lymph nodes was examined, and blind samples were predicted for verification.

Materials and Methods

Patients and Tumor Samples. A total of 160 breast cancer patients operated at St. Olavs University Hospital, Trondheim, or Molde Hospital, Molde, in Norway between 1999 and 2006 were included in this study. All patients had been diagnosed with IDC, and none of the patients had received neoadjuvant therapy. Breast cancer tissue specimens were frozen in liquid nitrogen immediately after removal. Histological tumor nuclear grade was determined according to guidelines of the Norwegian Breast Cancer Group, which are based on the Bloom and Richardson classification system.[13] Hormone receptor status was determined by immunohistochemistry, and samples with ≥10% staining cancer cells were considered receptor positive. Axillary lymph nodes were removed during surgery, either by axillary lymph node dissection (ALND) or by sentinel lymph node biopsy (SLNB), and considered positive if one or more lymph nodes analyzed by standard histopathology contained cancer cells. More detailed patient and tumor characteristics are shown in Table 1.

The study was approved by the Regional Committee for Medical and Health Research Ethics, and informed written consent was obtained from all included patients.

HR MAS MRS. Tissue samples were cut to fit a MAS rotor (50 μL, mean sample weight 17.7 mg) with added phosphate-buffered saline (PBS, 40 μL) based on D_2O with trimethylsilyl 3-propionic acid sodium salt (TSP, 1.0 mM). HR MAS MRS was performed on a Bruker Avance DRX600 spectrometer (Bruker BioSpin GmbH, Germany) equipped with a $^1H/^{13}C$ MAS probe with gradient aligned with the magic angle axis. Samples were spun at 5000 Hz and proton spectra were acquired using a water and lipid suppressing spin−echo Carr−Purcell−Meiboom−Gill sequence (cpmgpr; Bruker) with 2 s water-suppression prior to a 90° excitation pulse. The spin−echo sequence for suppression of broad peaks was performed using a delay of 1 ms repeated 136 times, resulting in an effective echo time of 285 ms. A total of 128 scans over a spectral region of 10 kHz were collected into 32k points during 1.64 s. All experiments were performed at 4 °C to minimize tissue degradation.

Histopathologic Examinations. After the MR experiment, the samples were fixed in 10% formalin and embedded in paraffin blocks. A section of 5 μm was cut from the middle of each paraffin block and stained with hematoxylin, erythrosine and saffron. The stained sections were examined microscopically and the relative areas of normal and neoplastic epithelial elements, necrotic tissue, fat and fibrous connective tissue were scored. Only samples containing ≥5% tumor cells were included in further analyses.

Samples were consecutively analyzed by HR MAS MRS and histopathologic examinations were performed. The resulting data were collected in a database. By searching the database, 213 spectra from 160 patients fulfilling the previously described criteria were chosen. Some tumor samples have been analyzed more than once by analyzing several parts of the same tumor; therefore, the number of spectra is greater than the number of patients.

Data Preprocessing and Modeling. The MR spectra were Fourier transformed into 128 K after 0.3 Hz exponential line broadening. Chemical shifts were referenced to the TSP peak at 0 ppm, and the region between 4.63 and 1.46 ppm was transferred to ASCII format. The spectral regions upfield from 1.46 ppm and downfield from 4.63 ppm, containing signals from lipid residues and residual water, were excluded from further analysis. The spectra were peak aligned using previously described algorithms.[14,15] Regions between 2.96 and 2.66 ppm and 2.23−1.96 ppm contained lipid residues and were deleted together with the ethanol contaminant between 3.68 and 3.63 ppm. The resulting region of interest was described by 5078 points. Baseline correction was performed by subtracting the value of the lowest point from all the variables in each sample, and the spectra were normalized by scaling the spectral data of a sample to achieve an equal total area for each spectrum.

The data were first explored using principal component analysis (PCA) in order to examine the variance of the data set without forcing the data into a model. PCA was performed using full cross-validation. On the basis of the PCA score plot and residuals, and manual inspection of the MR spectra, four samples were considered outliers and removed from further analyses. PCA analysis was performed using PLS_Toolbox 5.2.2 for Matlab (Eigenvector Research).

Scaling of the variables may be performed to bring all variables into the same range, and thereby regulate the relative importance of each variable. Our data set was variable stability (VAST) scaled in a supervised manner, a method giving focus to the stable variables of the data set by downweighting the least stable variables.[16] A good biomarker should have high interclass variation and low intraclass variation. To give focus to the regions of the spectra fulfilling these criteria, supervised VAST scaling was performed, that is, a set of variation coefficients was calculated for each class and the mean of the variation coefficients for the two classes were used for scaling. Scaling weights were based on the training set only.

It is important that the training data are representative of the whole data set. To achieve this, sample selection algorithms were used to divide the data set into training and test sets. Both the unsupervised Kennard Stone sample selection[17] and supervised SPXY[18] were used in this study. The training and test sets were manually corrected so that the test set had the same proportion of positives and negatives as the training set. Each test set consisted of 50 samples that were kept out of all training and used for validation only.

We used minimum redundancy, maximum relevance (mRMR) for variable selection. This method aims to select the variables that are relevant to the property of interest (maximum relevance) and at the same time do not contribute with the same information as other variables selected (minimum redundancy).[19,20] Variable selection was performed based on the training sets only.

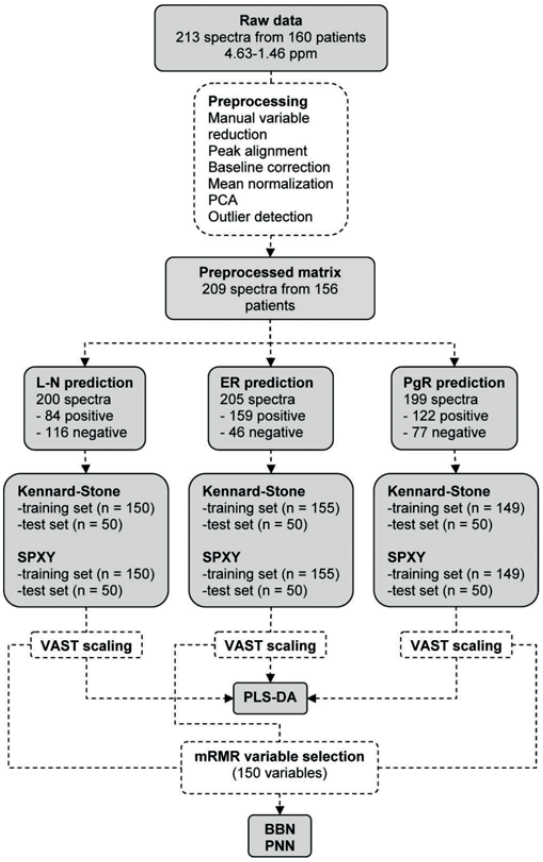

Figure 1. Flowchart of preprocessing and classification. White boxes represent the preprocessing performed on the data, while gray boxes represent the resulting data sets and multivariate models. L-N, lymph node; ER, estrogen receptor; PgR, progesteron receptor.

To investigate the relationship between the different metabolites of the MR spectra and breast cancer prognostic factors, PLS-DA, BBN and PNN were performed. Figure 1 summarizes the preprocessing performed and classification models built on the data.

Partial Least-Squares Discriminant Analysis. In PLS, the latent variables (LVs) are derived to maximize the covariance between the spectra and the class assigned target values. PLS-DA is a special case of PLS that attempts to discriminate between distinct classes.[21] PLS-DA was performed using full cross-validation with the VAST scaled MR spectroscopy data as the dependent *X*-variables and class as *Y*-variable. The number of latent variables to retain in the model was determined by choosing the number giving the smallest error of prediction for cross-validation. The retained PLS-DA model was used to predict blind samples kept out of all training. A PLS-DA threshold discriminating between positive and negative status was estimated using Bayes Theorem and the training data in order to minimize classification error.[22] PLS-DA was performed using PLS_Toolbox 5.2.2 for Matlab (Eigenvector Research).

Bayesian Belief Networks. BBNs use probability theory to classify samples to different groups. As opposed to PLS that is a linear method, BBNs can model complex nonlinear relationships.[23] The graphical structure of a BBN consists of nodes, one for each variable, and directed edges indicating conditional relations between the nodes.[24,25] Each node is associated with a conditional probability table that specifies the probability that a variable takes a certain value given the value of connected nodes.[26] Most BBN algorithms can only handle discrete variables so the variables have to be discretized before analysis.[27] In this study, a naïve Bayes model was used. Naïve Bayes models combine the unconditioned probabilities with the conditional probabilities in a single formula. The naïve Bayes classifier will have several nodes representing the chemical shifts, and one node representing the property of interest. There are no edges between the chemical shift nodes of a naïve Bayes classifier; hence, the spectral variables are assumed to be independent of each other. Despite this incorrect assumption, Naïve Bayes classifiers have shown good results in practice.[24] The BBN was built by making the network learn from a data set in a supervised manner. Input was 150 variables chosen by mRMR variable selection, and blind samples kept out of the model building were used for prediction. BBN was performed in Netica (Norsys Software Corp, Canada).

Probabilistic Neural Networks. PNNs have four layers: input, pattern, summation and output.[28] The input layer has several neurons, one for each variable needed to describe the samples to be classified. In this study, the input parameters are the NMR shifts selected by mRMR variable selection. PNNs operate by defining a probability density function (pdf) for each data class based on the training data set and an optimized kernel width parameter, also optimized by the genetic analysis.[29] Each pdf is estimated by placing a Gaussian-shaped kernel at the location of each pattern in the training set such that the pdf defines the boundaries for each data class, while the kernel width determines the amount of interpolation that occurs between adjacent kernels. The probability that a pattern vector will be classified as a member of a given output data class increases the closer the pattern vector is to the center of the pdf for that class. When an input test vector is presented, the pattern layer computes distances from the input vector to the training input vectors and produces a vector whose elements indicate how close the input is to a training input. The summation layer sums these contributions for each class of inputs to produce as its net output a vector of probabilities. Finally, a complete transfer function on the output of the second layer picks the maximum of these probabilities. PNN was performed using NeuroShell Classifier (Ward Systems Group) which uses a complex version of PNNs adapted around a genetic algorithm.

Results

Figure 2 shows a score plot of the three first principal components (PCs) from PCA of the whole initial data set. The data set was peak aligned, baseline corrected and normalized prior to PCA. Clusters of samples from patients that are ER positive (ER^+) and ER negative (ER^-) can be seen in a score plot of PC1, 2, and 3, although a considerable number of samples overlap. Samples could not be clustered according to PgR and lymph node status in the PCA score plot. The four samples considered outliers are annotated in Figure 2. The samples were considered outliers based on the score plot, sample residuals and observations of the MR spectra, and were removed from further analyses. Removing the outliers did not improve discrimination of the prognostic factors by PCA.

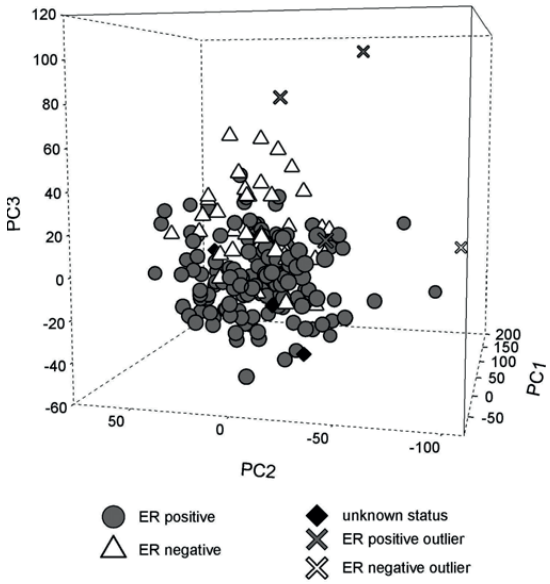

Figure 2. PCA score plot of the data set. Samples considered outliers are marked in the plot.

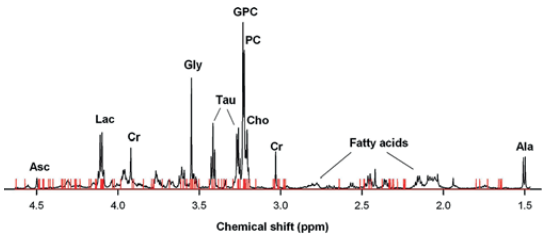

Figure 3. Representative HR MAS MR spectrum with mRMR selected variables marked in red. The variables shown here were selected from the Kennard-Stone training set based on lymph node status. The two fatty acid regions between 3.0 and 2.0 ppm were not part of the multivariate analysis; hence, no variables were chosen from these regions.

A representative HR MAS MR spectrum is shown in Figure 3. The spectrum is baseline corrected and normalized. A total of 150 variables chosen by mRMR variable selection are marked in the spectrum. The variables shown here were selected from the Kennard-Stone training set based on lymph node status. Variables chosen from the other data sets appeared similar. Figure 3 shows that the selected variables mainly represent a metabolite peak and that all visible metabolites are represented by several variables, though some variables have been chosen from what appears to be less important areas of the spectra.

Axillary Lymph Node Status. The results from prediction of axillary lymph node status of 50 blind samples by PNN, BBN and PLS-DA are shown in Table 2. Input for PNN and BBN was 150 variables chosen by mRMR variable selection. Axillary lymph node status was best predicted by BBN. However, all methods gave an unacceptably high classification error.

ER Status. Results from blind sample prediction of ER status are shown in Table 3. Neural network predictions were performed using a customized fitness function in order to correct for the uneven proportion of ER positive and negative

Table 2. Results from Prediction of Axillary Lymph Node Status[a]

	PLS-DA (1 LVs)	BBN	PNN
Kennard-Stone			
-correct classification	30/50	26/50	33/50
-sensitivity (%)	71	43	62
-specificity (%)	52	59	69
SPXY			
-correct classification	31/50	**34/50**	30/50
-sensitivity (%)	62	**52**	38
-specificity (%)	62	**79**	76

[a] The best predictions are emphasized in bold. Correct classification: number of samples in the test set predicted to have the correct lymph node status. Sensitivity: the number of lymph node positive samples correctly classified. Specificity: the number of lymph node negative samples correctly classified.

Table 3. Results from Prediction of ER Status[a]

	PLS-DA (3 LVs)	BBN	PNN[b]
Kennard-Stone			
-correct classification	**44/50**	39/50	40/50
-sensitivity (%)	**90**	95	82
-specificity (%)	**82**	18	73
SPXY			
-correct classification	42/50	41/50	42/50
-sensitivity (%)	87	97	90
-specificity (%)	73	38	64

[a] The best predictions are emphasized in bold. [b] Using customized fitness function. Correct classification: number of samples in the test set predicted to have the correct ER status. Sensitivity: the proportion of ER positive samples correctly classified. Specificity: the proportion of ER negative samples correctly classified.

samples. ER status was best predicted by PLS-DA. Permutation testing was performed ($n = 10\,000$), and the prediction results were significantly different than random for both the Kennard-Stone ($p < 0.0001$) and SPXY ($p = 0.0001$) test sets.

A PLS-DA model of the whole data set with three LVs explains 43.8% of the *X*-variance and 42.7% of the *Y*-variance. The score values for ER^+ and ER^- samples are significantly different for all three LVs (*t* test, $p < 0.001$), and it is possible to discriminate between ER^+ and ER^- samples in a score plot of LV1, LV2 and LV3 (Figure 4a). ER^+ and ER^- samples are mainly separated on the first LV that represents 70% of the *Y*-variance explained by the model, and ER^- samples have higher score for LV1 than ER^+ samples. The loading profile for LV1 reveals that samples with higher score for LV1 have more of the metabolites glycine (Gly), glycerophosphocholine (GPC), choline (Cho) and alanine (Ala), and less ascorbate (Asc), creatine (Cr), taurine (Tau) and phosphocholine (PC) than samples with lower LV1 scores (Figure 4b). The regression vector of the PLS-DA model gives an indication of the overall influence of the variables based on all three LVs. The regression vector of ER^- samples appears similar to LV1, and shows the same metabolic patterns. In addition, lactate (Lac) appears to be more expressed in ER^- samples.

PgR Status. Results from blind sample prediction of PgR status are shown in Table 4. PgR status was best predicted by PLS-DA. Permutation testing ($n = 10\,000$) showed that the prediction results were significantly different than random for both the Kennard-Stone ($p = 0.0001$) and SPXY ($p = 0.0018$) test sets.

PLS-DA with three LVs explains 41.0% of the *X*-variance and 34.1% of the *Y*-variance. It is possible to partly discriminate between PgR^+ and PgR^- samples on a score plot of LV1, LV2

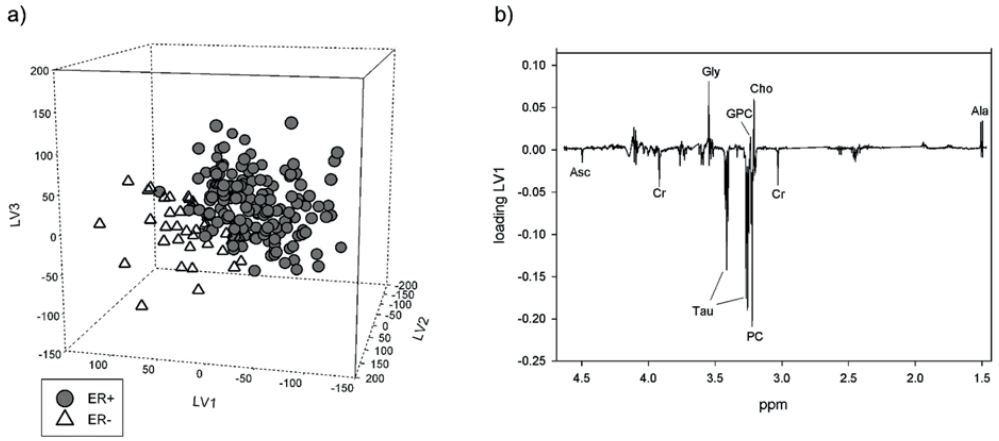

Figure 4. Results from PLS-DA of ER status. (a) Score plot of the first three latent variables; (b) loading profile of the first latent variable.

Table 4. Results from Prediction of PgR Status[a]

	PLS-DA (3 LVs)	BBN	PNN
Kennard-Stone			
-correct classification	**39/50**	35/50	35/50
-sensitivity (%)	**81**	77	71
-specificity (%)	**74**	58	68
SPXY			
-correct classification	36/50	36/50	36/49[b]
-sensitivity (%)	77	84	80
-specificity (%)	63	53	63

[a] The best predictions are emphasized in bold. [b] One row not classified. Correct classification: number of samples in the test set predicted to have the correct PgR status. Sensitivity: the number of PgR positive samples correctly classified. Specificity: the number of PgR negative samples correctly classified.

and LV3 (Figure 5a), and the scores for PgR^+ and PgR^- are significantly different for all three LVs (*t* test, $p < 0.001$). PgR^+ and PgR^- samples are mainly separated on the first latent variable that represents 62% of the *Y*-variance explained by the model. PgR^- samples have higher scores for LV1, and the loading plot for LV1 (Figure 5b) shows that samples with higher LV1 scores have more Asc, Lac, Gly, GPC, PC, Cho, Cr and Ala than samples with lower LV1 scores. The regression vector for PgR^- samples appears similar to that of ER^- samples.

For all prognostic factors, PLS-DA models were also made based on the mRMR variable reduced matrix in order to examine if PLS-DA gave better predictions simply because it has access to more variables. However, the prediction results using variable reduced data sets were approximately the same as when using all variables.

Discussion

Axillary lymph node status was best predicted by BBN with 34 of 50 blind samples correctly classified. However, this was only true for the samples chosen by SPXY sample selection, and the same number of correctly classified samples was not achieved using Kennard-Stone sample selection. PLS-DA and BBN gave similar results, and overall, all three methods gave unacceptably high classification errors. However, the number of correctly classified samples was better than expected by chance for all methods. This indicates that there is a difference between the MR spectra of lymph node positive and negative patients, and that the metabolic profile is altered in patients with lymphatic spread compared to patients without spread. The difference may, however, not be robust enough to reliably separate positive and negative lymph node status based on MR spectra. This may be due to other metabolic changes appearing in cancer tissue that partly masks metabolic changes from lymphatic spread. It is also possible that metabolic changes appear prior to lymphatic spread, making it difficult to separate patients with lymphatic spread from patients in risk of spread.

The lymph node status of patients that were sampled during the first period of this study was determined by ALND. This method was later replaced by SLNB, where only the first lymph node(s) to which metastasizing cells are likely to spread is examined. Further ALND is only done if the sentinel lymph node is positive. For ALND, only one slice per lymph node is examined. It is possible that metastatic cells may be present in parts of the lymph nodes not examined. For SLNB, several sectional slices of the sentinel node are examined; however, there is a possibility that micrometastasis may have passed the sentinel node. Thus, it is possible that samples classified as false positives actually may have undetected lymphatic spread.

Mountford et al.[30] have previously predicted the lymph node status of breast cancer patients using MR spectroscopy with a sensitivity and specificity of 96% and 94%, respectively, using intraoperative fine-needle aspiration biopsies. It can be assumed that fine-needle aspirates contain less fat than the biopsies used in our study. Their study was, however, not validated on blind samples, and the same samples ($n = 61$) were used both for building and testing the model. It is likely that this may have led to overoptimistic results in predicting the lymph node status.

ER and PgR status were best predicted by PLS-DA. For ER status, the number of correctly classified blind samples were 44/50 and 42/50 for Kennard-Stone and SPXY sample selection, respectively, while PgR status had a correct blind sample classification of 39/50 for the Kennard-Stone test set and 36/50 for SPXY. Similar results for both Kennard-Stone and SPXY sample selection indicate robust classification by PLS-DA. The sensitivity and specificity of classification were approximately equal; this in contrast to the results of PNN and BBN where the sensitivity was higher than the specificity. The higher sensitivity may be due to the fact that, especially for ER status, there are more positive than negative samples. This could lead

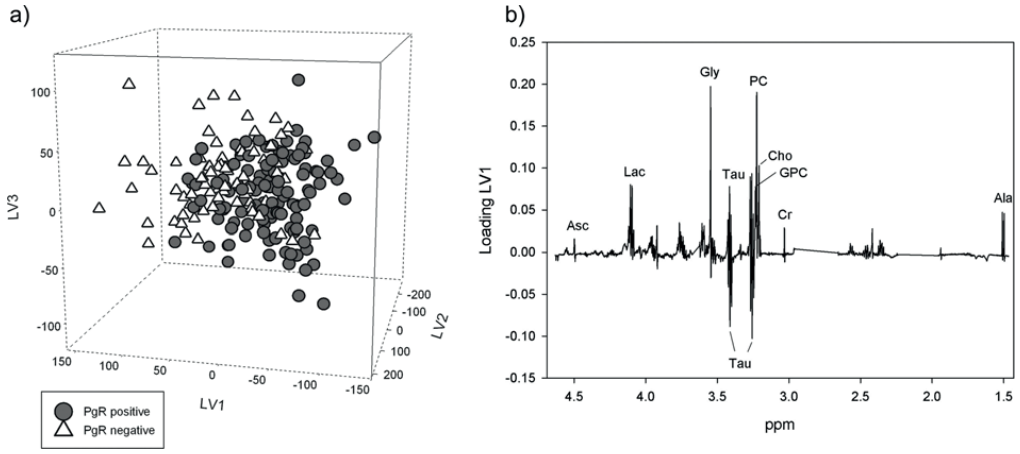

Figure 5. Results from PLS-DA of PgR status. (a) Score plot of the first three latent variables; (b) loading profile of the first latent variable.

to networks that are more specialized in recognizing positive than negative samples. Since the probability of a sample being positive is much higher than the probability of it being negative, the network achieves a greater number of total correct classified samples by classifying most of the samples as positives. In PNNs, this can be partly overcome by the customized fitness function, allowing the user to insert a penalty whenever a negative sample is classified incorrectly. In this study, the same penalty was used for both the Kennard-Stone and the SPXY training and test sets. Although this improved the classification ability of the networks compared to networks without penalty, the classification error was still higher than that achieved by PLS-DA.

The threshold for classifying a sample in PLS-DA as either positive or negative was, as previously described, calculated using Bayes Theorem based on the training data. A normal distribution is fitted to the predicted values of the training data, and based on this, each blind sample is given a probability of belonging to each class.[22] The standard deviation of the two groups does not have to be equal, and thereby the threshold may be set to classify a larger portion of *y* predicted values to one group than to the other. In this way, PLS-DA manages to overcome the problem of having a larger portion of samples belonging to one group, and PLS-DA is able to predict ER and PgR status from MR spectra with high sensitivity and specificity.

The loading profiles and regression vectors of a PLS-DA give an indication of the variables that contribute in discriminating the different groups. However, the size of the regression coefficients will also depend on the size and range of variation of the variables, and is therefore not straightforward to interpret. ER^- samples, that have a high score on LV1, appear to have more Gly, GPC, Cho, and Ala, and less Asc, Cr, Tau and PC than ER^+ samples. The same metabolic pattern is seen in the regression vector of ER^- samples, where in addition ER^- samples appear to have more Lac. Different molecular subclasses of breast cancer have been described based on gene expression profiles.[31] Most patients having ER^- tumors are classified to the basal-like molecular subtype, while ER^+ tumors are mostly luminal-like.[32,33] Our findings correspond with preclinical studies of basal-like and luminal-like molecular subtypes, where the basal-like animal model had significantly lower concentration of PC and significantly higher concentrations of Gly and GPC than the luminal-like subtype.[34] PC and GPC are, together with Cho, involved in the synthesis of phosphatidylcholine, the most abundant phospholipid in biological membranes.[35,36] Aberrant choline phospholipid metabolism has been observed in several cancers, and this has been related to phenotypic characteristics of tumors.[35]

The regression vector of PgR^- samples appears similar to that of ER^- samples. This may be due to the fact that most patients have the same ER and PgR status; either positive for both or negative for both. However, LV1 indicates that PgR^- samples have more Asc, Lac, Gly, GPC, PC, Cho, Cr and Ala than PgR^+ samples, and thereby a different metabolic profile than ER^- samples. Expression of PgR is regulated by ER, and only 1−12% of breast cancer patients are ER^-/PgR^+.[37] In this study, two patients had tumors that were ER^-/PgR^+ while 23 patients (represented by 33 spectra) had ER^+/PgR^- tumors. The differences between the loading profiles of ER and PgR may indicate that ER^+/PgR^+ tumors have a different metabolic profile than tumors that are ER^+/PgR^-. Liu et al.[5] have previously shown that PgR status adds significant prognostic value beyond that obtained by ER status alone. Our findings suggest that PgR status is represented by a metabolic profile different from that of ER status, and this might explain why PgR status reflects a different prognostic pattern than ER status alone.

Patients who had received neoadjuvant therapy, that is, treatment given before surgery, were excluded from this study. It can be assumed that neoadjuvant therapy affects the metabolic profile and makes this patient group more heterogeneous. Patients who receive neoadjuvant therapy generally have worse prognosis than those not receiving it; thus, this study was based on patients with a better prognosis. It is possible that other, or more distinct, differences would be present in the metabolic profiles of the prognostic factors if patients with a worse prognosis had not been excluded.

Large regions of an MR spectrum may contain uninformative data, and including all variables may add noise to the model. In addition, using too many variables may lead to overfitting. The input variables for PNNs and BBNs were mRMR selected variables. mRMR is a multivariate variable selection method that takes into account interactions between variables while

retaining the original representation of the variables.[38] The mRMR variable selection method was initially developed for microarray data, but is also applicable to spectral data.[19] Since Naïve Bayes classifiers assume independency between the variables, it is preferable to reduce the number of variables prior to analysis in order to reduce the colinearity of the data. Also PNNs appear to work best on fewer variables. Several variable selection methods exist, and it is likely that different methods under investigation would select different variables. However, it can be assumed that most methods would select variables from similar metabolite regions and thereby give a similar multivariate model.

In conclusion, ER and PgR status were successfully predicted by MR metabolomics. There is also a relationship between metabolic profile and lymph node status, although prediction of lymph node status based on MR spectra did not reach a reliable level of correctly classified samples. By combining MR spectroscopy with multivariate modeling, the biological differences between different metabolic profiles could be revealed. Here we showed that hormone receptor negative patients appear to have more of the metabolites Gly, GPC and Cho than receptor positive patients. The data also indicate different metabolic profiles between ER status and PgR status. Thus, this study has shown that MR profiles contain prognostic information that may be of benefit in treatment planning and patient follow-up, and MR metabolomics may become an important tool for clinical decision-making in breast cancer patients.

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83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

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92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

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104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.
109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

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110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANCER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

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124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs

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132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.

133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.
139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

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141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
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160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.

162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.
168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT

192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
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197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.

217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
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227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
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233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA

244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS
251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT

272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT
273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY
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