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Identification and characterization of prognostic factors in breast cancer using MR metabolomics

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
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Identifisering og karakterisering av prognostiske faktorer i brystkreft ved bruk av MR metabolomics

Brystkreft er den vanligste kreftsykdommen blant kvinner, og omtrent en av 11 kvinner vil få diagnosen i løpet av livet. Sykdomsforløpet og prognosen kan variere mye fra pasient til pasient, og ikke alle vil ha samme nytte av en gitt behandling. Det arbeides derfor med å kunne tilby hver enkelt pasient den behandling som er optimal for pasientens brystkreftsykdom. Arbeidet i denne avhandlingen omhandler bruken av magnetisk resonans (MR) spektroskopi og fagfeltet metabolomics for bedre vurdering av brystkreftpasienters prognose og økt forståelse av de biologiske faktorene som ligger bak. Motivasjonen er å finne nye metoder for diagnostisering og vurdering av prognose som et tilleggsverktøy til dagens kliniske faktorer.

Kreftceller deler seg raskt, og har derfor en metabolisme som er forskjellig fra den av normale celler. De kjemiske produktene av metabolisme, kalt metabolitter, vil reflektere de biologiske prosesser som pågår. MR spektroskopi kan gi et bilde av nivået av de ulike metabolittene i en vevsprøve. I dette arbeidet ble kreftbiopsier fra brystkreftpasienter analysert ved bruk av MR spektroskopi. MR spekterene ble analysert ved bruk av multivariate statistiske metoder, og arbeidet inkluderte også å optimalisere bruken av de ulike metodene.

Det ble i dette arbeidet funnet tydelige metabolske forskjeller i tumorer som utrykker hormonreseptorer og tumorer som har mistet dette utrykket. Hormonreseptorer er viktige prognostiske faktorer, og kan også forutsi en pasients respons til endokrin behandling. En trend til forandret metabolisme ble detektert i pasienter med lymfeknutespredning. Videre kunne utrykket av spesifikke metabolitter knyttes opp mot 5-års overlevelse. To metabolitter, laktat og glysin, skilte seg ut som potensielle biomarkører for prognose. Det samme ble observert i en gruppe pasienter med lokalavansert brystkreft som ble behandlet med kjemoterapi før operasjon. Her hadde pasienter som døde innen fem år økt laktatnivå etter behandling, mens pasienter som overlevde mer enn fem år hadde en nedgang i glysin.

Forskningsarbeidet som utgjør denne avhandlingen består av fire deler. I det første arbeidet ble metabolske profiler relatert til kliniske prognostiske faktorer, og i tredje arbeid ble metabolske forandringer knyttet opp mot overlevelse. Disse arbeidene er basert på en regional brystkreftbiobank med prøver samlet inn fra pasienter i midt-Norge. I det andre arbeidet ble optimal preprosessering av MR spekterne før multivariat analyse undersøkt, og resultater fra dette arbeidet ble benyttet til å optimalisere analysene i det tredje og fjerde arbeidet. Det fjerde arbeidet omhandlet pasienter diagnostisert med lokalavansert brystkreft. Disse pasientene har større tumorer og mer utbredt lymfeknytespredning enn pasientene i de foregående arbeidene. Tumorens metabolske respons til kjemoterapi ble relatert til overlevelse. De to første arbeidene er publisert i internasjonale tidsskrifter, mens de to siste er innsendt for vurdering.

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close friends (you know how you are) for your unlimited support and for always believing in me. Thanks also to my nephew Marius for attractive distractions from the work. Last, but not least, I want to thank my dear husband and best friend Magnus for your love and support.

Summary

Breast cancer is a heterogeneous disease with a varying prognosis. Today's clinical and pathological diagnostic tools are not sufficient for accurately predicting the prognosis of a breast cancer patient, or for predicting who will benefit from a certain treatment. The identification of new factors for diagnosis and prognosis evaluation could lead the way for improved individualized treatment of breast cancer patients.

Cancer cells have changed metabolism compared to normal cells due to high proliferation rates and malignant transformation. An increased uptake of glucose is frequently observed in cancer cells, in addition to a high degree of lactate production from glucose even in the presence of oxygen. In addition, altered phospholipid metabolism is commonly observed in cancers. The chemical bi- or end-products of metabolism are referred to as metabolites. The levels of metabolites in biological materials can be studied using magnetic resonance (MR) spectroscopy, and high resolution magic angle spinning (HR MAS) MR spectroscopy provides highly resolved spectra from solid samples. The aim of this work was to examine the metabolite profiles of tissue from breast cancer tumors, and to relate these profiles to diagnostic and prognostic factors. A diverse selection of methods for preprocessing and multivariate modelling of MR spectra was optimized and used for analyzing the data. The patients included in this work represent a cohort with varying prognosis, from small, localized tumors to larger tumors with extensive lymph node involvement. Tissue biopsies from the tumors were excised before or during breast cancer surgery and analyzed by MR spectroscopy.

A clear connection between metabolite profiles and hormone receptor status was shown, and a trend of metabolic differences related to lymphatic spread was observed. Hormone receptors and lymph node status are important prognostic factors, and the presence of hormone receptors is also predictive of response to endocrine treatment. Differences in the metabolite profiles of patients surviving more than five years and deceased patients were found, and increased levels of the metabolites lactate and

glycine were associated with a poor prognosis. Analysis of the metabolite profiles of tumor biopsies excised before and after pre-surgical chemotherapy showed that the tumor's metabolic response to treatment could be indicative of prognosis. Patients that died within five years after diagnosis experienced increased lactate levels after chemotherapy, while patients surviving more than five years had stable lactate levels and decreased levels of glycine.

The importance of proper preprocessing of MR spectra has also been illustrated in this thesis, and different multivariate analysis methods have been assessed for their feasibility of analyzing MR spectra. Overall, the work in this thesis has provided an increased insight into the complex mechanisms of cancer progression. MR spectroscopy is a promising tool for stratification of patients into clinically useful prognostic groups.

Symbols and abbreviations

2HG	2-hydroxyglutarate
ALND	axillary lymph node dissection
B_0	the static magnetic field
BBN	Bayesian belief network
CDP-Cho	cytidine diphosphocholine
Cho	free choline
COW	correlation optimized warping
cpmg	Carr Purcell Meiboom Gill sequence
CV	coefficient of variation
IDC	invasive ductal carcinoma
ER	estrogen receptor
ERETIC	electronic reference to access in-vivo concentrations
fastpa	peak alignment by beam search
FID	free induction decay
FFT	fast fourier transform
GLUT	glucose transporter
GPC	glycerophosphocholine
HER-2	human epidermal growth factor receptor 2
HES	hematoxylin, erythrosine and saffron
HIF	hypoxia inducible factor
HMDB	human metabolome database
HR MAS	high resolution magic angle spinning
I	nuclear spin
IDH	isocitrate dehydrogenase
ILC	invasive lobular carcinoma
LOO	leave one out
M_0	net equilibrium magnetization
mRMR	minimum redundancy maximum relevance
MRS	magnetic resonance spectroscopy

MS	mass spectrometry
NAC	neoadjuvant chemotherapy
NBCG	Norwegian breast cancer group
NIPALS	non-iterative partial least squares
OPLS-DA	orthogonal partial least squares discriminant analysis
PAFFT	peak alignment by fast Fourier transform
PARS	peak alignment using reduced set mapping
PBS	phosphate buffered saline
PC	principal component
PCho	phosphocholine
PCA	principal component analysis
pdf	probability density function
PgR	progesterone receptor
PLS	partial least squares
PLS-DA	partial least squares discriminant analysis
PNN	probabilistic neural network
ppm	parts per million
PtdCho	phosphatidylcholine
PTW	parametric time warping
RAFFT	recursive alignment by FFT
RF	radio frequency
RSPA	recursive segment-wise peak alignment
SCW	self-calibrated warping
SLNB	sentinel lymph node biopsy
SPXY	sample set partitioning based on joint x–y distances
T_1	longitudinal relaxation time
T_2	transversal relaxation time
T_2^*	apparent transversal relaxation time
TCA	tricarbocylic acid cycle
tCho	total choline signal
TE	echo-time
TNM	tumor node metastasis

TSP	trimethylsilyl 3-propionic acid sodium salt
VAST	variable stability scaling
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPdtw	variable penalty dynamic time warping

List of papers

Paper I

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

Giskeødegård GF, Grinde MT, Sitter B, Axelson DE, Lundgren S, Fjøsne HE, Dahl S, Gribbestad IS, Bathen TF.

Journal of Proteome Research, 2010; 9(2): 972-979.

Paper II

Alignment of high resolution magic angle spinning magnetic resonance spectra using warping methods

Giskeødegård GF*, Bloemberg TG*, Postma G, Sitter B, Tessem MB, Gribbestad IS, Bathen TF, Buydens LMC. *Shared first authorship

Analytica Chimica Acta, 2010; 683(1): 1-11.

Paper III

Glycine and lactate- potential MR biomarkers of breast cancer prognosis

Giskeødegård GF, Lundgren S, Sitter B, Fjøsne HE, Postma G, Buydens LMC, Gribbestad IS, Bathen TF.

Submitted manuscript

Paper IV

Prognostic value of metabolic response in breast cancer patients receiving neoadjuvant chemotherapy

Cao MD*, Giskeødegård GF*, Bathen TF, Sitter B, Bofin A, Lønning PE, Lundgren S, Gribbestad IS. *Shared first authorship

Submitted manuscript

Contents

1 Introduction

1.1 Cancer

Cancer is a collection of diseases characterized by uncontrolled proliferation of cells. While normal cells are strictly controlled by regulatory signals, cancer cells have become immortal by genetic alterations, or *mutations*, giving errors in the regulatory pathways. Additional alterations enable cancer cells to invade surrounding tissue and metastasize to other locations in the body. As an accumulation of several genetic alterations are required for cancer to develop, the risk of cancer increases with age. These genetic alterations may arise spontaneously during cell division, or they are caused by carcinogens such as chemicals and radiations that damage the DNA of a cell. In some cases, genetic alterations are inherited, giving a predisposition for cancer.[1]

The multistep development of cancer was in 2000 summarized by Hanahan et al. through the six 'hallmarks of cancer'; cancer cells must evade apoptosis and growth suppressors, be self-sufficient of growth signals, enable replicative immortality, sustain angiogenesis and activate invasion and metastasis.[2] Metastasis to vital organs is the leading cause of death in cancer patients. In addition to growth invasion of surrounding tissue, cancer cells can spread via the blood vascular system or via the lymphatic system.[3] Based on progression in cancer science the last decades, two additional hallmarks have recently been proposed; namely reprogramming of energy metabolism and evading immune destruction.[4] It has been suggested that most if not all cancers require these functions via distinct mechanisms and at various times during the course of the multistep tumorigenesis.

Treatment of cancer is based on removal or killing of cancer cells. Treatment can be local (surgery and radiation therapy) or systemic (chemotherapy and hormone treatment), and often a combination of treatments is used.

1.1.1 Breast cancer

Breast cancer is the most common cancer diagnosis among women in Norway, with more than 2700 new cases every year. In 2009, breast cancer accounted for 22% of all diagnosed cancers among Norwegian women.[5] Figure 1.1 shows the anatomy of the breast. The lobules contain several milk-producing alveoli, and are connected to the lactiferous ducts which transport milk to the nipple. Invasive ductal carcinoma (IDC) is the most common type of breast cancer, accounting for 70-80% of all invasive breast cancers. IDC starts in the milk ducts where it breaks through the duct walls and invades the surrounding tissue. Another common type of invasive breast cancers is invasive lobular carcinoma (ILC) originating from the lobules. ILC accounts for approximately 10% of invasive breast cancers, and is biologically distinct from IDC.[6] Lobular and ductal carcinoma in situ are non-invasive breast cancers with good prognosis. They may however become invasive if left untreated.

Breast cancer is a heterogeneous disease with differing progress and prognosis.[7] 5-year survival rates of breast cancer patients in Norway are ranging from 19-95% depending on the stage of the disease, with an overall survival rate of 88%.[5] The mortality rates have decreased in industrialized countries during the last decades, probably due to better treatment and earlier diagnosis by mammographic screening.[8] In Norway, mammographic screening is offered to all women between 50 and 69 years of age.

Factors that will influence a patient's prognosis and treatment regime include tumor size, lymphatic involvement and metastatic state (summarized as the TNM status), estrogen receptor (ER) and progesterone receptor (PgR) status, human epidermal growth factor receptor 2 (HER-2) status, histological grade, and age.[9-10] Patients with tumors lacking the expression of ER and/or PgR have a worse prognosis,[11] while an amplification of HER-2 induces enhanced malignant growth and is associated with lower survival rates.[12] HER-2 and hormone receptor status are also predictive of treatment response, as patients that are receptor positive or have HER-2 overexpression will be suitable for endocrine treatment or treatment with Trastuzumab, respectively.

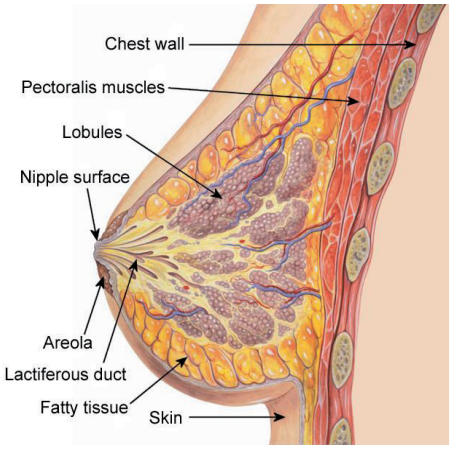

Figure 1.1: Anatomy of the breast. Adapted from Wikimedia Commons, with credits to Patrick J. Lynch and C. Carl Jaffe, MD.

Locally advanced breast cancer

Approximately 10-20 % of breast cancer patients are diagnosed with locally advanced breast cancer in industrialized countries, whereas it constitutes as much as 75% of all diagnosed breast cancers in developing countries.[13] This type of breast cancer is characterized by large tumors (T3-T4: largest dimension > 5 cm, or extensions to the skin or chest wall) and/or extensive regional lymph node involvement (N2-N3) but without distant metastases.[14] Locally advanced breast cancers are primarily inoperable cancers with varying prognosis. The patients with this diagnosis are treated with chemotherapy prior to surgery (neoadjuvant chemotherapy, NAC) in order to decrease the tumor size and make the tumor operable. No survival advantages have been demonstrated for NAC treatment,[15] however NAC allows more breast-conserving surgeries without any significant increase in local or distal recurrence.[16] Patients with a pathological complete response to NAC have improved outcome compared to patients with residual disease, thus treatment response can be useful as a prognostic indicator.[17-18] In Norway, the treatment regime of patients with locally advanced breast cancer is recommended by the Norwegian Breast Cancer Group (NBCG[19]), and consists of NAC followed by surgery and radiotherapy. Patients will first receive four cycles of FEC (5-

fluorouracil, epirubicin, and cyclophosphamide). If the longest diameter of the tumors is reduced by ≥ 80% after the four cycles, two additional cycles of FEC is provided. If the reduction in the longest tumor diameter is < 80%, the patient proceeds to 12 weeks of treatment with either docetaxel or paclitaxel before surgery.

1.1.2 Tumor metabolism

Cells need energy in the form of ATP in order to replicate and proliferate. Conversion of glucose, a main source of energy, into pyruvate through the glycolysis yields a small amount of ATP. Under normal aerobic conditions, pyruvate is further oxidized in the tricarbocylic acid cycle (TCA or Krebs cycle) followed by oxidative phosphorylation in the mitochondria of the cell. This process provides the vast majority of energy used by aerobic cells; more than 95 % of all energy in humans. Oxidative phosphorylation is dependent on oxygen, and under hypoxic conditions pyruvate will be catabolized to lactate in an anaerobe manner (Figure 1.2). This process will produce far less ATP than aerobe metabolism of glucose.[20]

Cancer cells require large amounts of energy due to increased proliferation and survival, and have developed alternative metabolic strategies in order to achieve this. An enhanced rate of glucose uptake is observed in a majority of tumors.[21] This is achieved through upregulation of glucose transporters (GLUT), primarily GLUT1, a phenomena observed in most cancers.[22] Hypoxia is a common feature of solid tumors due to poor blood supply, and as a consequence glucose is catabolized to lactate in the lack of oxygen.[23] Cancer cells are highly adaptive to hypoxia as hypoxia induces the transcription factor hypoxia inducible factor (HIF-1) which in turn upregulates multiple genes involved in glucose metabolism and angiogenesis.[24] Also under conditions with sufficient oxygen levels, cancer cells may convert glucose to lactate instead of oxidative phosphorylation. This process of aerobic glycolysis, known as the Warburg effect[25], is a relatively inefficient way of producing ATP. One possible explanation of aerobic glycolysis is that proliferating cells have metabolic requirements that extend beyond ATP production. Due to increased glycolytic uptake in cancer cells, aerobe glycolysis may provide sufficient amounts of ATP while also providing essential precursors for

biosynthesis. The resulting increase in lactate levels may enhance the invasiveness of tumor cells by disrupting the architecture of surrounding normal tissue, and reduced pH in the environment may help tumor cells evading the tumor-attacking immune cells.[21]

In addition to a switch in the metabolism of glucose, several studies have shown altered phospholipid metabolism in cancers.[26-28] Phospholipids are major components of the cell membranes where they form lipid bilayers. Phosphatidylcholine (PtdCho), a phospholipid with a choline head group, is the most abundant phospholipid in eukaryote cell membranes.[29] Figure 1.3 shows the biosynthesis of PtdCho. Free choline (Cho) is transported into the cell and converted to phosphocholine (PCho) through the action of choline kinase. PCho is further converted into cytidine diphosphocholine (CDP-Cho) in a step that is tightly regulated in normal cells.[30] PtdCho is synthesized through the combination of CDP-Cho and 1,2-diacylglycerol. Glycerophosphocholine (GPC) and 1-acylglycerophosphocholine are degradation products of PtdCho. Using *in vivo* MRS, signals from GPC, PCho and Cho will be detected as one signal termed total choline (tCho). Increased levels of tCho compared to normal tissue have been detected in breast cancers.[31-34] *Ex vivo* studies have suggested that the increased levels of tCho are mainly due to increased PCho.[28] A reduction in the concentration of tCho has been suggested as an *in vivo* marker for response to cancer treatment.[35-37]

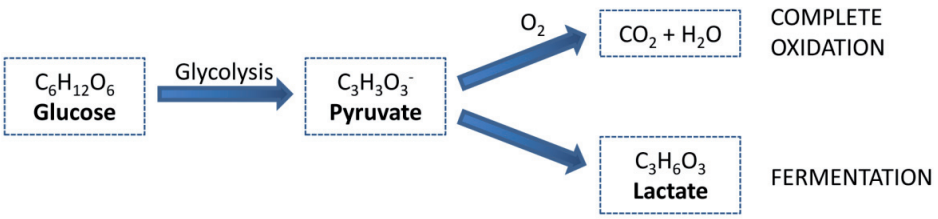

Figure 1.2: The different fates of glucose. In the presence of oxygen, pyruvate is completely oxidized through the TCA cycle and oxidative phosphorylation. In anaerobe conditions, pyruvate is fermented to lactate. Cancer cells may convert pyruvate to lactate also under aerobe conditions.

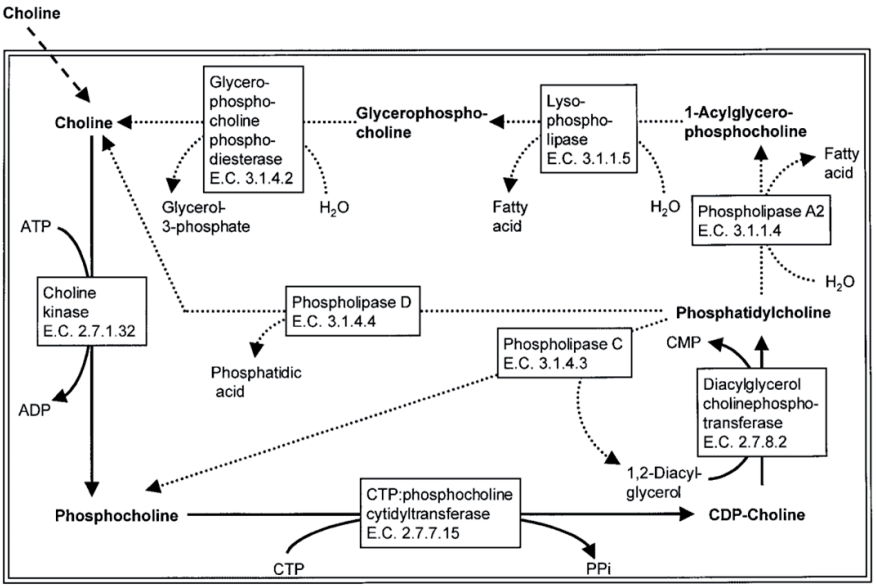

Figure 1.3: Biosynthesis (solid lines) and catabolism (dotted lines) of phosphatidylcholine. Altered phospholipid metabolism is frequently observed in cancers. The figure is reprinted from Ackerstaff et al.[28] with permission.

Changed levels of different amino acids have been detected in cancers when comparing with non-cancerous state, and may be a reflection of the high proliferation rate of cancers. Increased levels of the amino acid taurine have been found in cancerous compared to normal tissue in studies of cervix[38], prostate[39] and colon[40] tissues. For breast cancer, increased levels of taurine have been detected in cancer tissues[41] while the levels were decreased in serum samples of cancer patients compared to healthy volunteers.[42] Taurine levels have also been studied for further characterization of cancers, and were found to significantly correlate with apoptotic cell density in gliomas.[43] Taurine is a poly-functional molecule which is involved in a variety of cell functions including osmoregulation, cardioprotection, hypertension and neurotransmitting.[44] The exact actions of taurine are however not fully mapped, and the role of taurine in cancer development is currently unknown. Glycine is another amino acid proposed to contribute to cancer development and progression. Glycine concentrations have been shown to positively correlate with tumor aggressiveness in

brain tumors.[45-46] As for taurine, the exact role of glycine in cancer development is unknown. Glycine is synthesized through several pathways. It is mainly derived from the glycolysis through the formation of its precursor serine from 3-phosphoglycerate.[20] In addition, glycine can be derived from Cho through its precursor sarcosine. Preclinical studies of the basal-like and luminal-like breast cancer subtypes[7] showed an increased level of glycine in the basal-like model compared to the luminal-like model, with gene expression data suggesting a metabolic shift from PtdCho synthesis to glycine formation in the basal-like subtype.[47] Basal-like and luminal-like breast cancers are defined based on differences in gene expressions, with the basal-like model having a poor prognosis compared to luminal-like breast cancer.

Mutations in the genes encoding both isoforms of the Krebs cycle enzyme isocitrate dehydrogenase (IDH1/2) have been found in gliomas and leukemia.[48] More specifically, mutations have been detected in more than 70% of grade II and III gliomas.[49] While wild-type IDH1 catalyse the conversion of isocitrate to α-ketoglutarate, Dang et al. found that mutations in IDH1 resulted in production of 2-hydroxyglutarate (2HG).[50] These findings indicate that excess 2HG promotes tumor growth and malignant progression, leading to the proposal of 2HG being an 'oncometabolite'.

1.2 Metabolomics

Metabolism comprises the integrated network of biochemical reactions that supports life in a living organism. Chemical compounds that are intermediates or end-products of metabolism are referred to as metabolites. Unlike the human genome, the human metabolome is not an easily defined entity. More than 6000 metabolites have been described in the Human Metabolome Database (HMDB) including lipids, amino acids, carbohydrates, fatty acids, and vitamins.[51] Systematic studies of these small-molecular compounds of metabolism are being referred to differently among disciplines, with terms such as metabolic profiling, metabolic fingerprinting, metabolomics, and metabonomics commonly used.[52-53] Metabolomics is probably the most widely used

designation. More precisely, metabolomics can be defined as the "non-biased identification and quantification of all metabolites in a biological system".[54]

Metabolomics is a relatively new field of research compared to the other "omics" approaches; genomics, transcriptomics and proteomics. The metabolome is the final downstream product of gene expression, and therefore closest to the phenotype of the biological system under study. As shown in Figure 1.4, the metabolome shows what is happening at the exact time of sampling.[55] Transcriptomics and proteomics, however, will not directly reflect the biological happenings of the biological system due to post-modifications and other regulatory mechanisms.

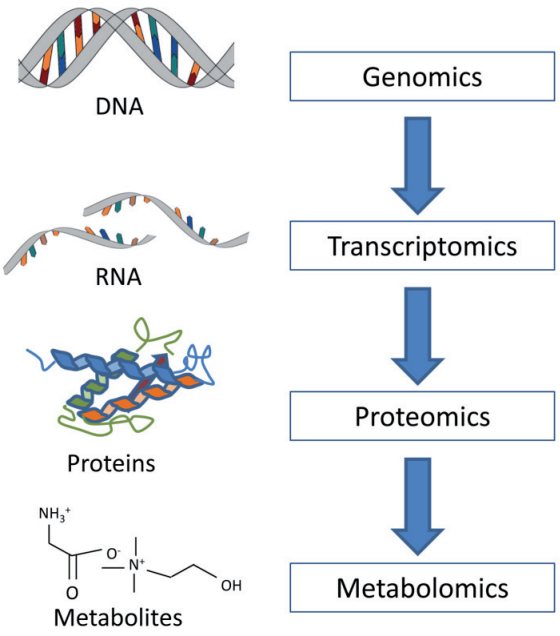

Figure 1.4: The "omics" cascade. Metabolites are the final downstream product of gene expression, and provide a picture of the biological happenings of a system at the time of sampling.

Metabolites exist with a variety of chemical and physical properties, and will be present in a wide range of concentrations. As opposed to for instance transcriptomics, there is not one single platform that can analyze all metabolites at once. The most commonly used tools for measuring the metabolic state of a biological system are nuclear magnetic resonance spectroscopy (MRS) and mass spectrometry (MS). MS is usually performed in combination with either gas or liquid chromatography for metabolite analyses.[54, 56] Both MRS and MS have their pros and cons. MS is a very sensitive technique, and can detect metabolites present in much lower concentrations than MRS. MRS, however, is highly quantitative and reproducible, and suitable for samples in a broad range of conditions independent of hydrophobisity and acidity.[57] In addition, MRS has the advantage of being a non-destructive technique requiring a minimum of sample preparation.[54]

1.2.1 MR spectroscopy

MRS is a common tool for examining the metabolic state of a biological system. All nuclei with non-zero spin ($I \neq 0$), i.e. nuclei with an uneven number of protons and/or neutrons, have an intrinsic magnetic moment and can be used in MRS. This includes ^{1}H, ^{13}C, ^{14}N, ^{15}N, ^{19}F, and ^{31}P, of which ^{1}H has the highest sensitivity and natural abundance and is most commonly used for MRS in biological systems. When placed in a magnetic field (B_0) the nuclei will orient in 2I+1 different energy levels by equilibrium processes, and they will precess with a frequency dependent on the type of nuclei and the strength of the magnetic field. ^{1}H has spin I = 1/2, and will thus be present in two energy levels; oriented either parallel or anti-parallel to B_0.

A small excess of spins will be oriented parallel to B_0, and this produces a net magnetization (M_0) along B_0. If a radio frequency (RF) pulse is applied, the nuclei in a lower energy level will excite to a higher energy level and thereby disrupt the equilibrium. The result is that M_0 will be tilted away from the direction of B_0 with an angle called the flip angle of the RF pulse. A 90^{o} pulse will flip M_0 with an angle of 90^{0}, from the z-direction to the x-y-plane. When the RF pulse is switched off, the excited nuclei will return to equilibrium via longitudinal (T_1) and transversal (T_2) relaxation,

thus the magnetization vector will return to its equilibrium state M_0. A signal called the free induction decay (FID) can be detected. The detected signal will decay faster than predicted from T_2. This is caused by additional dephasing due to inhomogeneities in B_0, and the effective T_2 is called T_2^*. The FID can be Fourier transformed into a frequency dependent spectrum where the frequencies are determined by B_0 and the gyromagnetic ratio of the nucleus.

Nuclei in different magnetic environments will experience slightly different magnetic fields due to shielding from surrounding electrons, and will therefore appear as peaks at different positions, or chemical shifts, of the spectrum. The chemical shifts are converted into parts per million (ppm) which is independent of the magnetic field strength. The nuclei of a molecule will also be influenced by the spins of closely located nuclei, resulting in splitting of peaks into multiplets.[58]

1.2.2 HR MAS MRS

Biological tissue can be considered a semisolid material, and resonating nuclei in tissues will be affected by interactions between nuclei due to restricted mobility of the molecules in the tissue. This gives rise to broader peaks in the MR spectra of tissues compared to liquid solutions when using conventional MRS. It is however possible to impose motion on the nuclei by spinning the sample.[59] Rapid spinning (typically 5 kHz) of the sample about an axis inclined 54.7^o (the magic angle) to the direction of the static magnetic field B_0 will reduce line broadening and thus provide spectra of high resolution (Figure 1.5). Spinning splits the broad resonance into a narrow line at the isotropic resonance frequency and spinning sidebands.[59-60] Magic angle spinning of solids was first described by Andrew et al.[61] and Lowe[62] in 1958, and high resolution magic angle spinning (HR MAS) MRS was applied to human tissues in 1997.[63-64]

HR MAS MRS is a non-destructive technique that requires a minimum of sample preparation. As the samples remain intact after analysis, they may be further analyzed by for instance histopathological examinations or gene expression profiling after MRS.[65] The resolution of the acquired HR MAS MR spectra from tissue samples is

comparable to that of tissue extracts, and more than 30 metabolites have been identified from breast cancer tissue using HR MAS MRS.[66] This includes lactate, glucose, and the choline-containing metabolites GPC, PCho and Cho, making HR MAS MRS an excellent tool for investigating the altered glucose and phospholipid metabolism of cancers. In addition, several amino acids and other organic acids can be detected (see annotated spectrum, Figure 1.5).

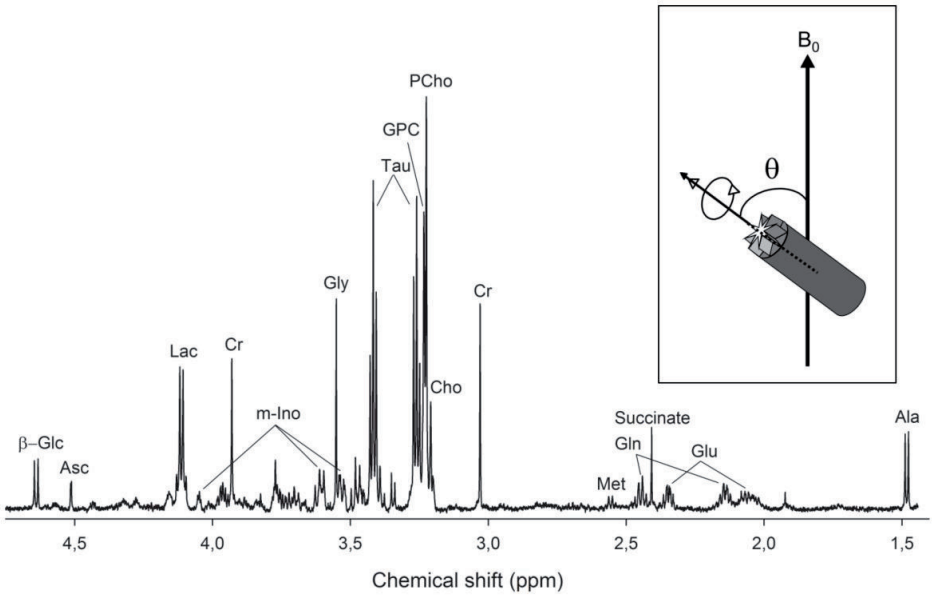

Figure 1.5: A representative HR MAS MR spectrum of breast cancer tissue showing some of the metabolites that are detectable by MR. In frame: Schematic representation of a sample in a MAS rotor inclined in the magic angle $\theta = 54.7^{o}$ to the direction of the static magnetic field B_0. Spinning a sample about an axis inclined at the magic angle reduces line broadening. β-Glc, β-glucose; Asc, ascorbate; Lac, lactate; Cr, creatine; m-Ino, myo-Inositole; Gly, glycine; Tau, taurine; Met, methionine; Gln, glutamine; Glu, glutamate; Ala, alanine. The MAS rotor is reprinted with permission from Beathe Sitter.

1.2.3 MRS acquisition

Biological materials consist of large amounts of water, and the water signals in an MR spectrum will be several orders of magnitude larger than signals from the metabolites. Sequences that suppress the water signal are therefore commonly used for MRS acquisition. Water can be suppressed using several types of pulse programs. Commonly used methods for acquiring MR spectra contain pre-saturation of the water signal by applying a low power continuous wave irradiation before the signal is acquired (for instance Noesy with preset as described by Beckonert et al. [67]). The metabolite signals may also be affected by lipids and large molecules giving broad peaks in the spectrum. As these large molecules have a short T_2-relaxation time, they can be suppressed by acquiring spectra using a long echo-time (TE) before acquisition. A much used sequence for water and fat suppression is the spin-echo Carr Purcell Meiboom Gill sequence (cpmg, Figure 1.6). After presaturation of the water signal, a 90^o pulse is applied followed by several 180^o pulses each after a delay τ. The 180^o pulses will refocus the spins that are dephased according to T_2*, resulting in an echo of the 90^o signal. For molecules with a long T_2, the magnetization in the x-y plane will be better preserved, thereby reducing the signal from molecules with a short T_2.

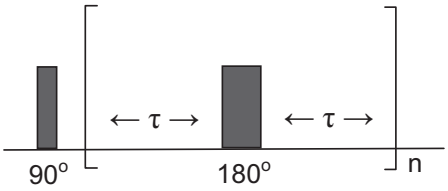

Figure 1.6: The spin-echo Carr Purcell Meiboom Gill sequence. After pre-saturation of the water signal, a 90^o pulse is applied. This is followed by several 180^0 pulses each after a delay τ. The 180^o pulses will refocus the spins, resulting in an echo of the initial 90^o pulse.

1.3 Preprocessing of MR spectra

Preprocessing of MR spectra prior to analysis can correct for variations in the data that are introduced by technical or environmental effects not related to the property of interest. Proper preprocessing may in some cases make the difference between a useful data model and no model at all.[68-69] Several forms of preprocessing exist; some performed on each separate sample and others performed on the variables. The different kinds of preprocessing may be applied individually or in combination with others, and in different orders.[70-71]

1.3.1 Baseline corrections

Baseline corrections can be applied to correct for baseline distortions of the spectra. A simple way to correct for differences in baseline offset is to set the lowest value of each spectrum equal to zero by subtracting the minimum point. This is a safe way of baseline correcting as it does not change the shape of the spectra. It will however give poor results if the spectrum contains negative peaks, and these artefacts should be dealt with before correcting the baseline. An example of a baseline correction method that intends to change the shape of the spectra is asymmetric least-squares baseline estimation, where a baseline is estimated using asymmetric least squares.[72] This estimated baseline is then subtracted from the spectrum in order to remove baseline noise.

1.3.2 Peak alignment

The peaks of an MR spectrum may be shifted due to differences in pH, temperature, ion-concentrations and metabolite-protein interactions. Misalignments between corresponding peaks in a data set may affect multivariate analysis, and should therefore be corrected for.[73-74] Misalignments can be dealt with by binning the data, or by alignment of the spectral peaks. In binning, the spectra are divided into regions and a sum of spectral intensities is calculated for each region. The size of the regions may vary, and each region may be of equal or unequal size.[71, 75-76] The penalty of binning is however loss of spectral resolution and the resulting loss of interpretability. Peak alignment, or *warping*, corrects for misaligned peaks without any loss of variables. A

plethora of different methods are available. Alignment is performed by optimizing the positions of spectral peaks based on a specific criterion, such as the correlation. Some algorithms work by dividing the spectra into segments which are aligned, while some align each spectrum as a whole. Most methods require a reference spectrum to which the other spectra are aligned.

Icoshift,[77] correlation optimized warping (COW[78-79]) and peak alignment by beam search (fastpa[80]) are examples of segmented alignment methods. The main difference between them is the way they perform the alignment. In icoshift, the segments are shifted sideways to achieve optimal alignment with the reference, while COW stretches or shrinks the segments. Fastpa however allows both shifting and stretching/shrinking for alignment of the segments. Rather than dividing the spectra into segments, variable penalty dynamic time warping (VPdtw[81]) aligns by shifting individual points of the spectra. The variable penalty function of the algorithm is based on a running maximum, and results in a high penalty for shifting variables in peaks, thereby reducing the risk of introducing artefacts in the spectra. A method that aligns the spectrum as a whole is parametric time warping (PTW[72, 82]). PTW models a global polynomial function of the misalignment. Using a limited number of higher order terms makes the polynomial modelling of PTW more restricted, but reduces the risk of introducing artefacts or overfitting the spectra.

The optimization criteria of the abovementioned algorithms differ. In icoshift and fastpa, the correlation per segment is optimized. Icoshift does this by calculating the cross-validation using fast Fourier transform (FFT). Fastpa however is based on a genetic algorithm routine by Forshed et al.[83], but with a faster beam search algorithm implemented instead of the more time-consuming genetic algorithm. Instead of the correlation per segment, COW optimizes the total correlation for the whole spectra by dynamic programming, while the optimization criterion for PTW is the weighted cross-correlation taking into account an area of neighbouring points.[84] In contrast to the correlation-based methods, VPdtw optimize the sum of the absolute differences, called the L1 norm, between the variables of the spectrum and the reference. Figure 1.7 shows a data set before and after alignment by COW.

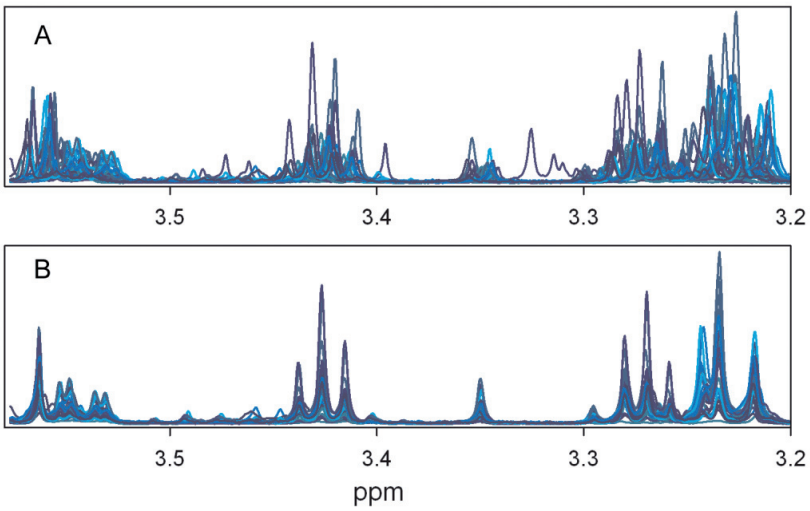

Figure 1.7: HR MAS MR spectra (A) before and (B) after alignment by correlation optimized warping. The peaks are clearly more overlapping after alignment.

1.3.3 Scaling and normalization

Scaling of spectra can be performed on the samples or on each individual variable of a data set. Scaling performed sample-wise will normalize the spectra. Normalization of spectra corrects for differences in dilutions or sample weight in order to make the spectra comparable. Several normalization algorithms for MR spectra exist. One frequently used is area normalization, where each spectrum is scaled to the same total integral.[70] Another commonly used normalization strategy is scaling all samples to a common range; a method known as range normalization.[85] Normalization to a "housekeeping" metabolite; a metabolite assumed to be stably expressed, have also been attempted. However, the assumption of stable expression of a metabolite may in many cases be wrong. This can be corrected for by determining the exact concentration of the metabolite and using this as a reference value.[70]

Scaling of the individual variables prior to data analysis can be performed to bring all variables into the same range, and thereby regulate the relative importance of each

variable.[86] Mean-centering, a method generally used for MR spectra, is done by subtracting the mean of each variable in a data set to all variables in the spectra. By mean-centering the variables, only the fluctuating variation will be in focus.[68] In autoscaling, the mean of the variables is subtracted before dividing the variable on its standard deviation. Although commonly used, autoscaling may not be optimal for MR spectra as all variables, including noise, is given the same potential to influence the model.[87] Variable stability (VAST) scaling is a method that gives focus to the stable variables of the data set by downweighting the least stable variables.[86] A coefficient of variation (CV), i.e. the ratio of the standard deviation and mean of each variable, is defined as the scaling factor. The CV may be calculated for each group in the data set, and the mean of the CVs may be used in scaling, thereby performing VAST in a supervised manner. In this way, prior class information is incorporated into the scaling.

1.3.4 Variable selection

Large regions of an MR spectrum may contain uninformative data, e.g. data representing technical noise or biological variation not related to the property of interest. Often only a small subset of the variables is necessary, and including all variables may add noise to the model.[88] In addition, some analysis methods cannot handle the full number of variables in a spectrum, and variable reduction is necessary. The minimum-redundancy-maximum-relevance (mRMR) method for variable selection was initially developed for microarray data, but is also applicable to spectral data. 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).[89-90] This is especially useful for dimension-reduction of spectral data, where variables in close distance of each other are highly correlated. The variables with maximum relevance are selected using F-statistics:

$$\max V_F, \quad V_F = \frac{1}{|S|}\sum_{i \in S} F(i,h) \qquad [1]$$

where S is the variable set, F is the F-test value and h is the class. Correlation, c, between to variables i and j, may be used to determine the redundancy of the variables:

$$\min W_c, \quad W_c = \frac{1}{|S|^2}\sum_{i,j}|c(i,j)| \qquad [2]$$

Euclidian distance may also be used. Redundancy and relevance using correlation may be combined by difference (max V- W) or quotient (max V/W).[89]

1.4 Multivariate analysis

MR spectra contain a vast amount of variables that are highly collinear. This makes MR spectra unsuited for standard statistical methods. Multivariate analysis methods, or *chemometric*s, can handle several variables simultaneously and are commonly used tools for the analysis of MR data.

1.4.1 Principal component analysis

Principal component analysis (PCA) is concerned with explaining the variance structure of a data set through linear combinations of the variables. These linear combinations are called principal components (PCs). By detecting the underlying structures of a data set, one can reveal relationships previously hidden in the data and thereby ease the interpretation of the data.[87, 91] PCA is an unsupervised method, and is well suited for exploration of the data without forcing on a model. The PCs are derived so as to maximize the span of variation, and the first PC will be the axis that describes most of the variance of the data (Figure 1.8). Subsequent components are orthogonal and derived to explain the residual variation. The first few PCs will usually describe the interesting aspects of the data, and the remaining components can be regarded as noise. Mathematically, a PCA can be described by:

$$\mathbf{X} = \mathbf{T}\mathbf{P}^T + \mathbf{E} \qquad [3]$$

where **X** is the original data set, **T** is the score matrix, **P** is the loading matrix and **E** is the matrix of residuals. Scores represent the coordinates of the samples in the new coordinate system defined by the PCs, and can be used to detect patterns or groupings among the samples. Loadings represent the weights needed to define the directions of the principal components in the original coordinate system, and can be useful for interpreting the biological meaning of the model.[87]

1.4.2 Partial least squares

Partial least squares (PLS) is a supervised method where the relationship between two matrices **X** and **Y** is modelled.[92] For MR spectroscopy, the **X**-matrix will be the spectra and the **Y**-matrix will consist of one or several properties related to each spectrum. PLS aims to find underlying structures, called latent variables (LVs), that maximize the covariance between **X** and **Y**.

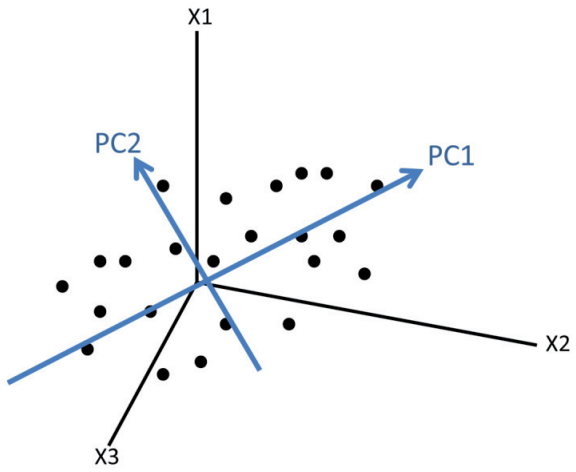

Figure 1.8: Principal component analysis of a mean-centered data set with three variables x1-x3. PC1 and PC2 are orthogonal, and derived to explain maximum variance.

The PLS model can be written as:

$$\mathbf{X} = \mathbf{TP}^T + \mathbf{E}$$
$$\mathbf{Y} = \mathbf{UQ}^T + \mathbf{F} \qquad [4]$$

where **T** and **U** are the score matrices for **X** and **Y**, respectively, **P** and **Q** are the loading matrices and **E** and **F** are the residuals. These PLS parameters can be calculated using different algorithms,[93] such as the original non-iterative partial least squares (NIPALS) algorithm or the faster SIMPLS.[94] For both algorithms, the scores are defined by weights that maintain orthogonality, and a vector of regression coefficients is calculated which relates the **X** and **Y**-scores. SIMPLS and NIPALS will give the exact same result for a univariate **Y**, while slightly different models are acquired for multivariate **Y**. The scores and loading of a PLS model can be interpreted in the same way as scores and loadings of PCA.

PLS discriminant analysis (PLS-DA) is a special case of PLS that attempts to discriminate between distinct classes using so-called 'dummy' variables representing each class.

1.4.3 Bayesian belief networks

Bayesian belief networks (BBN) use probability theory in order to classify samples to different groups. The method aims to find the most likely of the possible classifications. The graphical structure of a BBN consists of nodes, one for each variable, and directed edges indicating conditional relations between the nodes (Figure 1.9). The network is acyclic, i.e. no feedback-loops are allowed. If a directed edge is pointing from node A to node B, then A is called the parent of B and B is called the child of A.[95-96] Each node is associated with a conditional probability table that specifies the probability that a variable takes a certain value given the value of its parents. In this way, BBNs can model complex non-linear relationships.[97] Most BBN algorithms can only handle discrete variables, so for continuous data such as MR spectra the variables must be discretized before analysis.[98]

Naïve Bayes models combine the unconditioned probabilities, i.e. the probability that a variable is in a given state, with the conditional probabilities in a single formula. For MR spectra, a naïve Bayes classifier will have several parent nodes representing the chemical shifts, and one child node representing the property of interest. There are no edges between the parent nodes of a naïve Bayes classifier, hence the variables are assumed to be independent of each other. Despite this often incorrect assumption, Naïve Bayes classifiers have shown good results in practice.[99]

BBNs can be built in two ways; by using pre-existing knowledge about the probabilistic distribution of the variables, or by making the network learn from a data set in a supervised manner.[100] The latter will be most relevant for MR spectra.

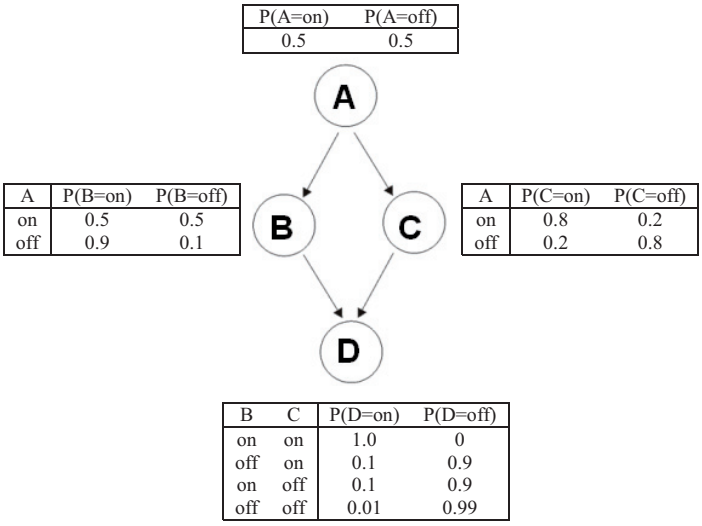

Figure 1.9: An illustrative example of a Bayesian belief network consisting of four variables, A, B, C and D, with two different states (on and off).

1.4.4 Probabilistic neural networks

Neural networks aim to mimic the brain's processes in solving problems, by applying knowledge gained from past experience to new problems.[101] Probabilistic neural networks (PNNs[102]) for classification will define a probability density function (pdf) for each class based on training data, and assign an unknown sample to the most probable class. PNNs have four layers: input, pattern, summation and output, and the neurons of the different layers are connected by weights (Figure 1.10). The pattern layer consists of one neuron for each sample in the training set. A kernel, typically of Gaussian shape, is defined for each neuron in the pattern layer. The summation layer of the network sums up the information from the pattern layer and produces an overall pdf. The kernels are placed 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. In this way, all classes are characterized by a pdf defined by the training data. The probability that a sample will be classified as a member of a given class increases the closer it is to the centre of the pdf for that class. The output layer picks the class of maximum probability to which the new sample is classified.

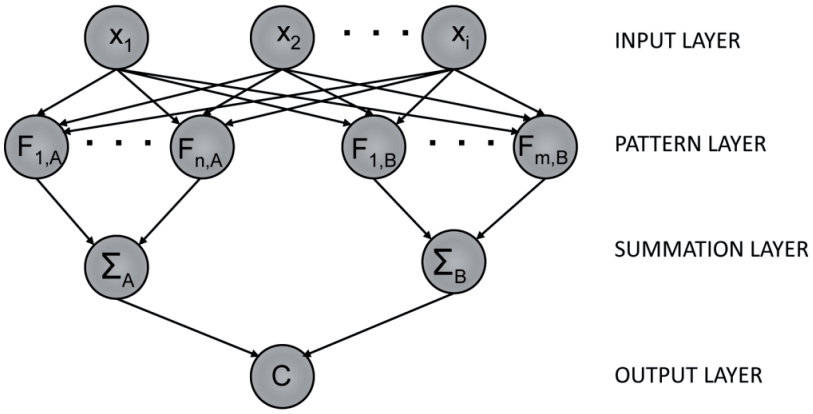

Figure 1.10: Probabilistic neural network for a two-class discrimination problem. The training data consist of n samples from class A and m samples from class B. The input layer has one node for each of the i variables. The predicted class C of a new sample is provided by the output node.

1.4.5 Multilevel analysis

One of the challenges in metabolomic studies is that the metabolic changes of interest may be small and subtle compared to the larger variations between subjects, making it difficult to extract the relevant information from the data. Taking advantage of the paired structure in a multilevel study, i.e. when interventions are evaluated on the same subject, can be beneficial.[103-104] This is comparable to a paired t-test, which will have increased statistical power over a regular t-test. In multilevel analysis, the *between* subject variation is separated from the *within* subject variation, and the two sources of variation can be analyzed separately. The between subject variation is described by the average of the two observations from one subject, whereas the within subject variation is described by the net difference between them.

If the observations for each subject at baseline (the control samples) is given by the matrix **A**, and the observations after intervention by the matrix **B**, then the between subject variation **M** is defined by

$$\mathbf{M} = \frac{1}{2}[\mathbf{A} + \mathbf{B}] \qquad [5]$$

while the within subject variation **W** is defined by

$$\mathbf{W} = \begin{bmatrix} -\mathbf{D} \\ \mathbf{D} \end{bmatrix} = \begin{bmatrix} \mathbf{A} - \mathbf{B} \\ \mathbf{B} - \mathbf{A} \end{bmatrix} \qquad [6]$$

The rows of **A** and **B** correspond to the same subjects in the study, i.e. row number 3 in both **A** and **B** describes subject number 3. The within and between subject variation can be analyzed separately using different multivariate methods, e.g. PCA or PLS-DA.

1.5 Validation of multivariate methods

Modelling of data sets with few samples compared to variables involves a risk of overfitting. Proper validation of the resulting model is therefore of outmost importance. In K-fold cross-validation, the samples are divided into K subsets. K-1 subsets are assigned to training of the model, while the remaining subset is used for testing. This procedure is repeated K times until all subsets have been used as a test set once.[105] The number of subsets to divide the data into will depend on the total sample size. A larger K results in a larger training set and thus less biased results, but at the same time the variance of the estimated error will increase.[106] When K equals the sample size of the data set, the procedure is called leave-one-out (LOO) cross-validation. LOO cross-validation may however give over-optimistic results for data sets with a large number of samples, and should only be used for small sample sets.

When cross-validation is used to optimize model parameters, such as the number of latent variables to use in a PLS-DA, the final model should be validated using an independent validation set. Samples to keep out for final validation may be chosen randomly, or by using sample selection algorithms such as Kennard-Stone[107] and SPXY[108] sample selection. These algorithms will make sure that the training data span the whole dimensional space. The advantage of using random validation sets is that the whole procedure can be repeated several times, each time keeping a different validation set out of the optimisation process. This will give less biased validation results. A dilemma of using several validation sets however is that the result is not one but several models, and there are no accepted criteria for the way of choosing a final model.[88] One possibility is to build a final model based on the full data set. It is not straightforward then to choose the number of latent variables to use for this final model if all models have been optimized for a different number of latent variables. It has also been suggested to use an average of multiple models for future prediction.[109] Validation by an independent validation set requires enough samples, and may not be feasible for small sample sets.

Permutation testing is a method for determining if the results achieved by either cross-validation or by using an independent validation set, are significantly different than random. The labels of all samples are permuted, and randomly assigned to the samples in the data set. The new data set, now with the wrong labels, are modelled in the exact same way as the original data set. This procedure is repeated several times, and the distribution of prediction results from the permuted samples is compared to the results of the original model.[109]

2 Objectives

The main objective of the research presented in this thesis is to evaluate the use of HR MAS MRS and multivariate analysis for determining the prognosis of breast cancer patients. More specifically to:

- Optimize preprocessing of MR spectra for multivariate analysis.
- Examine different multivariate methods for their feasibility in analyzing MR spectra.
- Investigate the relationship between metabolite profiles and clinical prognostic parameters.
- Examine the metabolic changes caused by neoadjuvant chemotherapy in breast cancer tumors, and to relate these changes to clinical treatment response and prognosis.
- Indentify potential biomarkers for breast cancer prognosis.

3 Materials and methods

3.1 Patients and data sets

The patients of paper I and III were enrolled in a regional collection of breast cancer biopsies in mid-Norway. The patients had undergone surgery at either St. Olavs University Hospital, Trondheim, or Molde Hospital, Molde, between 1999 and 2006, and the biopsies were excised during surgery. All patients were diagnosed with IDC, and did not receive NAC. After surgery, the patients were given the treatment considered optimal according to guidelines of the NBCG.

The patients of paper IV were operated at Bergen University Hospital, Bergen, between 1997 and 2003. These patients were diagnosed with locally advanced breast cancer, and were part of an open-label multicenter study where patients were randomly allocated to receive NAC with either epirubicin (90 mg/m^2) or paclitaxel (200 mg/m^2) monotherapy. Patients showing a non-satisfactory response were assigned to the opposite treatment. For each patient, an incisional open biopsy was taken before treatment with NAC and a post-treatment biopsy was excised during surgical removal of the tumor.

Paper II included data sets from patients diagnosed with either breast cancer, cervical cancer, or colon cancer. The breast cancer data set corresponds to the same data set as in paper I. The cervical and colon data have been described in previous studies.[38, 40]

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

3.2 Sample handling

Breast cancer tissue samples were frozen in liquid nitrogen immediately after dissection and stored until HR MAS MRS analyses. Samples from the cervix were obtained as soon as possible after surgery from the uterus that was kept in saline compresses after

excision, and further stored in liquid nitrogen. Colon samples were collected immediately after surgery and stored at -80^{o}C. Sample preparations before HR MAS MRS were performed on ice in order to prevent tissue degrading (Figure 3.1).

3.3 HR MAS MRS protocol

The tissue samples were analyzed on a Bruker Avance DRX600 (Bruker BioSpin GmbH, Germany) equipped with a $^{1}H/^{13}C$ MAS probe with gradient aligned with the magic angle axis. The breast cancer samples of the biobank (paper I, II and III) were cut to fit a 50 µL MAS rotor added phosphate buffered saline (PBS, 40 µL) based on D_2O with trimethylsilyl 3-propionic acid sodium salt (TSP, 1.0 mM) added as a reference for chemical shift calibration. The cervical samples were analyzed similarly, but without PBS buffering. The breast cancer samples of paper IV and the colon samples were analyzed in disposable Kel-F HR MAS inserts added 3 µL PBS based on D_2O with TSP (breast: 98.2 mM, colon: 4.5 mM). ^{1}H spectra were acquired using a water and lipid suppressing spin-echo cpmg (Bruker) sequence as specified in Table 3.1. An exponential line broadening (breast and colon: 0.3 Hz, cervix: 0.7 Hz) was applied to the data prior to Fourier transformation.

Table 3.1: Parameters for acquisition of HR MAS MR spectra

	Breast spectra	**Cervix spectra**	**Colon spectra**
Temperature	4^{o}C	Room temp.	4^{o}C
Spin rate	5 kHz	6 kHz	5 kHz
Echo time	285 ms	285 ms	272 ms
Number of scans	128	128	128
Collected region	10 kHz	10 kHz	10 kHz
Number of points	32k	32k	64k
Acquisition time	1.64 s	1.64 s	3.27 s

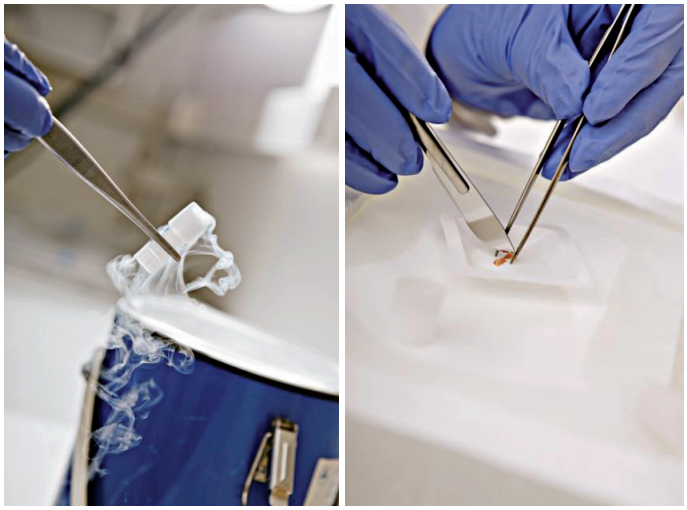

Figure 3.1: Preparation of a tissue sample for HR MAS MRS. Left: The breast cancer samples were frozen in liquid nitrogen immediately after dissection and stored until MRS analysis. Right: The tissue sample is cut to fit a MAS rotor before MRS acquisition. Photo: The medical faculty, NTNU/Geir Mogen.

3.4 Data analysis

Preprocessing of spectra was performed in XWinNMR, Matlab R2008b (The Mathworks, Inc., Natick, USA) and R, version 2.9.2. PCA and PLS-DA were performed in Matlab using PLS toolbox (Eigenvector Research,Wenatchee, USA). PLS-DA was performed using the SIMPLS algorithm. BBN analyses were performed in Netica (Norsys Software Corp, Canada). A naive Bayes classifier was built by making the data learn in a supervised manner. Input for the BBN was spectral variables chosen by mRMR variable selection. PNN analyses were performed in NeuroShell Classifier (Ward Systems Group, USA). The Neuroshell Classifier uses a version of PNNs adapted around a genetic algorithm in order to find the optimal combination of variables. Genetic algorithms solve optimization problems using the concepts of evolutionary theory. A population of possible solutions to a problem is created, where each individual in the population carries chromosomes that are values of variables of the problem. The genetic algorithm lets the less fit individuals die out, while the fit ones are selected to ‘mate’ in a process called cross-over. In addition, mutations are allowed by

random change of variables to avoid getting trapped in local minima. After 100 generations without any improvement, the algorithm was stopped and the most fit individual was chosen as a solution to the problem. Input for the PNN was spectral variables chosen by mRMR variable selection. The split-up of variation for multilevel analyses in paper IV was done using algorithms made available by van Velzen et al.[103] Kaplan Meier analyses and ROC statistics were performed in SPSS 17.0 (SPSS Inc, Chicago, USA).

3.5 Histopathology and clinical diagnostics

Routine histopathology was performed on tissue samples excised during surgery. For the breast cancer samples, histological tumor nuclear grade was determined according to guidelines of the NBCG, which are based on the Bloom and Richardson classification system.[110] 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. Histopathological examinations of the biobank samples were performed on the tissue after HR MAS MRS analyses. All tissue samples were fixed in 10 % formalin and embedded in paraffin after HR MAS MRS. A section of 3-5 μm was cut from the middle of each paraffin block and stained with HES (hematoxylin, erythrosine and saffron) for pathology reading. The stained sections were examined microscopically by an experienced pathologist and the relative areas of normal and neoplastic epithelial elements, necrotic tissue, fat and fibrous connective tissue were scored (Figure 3.2). Samples containing < 5% tumor cells were not included in further analyses. The breast cancer samples in paper IV were analyzed by imprint cytology prepared prior to HR MAS MRS, and stained with May-Grünwald-Giemsa stain to evaluate the tumor cell content. Samples not containing any tumor cells were removed from further analyses.

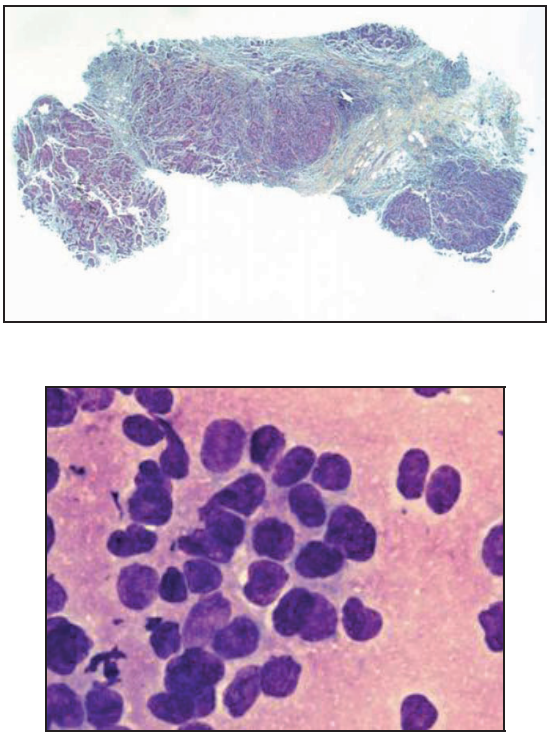

Figure 3.2: Histopathological examinations of tissue samples. Top: A sliced tissue sample has been stained with hematoxylin, erythrosine and saffron after HR MAS MRS. The sample was pathologically determined to contain 70 % tumor cells and 30 % connective tissue. Bottom: A tissue sample analyzed by imprint cytology before HR MAS MRS. The presence of cancer cells was confirmed by May-Grünwald-Giemsa stain.

4 Summary of papers

Paper I

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

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 could be predicted from the MR spectra, and were best predicted by PLS-DA with a correct classification of 43 of 50 and 39 of 50 samples, respectively. Lymph node status was best predicted by BBN with 36 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.

Paper II

Alignment of high resolution magic angle spinning magnetic resonance spectra using warping methods

The peaks of magnetic resonance (MR) spectra can be shifted due to variations in physiological and experimental conditions, and correcting for misaligned peaks is an important part of data processing prior to multivariate analysis. In this paper, five warping algorithms (icoshift, COW, fastpa, VPdtw and PTW) are compared for their feasibility in aligning spectral peaks in three sets of high resolution magic angle spinning (HR-MAS) MR spectra with different degrees of misalignments, and their merits are discussed. In addition, extraction of information that might be present in the shifts is examined, both for simulated data and the real MR spectra. The generic evaluation methodology employs a number of frequently used quality criteria for evaluation of the alignments, together with PLS-DA to assess the influence of alignment on the classification outcome.

Peak alignment greatly improved the internal similarity of the data sets. Especially icoshift and COW seem suitable for aligning HR-MAS MR spectra, possibly because they perform alignment segment-wise. The choice of reference spectrum can influence the alignment result, and it is advisable to test several references. Information from the peak shifts was extracted, and in one case cancer samples were successfully discriminated from normal tissue based on shift information only. Based on these findings, general recommendations for alignment of HR-MAS MRS data are presented. Where possible, observations are generalized to other data types (e.g. chromatographic data).

Paper III

Glycine and lactate- potential MR biomarkers of breast cancer prognosis

Breast cancer is a heterogeneous disease with a varying prognosis. Today's clinical factors provide some information about the prognosis of a breast cancer patient; however there is a need for additional information to stratify patients for improved and more individualized treatment. The aim of this study was to examine the relationship between the metabolic profiles of breast cancer tissue and 5-year survival. Biopsies from breast cancer patients (n = 98) were excised during surgery and analysed by high resolution magic angle spinning MR spectroscopy (HR MAS MRS). The data were analyzed by multivariate principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), and findings of important metabolites were confirmed by spectral integration of the metabolite peaks. Predictions of 5-year survival using metabolite profiles were compared to predictions using clinical parameters. Based on the metabolite profiles, estrogens receptor (ER) positive breast cancer patients (n = 71) were separated into two groups with significantly different survival rates ($p = 0.024$). Higher levels of glycine and lactate were found to be associated with lower survival rates both by multivariate analyses and spectral integration, and are suggested as biomarkers for breast cancer prognosis. Similar metabolic differences were not observed for ER negative patients. Predictions of 5-year survival of ER positive patients using metabolite profiles gave better and more robust prediction results than using traditional clinical parameters. This shows that the metabolic state of a tumor may provide additional information concerning breast cancer prognosis. Metabolomics may serve as an additional tool for determining the prognosis and treatment strategy of breast cancer patients.

Paper IV

Prognostic value of metabolic response in breast cancer patients receiving neoadjuvant chemotherapy

Today's clinical diagnostic tools are insufficient for giving accurate prognosis to breast cancer patients. The aim of our study was to examine the tumor metabolic changes in patients with locally advanced breast cancer caused by neoadjuvant chemotherapy (NAC), relating these changes to clinical treatment response and long-term survival.

Patients (n=89) participating in a randomized clinical trial were allocated to receive either NAC as epirubicin or paclitaxel monotherapy. Biopsies were excised pre- and post-treatment, and analyzed by high resolution magic angle spinning magnetic resonance spectroscopy (HR MAS MRS). The metabolite profiles were examined by paired and unpaired multivariate methods and findings of important metabolites were confirmed by spectral integration of the metabolite peaks.

All patients had a significant metabolic response to NAC, and pre- and post-treatment spectra could be discriminated with 87.9%/68.9% classification accuracy by paired/unpaired partial least squares discriminant analysis (PLS-DA) ($p<0.001$). Similar metabolic responses were observed for the two chemotherapeutic agents. The metabolic responses were related to patient outcome. Non-survivors (<5 years) had increased tumor levels of lactate ($p=0.004$) after treatment, while survivors (≥5 years) experienced a decrease in the levels of glycine ($p=0.047$) and choline-containing compounds ($p\leq0.013$) and an increase in glucose ($p=0.002$) levels. The metabolic responses could not be related to clinical treatment response.

The differences in tumor metabolic response to NAC were associated with breast cancer survival, but not to clinical response. Monitoring metabolic responses to NAC by HR MAS MRS may provide information about tumor biology related to individual prognosis.

5 Discussion

In this thesis the use of MR metabolomics as a clinical tool for breast cancer diagnostics and evaluation of prognosis and treatment response has been evaluated. A diverse selection of multivariate methods for preprocessing and modelling of MR spectra has been optimized and used for analyzing the data.

In paper I the relationships between metabolite profiles and the status of the clinical prognostic factors ER, PgR and lymph node involvement were examined. Hormone receptor status was successfully predicted from the MR spectra, and a trend of different metabolite profiles in lymph node positive and negative tumors was detected. To further explore the prognostic information of the MR spectra, metabolic differences between breast cancer survivors and non-survivors were examined in paper III. Two patient groups with significantly different survival rates were defined based on the metabolic patterns, and glycine and lactate were found to be potential prognostic biomarkers. Classification of patients as survivors or non-survivors by multivariate methods gave better and more robust results when using MR spectra as input compared to using clinical parameters. In paper IV, the tumor metabolic responses to NAC in breast cancer patients were explored and related to clinical treatment response and breast cancer survival. In contrast to the lower stage breast cancer patients included in paper I and III, this patient cohort consisted of locally advanced breast cancer patients with large tumors. The results showed that all patients had a metabolic response to the treatment. The metabolic responses could not be related to clinical treatment response. However, the metabolic changes were different between 5-year survivors and non-survivors, with non-survivors having an increase in lactate levels and survivors experiencing decreased levels of glycine and choline-containing metabolites after treatment. The impact of the chosen methodology for preprosessing of MR spectra was assessed in paper II. Five peak alignment algorithms were examined for their feasibility of aligning HR MAS MR spectra. The results from paper II were used to achieve optimal alignment of the data in paper III and IV.

5.1 Metabolite profiles of breast cancer

Identifying the metabolic patterns that are differently expressed in a diseased state can give insight into the biological mechanisms leading to the development and progression of the disease. This makes it possible not only to achieve a better understanding of the tumor biology, but also to target therapies directly towards the disease mechanisms. In addition, clearly expressed metabolic changes within a diseased state could make MR metabolomics a feasible clinical tool for diagnostics and for stratifying patients into subgroups of clinical value. Previous studies have suggested a relationship between the metabolite profiles and clinical prognostic factors of breast cancer in smaller patient cohorts.[111-112] This was further explored in these studies, where we revealed differences in the metabolite profiles and metabolic responses to NAC treatment in breast cancer patients from different prognostic groups.

Glycine and lactate were identified as potential biomarkers for prognosis in paper III, where the tumors of non-survivors had higher levels of glycine and lactate than tumors of patients surviving more than five years. The same trend was observed in paper IV, where non-survivors experienced increased tumor levels of lactate during NAC whereas survivors had decreased levels of glycine. These differences in metabolic response between survivors and non-survivors were reflected in the metabolite profiles of post-treatment biopsies, where non-survivors had higher levels of both glycine and lactate compared to survivors. Results from paper I showed that also ER and PgR negative tumors had higher levels of glycine and lactate than hormone receptor positive tumors. ER and PgR expressions are predictive markers for response to treatment, as tumors that are insensitive to hormones will not benefit from endocrine therapy. ER and PgR positive status has also been associated with a higher rate of overall and disease-free survival.[113-114] This indication of a better prognosis among patients with tumors that are sensitive to hormones has been observed in both patients receiving and not receiving adjuvant endocrine treatment, and is therefore not only an effect of the treatment.[115-116] However, this effect may be limited to the first five years after diagnosis.[116] As a lack of hormone receptor expression is associated with a worse prognosis, the finding of higher levels of lactate and glycine in receptor negative tumors is in accordance with the

findings for 5-year survival in paper III and IV. The detection of glycine and lactate as potential prognostic biomarkers for survival in paper III was based on ER positive patients only, and is therefore not simply reflecting the difference in hormone receptor status of the tumors.

As described in section 1.1.2, lactate may be related to cancer progression through both anaerobe (hypoxia) and aerobe (the Warburg effect) mechanisms. Elevated levels of lactate have been observed in several cancers, including breast[117] and prostate[118-119] cancer, and studies have shown a positive correlation between lactate levels and poor survival rates in cervical,[120] lung,[121] and head and neck cancers.[122] Increased levels of lactate may reflect enhancement of aerobe glycolytic activity that confer higher tumor malignancy and poor prognosis. The relation of the amino acid glycine to cancer progression is more unclear. Glycine is mainly synthesized from an intermediate in the glycolysis, and high levels of glycine may also be a reflection of enhanced glycolytic activity. As glycine can also be synthesized from Cho, the different glycine levels may additionally reflect the altered phospholipid metabolism of cancers. Elevated levels of glycine have previously been related to prognosis and tumor aggressiveness in brain[45, 123] and breast[124] tumors, and its role in cancer progression is being further investigated.

Results from paper I showed lower levels of PCho and higher levels of Cho and GPC in ER-negative tumors compared to ER-positives. Similar findings have been detected in pre-clinical studies, where the ER-negative basal-like model had significantly lower levels of PCho and higher levels of GPC and glycine than the ER-positive luminal-like model.[47] As basal-like breast cancer has a poor prognosis this may indicate that specific patterns of choline metabolism reflect more aggressive and highly proliferating tumors. These finding are not in accordance with *in vitro* studies on human mammary epithelial cells indicating that the level of PCho increases with malignancy.[26] However, this discrepancy may be attributed to the more complex microenvironment and interactions of solid tumors. Conditions such as hypoxia and pH may be different *in vivo* compared to *in vitro*, and this may impact the expression of choline kinase and affect the levels of choline-containing compounds in the tissue.[125]

None of the choline-containing metabolites were shown to be related to prognosis in the survival analyses in paper III, but for the locally advanced breast cancer patients in paper IV non-survivors appeared to have lower levels of GPC and Cho after NAC compared to survivors. These findings were not significant for the integrated spectral intensities of the metabolites, but a trend of lower tCho levels in survivors was seen. In addition, survivors had a significant decrease in all choline-containing metabolites as a response to NAC treatment, while this increase did not reach statistical significance in non-survivors. In accordance with our findings in paper IV, a study by Cao et al. of patients with locally advanced breast cancer treated with doxorubicin showed decreased levels of GPC after treatment to be associated with long-term survival.[126] Further clinical studies are required to determine the potential of choline-containing metabolites as prognostic markers in breast cancer.

The patients included in paper III were all diagnosed with ER-positive invasive ductal carcinoma, and none of the patients had received neoadjuvant treatment. These inclusion criteria will exclude the larger and more aggressive tumors, resulting in a cohort with a good prognosis.[127] This is in contrast to the patients of paper IV who had larger and more malignant tumors, and a worse overall prognosis. It was not possible to extract any information from the biopsies excised before treatment for the locally advanced breast cancer patients, thus all prognostic information could be related to treatment responses. For the patients in paper III however, the metabolite profiles of untreated biopsies could be related to outcome. Moreover, the differences in metabolite profiles between hormone receptor positive and negative tumors that were seen in paper I were not present in the locally advanced breast cancer group (results not shown). These results demonstrate the large differences between patients with locally advanced breast cancer and those diagnosed with a lower stage breast cancer, and indicate that biopsies acquired at an earlier stage of the disease contain more prognostic information.

Predictions of survival in paper III were performed separately on subgroups of patients that were either ER negative or ER positive. The subgrouping was based on the results from paper I showing clear differences in the metabolism of ER positive and negative tumors. Subgrouping of the patients revealed metabolic differences of prognostic value

in ER positive patients that could not be seen when examining the whole data set. Neither could any metabolic differences be identified in the tumors of survivors and non-survivors lacking the expression of ER receptors. These results demonstrate the importance of not handling the data from all patients similarly. Especially in a heterogeneous disease such as breast cancer, there may be large differences among patients masking the smaller metabolic differences of clinical value. In addition, the biological mechanisms of the disease states may not be equal. As the ER status itself is a prognostic factor, the mechanisms for disease progression and aggressiveness in ER negative patients may be different from those of ER positives. Thus subgrouping of the data for analytical purposes was highly beneficial.

Metastasis to the lymph nodes via the lymphatic vessels is a common step in the spread of solid tumors, and lymph node status is acknowledged to be one of the most important prognostic factors of breast cancer.[128] In paper I, we could only see a trend of differences in the metabolite profiles of patients with and without lymphatic spread, and no reliable classification of lymph node status was achieved. Prediction of lymph node status from MR spectra has also been examined by Mountford et al., achieving a classification accuracy of 94%.[129] However, the authors used the same samples both for building and testing the classification model, and this may have led to overoptimistic results. The resulting model should be validated using an independent test set in order to assess the true predictive value of the model. The mechanisms that underlie the growth of lymphatic vessels (lymphangiogenesis) and lymphatic spread through new or pre-existing lymphatic vessels are not fully mapped. The growth factors vascular endothelial growth factor (VEGF)-C and VEGF-D have been shown to induce lymphangiogenesis through activation of the growth factor receptor VEGFR-3,[130-131] and high expression of these factors has been linked to poor survival rates in esophageal[132] and cervical[133] cancer. Thus the metabolic effects of the multistep mechanisms leading to lymphatic spread may be present before the spread is clinically detectable, making it difficult to correlate metabolite profiles with histopathological results. In a study by Cote et al., immunohistochemical examinations showed micrometastases in the lymph nodes of 20 % of 736 patients classified as lymph node negative by routine histology.[134] In a similar study, Kahn et al. detected

micrometastases in 14 % of 214 lymph node negative patients.[135] The prognostic value of micrometastases in axillary lymph nodes is controversial. Some studies show no prognostic value of detected micrometastases in node negative patients,[135-137] whereas others find micrometastases to be associated with a poorer prognosis.[134, 138] In a meta-analysis of 58 studies with a total of 297 533 patients, de Boer et al. found the presence of micrometastases of 2 mm or less in diameter to be associated with a poorer disease-free and overall survival.[139] The mechanisms giving rise to metastases of either micro or macro size may be similar, making it difficult to distinguish between lymph node positive patients and patients with clinically undetectable micrometastases classified as lymph node negative. In addition, the method for determining lymph node status was changed from ALND to SLNB in Norway in 2000, and our study includes both methods. In ALND all axillary lymph nodes are removed and one slice per node is examined for tumor cells, whereas the SLNB method removes only the sentinel node(s) and examines it by multiple sectional slides. The number of lymph nodes removed has been shown to affect the false-negative rates,[140] and the method used may therefore have affected the classification.

Predicting who will benefit from a given treatment at an early stage could reduce overtreatment and shorten the time between diagnosis and surgery. Monitoring a tumor's response to NAC treatment is however challenging. The *in vivo* concentration of tCho measured by MR imaging has been suggested as a marker for tumor response, however studies have shown varying results.[37, 141] In paper IV, we examined the correlations between tumor metabolite profiles and clinical response to NAC treatment in patients with locally advanced breast cancer. We found no metabolic differences in the pre-treatment biopsies of patients with a stable disease (< 50% reduction to ≤ 25% increase in tumor volume) and partial responders (≥ 50% but < 100% reduction in tumor volume). Interestingly, the two response groups had indistinguishable metabolic responses to the treatment, and the patients had a general decrease in tCho levels. This could be because also patients with a stable disease can have up to 50% reduction in tumor volume, and therefore have a biological response to the treatment. Further studies including also patients with a progressive disease (≥25% increase in tumor volume) are required to investigate the potential of HR MAS MRS in the assessment of clinical

response. However, we showed that the metabolic responses to NAC were associated with overall prognosis. This may be useful in a clinical setting as NAC may downstage the standard prognostic indications such as tumor size and lymphatic spread, making these factors less applicable for assessment of prognosis after NAC.

The oncometabolite 2HG was first described in glioblastomas in 2008, and has gained attention as a proposed solid link between mutations in metabolic genes and cancers.[142] Since the first discovery, 2HG and has been detected in both gliomas and leukemia. Preliminary examinations of spectra from our breast cancer biobank indicate that this metabolite is not present in breast cancer. However, further studies are necessary to investigate the potential role of 2HG in breast cancer and other cancers.

5.2 Preprocessing of MR spectra

As a plethora of methods for both preprocessing and modelling of biological data exist, it is not straightforward to select the methods that are most appropriate for the analysis of MR spectra. In this thesis, different methods for peak alignment, variable scaling and data modelling were examined. Of the five alignment algorithms examined in paper II, icoshift and fastpa are developed specifically for MR spectra, while COW, VPdtw and PTW were developed for other types of data. DTW was for instance initially developed for speech recognition, but is now frequently being used to align chromatograms. The results of paper II showed icoshift and COW to be the most appropriate methods for alignment of HR MAS MR spectra, demonstrating that the tools developed specifically for MR data not necessarily are the most optimal ones. Nevertheless, icoshift was remarkably faster than COW due to the FFT engine, making it more convenient for testing several parameters and possibly achieving a better alignment.

We used four numerical criteria in addition to visual inspection in order to evaluate the alignment results in paper II; correlation, simplicity value, peak factor, and classification results. There is no gold-standard for assessing alignment quality, but the abovementioned criteria cover the most widely used measures for optimization of alignment and final evaluations. The root mean square distance (RMS) is also being

used for optimization[72], and was tested as an additional criterion in paper II. However, the RMS results were superfluous as no additional information to that of the correlation was given. As expected, all alignment methods improved the mean correlation of the data when comparing with the unaligned data. In addition, PCA has been frequently used to assess alignment quality.[76, 83, 143] Aligning the peaks will increase the amount of variance explained by the PCA model and provide better clustering and less ambiguous loading profiles. The simplicity value is related to the amount of variation explained by PCA and will be larger when more variance is explained by the first PCs. As all of the data sets in our study represented two-class problems, the number of correctly classified samples by PLS-DA was used in addition as an overall measure of data modelling, since aligned peaks usually will be more optimal for multivariate analyses. Alignment did improve the classification results for the breast cancer and cervix cancer data sets. Although alignment improved both the correlation and the simplicity values for the colon cancer data, the classification results did not improve compared to the unaligned data. Nevertheless, the loading profiles were less distorted and the dispersive shape of the peaks was removed. This effect of peak alignment has also been shown by others,[144] and demonstrates that peak alignment greatly increases the interpretation of the resulting model. It is therefore reasonable to perform peak alignment prior to modelling even if it does not improve the classification results.

As not only the intensity but also the shape of the peaks reflects the concentration of a metabolite in an MR spectrum, it is important to preserve the shape as much as possible. This is in contrast to chromatographic data where the width of the peaks may be changed due to experimental settings.[78] Stretching and shrinking of the peaks may therefore be disadvantageous for MR spectra. The implemented peak factor measure in COW was effective for this purpose, and COW conserved the peak shapes much better than fastpa and PTW. Overall, the peak factor was a good numerical measure for final evaluation of the amount of change in peak shapes; however visual inspection of the final result was still highly necessary to detect potential artefacts in peak shapes. Icoshift that only shifts the segments without any stretching and shrinking, and VPdtw with a strict penalty function for shifting in peaks, were the best methods for conserving peak shapes.

We showed that the shifts of a spectrum may contain biological information. By using the warping functions as input for multivariate analysis, cervical cancer samples were discriminated from normal adjacent tissue with up to 90% correct classification. This was probably due to pH differences between cancerous and normal tissue reflecting hypoxic areas with a low pH in the tumor tissue. Although incorporating the warping functions did not improve the classification compared to using spectra only (possibly because the spectra already gave close to perfect classification results), the discovery of biological information in the shifts is an interesting aspect. The shift information may improve classifications for other biological data sets, and measuring biological samples without buffering might reveal biologically relevant differences that would be obscured otherwise.

The five alignment algorithms that were compared in paper II were all warping methods, and were chosen as they were considered 'state-of-the-art' or algorithms specific for MR data. However, several other alignments algorithms exist which may also be applicable. Peak alignment by fast Fourier transform (PAFFT[145-146]) by Wong et al. is claimed by the authors to perform alignment similarly to fastpa, but with the use of FFT cross-correlation instead of a beam search. However, PAFFT only aligns by shifting and does not stretch or shrink the spectra in any way. The FFT has the benefit of improved speed. In addition, the optimal shift is found by calculating the complete cross-validation for the whole spectrum instead of the correlation per segment. These modifications gave slightly better results for PAFFT compared to fastpa for alignment of the MS data tested in their paper. Additionally the authors describe a related alignment algorithm, recursive alignment by FFT (RAFFT), developed with the aim of eliminating the requirement of parameters. The minimal segment size is found automatically by recursive alignment from the full spectrum into progressively smaller segments until no further alignment is necessary. Another recursive alignment method is recursive segment-wise peak alignment (RSPA) by Veselkov et al., developed for the alignment of MR spectra.[143] The spectra are divided into segments consisting of multiple peaks (such as spin-coupled multiplets) that are aligned to a reference. The recursion starts by shifting the peaks in a segment as a whole and then progressing to smaller subsegments until the optimal alignment is achieved as measured by the

maximum FFT cross-correlation. The subsegments are linearly interpolated at the boundaries and the aligned segments are joined together. RSPA was shown to outperform COW and PAFFT for the alignment of urine MR spectra.

A different type of alignment methods are based on detection of peaks in the spectra prior to alignment. An example is peak alignment using reduced set mapping (PARS[147-148]). The method starts by detecting the peaks in a spectrum using one of several algorithms, followed by corrections of the baseline in order to conserve the area under the peaks. Finally, the peaks are sparsely represented as a vector of zeros except for the position of the peaks, and this reduced peak set is used for alignment. This has the advantage of greatly reducing the dimensions of the data. The drawback of such methods is that small or overlapping peaks may be difficult to detect, and the consequence may be misalignments or loss of information. Self-calibrated warping (SCW) is a recently proposed method combining peak detection and warping.[149-150] The algorithm consists of three steps; identification of peaks by detecting the sign changes of the signals derivate in a constrained manner, alignment of the chosen peaks to the peaks of a reference by maximizing the correlation, and finally defining a warping function for the whole spectrum by weighted least squares fitting of the warping values in the first two steps. A threshold for detection of peaks is defined that is based on the magnitude of the peaks. In that way, only the larger peaks will define the warping function and the algorithm will be less sensitive to small and dense peaks and noise. This may reduce possible misalignment in dense spectral areas without risking loss of information. SCW was compared to COW, PTW and RAFFT for alignment of MS spectra and found to perform equally or better.

5.3 Multivariate data analysis applied to MR spectra

Multivariate analysis methods are frequently applied when examining disease characteristics, as one biomarker on its own often is insufficiently specific for a given condition.[151] The advantage of multivariate methods is their ability to identify patterns of several metabolites simultaneously. In addition, multivariate methods can be applied to the whole MR spectra, requiring no quantification of the metabolites prior to analysis.

Linear modelling methods have the advantage that the important metabolites easily can be defined from the resulting model. For non-linear methods, interpretations may be challenging as most methods are so-called 'black box' methods, revealing no further insight into the dependence of classification on the variables. While the construction of such a model may be of interest for pure diagnostic purposes, most metabolomics studies will also aim for characterization of the disease for improved biological insight and targeted therapies. Achieving superior results using non-linear compared to linear methods may therefore be required in order to justify this disadvantage. In paper I, the classification performance of PLS-DA was compared to the non-linear methods BBN and PNN. PLS-DA provided better classification results for the prediction of ER and PgR status. This effect was observed both when using the whole spectra as input and when using variable reduced input. For lymphatic spread, PLS-DA gave a more balanced sensitivity and specificity ratio. Achieving an equal rate of sensitivity and specificity in discriminant analyses may be challenging if one group is much larger than the other, such as for predictions of ER status in paper I and survival in paper III. As an extreme, a high number of correctly classified samples may be achieved by simply classifying all samples to the largest group. This will however be reflected in the sensitivity/specificity ratio. The optimal classification threshold for PLS-DA was calculated using a Bayesian method,[152] where a normal distribution is fitted to the predicted values and the threshold corresponds to the intersection of the distributions. A new sample will be classified to the class for which the predicted value is greater than the threshold value. This appears to be a simple but efficient way of choosing a threshold that minimizes both the number of false positive and false negative samples. For the PNN analysis in Neuroshell Classifyer it is possible to define a customized fitness function that adds a penalty to the function whenever a sample from the smallest group was wrongly classified. Although this improved the results in paper I, the sensitivity of the smallest group was still much lower than the specificity for the test data. In general, both PNN and BBN gave almost perfect classification for the training data but not for the test data, indicating that the algorithms used were prone to overfitting. The same superior results for PLS-DA compared to PNN and BBN were observed for classification of survival in paper III, although only the results from PLS-DA were presented.

Paper IV explored the use of multivariate paired data analysis, taking advantage of the multilevel structure of the data. In order to examine the variation resulting from the given treatment, the multilevel analysis simply uses the net difference before and after treatment as input for classification. Discrimination of pre and post-treatment spectra by PLS-DA was performed using both paired spectra and the original data. Taking advantage of the multilevel structure was clearly beneficial; the average number of correctly classified samples increased from 69 % to 88 % when using paired analyses (Figure 5.1). The same beneficial effect was observed by Westerhuis et al. when comparing multilevel PLS-DA and orthogonal PLS-DA (OPLS-DA) for the discrimination of urine samples taken before and after black tea consumption.[104] The metabolic changes resulting from the treatment are probably more subtle than the larger variations between the patients, and splitting these sources of variation will therefore enhance the predictive strength of the classifier.

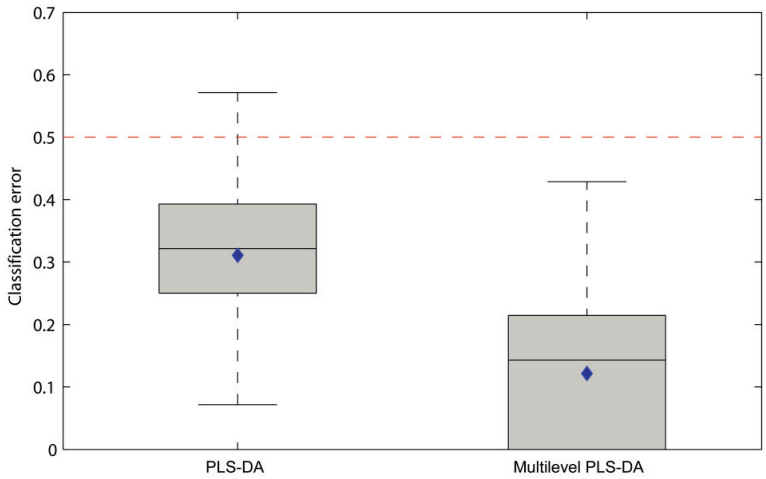

Figure 5.1: Boxplots showing the distributions of classification errors using unpaired PLS-DA and paired multilevel PLS-DA. Talking advantage of the multilevel structure of the data is clearly beneficial, and the error distributions are significantly different ($p < 0.001$)

The use of multivariate methods has a great advantage over univariate statistics requiring quantification of metabolites prior to analysis. The integrals of overlapping peaks from HR MAS MR spectra of tissue samples are most accurately measured by peak-fitting, which is a subjective and time-consuming process. Therefore integration of the metabolites spectral regions is often used, at the expense of less accurate results as some integrals contain signals from more than one metabolite. Quantification is also challenging due to the lack of a reliable internal reference. TSP has been used as an added internal reference in HR MAS MRS.[112, 118] The use of TSP is however not straightforward as it will bind to proteins in the tissue,[153] affecting the T2 relaxation times. Accurate quantification of HR MAS spectra will therefore require an external standard, such as standard curves or the electronic reference to access in-vivo concentrations (ERETIC[154]) method. Creatine has been used as a reference for relative quantification using metabolite ratios in brain tumors;[155] however the assumption of constant creatine levels in breast cancer tissue is probably not valid. For instance, the results from paper I indicated a correlation between the creatine levels of tumors and hormone receptor status. Thus relative quantification using metabolites ratios has the drawback that variations may be hidden if the metabolites are positively correlated. Integration of peak areas of spectra normalized to equal areas has been used as a measure of metabolite levels.[156-158] It is then assumed that the normalization corrects for differences in sample size. This method was used in paper III and IV for further validation of important metabolites from the multivariate analyses. To correct for differences in tumor cell content, the lipid residuals were removed prior to normalization. Although these integrated values may be inaccurate due to overlapping peaks and the lack of an internal reference, the combination of multivariate modelling and univariate testing of integrated intensities may give increased confidence in the importance of the metabolites.

Table 5.1 summarizes the preprocessing and modelling methods that have been found suitable for HR MAS MR spectra during the work of this thesis.

Table 5.1: Data analysis of HR MAS MR spectra.

Purpose	**Methods**
Baseline correction	• Giving the spectra equal minimum points will correct for baseline offsets. Artefacts such as negative spikes must first be removed. • Baseline correction by asymmetric least squares removes baseline trends, but should be used with caution as overfitting of the estimated baseline will remove spectral information.
Peak alignment	• The spectra should be shift referenced to one peak (for instance TSP) before applying peak alignment algorithms, as this will reduce the amount of alignment necessary. • Segmented alignment methods are most appropriate for aligning MR spectra, but segment boundaries inside peaks should be avoided. Icoshift is rapid and provides good alignment results of MR spectra. COW is a good alternative for crowded data or when the results from icoshift are not satisfying. Some effort should be put into optimizing the parameters and finding a good reference spectrum.
Removal of unimportant signals and contaminations	• Water residuals from incomplete water suppression should be removed from the spectra • The spectra should also be examined for contaminations, such as ethanol and acetone contaminations from cleaning of the lab equipment. • Lipid residuals may contain valuable information, but they may also disturb the analysis. Consider removing the lipid residuals prior to multivariate analysis.
Normalization	• Normalizing all spectra to equal area corrects for differences in sample weight. To correct for differences in lipid content in the samples, lipid residuals should be kept out when calculating the normalization parameters.

Scaling	• Supervised VAST scaling is suitable for upweighting possible biomarkers between groups. We used VAST-scaling without prior autoscaling in order to reduce baseline noise.
Variable selection	• Using the whole spectra provides the best interpretability of the resulting model. • mRMR variable selection is suitable for methods where the large dimensions of data are problematic.
Multivariate modeling	• PCA is useful for examining the data without forcing it into a model, and may help detect outlying samples. • PLS-DA is suitable for classification of MR spectra. • Multilevel PLS-DA is a good choice when the data has a paired structure.

6 Conclusions and future perspectives

In this thesis, differences in the metabolite profiles of tumors from breast cancer patients with different prognosis were identified. Hormone receptor positive tumors have different metabolic patterns than hormone receptor negative tumors, and the patients could be successfully discriminated according to their hormone receptor status. Differences in the tumor metabolism of patients with and without spread to axillary lymph nodes were also detected. These differences were however not prominent enough to successfully classify the patients according to their lymph node status. Further studies could possibly explain if the lymph node negative patients misclassified as false positives had micrometastases to the lymph nodes and thereby a tumor metabolism similar to that of lymph node positive patients. As an overall measure of prognosis, 5-year breast cancer survival was examined, and metabolic traits of good and poor prognosis were detected. Glycine and lactate levels were increased in non-survivors, and may serve as clinical biomarkers for poor prognosis. Tumor metabolic responses to NAC were explored, and different metabolic response patterns were related to breast cancer outcome. High levels of glycine and lactate after treatment were also here associated with a worse prognosis. These findings should be further validated in a larger patient cohort, and the clinical value of these potential biomarkers should be assessed.

We used breast cancer biopsies excised either during surgery or by open biopsy. Needle biopsies excised when the patient is diagnosed would probably provide the same metabolic information. Using today's histological methods, hormone receptor status is determined at the time of diagnosis, while an exact lymph node status can only be determined pathologically after lymph node removal during surgery. Being able to stratify the patients according to prognosis at an earlier stage could be beneficial for improved individualized treatment of breast cancer patients. A combination of histological examinations and MR metabolomics could provide additional prognostic information at an early stage. Although MR metabolomics is not yet used in the clinic, this thesis together with several other papers have shown promising prospects, and research groups at Imperial College, London, and Hautepierre hospital, Strasbourg,

have already implemented HR MAS MRS in the surgery theatre as a trial for rapid real-time analysis of excised tissue.[159] In addition to being cost efficient, this could more importantly reduce the amount of time that patients spend on the surgery table.

One of the great advantages of MR is the *in vivo- ex vivo* connection. MRS is being used together with MR imaging to examine metabolic patterns of tissues *in vivo*. Today's clinical MR scanners are primarily of field strength 1.5 T or 3 T, which is much lower than the 14.1 T scanner used for MR analyses in this thesis. It is therefore not possible to separate all the metabolites *in vivo*, and generally only one peak representing tCho levels is detected in addition to water and lipid signals in breast tumors. Nevertheless, future clinical MR scanners of higher field strengths, including technical improvements in coil design and pulse sequences, can enable the translation of previous *ex vivo* findings into *in vivo* clinical use.

The importance of proper preprocessing of MR spectra has been illustrated in this thesis, and different multivariate analysis methods have been assessed for their feasibility of analyzing MR spectra. Overall, the work in this thesis has shown promising results concerning MR metabolomics as a tool for evaluation of prognosis, and has provided an increased insight into the metabolic changes of progressing breast cancer. These findings can hopefully contribute to improved personalized medicine for breast cancer patients.

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Paper II

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Alignment of high resolution magic angle spinning magnetic resonance spectra using warping methods

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ABSTRACT

The peaks of magnetic resonance (MR) spectra can be shifted due to variations in physiological and experimental conditions, and correcting for misaligned peaks is an important part of data processing prior to multivariate analysis. In this paper, five warping algorithms (icoshift, COW, fastpa, VPdtw and PTW) are compared for their feasibility in aligning spectral peaks in three sets of high resolution magic angle spinning (HR-MAS) MR spectra with different degrees of misalignments, and their merits are discussed. In addition, extraction of information that might be present in the shifts is examined, both for simulated data and the real MR spectra. The generic evaluation methodology employs a number of frequently used quality criteria for evaluation of the alignments, together with PLS-DA to assess the influence of alignment on the classification outcome.

Peak alignment greatly improved the internal similarity of the data sets. Especially icoshift and COW seem suitable for aligning HR-MAS MR spectra, possibly because they perform alignment segment-wise. The choice of reference spectrum can influence the alignment result, and it is advisable to test several references. Information from the peak shifts was extracted, and in one case cancer samples were successfully discriminated from normal tissue based on shift information only. Based on these findings, general recommendations for alignment of HR-MAS MRS data are presented. Where possible, observations are generalized to other data types (e.g. chromatographic data).

1. Introduction

Nuclear magnetic resonance spectroscopy, or just magnetic resonance spectroscopy (MRS) in a medical context, is a highly reproducible and robust technique for examining the metabolic profiles of fluids or tissue specimens. By using high resolution magic angle spinning (HR-MAS) MRS, intact tissue samples can be analysed while peak broadening caused by anisotropic interactions is reduced [1]. The result is well-resolved spectra in which the metabolites are represented by sharp peaks. The peak positions in an MR spectrum may, however, be shifted, or misaligned, among spectra in a data set. In general, two types of misalignment are conceivable: non-systematic and systematic misalignments. Non-systematic misalignments can be caused by differences in temperature, intermolecular interactions and other variations due to imperfect control of experimental conditions [2–6], while systematic misalignments contain information about the biological origin of the sample. It has for instance been shown that tumour tissue has a lower pH than normal tissue, possibly due to the Warburg effect [7]. A different pH or ionic strength of samples influences the ionization state of basic or acidic groups and thus their associated chemical shifts [4,8,9]. Metabolite–protein interactions are another possible source of misalignment [10] which is especially important to consider when dealing with HR-MAS data of whole-tissue samples. In general, chemical interactions between substances and different background matrices might provide circumstantial evidence for differences between samples by systematic changes in chemical shifts [4,10,11].

Misalignments between corresponding peaks will affect multivariate analysis of the data. Therefore, it is generally recommended to correct for them [3,5,8,12]. Minor misalignment problems can be overcome by binning the data (typically using a bin width of 0.04 ppm), or by using more sophisticated peak alignment algorithms. An important disadvantage of binning is the loss of resolution and the resulting loss of interpretability [9]. For major misalignments, binning is not a feasible approach due to the resulting loss of resolution, and alignment would be preferable. Several different alignment methods exist. Amongst these, the so-called *warping* methods are most prominent [13–19], but other methods

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Table 1
Characteristics of alignment algorithms.

Alignment method	Optimization criterion	Optimization method	Alignment unit	Aligns by	Parameters to optimize
Icoshift	Correlation per segment	Cross-correlation by FFT	Segments	Shifting	2
COW	Total correlation	Dynamic programming	Segments	Stretching/shrinking	2
Fastpa	Correlation per segment	Beam search	Segments	Shifting and stretching/shrinking	3
VPdtw	L_1 norm	Dynamic Programming	Points	Shifting	2
PTW	Weighted cross-correlation	Nelder–Mead[a] simplex [46]	Complete spectrum	Polynomial model	2

[a] Default, different optimization algorithms are available.

have been described as well [6,20–22]. Most algorithms have their roots in chromatography, but some were specifically developed for MRS. It is not clear, however, which algorithm is the best choice for aligning HR-MAS MRS data and whether the methods originating in chromatography are indeed less suited for this type of data.

In this study, we investigated the suitability of five different warping algorithms—icoshift [13], COW [14,15], fastpa [16], VPdtw [17], and PTW [18]—for aligning HR-MAS MRS data. Of these, icoshift and fastpa were developed specifically for MRS data. The performances of COW and PTW have previously been compared for chromatographic data [23] and capillary electrophoresis (CE) data [24]. The performance of VPdtw for chromatographic data was briefly compared with that of PTW in the original VPdtw paper [17], and the original icoshift [13] paper discusses comparisons with COW and a number of fastpa-related methods for MRS data. We used three different cancer-related HR-MAS MRS data sets for evaluation, all containing samples from two distinct classes. All three data sets represent complex biological samples with varying degrees of misalignments. The various algorithms are compared, and their pros and cons will be discussed in this paper. Because there is no gold standard for assessing alignment quality, the alignments were evaluated with a number of commonly used criteria describing the similarity of the spectra and quantifying their change due to alignment. In addition, the data were classified using partial least squares discriminant analysis (PLS-DA) [25,26] to investigate the effect of alignment on the classification outcome. Although we limited our evaluation to MRS data, the presented evaluation methodology is generic and equally valid for other types of data.

Apart from the warping algorithm, the spectrum to use as the reference for aligning might influence the end result. Therefore, in all evaluations a number of different references were considered and their influence will be discussed.

A possible drawback of correcting for misalignments is that any information that might be present as systematic misalignments in the chemical shifts is lost from the spectra. In that case, correction via alignment or binning might be counterproductive. At the same time, it may be possible to align the spectra while extracting potential shift information from the warping path that describes the transformation from unaligned into aligned spectra. This possibility will be discussed in this article.

To evaluate the effect of alignment on data that display systematic shifts, a number of simple data sets were simulated in which class information was added as intensity differences, shift differences or a combination of the two. These data were aligned, and classification was performed on both the raw and aligned data, as well as on the shift information (i.e. the coefficients resulting from the alignment procedures) and to a combination of these with the aligned spectra. The insights from this procedure were subsequently used in an attempt to enhance the classification results for the real data.

Based on the results from this study, general recommendations for choosing an alignment method for HR-MAS MRS data and getting the optimal alignment are described. The validity of these results and recommendations for other types of data will be discussed.

2. Experimental

2.1. Description of the alignment algorithms

Five different alignment methods were used in this study, and will be elaborated here. Characteristics of the different alignment algorithms are summarized in Table 1. Fig. 1 shows typical warping paths, or warping *functions*, (the new *x*-axes as a function of the old *x*-axis) for all five methods. For clarity, the differences between the new *x*-axes and the old x-axis are drawn, rather than just the new *x*-axes.

2.1.1. Interval correlated shifting (icoshift)

Interval correlated shifting was developed specifically for MRS data [13]. It divides spectra into segments, and aligns these to the corresponding segments of a reference spectrum. The alignment is performed by shifting the segments sideways so as to maximize their correlation. In practice, this involves calculating the cross-correlation between the segments by a fast Fourier transform (FFT) engine that aligns all spectra of a data set simultaneously. The segments can be user-defined or of constant length. Missing parts on the segment edges are either filled with 'missing values', or by repeating the value of the boundary point. The maximum shift correction of the segments can either be equal to a constant defined by the user, or the algorithm can search for the best value for each segment [13]. Icoshift is available as a tool for Matlab from Ref. [27].

2.1.2. Correlation optimized warping (COW)

Correlation optimized warping [14,15] is another segmented warping method. It aims to optimize the overall correlation between two spectra. The spectra are aligned by shrinking or stretching the segments, rather than by shifting them as in icoshift.

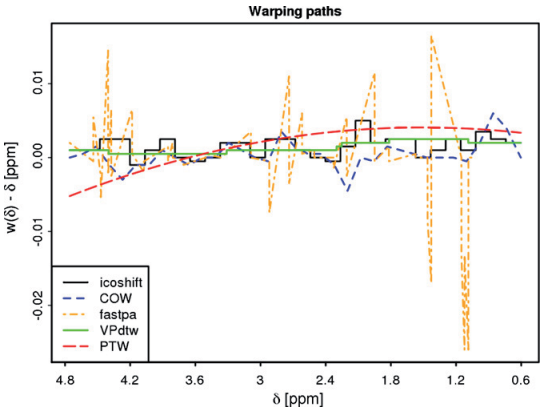

Fig. 1. A comparison of the warping paths of the different alignment methods. Warping paths depicting the fine structures of the different alignment methods are shown. The same query and reference spectra were used for all methods. For clarity, the *y*-scale is set to the difference between the warping paths proper and the original *x*-axis.

Another difference with icoshift is that the optimization takes all segments into account instead of aligning each segment separately, i.e. stretching a segment causes subsequent segments to shift. The maximum allowed change in segment length is determined by the so-called slack parameter defined by the user. In addition, the user must specify the segment length.

The alignment is performed using a dynamic programming algorithm [28]. The algorithm uses linear interpolation to create stretched (or shrunken) versions of the individual segments within the limits determined by the slack parameter. It calculates the sums of the individual correlation coefficients for all combinations of the stretched segments and picks the combination that leads to the largest sum (and hence the largest overall correlation) to construct the aligned spectrum. As all possible combinations are considered, dynamic programming will always yield the global optimum for the chosen parameters [14,15].

The slack and segment length parameters of COW can be optimized by a discrete simplex-like optimization routine described by Skov et al. [29]. An optimization space for both parameters must be specified, and the initial search is defined by a 5×5 grid in both parameter directions. Each of the 25 parameter combinations is evaluated by calculating the sum of the simplicity value and average peak factor (see Section 2.5) of the corresponding trial alignment of the data set. By default, the three best combinations are used as starting points for further simplex optimization. COW is available as a tool for Matlab from Ref. [27].

2.1.3. Peak alignment by beam search (fastpa)

Like icoshift, peak alignment by beam search was developed for MRS data [16]. It also divides the spectra into segments, but aligns these by both shifting and stretching/shrinking them to maximize their respective correlations. Fastpa is based on a routine by Forshed et al. [30] where the segments are chosen automatically to avoid cutting in a peak. However, instead of Forshed's genetic algorithm, fastpa uses a faster beam search [31,32] as the optimization routine for finding the optimal alignment. This change of optimization algorithm is possible because the segments are aligned independently, as opposed to COW.

Fastpa requires three input parameters to be specified: the maximum number of segments, the maximum range of shifting, and the maximum range of stretching or shrinking. In addition, the beam width k [31,32] has to be specified as either 1 or 2. From the viewpoint of optimization, a larger beam width is always preferable [32], and we considered k to be constant at a value of 2.

After choosing segments [30], the algorithm starts by adapting an initial trial solution of stretches and shifts for the individual segments. The 2 best adaptations are used as the next trial solutions in the algorithm. This is repeated until the optimal solution within the beam search space is found [16]. Fastpa is available as a Matlab tool upon request from the authors [16].

2.1.4. Variable penalty dynamic time warping (VPdtw)

Dynamic time warping (DTW) [33] is generally considered to be the first full-fledged warping method that has been developed. It works by shifting individual points of the query spectrum, rather than complete segments, as in icoshift. Many different sets of rules exist for allowed shifts [33,34]. Variable penalty DTW is a recent implementation of asymmetric DTW [17]. Instead of optimizing the correlation between the spectra, VPdtw tries to optimize the L_1 norm, i.e. the sum of the absolute differences between the variables in the spectra.

Regular DTW is notorious for causing artifacts in aligned data, by allowing too many shifts [17,35]. The variable penalty in VPdtw aims to prevent these from occurring by adding a penalty to the L_1 norm for each shift. Clifford and Stone [17] propose to use a morphological dilation (i.e. a running maximum) of the reference spectrum as a penalty. This results in a high penalty being added to the L_1 norm for a shift at or near the position of peaks, whereas in a baseline region, it would result in almost no extra increase. A maximum allowed shift and the penalty must be specified by the user [17]. VPdtw is available as a package in R from Ref. [36].

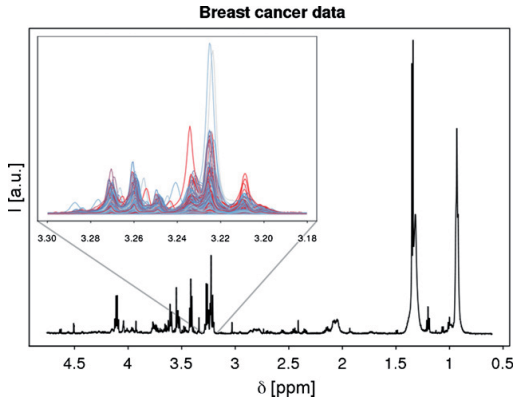

Fig. 2. Breast cancer data. A representative HR-MAS MR spectrum from the breast cancer data set. The inset shows the misalignment for the peaks between 3.18 and 3.30 ppm. a.u., arbitrary units.

2.1.5. Parametric time warping (PTW)

Rather than point-wise shifting, or dividing the spectra into segments that can subsequently be shifted and/or stretched, PTW [18] explicitly produces a global polynomial model (the warping function) of the misalignment:

$$w(t) = \sum_{k=0}^{K} a_k t^k$$

The first two coefficients, a_0 and a_1, in the warping function can readily be interpreted as an overall shift and stretch/shrinkage, respectively. Further coefficients correspond to higher order stretching or shrinking that are useful to model changes in the misalignment along the retention time axis. Bloemberg et al. recently proposed to use the weighted cross-correlation (WCC) [37] as the optimization criterion in PTW [19]. In their implementation, the user has to specify the order of the warping function and the width of the triangular weighting function for the WCC.

The continuity and smoothness of the PTW warping function imply that PTW does not lead to artifacts like 'decapitated' peaks. In the absence of many high-order terms, the polynomial model makes PTW a somewhat restrained method. This means that it may have difficulties in correcting strongly nonlinear misalignments, but also that overfitting is very unlikely to occur [18,19]. PTW is available as a package in R from Ref. [38].

2.2. Data

Three different cancer-related data sets were used in this study: data from cervix, breast, and colon tissue. All three data sets contain data from two biologically distinct classes of tissue. The data sets represent different degrees of misalignment: the cervical cancer data display minor misalignments, colon cancer has major misalignments and the breast cancer data show something in between. Fig. 2 shows the MR spectra from the breast cancer set as an example. In addition to these HR-MAS MRS data sets, simulated data sets with varying degrees of misalignment were generated.

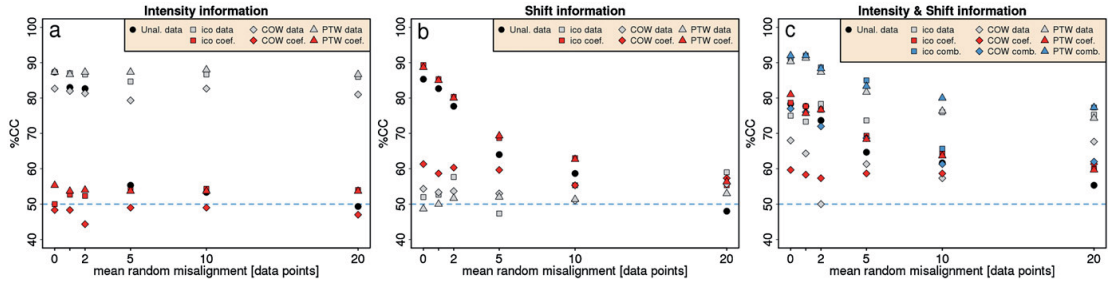

Fig. 3. Classification results of simulated data. The results are the averages of three replicates. (a) Class information is contained in the intensities; (b) class information is contained in the peak shifts; (c) class information is contained in both intensities and shifts. Abbreviations: Unal.: unaligned; coef.: coefficients; comb.: combined; %CC: percentage of correctly classified samples.

2.2.1. Simulated data

A large number of simple data sets were simulated. Each set consists of 100 spectra—50 of class 1 and 50 of class 2—with a spectral width of 1000 variables and two peaks only of width ~10 points. Bivariate class information was added as intensity differences (mean differences ranging from 1% to 10% of peak height), shift differences (means ranging from 1 to 25 points) or a combination of the two. For each set, six copies were produced with increasing non-systematic shifts (see Fig. 3), but exactly similar simulation parameters otherwise. Furthermore, all simulations were performed in triplicate; all reported results are the averages of the results obtained on three sets with similar settings for the randomly generated normal distributions that were used to generate realistic intensity and shift distributions.

2.2.2. Cervical cancer data

This data set is fully described in Ref. [39]. In short, cervical tissue samples ($n = 16$) were collected after hysterectomy of cervical cancer patients ($n = 8$) and patients with non-malignant disease ($n = 8$). The samples were analysed by HR-MAS MRS on a Bruker Avance DRX600, using a water and lipid suppressing spin-echo sequence (cpmgpr, Bruker BioSpin GmbH, Germany). All experiments were performed at room temperature and without buffering. The chemical shifts were referenced to the lactate doublet at 1.32 ppm, and the spectral region between 4.7 and 0.5 ppm was saved in a matrix of 16×3736 variables. The spectra were baseline corrected using asymmetric least squares [18] with parameters $\lambda = 1e5$ and $p = 0.0001$, and the minimum value of each spectrum was set to zero by subtracting the lowest value. The spectra were normalized to equal total area.

2.2.3. Breast cancer data

This data set is fully described in Ref. [40]. Breast cancer tissue samples ($n = 208$) were excised from estrogen receptor (ER) positive ($n = 161$) and negative ($n = 47$) patients. The samples were analysed by HR-MAS MRS using a cpmgpr sequence. All experiments were performed at 4 °C, and the samples were buffered with phosphate-buffered saline (PBS). Chemical shifts were referenced to the TSP peak at 0 ppm. The spectral region between 4.8 and 0.6 ppm, represented by 8251 variables, was extracted for further analyses. The spectra were baseline corrected by subtracting the lowest value of each spectrum, and normalized to equal total area.

2.2.4. Colon cancer data

Colon tissue samples ($n = 32$) were excised from the tumour area ($n = 17$) and normal mucosa ($n = 15$) of colon cancer patients, and the samples were analysed by HR-MAS MRS using a cpmgpr sequence. These samples are part of a larger patient cohort described in Ref. [41]. All experiments were performed at 4 °C, and the samples were buffered with phosphate-buffered saline. In order to induce random misalignments in the data, this data set was not chemical shift referenced. The spectral region between 4.8 and 0 ppm, represented by 9661 variables, was extracted for further analyses. The spectra were baseline corrected by subtracting the lowest value of each spectrum, and normalized to equal total area (excluding polyethylene glycol pollution at 3.71 ppm).

2.3. Alignment of simulated data

All simulated data sets were aligned using the five warping methods and alignment was performed using the first spectrum as the reference. Icoshift was set to align the data in two segments, whereas PTW was set to align using a linear warping function, corresponding to an overall shift and stretch. Unexpectedly, COW ran into memory problems when the segment length was chosen to be half the spectral width (500 points), and the segment length was set to one tenth of the spectral width (100 points). VPdtw was unable to produce well-aligned data consistently and the fastpa algorithm was too unstable in its current form to allow high-throughput analysis of a large number of data sets.

The obtained warping coefficients correspond to two (integer) shifts for icoshift (one shift coefficient per segment), a shift and a stretch coefficient for PTW and ten segment endpoints for COW. For classification purposes, the icoshift coefficients were used 'as is', whereas the stretch coefficient for PTW was multiplied by the number of data points (1000) after subtracting 1 (the default for 'no alignment') from it, as described in Ref. [19]. In this way, the shift and stretch coefficients are on comparable scales. For COW, the original segment endpoints were subtracted from the new endpoints, so as to provide the differences between them.

2.4. Alignment of real data

The cervix, colon, and breast cancer data sets were aligned using the five different warping methods, as described below. Ten different reference spectra were used subsequently for aligning the data; this in order to examine the influence of choosing different references and also to examine the robustness of the warping methods. Four spectra were chosen from each of the two classes in a data set: two randomly chosen ones and the two spectra having the highest average correlation with the other spectra in the data set. In addition, the mean and the median spectra were used as references.

2.4.1. Icoshift

The optimal number of segments for icoshift was determined by visual inspection of trial alignments and by the average overall correlation, and ranged from 20 to 150 for the different data sets and references. The maximum allowed shifts were determined by the

algorithm. For some matrices this led to artifacts, and the maximum allowed shift was manually determined instead. A full spectrum correction was performed prior to alignment of the segments. Missing parts on the segment edges were replaced by repeating the value of the boundary point. Both user-defined segments and segments of constant length were tested.

2.4.2. COW

Parameters for COW were determined using the optimization routine by Skov et al. [29]. The search interval for segment length was based on the average peak width, as suggested by the authors, and the slack size search space ranged from 1 to 15. The search space was increased if the limit values were chosen as the optimal parameter. Optimal segment length ranged from 30 to 300 variables.

2.4.3. Fastpa

Fastpa parameters were optimized by visual inspection of trial alignments, and the overall average correlation. The spectra were zero-padded prior to alignment to avoid cutting off peaks at the spectrum edges. This was also done because fastpa does not align the last segment of the spectra. The ranges of segment number, sideways movement and interpolation were 40–170, 10–150 and 10–100, respectively.

2.4.4. VPdtw

Alignments were performed on the normalized data, after additional square-root scaling, as this turned out to provide better alignment results than for unscaled data. The resulting warping paths were applied to the normalized data. The penalties that were used for the alignment were morphological dilations of the data, as described in Ref. [17]. Penalties were determined by trial and error until satisfactory alignment results were produced. The maximum allowed shift (the width of the Sakoe-Chiba band [33]) ranged from 100 to 300 variables.

2.4.5. PTW

Prior to alignment, the data were zero-padded, as described in Ref. [19]. The resulting warping coefficients were transformed accordingly and applied to the unpadded data. Triangle widths for the weighted cross-correlation measure were on the order of the largest misalignment in the data, as determined by visual inspection and ranged from 2 to 100 variables.

2.5. Evaluation criteria

The alignment results were assessed based on different measures:

2.5.1. Correlation

The spectra of a data set will be more uniform after successful alignment, and thereby have a higher correlation. The correlation between all the spectra of a data set was calculated before and after alignment.

2.5.2. Simplicity value

The simplicity value is related to principal component analysis (PCA) by singular value decomposition (SVD) of a matrix, where the singular values state how much variance is explained by each component. Aligned spectra will have more variance explained by the first components. The simplicity value of a matrix is defined as the sum of all singular values of the matrix—scaled to a total sum of squares of one—taken to the fourth power, and will be larger when more variation is explained by the first components [29].

$$\text{Simplicity} = \sum \left(\text{SVD} \left(\frac{X}{\sqrt{\sum_i \sum_j x_{ij}^2}} \right) \right)^4$$

2.5.3. Peak factor

The peak factor gives an estimate of how much the area and the shape of the peaks have changed in a spectrum after alignment. It compares the Euclidian length, or norm, of a spectrum before and after alignment. If the peak area and shape stay almost the same, the difference between the norms before and after alignment will be small [29]. The optimal value for the peak factor is 1, meaning that there is no change in peak shape.

$$\text{Peak factor} = \frac{\sum_{i=1}^{I} (1 - \min(c_i, 1)^2)}{I}$$

where

$$c_i = \left| \frac{\text{norm}(x_{i,\text{after}}) - \text{norm}(x_{i,\text{before}})}{\text{norm}(x_{i,\text{before}})} \right|$$

2.5.4. Classification

Correcting for misalignments should improve the classification results for data sets distorted by random shifts. However, it is also possible that information arising from biological differences between different classes may be removed when the spectra are aligned. In PLS, latent variables (LVs) are derived to maximize the covariance between the spectra and a quantity to be modelled. PLS-DA is a special case of PLS that attempts to discriminate between classes, represented by discrete numbers. Here, PLS-DA was used to evaluate the classifiability of aligned and unaligned data. In addition, the warping path or warping parameters were used as input to investigate possible shift information.

2.5.5. Visual inspection

Quantitative measures are valuable means for comparing specific characteristics of large sets of data at a glance, but they also have their limits. The human eye and brain are still unsurpassed as a pattern recognition tool. In the context of alignment, especially the assessment of alignment quality and detection of artifacts benefit from visual inspection.

2.6. Classification of simulated sets

Each data set was classified using PLS-DA. Classification was performed on the unaligned spectra, the aligned spectra, the warping coefficients and a combination of the aligned spectra and the coefficients. The latter was achieved by simply concatenating the spectra with the coefficients and multiplying the latter with a large number (on the scale of the average-scaled spectra, typically 100 was used) to make sure they would be contained in the first latent variables of the PLS model.

PLS-DA, including mean centering, was performed using full leave-one-out cross-validation (LOO-CV), and the number of LVs giving the first minimum in prediction error was chosen for the model. PLS-DA was performed in Matlab 7.7.0.471 (R2008b, The Mathworks, Inc., Natick, USA) using PLS_toolbox 5.5.1 (Eigenvector Research, Wenatchee, USA).

2.7. Classification of real data

The data sets were classified using PLS-DA, as described for the simulated data. Classification was performed on the unaligned spectra, the aligned spectra, the warping coefficients, and a combination of aligned spectra and coefficients (multiplied by 100).

Pollutions from ethanol and fatty residuals, and from polyethylene glycol for the colon cancer data, were removed from the spectra prior to classification. Single outlying spectra were removed from the colon cancer and breast cancer data sets. For the breast cancer data, the spectra were square-root scaled prior to analysis.

3. Results and discussion

3.1. Simulated data

Fig. 3a shows the average classification results for the sets in which classes are coded as intensity differences. Fastpa and VPdtw were not capable of correctly aligning the simulated data. As expected, classification rates for unaligned data decreased as random misalignment increased. Aligned data, on the other hand, delivered stable classification results. The warping coefficients did not contain class information, as expected, since the simulated shifts were completely random.

The average classification results for three data sets where class information is purely contained as peak shifts are depicted in Fig. 3b. As expected, the situation here was completely opposite to the previous one. The raw data gave better results than the aligned data; alignment removes the information of interest from the spectra. Now, the coefficients *do* contain information and in this case the coefficients give even better classification results than the raw data themselves. This is most likely due to less noise being present in the coefficients than in the data. Because classification is based on intensities, the intensity noise in the unaligned data will have a negative influence on the result. By extracting the positional information of the peaks into the warping coefficients, it effectively becomes available as information without the intensity noise that was present in the spectra.

When class information is present in both the shifts and the intensities, as might be expected with real data, the situation will be somewhere in between the two extremes discussed above. Where exactly depends on the data at hand. In that respect, any simulation is rather arbitrary and we should not over-interpret the results. It is clear from the example in Fig. 3c however, that—if discrimination is the main interest—there are situations in which aligned data on their own can be a sub-optimal choice as input for multivariate analyses when there is information present in the shifts. In these cases, for icoshift and PTW, the combination of aligned data with the warping coefficients delivered the best classification results. The COW results for the combination of data and coefficients were a lot worse than those of the other two methods; this may have to do with the sub-optimal parameter settings that were used to prevent the program from running into memory problems. Furthermore, it is likely that the good results for PTW are due to the simplicity of the data and the resulting suitability of a warping function of degree 1 for modelling the misalignments. Bearing in mind the conclusions from Refs. [23,24], it is to be expected that individual shifts in more complex MR data cannot be modelled very well with the global PTW model. Icoshift and COW are more likely to extract positional information in a way that is suited for multivariate analyses, although the cumulative character of the COW warping path might 'smear out' misalignment information over several segments, making it harder to interpret.

3.2. Real data

3.2.1. Correlation and simplicity value

A plethora of similarity and distance measures are used as optimization criteria in different alignment algorithms. Icoshift, COW and fastpa are all optimized using correlation as a criterion. DTW is available with various distance measures [34] and VPdtw employs the L_1 norm as a criterion for optimization. PTW optimization was originally based on the root mean square difference (RMS) between spectra [18], but the current implementation uses the weighted cross-correlation as a similarity measure [19]. There is still no generally accepted gold standard measure for assessing alignment quality. However, the combination of simplicity value and peak factor introduced by Skov et al. [29] is an interesting choice. Both measures, together with the correlation, were used to assess alignment quality in this study. In addition, the RMS and WCC criteria were examined, but these measures did not provide extra information. It should be kept in mind that methods optimizing the correlation will very likely be biased towards that measure and it is not certain that the results for the simplicity value will be completely independent.

Fig. 4 shows box plots of the mutual correlations between all samples in the three HR-MAS data sets, before alignment and after alignment with each of the five warping methods for ten different references. It is clear that for all methods, the correlation distributions of the aligned data are better than for the unaligned data. This is especially pronounced for the colon cancer data set which has the largest misalignments. Here, all warping methods greatly improved the correlations, with PTW scoring lower than the other methods. For the cervical cancer data, where the unaligned data displayed only minor misalignments, VPdtw resulted in the lowest correlation values, while the other methods performed comparable.

The simplicity values for the raw and aligned data in Fig. 4 convey the same general picture as the correlations. There are some differences, however. The most striking ones are the PTW and VPdtw results for the colon cancer data set. When looking at the correlations, the PTW correlations are clearly lower than the ones for icoshift, COW, and fastpa, and comparable to the VPdtw correlations. The PTW simplicity values, however, are comparable to those of icoshift, COW, and fastpa, whereas the VPdtw simplicity values are much lower. The simplicity value is influenced by the intensities of the peaks, and peaks with high intensities influence the value more than low intensity peaks. The colon cancer data displayed a high intensity peak at 3.71 ppm, resulting from polyethylene glycol. When the peak was removed from the data set, the simplicity values were more in accordance with the correlations. This example shows a weakness of the simplicity value, and it might be advisable to scale the data prior to simplicity calculations.

Fig. 4 also shows that the choice of reference can have a large influence on the alignment result for one-dimensional HR-MAS MR spectra, contrary to the observation made in Ref. [21] for LC–MS data. The breast cancer data generally provided stable results, but demonstrated that a bad choice of reference had a larger influence on the correlations than the particular warping method that is used. Icoshift, COW and fastpa, which are all segmented warping methods, appeared to be less influenced by the choice of reference, whereas PTW gave worse results than the unaligned data for some of the randomly chosen reference spectra. It is therefore advisable to try different references when aligning. Using the spectrum that has the highest average correlation to the other spectra does not seem to be a bad choice; however, it does not always give the optimal alignment. For data sets consisting of two or more classes, it is conceivable that the alignment will be affected by the class the reference belongs to. Trying references from both classes may therefore be advisable. Using the mean or median spectrum as a

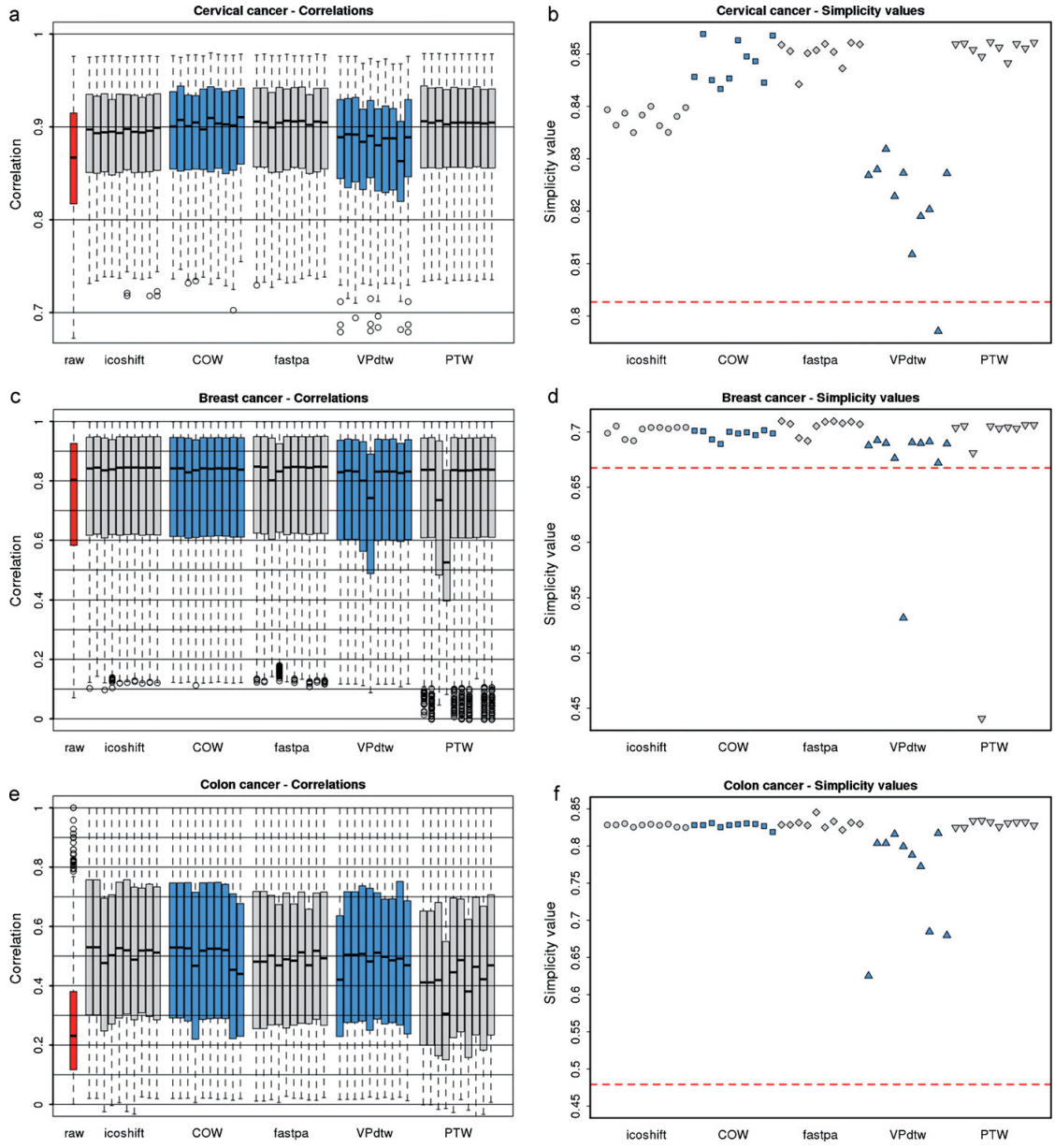

Fig. 4. Correlation and simplicity values for different alignment methods. In all plots, for each method, results from 10 different reference spectra are shown. From left to right: the spectrum having the highest average correlation with all other spectra, the (on average) second most highly correlated spectrum from the same class, and two random spectra from the same class; the most highly correlated spectrum, the second most highly correlated spectrum, and two random spectra from the other class, and the overall mean and median spectra. The box plots show the distributions of correlation values for the data sets; the red box stands for the unaligned data. The scatter plots show the simplicity values of the data sets; the red line depicts the simplicity value of the unaligned data. (a) Correlations of the cervical cancer data; (b) simplicity values of the cervical cancer data; (c) correlations of the breast cancer data; (d) simplicity values of the breast cancer data; (e) correlations of the colon cancer data; (f) simplicity values of the colon cancer data.

reference is also an option. This may not be a good choice for data sets with big misalignments though, as the mean/median spectrum will have broad peaks and may not resemble a real spectrum. This can be overcome by using an iterative procedure, i.e. by aligning the data and then recalculating the reference spectrum. This was tested for the colon cancer data in this study, and the resulting alignments then resembled those for the other references (results not shown).

In some cases, similarity measures can give the wrong impression of the alignment quality. An example of this is when peaks are badly deformed in order to give a high correlation, as in unpenal-

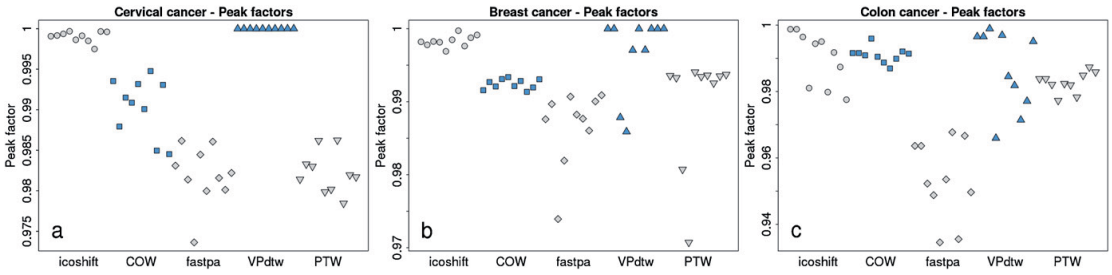

Fig. 5. Peak factors for different alignment methods. Results from the same 10 different references as in Fig. 4 are shown (a) peak factors for the cervical cancer data; (b) peak factors for the breast cancer data; (c) peak factors for the colon cancer data.

ized DTW [17,35]. Other examples were encountered when using icoshift: when using segments of constant length, the segment edges would sometimes be located in a peak, leading to major artifacts in the peak shapes, while the correlation of the spectra remained high. For some parameter choices for icoshift, the peaks were displaced to the wrong position, but again correlations remained high. Therefore, visual inspection of the data after alignment remains of the utmost importance. This will however put a limit to the complexity of the data of interest. While HR-MAS spectra of tissues have quite well-defined peaks, MR spectra from fluids may be more crowded, making visual inspection of the aligned data challenging. For these data, other methods may be more suitable, for instance the one described by Alm et al. [6].

3.2.2. Peak factor

Peak factor calculations for the different warping methods are shown in Fig. 5. The differences between the methods are small, and overall, all methods performed well. Icoshift and VPdtw gave the highest peak factors. This result was as expected for icoshift, as it only shifts segments of the spectra, as opposed to fastpa, COW and PTW that do shrinking and stretching. For VPdtw, the high peak factors are noteworthy, given that DTW has a history of strongly deforming peaks. Clearly, the variable penalty of VPdtw does what it is intended to do.

COW's parameters are optimized based on peak factor, and COW gave the best results of the warping methods that do shrinking and shifting. On average, fastpa had the lowest peak factor values, and was thus the method that changed the data most after alignment. This is also obvious from the warping functions in Fig. 1; fastpa typically had the most extreme warping path. As for correlation and simplicity value, icoshift and COW provided stable results for different reference spectra. The results for fastpa and PTW varied more, and it appears that the choice of reference is more critical for these methods.

3.2.3. Classification results

The classification results for unaligned and aligned data are shown in Fig. 6. For the cervical cancer data, the unaligned data already gave good results, with only one out of 16 samples misclassified. In general, the aligned cervical cancer data gave better classification results for all methods. For the breast cancer data, the average classification results, based on the 10 different references, improved for all methods except PTW. Here, the use of some reference spectra improved the classification results while others gave worse results. Despite the fact that both the correlation and the simplicity value of the colon cancer data greatly improved by aligning, the classification results did not improve. After alignment the average classification results decreased by one or two additional samples. It is not unlikely that the results for the unaligned data are better simply by chance. In theory, it is also possible that peaks have been aligned to the wrong peaks of the reference spectrum. This is unlikely however, as visual inspection of the alignment results gave no indication of wrong alignments.

Another possibility is that the shifts of the colon cancer data contained information, resulting from systematic misalignments of the spectra. Information present in the shifts would get lost after alignment. This was investigated for all the data sets by classification of the warping parameters and a combination of spectra and parameters. It should be noted that the prediction error from cross-validated PLS-DA was based on the same data set as the one used for choosing the optimal number of LVs. Thus, the prediction error will be slightly biased towards values lower than 0.5, and only results that differed strongly from 0.5 were considered important. Classification of the warping parameters alone did not give reliable predictions for the breast cancer and the colon cancer data. Furthermore, combining the warping parameters with the spectra did not improve classification. As previously described, cancer tissue can have a different pH than normal tissue, and the pH of a sample is an important source of shift variation. For the breast cancer samples, there are no established hypotheses for pH differences between ER positive and negative samples, and the results were as expected. For colon cancer, the samples in the data set were from either normal or cancer tissue. Therefore, differences in pH are more likely, even though the samples were buffered prior to HR-MAS analysis [42]. However, it is likely that shift information that might have been present in the data was masked by the major random shifts of the data set, similar to what is shown in Fig. 3b and c for the simulated data.

For the cervical cancer data, the results clearly indicate that the warping parameters of icoshift, COW and fastpa contain class information. Classification of the parameters gave an average correct classification of 87%, 84% and 76% for icoshift, COW and fastpa, respectively (Fig. 6d). Combining the spectra with the parameters was not beneficial for the overall classification. Thus, the information from the parameters was redundant. Nevertheless, the fact that shift information alone can discriminate between normal cervical tissue and cancerous tissue is very interesting. The cervical samples were analysed by HR-MAS without buffering, and therefore pH differences related to cancer-induced changes in tissue may be more pronounced here than for the colon cancer samples. So despite the redundancy of the shift information for the cervical data, this result indicates that measuring biological samples without buffering might reveal biologically relevant differences that would be obscured otherwise.

It is not hard to see why icoshift and fastpa provided warping coefficients that are suitable for subsequent multivariate analysis. These MRS-oriented methods align their segments independently; therefore corresponding shifts will always occur at the same position in the spectra. Opposed to that, for VPdtw, the effect of warping is cumulative, and the actual stretching occurs at slightly different places for different spectra even if they have similar misalignments (results not shown). The alignment of COW is also cumulative, but

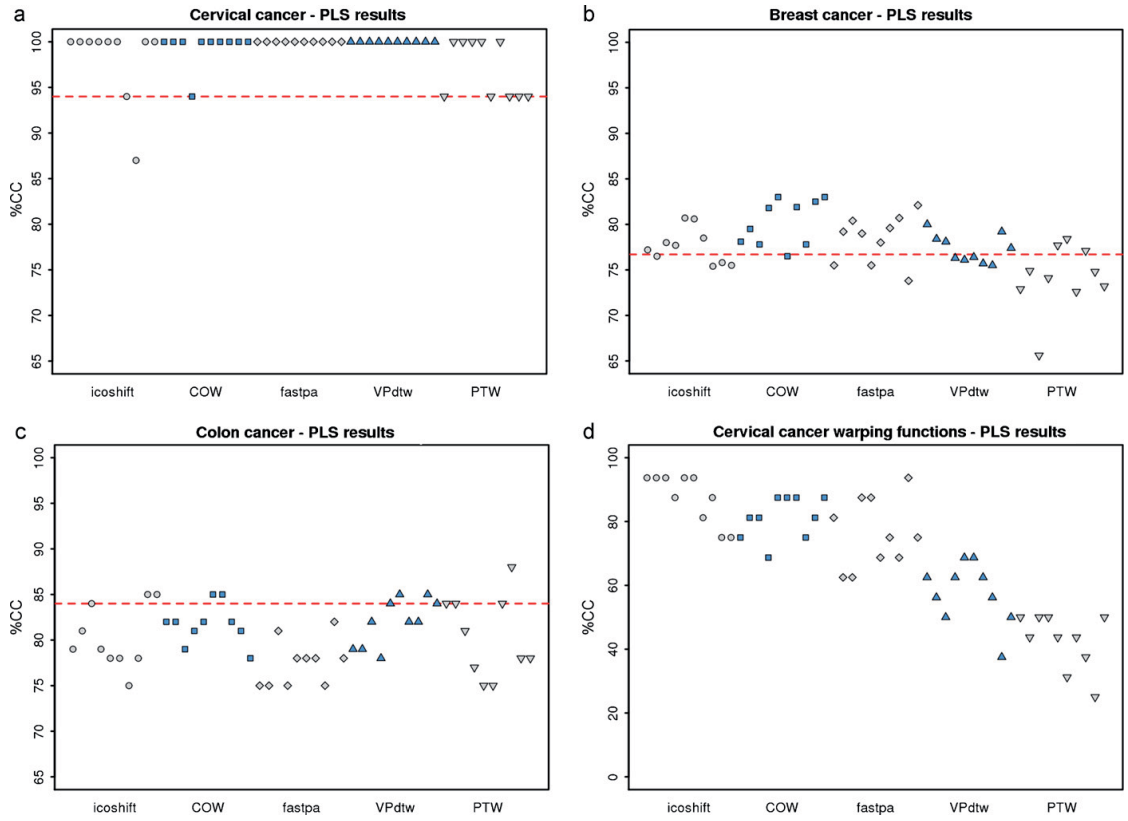

Fig. 6. PLS-DA classification results for different alignment methods. Results from the same 10 different references as in Fig. 4 are shown. The red line denotes classification results of the unaligned data (a) classification results of the cervical cancer data; (b) classification results of the breast cancer data; (c) classification results of the colon cancer data; (d) classification results of the cervical cancer warping coefficients.

its segmented nature largely prevents it from showing many local differences. Thus, it is not surprising that COW's warping coefficients for the cervical cancer data also led to good classification results. Both for VPdtw and COW, classification was also attempted using the cumulative sums of their warping functions instead, to (further) alleviate the local differences, but this did not improve the results. For PTW, alignment was performed using a quadratic function, and it can be assumed that the relevant shifts in the spectra were too complex to be modelled well by this function.

To summarize, the results presented here show that alignment of the data using the warping methods examined in this work not always improves the classification results compared to unaligned data. However, alignment improved the interpretability of the resulting model by providing less ambiguous loading profiles for PLS-DA, similar to the observations in Refs. [43–45]. This is especially important in situations where discriminating between two classes is not the only interest, but where one is also interested in looking at the differences in metabolic profiles to interpret biological incidences in the tissue. For that purpose, alignment will always be preferable.

3.2.4. Algorithms

Table 2 summarizes the evaluations of the different warping algorithms. Overall, icoshift and COW gave good alignment results and preserved the peak shapes. For COW, the optimization routine has a large part in this. Icoshift required quite some manual

Table 2
Evaluation of alignment methods[a].

Alignment method	Programming stability	Memory efficiency	Speed	Optimization of parameters	Peak conservation	Artifact- free	Alignment quality[c]
Icoshift	+	+	++	0	++	–	+
COW	+	–	–	+	+[b]	++	++
Fastpa	– –	+	0	–	0	0	+
VPdtw	+	+	+	0	+	0	0
PTW	+	+	0	0	0	++	0

[a] ++, very good; +, good; 0, moderate; –, improvement advisable; – –, improvement necessary.
[b] COW parameters were optimized to conserve peak shape prior to alignment.
[c] The alignment quality is assessed based on the end result after optimization of the parameters.

Table 3
Benchmarks. Time consumptions for alignment of the breast cancer data set using the same reference spectrum.

Alignment method	Time (s)
Icoshift	3.73
COW	292
Fastpa	87.0
VPdtw	42.8
PTW	149

optimization, but this was not considered a problem because of its speed, and the end results were satisfactory. For the parameter combinations allowed by the algorithm, fastpa also gave good alignment results, but at the expense of larger peak shape changes. This is not surprising when looking at Fig. 1: fastpa's warping paths were typically more extreme than those of the other algorithms. The segmentations of the spectra offered by fastpa and its predecessor [30] were typically good, however. A combination of this part of the algorithm with the alignment power of COW or icoshift may be worthwhile. Both VPdtw and PTW delivered variable results. The variable penalty in VPdtw clearly prevents the algorithm from deforming the spectra, but optimizing the resulting alignments is not trivial. The polynomial warping function of PTW is probably not flexible enough to model the local shifts occurring in NMR spectra very well.

The time consumption for alignment varies a lot among the methods. Table 3 shows the benchmarks for alignment of the breast cancer data set using the same reference spectrum. The benchmarks were obtained on a Dell Latitude E6400 laptop, equipped with an Intel Core 2 Duo P9500 processor running at 2.53 GHz and 3.48 GB of RAM. The operating system was Microsoft Windows XP SP3 (32 bit). Alignments with icoshift, COW and fastpa were performed in Matlab, version 7.7.0.471 (R2008b), while VPdtw and PTW alignments were performed in R, version 2.9.2.

Icoshift was the fastest warping method, and alignment of a data set of 209 spectra was done in a few seconds. COW, on the other hand, was the most time-consuming of the methods tested here, and used minutes to perform the same alignment. Also, COW sometimes runs into memory problems for large data sets. This can be overcome by choosing different parameters, or by dividing the data set in smaller subsets. Obviously, this may result in a final alignment that is not the optimal one for the data set.

The benchmarks shown here do not include optimization of the parameters, as that largely depends on the effort put into the procedures by the user. For fastpa, three parameters have to be optimized, as opposed to the other methods with only two parameters. This made fastpa more time-consuming to optimize. In addition, some combinations of fastpa parameters will give an error without any obvious reason. COW has an automatic optimization procedure that is time-consuming; however, it requires a minimum of effort from the user.

Although the evaluations in this paper were limited to MRS data, some of the observations above can safely be generalized to other types of data. Together with the conclusions in Refs. [23,24] it is clear that the rigidness of PTW's polynomial warping function limits its general applicability compared to COW. (At the same time, this is not to say that there are no situations in which such a rigid but also relatively simple warping function may be preferable; the data in the original PTW-paper were aligned in a satisfactory manner, for instance.) The 'wild' behaviour of fastpa is also something that seems to be inherent to that method and is expected to be independent of the exact type of data. Time consumption of alignments will mostly depend on data size and data complexity; the benchmarks in Table 3 can thus safely be used as an indication, regardless of the origins of the data.

3.2.5. General recommendations

Based on the observations in this study, we have the following recommendations for the alignment of HR-MAS MRS data from tissue samples:

- As a default method, icoshift is a good choice. It is fast, stable and gives good results. Its results should be thoroughly checked by visual inspection, though, and it may require some trial and error to prevent peaks from disappearing or artifacts to occur. However, its speed makes this feasible.
- When large local shifts occur in crowded data, or the results do not get satisfactory, COW is a good alternative. Although it is rather slow and memory intensive, this problem is alleviated somewhat by the computational power of current computers. COW robustly provides good alignment results and because it uses stretching instead of independent shifting for alignment, it is well suited to provide alignments exactly when icoshift runs into trouble. The pre-alignment optimization of the slack and segment length parameters ensures that peak shapes will be minimally affected.
- It is a good idea to try out a number of references for alignment. Although choosing the sample with the highest average correlation never seems to give bad results, it does not necessarily lead to the optimal result. Trying more references is a small effort and gives an idea of the variability of the results. Moreover, it is likely to provide a result close to the optimum that can be achieved.
- In general, a truly automatic warping procedure does not exist. The best alignment will not be achieved without putting some effort into optimizing alignment parameters, scaling, and finding a good reference.
- Visual inspection of the end result is an absolute necessity. Numerical measures can indicate a good result even if artifacts are present. At the same time, it should be kept in mind that visual inspection on its own is not infallible, since it is prone to subjective judgment.

4. Conclusion

In this paper, we investigated the suitability of five warping algorithms for aligning HR-MAS MR spectra to make them amenable to further multivariate analysis. Furthermore, we extracted shift information from the spectra and tested if it can be used in multivariate analysis.

Alignment of the data sets greatly improved their internal similarity compared to unaligned data. The differences in alignment quality between the algorithms examined in this study were not very large in general. Icoshift, COW and fastpa gave a good overall alignment result for HR-MAS data, but fastpa currently has too many drawbacks for general use. Both icoshift and COW also conserved the peak shapes of the spectra. Whether the algorithms were designed for chromatographic data or MR spectra did not seem to have an influence in general on their suitability for aligning MRS data. Comparison of our results with previous studies on chromatographical and CE data allowed generalization of some observations.

Both the choice of reference and the effort that is put into aligning are important factors in reaching the optimal alignment result. It is therefore advisable to try a number of different spectra as references and to optimize the parameter settings of the algorithms.

Based on the previous evaluations, general recommendations for aligning HR-MAS MRS data were proposed, including a suggestion for the algorithms to choose. The evaluation methodology discussed in this paper is generic and appropriate for assessing the suitability of warping methods for other types of data.

Finally, the extraction of shift information from spectra by means of the five warping algorithms has been demonstrated in

this paper. Cancer samples and samples from normal tissue in the cervical cancer data could successfully be discriminated based on the shift information, but this did not provide extra information next to the intensities in the aligned spectra.

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Paper III

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Paper IV

Prognostic value of metabolic response in breast cancer patients receiving neoadjuvant chemotherapy

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Running title: Metabolic treatment response in breast cancer patients

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Translational relevance:

Patients with locally advanced breast cancer have large tumor burdens and a poor prognosis. Molecular characterization of tumors may help stratify patients for individualized treatment, thereby achieving better prognosis. Our project aims to provide novel understanding of breast cancer biology in response to treatment. We have used high-throughput metabolomic analyses by high resolution magic angle spinning magnetic resonance spectroscopy (HR MAS MRS) to investigate the metabolic responses to neoadjuvant chemotherapy associated with patient outcome and clinical response. Findings from this study show that MR metabolomics can assist the identification of patients at high risk of breast cancer death, and help identify pathways for novel targeted treatment.

Abstract:

Purpose: Today's clinical diagnostic tools are insufficient for giving accurate prognosis to breast cancer patients. The aim of our study was to examine the tumor metabolic changes in patients with locally advanced breast cancer caused by neoadjuvant chemotherapy (NAC), relating these changes to clinical treatment response and long-term survival.

Experimental Design: Patients (n=89) participating in a randomized clinical trial were allocated to receive either NAC as epirubicin or paclitaxel monotherapy. Biopsies were excised pre- and post-treatment, and analyzed by high resolution magic angle spinning magnetic resonance spectroscopy (HR MAS MRS). The metabolite profiles were examined by paired and unpaired multivariate methods and findings of important metabolites were confirmed by spectral integration of the metabolite peaks.

Results: All patients had a significant metabolic response to NAC, and pre- and post-treatment spectra could be discriminated with 87.9%/68.9% classification accuracy by paired/unpaired partial least squares discriminant analysis (PLS-DA) ($p<0.001$). Similar metabolic responses were observed for the two chemotherapeutic agents. The metabolic responses were related to patient outcome. Non-survivors (<5 years) had increased tumor levels of lactate ($p=0.004$) after treatment, while survivors (≥5 years) experienced a decrease in the levels of glycine ($p=0.047$) and choline-containing compounds ($p\leq0.013$) and an increase in glucose ($p=0.002$) levels. The metabolic responses could not be related to clinical treatment response.

Conclusions: The differences in tumor metabolic response to NAC were associated with breast cancer survival, but not to clinical response. Monitoring metabolic responses to NAC by HR MAS MRS may provide information about tumor biology related to individual prognosis.

Introduction

The prognosis of patients with locally advanced breast cancer varies largely due to the heterogeneity of the disease, and 5-year survival rates from 50-80% have been reported (1). Neoadjuvant chemotherapy (NAC) has been established as a standard treatment for locally advanced breast cancer, with anthracyclines and taxanes being among the most frequently used agents. NAC is provided to make primarily inoperable tumors resectable, and will also increase the rate of breast-conserving surgery without any significant increase in local or distal recurrence (2, 3). Studies investigating the metabolic responses and chemoresistance to single or a combination of drugs are important for effective treatment and better patient outcome.

Patients with a pathological complete response (pCR) after NAC have improved outcome compared to patients with residual disease, thus treatment response is a prognostic indicator. However, only ~20% of patients will achieve a pCR to NAC (4). Other prognostic factors of breast cancer include axillary lymph node status, tumor size, Her-2 overexpression, histopathological grade, and hormone receptor status. The status of Her-2 and hormone receptors is also predictive of treatment response. Identification of other markers for prognosis and treatment response may help stratify patients for better individualized treatment.

Several studies have shown altered metabolism in cancer compared to normal tissue. Elevated levels of total choline-containing compounds (tCho) are frequently observed in cancer, and may serve as magnetic resonance spectroscopy (MRS) markers for malignancy, both *in vivo* and *ex vivo* (5, 6). The tCho signal constitutes signals from glycerophosphocholine (GPC), phosphocholine (PC) and free choline (Cho) which are involved in phospholipid metabolism through the Kennedy pathway. A decreased level of tCho detected by *in vivo* MRS has been suggested as a possible marker for treatment response (7, 8). Altered concentrations of other tissue metabolites, such as increased levels of lactate, have also been associated with malignancy (9, 10). Elevated lactate levels may be related to hypoxia, a common feature of solid tumors where glucose is catabolised to lactate due to the lack of oxygen. Also under conditions with sufficient oxygen levels, cancer cells may convert glucose to lactate, described as the Warburg effect.

High resolution magic angle spinning (HR MAS) MRS is a non-destructive technique providing highly resolved MR spectra of intact tissues with minimal sample preparation. HR MAS MR spectra provide an overview of the different metabolites that are present in a tissue sample, and can give insight into the complex processes leading to cancer and other diseases. More than 30 metabolites have been identified in breast tissue using HR MAS MRS (11). Systematic studies of the metabolic state of biological systems using multivariate analysis methods are referred to as metabolomics. MR metabolomics studies of breast cancer have revealed correlations between tissue metabolic profiles and clinical prognostic factors such as hormone receptor status, grade and lymphatic spread (12-14). Long-term survival of breast cancer patients has been successfully predicted from breast cancer tissue using multivariate classification models (15, 16). The purpose of this study was to examine the metabolic changes in breast cancer tissues resulting from treatment with NAC, and to relate these changes to treatment response and long-term survival. This is the first study to investigate the metabolic response of NAC in a large breast cancer cohort using *ex vivo* MRS.

Materials and methods

Patient and tumor characteristics

We examined a subcohort of breast cancer patients (n = 89) from a larger open-label multicenter study where patients were randomly allocated to receive NAC treatment with either anthracycline (epirubicin, 90 mg/m^2) or taxane (paclitaxel, 200 mg/m^2) monotherapy (17). The patients were given subsequent adjuvant endocrine treatment according to guidelines from the Norwegian Breast Cancer Group. The inclusion criteria and treatment protocol are fully described elsewhere (17). Briefly, female breast cancer patients at pre/post menopausal age (≤ 70 years) with locally advanced (stage III, $T_{3/4}$ and/or N_2) non-inflammatory breast cancer with or without limited distant metastasis were recruited in the period 1997-2003. The patients were treated every third week for four cycles. Patients showing a non-satisfactory response were assigned to the opposite treatment. From each patient, an incisional biopsy was taken before treatment with NAC and a post-treatment biopsy was excised during surgical removal of the tumor. The biopsies were immediately snap-frozen and stored in liquid nitrogen in a biobank until use. A part of the pre-treatment tumor biopsy was obtained for routine pathological diagnosis and hormone status assignment. Estrogen (ER) and progesterone receptor (PgR) status were determined by immunohistochemical staining (positive ≥ 10% staining cells). The study was approved by The Regional Committee for Medical and Health Research Ethics (Norwegian Health Region III) and informed written consent was obtained from all patients

Response and survival evaluation

Response to treatment was evaluated using the WHO criteria by the UICC system (18). Treatment response was assessed clinically by comparing caliper measurements prior to NAC treatment and after the last cycle. In the subcohort included in this study, the patients were classified to have either partial response (≥ 50 % reduction in tumor size (the product of the two largest tumor diameters), but not complete response) or stable disease (< 50 % reduction to ≤ 25 % increase in tumor size). Patients deceased within 5 years after diagnosis were classified as non-survivors whereas patients surviving 5 years or more were classified as survivors.

Histopathological examinations

Prior to HR MAS MRS analysis, imprint cytology smears were prepared from the tissue samples and stained with the May-Grünwald-Giemsa stain (Color-Rapid, Med-Kjemi, Norway). Confirmation of tumor cell content was determined microscopically by a cytopathologist.

HR MAS MRS experiments

HR MAS MRS analyses were performed on a Bruker Avance DRX600 spectrometer (Bruker Biospin GmbH, Germany) equipped with a $^{1}H/^{13}C$ MAS probe with gradient. The run order of the samples was randomized (www.random.org) and blindly analyzed during 18 days. Each sample (15.1±2.8 mg) was cut to fit a 30 µl leak-proof disposable insert (Bruker Biospin Corp, USA) and added phosphate buffered saline (PBS, 3 µl) in D_2O containing trimethylsilyl tetradeuteropropionic acid (TSP, 98.2 mM) for chemical shift referencing. Samples were spun at 5 kHz and spectra were recorded within 31 minutes per sample at 4°C to minimize tissue degradation. Spin-echo spectra (cpmgpr; Bruker) were recorded as previously described (14).

Data preprocessing

Twenty eight spectra were excluded from further studies due to low tumor cell content. The resulting data set consisted of 150 spectra from 85 patients (80 pre-treatment and 70 post-treatment spectra). Characteristics of the included patients and tumors are listed in Table 1. 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. The spectral region between 4.69-1.45 ppm, excluding the water peak and large lipid residuals, was chosen for analysis. Signals from ethanol pollutions between 3.69-3.57 ppm were removed together with lipid residual signals between 3.01-1.52 ppm. The spectra were baseline corrected using asymmetric least squares (19) with parameters λ = 1e7 and p = 0.0001, and the minimum value of each spectrum was set to zero by subtracting the lowest value. The spectra were normalized to equal total area, and peak aligned using icoshift (20).

Multivariate data analysis

Partial least squares (PLS) analysis is a regression method for analysis of collinear data with numerous variables. The method is based on extraction of underlying structures, or latent variables (LVs), that maximize the covariance between X (the spectra) and a response

variable Y (21). PLS discriminant analysis (PLS-DA) attempts to discriminate between distinct classes. PLS-DA was performed in Matlab R2009a (The Mathworks, Inc., USA) using PLS_Toolbox 6.2.1 (Eigenvector Research, USA). A PLS-DA model was built on mean-centred spectra from randomly chosen training samples (90 % of the patients) and used to predict the status of test samples (the remaining 10 %). This procedure was repeated 20 times and the average classification results were calculated. The number of LVs to use was chosen by cross-validation of the whole data set and used for all repetitions to avoid biased results. The importance of each variable in the loadings of the PLS-DA was evaluated by variable importance in the projection (VIP) scores (22). The VIP score positively reflects the variable's influence on the classification, and variables with a score greater than one are generally considered important (22, 23). To evaluate the statistical significance of the classification results, permutation testing was performed (24). In permutation testing, the class labels are permuted to resemble random classification. It is then possible to examine if the achieved prediction results of the original data set are significantly different than random predictions. The data set with the permuted class label was divided into training and test sets repeated 20 times as described for the original data set, and the average results were calculated. The permutation procedure was repeated 1000 times, and the prediction error of the original data set was compared to the distribution of prediction errors from the permutation. P-values ≤ 0.05 were considered significant.

Multilevel PLS-DA (25, 26) is an extension of ordinary PLS-DA which can be used as a paired analysis for multivariate data. This analysis can only be used when the data has a multilevel structure, i.e. when interventions are evaluated on the same subject. In multilevel PLS-DA, the *between* subject variation is separated from the *within* subject variation. This is useful in metabolic profiling as the variation between subjects, resulting from differences in age, disease state, genetics and other factors, can obscure the metabolic changes caused by the intervention. The between subject variation is described by the average of the two observations from one subject, whereas the within subject variation is described by the net difference between them. Multilevel PLS-DA was used to examine metabolic changes in the spectra resulting from NAC treatment. The split-up of variation was done using algorithms made available by van Velzen et al (25). Further PLS-DA classifications of the within subject variation were performed using PLS_Toolbox as described for the unpaired analyses. The net difference of the spectra pre minus post treatment (positively representing the metabolites

higher expressed before treatment) is annotated as control, while the net difference post minus pre treatment (positively representing the metabolites higher expressed after treatment) is annotated as treatment. More specifically,

$$\text{control} = \mathbf{A} - \mathbf{B}$$
$$\text{treatment} = \mathbf{B} - \mathbf{A} \qquad \text{(eq. 1)}$$

where the matrix **A** represents pre-treatment spectra, and matrix **B** represents the post-treatment spectra.

Univariate data analysis

To further validate the important metabolites from the PLS-DA models, relative intensities were found by integrating the peak areas of spectra normalized to equal total areas after removal of lipid residuals (Matlab R2009a, The Mathworks, Inc., USA). Normalization of spectra with the lipid residual signals removed will correct for differences in sample size and tumor cell content, as it can be assumed that most of the lipid signals from breast samples do not originate from cancer cells. Group differences were statistically tested by Wilcoxon rank sum tests or Wilcoxon sign rank for paired analyses, and considered significant if the p-values were ≤ 0.05.

Results

Metabolic response to neoadjuvant chemotherapy

All classification results are summarized in Table 2. An unpaired PLS-DA of the pre- and post-treatment spectra of the whole data set showed a significant difference in the metabolite profiles in response to NAC treatment, indicating a metabolic response to NAC in all patients. However, the specificity of the classification was low (57.1%). When comparing the classification errors of PLS-DA and paired multilevel PLS-DA from 20 different test sets, the multilevel PLS-DA with split-up of the variation decreased the classification error significantly (Wilcoxon rank sum test, $p < 0.001$), showing the beneficial effect of the paired analysis. Treatment and control spectra could be separated with a sensitivity and specificity of 87.9%. Figure 1A shows the scores and loadings of the multilevel PLS-DA. Lactate and PC were of high importance for the discrimination according to the VIP scores in the loadings. The levels of lactate and glycine appear to be increased in response to treatment, while the levels of PC are markedly decreased for some patients. In addition, GPC levels were decreased in response to treatment. No clustering according to the given chemotherapeutic agents could be seen in the multilevel PLS-DA score plot (results not shown), thus the metabolic treatment effects of epirubicin and paclitaxel appear to be indistinguishable.

No differences in metabolic response between clinical response groups

The patients were divided into two groups according to their clinical response (partial response or stable disease), and multilevel PLS-DA was performed on each group separately in order to discover potential differences in metabolic treatment response between the groups. Both for patients with partial response and stable disease there was a significant change in the tumor metabolism in response to NAC treatment, and treatment spectra could be discriminated from controls with high sensitivity and specificity ($\geq$ 80.0%). The metabolic response to NAC as observed in the loading plots was similar for both subgroups, resembling the changes observed for the whole data set (results not shown). Thus, no difference in the metabolic response could be detected between patients with stable disease and partial response.

Different metabolic responses correlate with survival

Accordingly the patients were divided into two groups according to their survival status (5-year survivors or non-survivors). Both for survivors and non-survivors there was a clear change in the tumor metabolism in response to NAC treatment (Figure 1B and C), and treatment spectra could be discriminated from controls with high sensitivity and specificity (≥ 82.5%). However, the metabolic treatment response appears to differ between survivors and non-survivors.

The loadings showed unchanged lactate levels in response to treatment in survivors, while lactate increased in non-survivors with high importance for the discrimination according to the VIP scores. This was confirmed by comparison of the relative intensities from metabolite integrals, showing a significant increase in lactate levels in response to treatment in non-survivors ($p = 0.004$) but not in survivors (Table 3).

Glycine appears to be decreased in survivors according to the loadings, and the difference in relative intensities before and after treatment was significant ($p = 0.047$). For non-survivors, the glycine level appears to be high in some samples from both the control and the treatment group; hence the role of glycine in the loading plot is more difficult to interpret. The glycine change from integrated relative intensities was not significant in non-survivors, with a mean value close to zero.

GPC levels were decreased in the loading plot of survivors with VIP scores showing high importance, while changes in GPC levels in non-survivors were less important for the discrimination. Accordingly, the relative intensities of GPC were significantly lower in response to treatment in survivors ($p < 0.001$) but not in non-survivors.

The loadings show decreased levels of PC in response to treatment in both survivors and non-survivors. However, the change in PC relative intensities was only significant for survivors ($p < 0.001$), but not for non-survivors, possibly due to a high standard error.

Relative intensities of Cho levels were significantly decreased in survivors ($p = 0.013$) in response to treatment, but only a trend of decreased Cho levels was seen in non-survivors ($p =$

0.084). In addition, glucose was significantly increased in survivors ($p = 0.002$). Cho and glucose were not protruding in the loadings, possibly due to low intensity values.

As an overall measure of the partly overlapping choline-containing metabolite peaks (GPC, PC, and Cho), the changes in relative intensities of tCho were calculated. Survivors had a significant decrease in tCho levels in response to treatment ($p < 0.001$), while a trend of decreased tCho levels were detected in non-survivors ($p = 0.091$).

Metabolic traits at pre- and post-treatment

A PLS-DA of the post-treatment spectra showed a significant difference in the metabolite profiles of 5-year survivors and non-survivors after treatment with 70.1% correct classification (Table 2). According to the scores and loadings shown in Figure 2, the tumors of non-survivors appear to have more of the metabolites lactate and glycine, and less GPC and taurine than survivors post-treatment. PC appears to be present in high levels in some samples of both survivors and non-survivors. The glycine level was denoted to be of major importance according to the VIP scores, and the relative intensities of glycine were significantly higher in non-survivors compared to survivors post-treatment ($p = 0.033$, Table 4). Similarly, a trend of higher relative intensities of lactate was observed in non-survivors ($p = 0.089$). No significant differences in the relative intensities of taurine and GPC were observed, however differences in the levels of tCho approached significance ($p = 0.075$) with non-survivors having higher relative intensities than survivors post-treatment.

The metabolic differences between survivor and non-survivors were not seen pre-treatment as the multivariate model could not discriminate the two outcome groups (model not valid). None of the metabolites showed significant differences in relative intensities between survivors and non-survivors pre-treatment (Table 4).

No significant differences in the metabolite profiles at pre- or post-treatment were detected between patients with partial responders and stable disease by PLS-DA. Post-treatment spectra from patients treated with Epirubicin and Paclitaxel could not be discriminated by PLS-DA, further confirming the similarity of the metabolic response of the two chemotherapeutic agents that were used in this study.

Discussion

In this study we examined the metabolic effect of NAC treatment in patients with locally advanced breast cancer. By comparing MR spectra of biopsies taken pre- and post-treatment, significant metabolic changes in response to treatment were found both by paired and unpaired multivariate models. The results using paired multilevel PLS-DA were however superior to those of unpaired PLS-DA, thus taking advantage of the multilevel structure in the data set was clearly beneficial.

Epirubicin and Paclitaxel appear to affect the metabolism of the tumor cells in the same manners, as evidenced both by indistinguishable metabolic responses and similar metabolic traits of the post-treatment spectra. Anthracyclines work by interfering with the synthesis and function of DNA, while taxanes stabilize the microtubules; thereby inhibiting cell division (27, 28). However, both treatments will eventually result in cell death. This might explain why the two agents appear to have similar metabolic responses.

Interestingly all patient in our study cohort showed clear changes in the metabolite profiles in response to treatment, including also patients categorized to have a clinically stable disease. No differences in the metabolic responses of the clinical response groups were detected. However, when examining the metabolic changes in survivors and non-survivors independently, a difference in the metabolic response to NAC was seen. Non-survivors had a significant increase in lactate levels in response to treatment, while survivors showed no change in lactate levels. As a result, a trend of higher levels of lactate was detected in non-survivors compared to survivors post-treatment. Increased lactate levels may be a marker for tumor aggressiveness as high levels of lactate have been correlated with low survival rates, high incident of distant metastasis and recurrence, and increased risk of radiation resistance in several types of cancer (29-31). Modification of cell energy metabolism is typically observed in malignant tumors and is suggested as an emerging hallmark of cancer (32). Under normoxic conditions, cancer cells can reprogram their energy metabolism to largely depend on aerobe glycolysis as their primary energy pathway resulting in increased lactate production; the so-called Warburg effect. It is not fully known why cancer cells prefer aerobe glycolysis over complete oxidation as this would produce far more ATP. It has been

hypothesized that lactate may enhance the invasiveness of tumor cells and the resulting low pH may help tumor cells evading tumor-attacking immune cells (33).

In addition to aerobe glycoysis, breast cancer cells are often hypoxic due to poor blood supply (34). It can be assumed that the large tumors of patients with locally advanced breast cancer will be affected by hypoxia. Hypoxia can induce the transcription factor hypoxia inducible factor-1α (HiF-1α), which in turn upregulates multiple genes involved in the glycolytic pathway, angiogenesis, cell proliferation, and other mechanisms (34-36). Furthermore HIF-1α promotes transcription of lactate dehydrogenase (LDH) and lactate monocarboxylate transporters (MCT), and thus plays an important role in the production and efflux of lactate in cancer cells (37, 38). Inhibition of LDH by small interfering RNA (siRNA) in mouse breast tumors has been shown to reduce the glycolytic activity associated with a decrease in tumor proliferation and tumorigenic potential (39). Thus we can suggest that the increased levels of lactate after NAC treatment observed in non-survivors may reflect enhancement of aerobe glycolytic activity and/or hypoxic tumor responses that confer higher tumor malignancy and poor prognosis. In coherence, the glucose levels were increased in response to treatment in survivors but not in non-survivors. Increased glucose may be indicative of decreased aerobe glycolysis and tumor hypoxic response favorable of long term breast cancer survival.

Survivors had a significant decrease in glycine as a response to treatment, while it remained unchanged in non-survivors. This was reflected in the post-treatment spectra, showing significantly lower levels of glycine in survivors. In a previous study, we also found decreased glycine levels after NAC to be associated with long term breast cancer survival (15). The biological role of glycine in tumor malignancy is still unclear. Several studies have elucidated the biomarker potential of glycine in human brain tumors, where it was found to positively correlate with tumor grade (40, 41). Higher levels of glycine have also been detected in pre-clinical studies of the more aggressive basal-like breast cancer model compared to the luminal-like model (42). In patients, high glycine levels detected in malignant breast tumors have been correlated with poor prognosis (43). Glycine is mainly synthesized from 3-phosphoglycerate, an intermediate of the glycolysis. In addition, glycine can be synthesized from Cho through the glycine-betaine pathway. We can postulate that the decreased glycine levels after NAC treatment detected in survivors are caused by altered glycolysis and/or reduced Cho levels associated with reduced tumor aggressiveness.

A significant decrease of GPC, PC, Cho levels and the combined tCho level was detected in survivors in response to treatment, whereas non-survivors experienced only a trend of decrease in Cho and tCho levels. As a result, lower tCho levels in survivors compared to non-survivors post-treatment approached significance. In a previous publication, we showed that GPC and Cho concentrations significantly decreased in patients with long-term survival (≥ 5 years), while non-survivors (< 5 years) had no significant changes in choline phospholipid metabolites in response to NAC (15). Choline phospholipid metabolites are important biological compounds in cell membrane synthesis and turnover. In addition, tCho levels have been associated with increased malignancy and activation of oncogenic signaling in breast cancer cells (44, 45). Higher tCho concentrations have been detected in high-grade breast tumors and tumors with higher pharmacokinetic parameters measured with dynamic contrast enhanced MR imaging, indicating a correlation between choline phospholipid metabolism and tumor malignancy and angiogenesis (46, 47). As previously mentioned, cancer cells may undergo adaptive responses to hypoxia by inducing HiF-1α. Increased tCho levels and choline kinase alpha (CHKA) expressions has been detected in prostate cancer cells and xenografts models under hypoxic compared to normoxic conditions (48). In the same study, the authors found hypoxic tumor regions to be co-localized with regions of high tCho, which possibly occurred through the up-regulation of CHKA by HiF-1α. CHKA is known to play an important role in malignant transformation in several types of cancer (49). Overexpression of CHKA and elevated PC and tCho levels of breast cancer cells have been associated with increases invasiveness and drug resistance (50). Decreased choline phospholipid metabolism after NAC treatment may be associated with lower malignancy that potentially can be used as a predictor of breast cancer survival.

The metabolic responses to NAC treatment appear to be similar in patients with partial response and stable disease. None of the patients in this study had a progressive disease, whereas patients with a complete response would not have any tumor tissue left for a post-treatment biopsy. By definition the group with stable disease can have up to 50% reduction in tumor volume, and indeed only two patients in this study had an equal or increased tumor size after NAC. In that respect, almost all patients had a biological effect of the treatment although the tumor reduction was small for patients with a stable disease. It is conceivable that a cohort including also patients with progressive disease would reveal clearer differences in metabolic

response between the clinical response groups. It is however noteworthy that all patients in this study in general had a decrease in tCho after NAC, as tCho is suggested as an *in vivo* biomarker for clinical treatment response.

In this patient cohort, the prediction of overall survival was accomplished with 70.1% classification accuracy using post-treatment spectra, but no prognostic information could be extracted from the pre-treatment spectra. This shows that the difference between survivors and non-survivors post-treatment results from a metabolic response to the treatment. The observed higher levels of lactate and glycine in non-survivors compared to survivors support our previous studies postulating high lactate and glycine levels to be predictive of low breast cancer survival rates (< 5 years) (15, 16).

Prediction of survival in patients receiving NAC is challenging. As NAC will downstage and potentially completely remove the disease, standard prognostic indicators such as tumor size and lymph node status are no longer fully applicable after NAC. Several studies have shown that a pathological complete response after NAC is associated with better survival rates (4). However, approximately 80% of patients will have residual tumor in the breast after treatment (4). Our study shows that the metabolic response to treatment may be an indicator of patient prognosis.

Conclusion

By comparing HR MAS MR spectra from biopsies excised before and after NAC treatment, we have revealed significant metabolic changes in breast cancer tumors as a response to treatment. Different metabolic responses could be related to patient outcome, but did not separate patients with partial response from those with stable disease. Non-survivors had increased tumor levels of lactate after treatment, while survivors experienced a decrease in the levels of glycine and choline-containing compounds. These differences in tumor response may reflect tumor aggressiveness associated with breast cancer survival. Monitoring metabolic responses to NAC by HR MAS MRS may provide information about tumor biology related to prognosis, and help identify pathways for targeted therapies.

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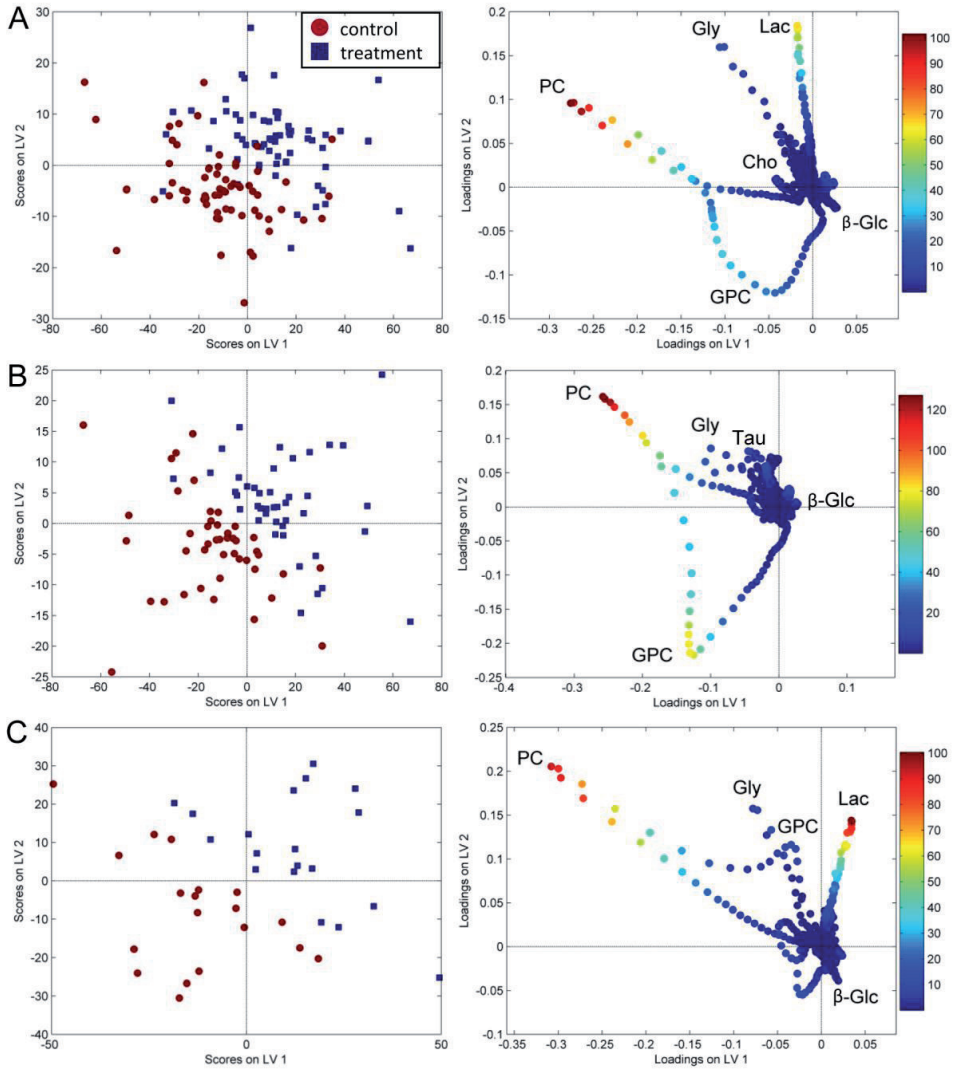

Figure 1 Scores and loadings from multilevel PLS-DA of treatment and control spectra from (A) the whole data set, (B) 5-year survivors, and (C) non-survivors. The variables in the loadings are colored according to VIP scores, indicating the importance of each variable in the discrimination. The control spectra equal the difference between pre- and post-treatment spectra, while the treatment spectra equal the post-pre treatment difference. Lac, lactate; Gly, glycine; β-Glc, β-glucose.

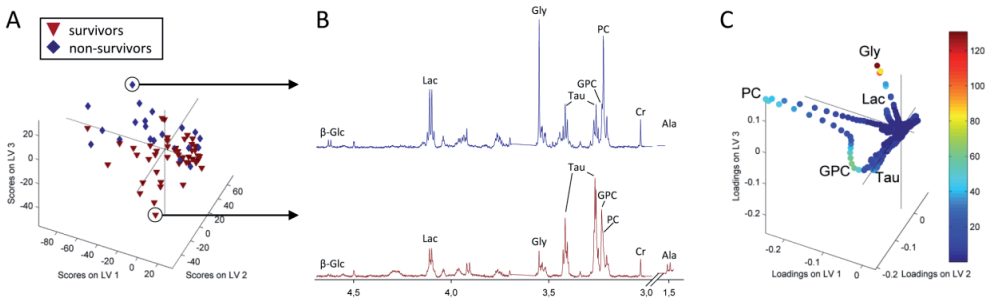

Figure 2 PLS-DA of the MR spectra from biopsies excised post-treatment. (A) A score plot discriminating survivors and non-survivors, (B) Representative spectra showing the metabolic differences of the tumors of survivors and non-survivors. (C) The loadings of the PLS-DA model with variables colored according to the VIP scores. β-Glc, β-glucose; Lac, lactate, Gly, glycine; Cr, creatine; Ala, alanine

Table 1 Patient and tumor characteristics

		Survivors (n=60)	Non-survivors (n=23)	NA* (n=2)
Mean age (±SD)	years	51.1±10.6	49.3±8.3	46.4±2.6
Mean tumor dimensions (mean ±SD)	mm	67.9x67.9 ±18.0x19.8	78.6x77.3 ±22.0x24.0	65.0x51.5 ±7.1x26.2
NAC treatment	Epirubicin	25	8	-
	Paclitaxel	23	5	2
	Both[1]	12	10	-
Treatment response	Partial response	40	11	1
	Stable disease	20	12	1
AJCC	IIB	21	7	1
	IIIA	25	11	1
	IIIB	12	2	-
	IV	2	3	-
ER status	+	41	7	2
	-	19	15	-
	unknown	-	1	-
PgR status	+	32	14	2
	-	28	18	-
	unknown	-	1	-
Nodes	+	32	14	1
	-	28	9	1
Metastasis	+	2	2	0
	-	58	21	2

*NA, not applicable - One patient without following up and one patient dead by other causes; AJCC, American Joint Committee on Cancer; Both[1], sequential treatment with Epirubicin and Paclitaxel

Table 2 Classification results from PLS-DA and multilevel PLS-DA

				Class. model	No. of LVs	Variance X/Y (%)	Sensitivity/ Specificity (%)	Class. accuracy (%)	Permutation p-value
Paired data	All samples	Control vs Treatment	(n=65p)	Multilevel PLS-DA	2	58.9/50.9	87.9/87.9	87.9	<0.001
	Partial response	Control vs Treatment	(n=37p)	Multilevel PLS-DA	2	55.7/61.7	80.0/80.0	80.0	<0.001
	Stable disease	Control vs Treatment	(n=28p)	Multilevel PLS-DA	2	63.2/61.3	88.7/88.7	88.7	<0.001
	Survivors	Control vs Treatment	(n=44p)	Multilevel PLS-DA	2	60.0/50.5	83.0/83.0	83.0	<0.001
	Non-survivors	Control vs Treatment	(n=19p)	Multilevel PLS-DA	2	70.6/63.9	82.5/82.5	82.5	0.006
Unpaired data	All samples	Pre- vs Post-treatment	(n=65p)	PLD-DA	2	50.3/29.2	80.7/57.1	68.9	<0.001
	Pre-treatment	Partial response vs Stable disease	(n=48) (n=32)	PLS-DA	2	51.5/19.3	56.7/60.2	58.4	0.231
	Post-treatment	Partial response vs Stable disease	(n=41) (n=29)	PLS-DA	NaN	-	-	-	-
	Pre-treatment	Survivors vs Non-survivors	(n=57) (n=20)	PLS-DA	NaN	-	-	-	-
	Post-treatment	Survivors vs Non-survivors	(n=47) (n=21)	PLS-DA	3	62.8/32.1	58.4/75.3	70.1	0.009
	Post-treatment	Epirubicin vs Paclitaxel	(n=29) (n=23)	PLS-DA	2	45.7/26.8	45.0/48.3	46.5	0.245

The sensitivity is for detecting a treatment/stable disease/non-survivor/Paclitaxel spectrum; Variance X/Y, amount of variance from X/Y explained by the model; NaN, no valid model; p, pairs.

Table 3 Changes in relative intensities of metabolites in response to NAC

		Survivors		Non-survivors	
Metabolite	**ppm**	**Mean ± SE**	**p-value**	**Mean ± SE**	**p-value**
Lactate	4.08-4.13	2.8 ± 15.3	0.815	97.1 ± 26.4	0.004**
Glycine	3.54-3.56	-19.6 ± 8.0	0.047*	0.8 ± 14.3	0.601
GPC	3.22-3.24	-59.6 ± 14.6	< 0.001**	-11.8 ± 15.7	0.469
PC	3.21-3.22	-95.4 ± 24.3	< 0.001**	-67.4 ± 44.2	0.227
Cho	3.20-3.21	-16.6 ± 6.3	0.013*	-17.8 ± 6.3	0.084
tCho	3.20-3.24	-167.2 ± 36.7	< 0.001**	-95.3 ± 57.5	0.091
Taurine	3.40-3.43	-0.8 ± 13.2	0.861	6.9 ± 12.5	0.398
β-Glucose	4.61-4.64	17.8 ± 5.1	0.002**	-0.2 ± 8.8	0.841

The values (post- minus pre-treatment) of relative intensities are integrated peak areas from spectra normalized to equal total areas. Wilcoxon sign rank tests were used for paired statistical analyses. *$p < 0.05$, **$p < 0.01$.

Table 4 Relative intensities of metabolites at pre- and post-treatment

		Pre-treatment (mean ± SE)			Post-treatment (mean ± SE)		
Metabolites	**ppm**	**Survivors**	**Non-survivors**	**p-value**	**Survivors**	**Non-survivors**	**p-value**
Lactate	4.08-4.13	185.8 ± 11.4	164.6 ± 13.8	0.534	196.2 ± 12.7	250.6 ± 26.0	0.089
Glycine	3.54-3.56	115.0 ± 6.4	120.8 ± 11.0	0.542	91.2 ± 4.1	111.4 ± 8.4	0.033*
GPC	3.22-3.24	167.3 ± 12.4	170.1 ± 23.9	0.949	115.6 ± 6.7	153.9 ± 24.1	0.144
PC	3.21-3.22	247.9 ± 17.0	285.4 ± 34.9	0.338	158.0 ± 16.1	205.5 ± 28.1	0.105
Cho	3.20-3.21	95.4 ± 3.9	108.7 ± 8.3	0.225	79.5 ± 3.7	85.9 ± 6.2	0.276
tCho	3.20-3.24	498.3 ± 26.4	551.3 ± 46.8	0.253	344.9 ± 22.3	434.6 ± 44.6	0.075
Taurine	3.40-3.43	242.6 ± 9.6	222.8 ± 12.4	0.393	248.0 ± 8.1	222.3 ± 9.3	0.144
β-Glucose	4.61-4.64	48.4 ± 3.3	49.3 ± 6.0	1.000	63.6 ± 4.3	53.5 ± 6.6	0.172

The values of relative intensities are integrated peak areas from spectra normalized to equal total areas. Wilcoxon rank sum tests were used for statistical analyses. *$p < 0.05$, **$p < 0.01$.

Dissertations at the Faculty of Medicine, NTNU

1977

1. Knut Joachim Berg: EFFECT OF ACETYLSALICYLIC ACID ON RENAL FUNCTION
2. Karl Erik Viken and Arne Ødegaard: STUDIES ON HUMAN MONOCYTES CULTURED *IN VITRO*

1978

3. Karel Bjørn Cyvin: CONGENITAL DISLOCATION OF THE HIP JOINT.
4. Alf O. Brubakk: METHODS FOR STUDYING FLOW DYNAMICS IN THE LEFT VENTRICLE AND THE AORTA IN MAN.

1979

5. Geirmund Unsgaard: CYTOSTATIC AND IMMUNOREGULATORY ABILITIES OF HUMAN BLOOD MONOCYTES CULTURED IN VITRO

1980

6. Størker Jørstad: URAEMIC TOXINS
7. Arne Olav Jenssen: SOME RHEOLOGICAL, CHEMICAL AND STRUCTURAL PROPERTIES OF MUCOID SPUTUM FROM PATIENTS WITH CHRONIC OBSTRUCTIVE BRONCHITIS

1981

8. Jens Hammerstrøm: CYTOSTATIC AND CYTOLYTIC ACTIVITY OF HUMAN MONOCYTES AND EFFUSION MACROPHAGES AGAINST TUMOR CELLS *IN VITRO*

1983

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1984

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13. Terje Terjesen: FRACTURE HEALING AND STRESS-PROTECTION AFTER METAL PLATE FIXATION AND EXTERNAL FIXATION.
14. Carsten Saunte: CLUSTER HEADACHE SYNDROME.
15. Inggard Lereim: TRAFFIC ACCIDENTS AND THEIR CONSEQUENCES.
16. Bjørn Magne Eggen: STUDIES IN CYTOTOXICITY IN HUMAN ADHERENT MONONUCLEAR BLOOD CELLS.
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33. Olav F. M. Sellevold: GLUCOCORTICOIDS IN MYOCARDIAL PROTECTION.
34. Terje Skjærpe: NONINVASIVE QUANTITATION OF GLOBAL PARAMETERS ON LEFT VENTRICULAR FUNCTION: THE SYSTOLIC PULMONARY ARTERY PRESSURE AND CARDIAC OUTPUT.
35. Eyvind Rødahl: STUDIES OF IMMUNE COMPLEXES AND RETROVIRUS-LIKE ANTIGENS IN PATIENTS WITH ANKYLOSING SPONDYLITIS.
36. Ketil Thorstensen: STUDIES ON THE MECHANISMS OF CELLULAR UPTAKE OF IRON FROM TRANSFERRIN.
37. Anna Midelfart: STUDIES OF THE MECHANISMS OF ION AND FLUID TRANSPORT IN THE BOVINE CORNEA.
38. Eirik Helseth: GROWTH AND PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN GLIOMAS AND BRAIN METASTASES - WITH SPECIAL REFERENCE TO TRANSFORMING GROWTH FACTOR BETA AND THE EPIDERMAL GROWTH FACTOR RECEPTOR.
39. Petter C. Borchgrevink: MAGNESIUM AND THE ISCHEMIC HEART.
40. Kjell-Arne Rein: THE EFFECT OF EXTRACORPOREAL CIRCULATION ON SUBCUTANEOUS TRANSCAPILLARY FLUID BALANCE.
41. Arne Kristian Sandvik: RAT GASTRIC HISTAMINE.
42. Carl Bredo Dahl: ANIMAL MODELS IN PSYCHIATRY.

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43. Torbjørn A. Fredriksen: CERVICOGENIC HEADACHE.
44. Rolf A. Walstad: CEFTAZIDIME.
45. Rolf Salvesen: THE PUPIL IN CLUSTER HEADACHE.
46. Nils Petter Jørgensen: DRUG EXPOSURE IN EARLY PREGNANCY.
47. Johan C. Ræder: PREMEDICATION AND GENERAL ANAESTHESIA IN OUTPATIENT GYNECOLOGICAL SURGERY.
48. M. R. Shalaby: IMMUNOREGULATORY PROPERTIES OF TNF-α AND THE RELATED CYTOKINES.
49. Anders Waage: THE COMPLEX PATTERN OF CYTOKINES IN SEPTIC SHOCK.
50. Bjarne Christian Eriksen: ELECTROSTIMULATION OF THE PELVIC FLOOR IN FEMALE URINARY INCONTINENCE.
51. Tore B. Halvorsen: PROGNOSTIC FACTORS IN COLORECTAL CANCER.

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52. Asbjørn Nordby: CELLULAR TOXICITY OF ROENTGEN CONTRAST MEDIA.
53. Kåre E. Tvedt: X-RAY MICROANALYSIS OF BIOLOGICAL MATERIAL.
54. Tore C. Stiles: COGNITIVE VULNERABILITY FACTORS IN THE DEVELOPMENT AND MAINTENANCE OF DEPRESSION.
55. Eva Hofsli: TUMOR NECROSIS FACTOR AND MULTIDRUG RESISTANCE.
56. Helge S. Haarstad: TROPHIC EFFECTS OF CHOLECYSTOKININ AND SECRETIN ON THE RAT PANCREAS.
57. Lars Engebretsen: TREATMENT OF ACUTE ANTERIOR CRUCIATE LIGAMENT INJURIES.
58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

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65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.
68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
76. Harold C Sexton: STUDIES RELATING TO THE TREATMENT OF SYMPTOMATIC NON-PSYCHOTIC PATIENTS.
77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
78. Terje Johannessen: CONTROLLED TRIALS IN SINGLE SUBJECTS.
79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

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82. Gunnar Bovim: CERVICOGENIC HEADACHE.
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
159. xxxxxxxxx (blind number)
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
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
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
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
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
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
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
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