Muhammad Riyas Vettukattil

Identification and Characterization of Biomarkers using Magnetic Resonance Metabolomics

– Metabolic portraits of cancers and aerobic fitness

Thesis for the degree of Philosophiae Doctor

Trondheim, September 2013

Norwegian University of Science and Technology
Faculty of Medicine
Department of Circulation and Medical Imaging

NTNU – Trondheim
Norwegian University of
Science and Technology

NTNU
Norwegian University of Science and Technology

Thesis for the degree of Philosophiae Doctor

Faculty of Medicine
Department of Circulation and Medical Imaging

ISBN 978-82-471-4587-6 (printed ver.)
ISBN 978-82-471-4589-0 (electronic ver.)
ISSN 1503-8181

Doctoral theses at NTNU, 2013:234

Printed by NTNU-trykk

Identifisering og karakterisering av biomarkører ved bruk av magnetisk resonans metabolomics

Sammendrag

Kreft og kardiovaskulære sykdommer er ledende dødsårsaker i industriland og i mange utviklingsland. De eksisterende kliniske og patologiske verktøy for disse sykdommene er ikke tilstrekkelige for å gi presis prediksjon av respons eller optimal individualisert behandling. Det er derfor et stort behov for å identifisere og implementere nye biomarkører for å oppnå bedre prediktiv, forebyggende og målrettet medisin.

Endring i cellenes stoffskifte er en viktig faktor i utviklingen av kreft og kardiovaskulær sykdom og derfor et viktig område innen biomedisinsk forskning. Studiet av små molekylære metabolitter i kroppsvæsker og vevsprøver (metabolomics), kan ved hjelp av magnetisk resonans spektroskopi (MRS) og multivariate dataanalyser, gi ny innsikt innenfor dette feltet. Identifikasjon av nye metabolske biomarkører for prediksjon, diagnose og behandlingsrespons av kardiovaskulær sykdom og kreft, har potensiale til å øke total overlevelse og pasientens livskvalitet, i tillegg til å spare samfunnet for store utgifter.

Økt forekomst av livsstilssykdommer er en trussel mot folkehelsen, og det er behov for mer effektive forebyggings- og behandlingsstrategier. Flere studier har vist at forekomsten av metabolsk syndrom og kardiovaskulær sykdom er relatert til kondisjonsnivå. Lav maksimal aerob kapasitet er foreslått som en prediktiv faktor for

kardiovaskulær død. De eksakte molekylære mekanismene bak dette er uklare. Studier basert på metabolomics har resultert i unike funn som kan gi informasjon om de underliggende mekanismene til koronar hjertesykdom, kreft, kosthold og livsstil. Innen kreftforskning har metabolomics også et potensiale som et ekstra verktøy i diagnostisering og risikovurdering. Videre vil det være et relevant verktøy for å finne optimal individualisert behandling, med andre ord kun behandle pasienter som med størst sannsynlighet har effekt av en spesifikk behandling og dermed kan unngå unødvendig behandling.

Hovedmålet med forskningen presentert i denne avhandlingen var å evaluere bruken av metabolomics basert på bruk av høyoppløselig MRS og multivariat dataanalyse for å identifisere og karakterisere mulige biomarkører for ulike helsetilstander. Avhandlingen består av tre artikler hvor anvendelsen av MR metabolomics til å identifisere biomarkører for kondisjon, astrocytom grad og endringer i bukhinne-/pleuravæske fra kreftpasienter etter kjemoterapi ble evaluert. Studiene ble utført i prøvematerialer fra et bredt spekter av mennesker, og spenner fra friske frivillige til pasienter med avansert kreftsykdom.

I den første studien ble kondisjonsavhengige forskjeller i serumnivåer av fritt kolin og fosfatidylkolin i en gruppe friske frivillige observert. Resultatene viser at kolinmetabolitter er potensielle tidlige markører for kardiovaskulær sykdomsrisiko og bør studeres nærmere. I neste studie ble muligheten for å differensiere diffuse hjernesvulster av Grad II og IV astrocytom basert på metabolske profil vist. I det siste

arbeidet ble metabolske markører for kjemoterapirelaterte endringer i bukhinnevæske fra pasienter med eggstokkreft identifisert.

Denne avhandlingen har vist nytten av MR metabolomics og multivariat dataanalyse i utredningsfasen av biomarkører. Videre har nytten av MR baserte metabolomics teknikker for å finne molekylære signaturer av kreft og kondisjon blitt studert. Dette kan bidra ytterligere til den vitenskapelige forståelsen av underliggende biologi av svulster og kondisjon.

Navn kandidat: Muhammad Riyas Vettukattil

Institutt: Institutt for sirkulasjon og bildediagnostikk

Veileder(e): Tone F. Bathen, Ingrid S. Gribbestad, Ulrik Wisløff

Finansieringskilde: NTNU

Ovennevnte avhandling er funnet verdig til å forsvares offentlig

for graden Philosophiae Doctor i medisinsk teknologi

Disputas finner sted i Auditoriet,Medisinsk teknisk forskningssenter,

Fredag 27.september 2013, kl 12.15.

Acknowledgements

The work presented in this thesis was performed at the MR Centre, Department of circulation and medical imaging, NTNU between October 2009 and September 2013. The financial support for my work was provided by the Norwegian University of Science and Technology (NTNU) and is hereby greatly acknowledged.

I would like to express my gratitude to my supervisors Prof. Tone Frost Bathen, Prof. Ingrid Susann Gribbestad and Prof. Ulrik Wisløff. Your enthusiasm, inspiration, support and valuable advices are sincerely appreciated. Thank you to all my co-authors for their contributions and critical discussions.

I wish to thank all my colleagues at MR center for creating such a brilliant environment and helping me get through the difficult times, entertainments and the caring they provided. Special thanks to my office mates Morteza and Kirsten for the discussions, encouragements and chitchats. "*Spesiell takk til Torill og Kirsten for norsk oversettelse av sammendraget*". Sincere thanks to Guro, Siver and Trygve for their willingness to provide feedbacks on my writing.

Finally, thanks to my family for their support and encouragement, specifically to my wife Sinu for her affection and patience.

Summary

Cancer and cardiovascular disease are the leading cause of mortality in the developed countries and in many developing countries. The existing clinical and pathological tools for both these diseases are insufficient for accurate response prediction, or for an individualized treatment. There is a compelling need for identification and development of new biological markers to achieve a new era of predictive, preventive and targeted medicine.

Altered cellular metabolism is an important factor in the pathogenesis of cancer and cardiovascular disease and has become a major area of biomedical research. Metabolomics, the study of small molecular metabolites present in biofluids and tissue samples using magnetic resonance spectroscopy may hold the power to bring new insights on this subject. Identification of metabolic biomarkers for cardiac disease and cancer risk prediction, diagnosis and treatment response could have the power to increase overall survival and the patient quality of life, in addition to saving huge expenses for the society.

Increased prevalence of lifestyle-related diseases is an impending threat to public health, and calls for effective prevention and treatment strategies. Several studies have indicated that the occurrence of metabolic syndrome and cardiovascular disease are related to the exercise capacity. Low maximal aerobic capacity is suggested as a predictive factor for cardiovascular deaths. However, the exact molecular mechanisms behind this are unclear and are difficult to explore. Metabolomics based approach may

provide potential information in this direction and has resulted in unique findings in relation to coronary heart disease, cancer, diet, and lifestyle. The potential benefits of metabolomics within cancer research would be to serve as an additional tool in diagnosis and risk evaluation. In addition, targeting of specific patients who are more likely to benefit from a specific treatment than those who may not benefit from it or may be harmed is highly relevant.

The main objective of the research presented in this thesis was to evaluate the use of high resolution magnetic resonances (MR) spectroscopy together with multivariate analysis based metabolomics for identifying and characterizing potential biomarkers of health-disease continuum. This thesis consists of three papers in which the applicability of MR metabolomics in identifying biomarkers of aerobic fitness, astrocytoma grading and chemotherapy dependent changes in malignant serous effusion was investigated. Metabolomic studies were performed on a broad range of subjects ranging from healthy volunteers to patients with advanced stage of malignancies.

In the first study, aerobic fitness dependent differences in serum levels of free choline and phosphatidylcholines in a group of healthy volunteers were observed. These choline metabolites are potential early markers of CVD risk and should be studied further. In the next study, the possibility of differentiating diffuse World Health Organization Grade II and IV astrocytoma based on their metabolic profiles were shown. In the third paper, metabolic markers of chemotherapy related changes in ovarian serous carcinoma effusions were identified.

The usefulness of MR metabolomics together with multivariate data analysis in the exploratory phase of biomarker discovery has been illustrated in this thesis. Furthermore, the usefulness of MR based metabolomic techniques in capturing molecular signatures of cancers and aerobic fitness has been explored and may contribute further to the scientific understanding of underlying tumor biology and aerobic fitness.

Symbols and abbreviations

2HG	2-hydroxyglutarate
A-II	astrocytoma grade II
ATP	Adenosine-5'-triphosphate
B_0	the static magnetic field
BHB	beta hydroxyl butyrate
CHD	coronary heart disease
Cho	free choline
COW	correlation optimized warping
CPMG	Carr Purcell Meiboom Gill sequence
CVD	cardiovascular disease
FID	free induction decay
GBM	glioblastoma
GPC	glycerophosphocholine
GSH	glutathione
HR-MAS	high resolution magic angle spinning
HUNT	the Nord-Trøndelag Health Study
IDH	isocitrate dehydrogenase
KPS	karnowsky performance status
ML-PLSDA	multilevel partial least squares discriminant analysis
MRS	magnetic resonance spectroscopy
MS	mass spectrometry
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
OC	ovarian carcinoma
PC	principal component
PCA	principal component analysis
PCho	phosphocholine
PLD	phospholipase D
PLS	partial least squares

PLS-DA	partial least squares discriminant analysis
ppm	parts per million
PPP	pentose phosphate pathway
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PTW	parametric time warping
RF	radio frequency
T_1	longitudinal relaxation time
T_2	transversal relaxation time
tCho	total choline signal
TE	echo-time
TSP	trimethylsilyl 3-propionic acid sodium salt
VO_{2max}	maximal oxygen uptake

List of papers

Paper I

Serum levels of choline-containing compounds are associated with aerobic fitness level: the HUNT-study.

Bye A[*], Vettukattil R[*], Aspenes ST, Giskeødegård GF, Gribbestad IS, Wisløff U, Bathen TF. *Shared first authorship

PLoS One. 2012;7(7):e42330.

Paper II

Differentiating Diffuse World Health Organization Grade II and IV Astrocytomas With Ex Vivo Magnetic Resonance Spectroscopy.

Vettukattil R[*], Gulati M[*], Sjøbakk TE, Jakola AS, Kvernmo NA, Torp SH, Bathen TF, Gulati S, Gribbestad IS. [*]Shared first authorship

Neurosurgery, 2013 Feb; 72(2):186-95

Paper III

Proton magnetic resonance metabolomic characterization of ovarian serous carcinoma effusions: chemotherapy-related effects and comparison with malignant mesothelioma and breast carcinoma

Vettukattil R, Hetland TE, Flørenes VA, Kærn J, Davidson B, Bathen TF.

Human Pathology, 2013 May; doi: 10.1016/j.humpath.2013.02.009. [Epub ahead of print]

Contents

1 Introduction

1.1 Motivation

Cancer and coronary heart disease (CHD) are the leading causes of death in the majority of developed and many developing countries in the world.[1,2] Despite the impressive progress in medical, scientific and technological achievements over the past decades, the prevalence of these diseases has reached alarming proportions. Existing diagnostic tools in the clinics are often insufficient for early diagnosis, risk stratification and in treatment response prediction. Hence, there is an urgent need to develop tools for early risk prediction, to actuate prevention strategies and to optimize the therapeutic regime to achieve a successful clinical outcome.

Biological mechanisms underlying cancers and CHD are complex and not fully understood. There is an intricate interplay of genetic, environmental, and lifestyle factors involved in the pathogenesis of these diseases. Unraveling the complex association between disease phenotype and the individual's genetic makeup is a challenging task. Recently, the use of molecular tools are gaining much attention in detecting and studying DNA, RNA, proteins and metabolites to understand the biological basis of cancers and heart diseases. It is now possible to perform comprehensive and non-targeted analysis of gene products (i.e. RNA, proteins, and metabolites) present in a specific biological sample. These high throughput analytical techniques generate enormous amount of data which needs sophisticated computational methods for interpretation. A new field of research called systems biology has emerged

which use a global top-down approach to elucidate the complex behavior which are difficult to explain by targeted experiments.[3]

Systems analysis of -omic (genomic, transcriptomic, proteomic or metabolomic) data helps to identify the biological processes and pathways which are most affected in the system being studied and help to highlight the key genes, proteins and metabolites as potential biological indicators and drug targets. These biological indicators are also known as biomarkers, and they help to identify differences in disease populations and mark response to therapeutic strategies. The complex and interconnected nature of biological processes which underlines health and disease responses offers unique opportunities for using systems approaches to identify novel biomarkers. A systems perspective on disease involves the integration of several elements, from genome through phenotype as depicted in Fig. 1.1

Within systems biology, metabolomics has become a key platform, allowing the comprehensive and high throughput study of small molecular weight substances in cells, tissues and/or whole organisms. Metabolites represent the downstream products of gene expression and are closer to the phenome than the proteome or transcriptome. Even subtle changes in the genes or proteins can give rise to as much as 10,000-fold change in the concentration of certain metabolites.[4] The future of metabolomics lies on its ability to capture even smaller perturbations in the metabolome which occurs prior to the gross phenotypic manifestation of the disease.

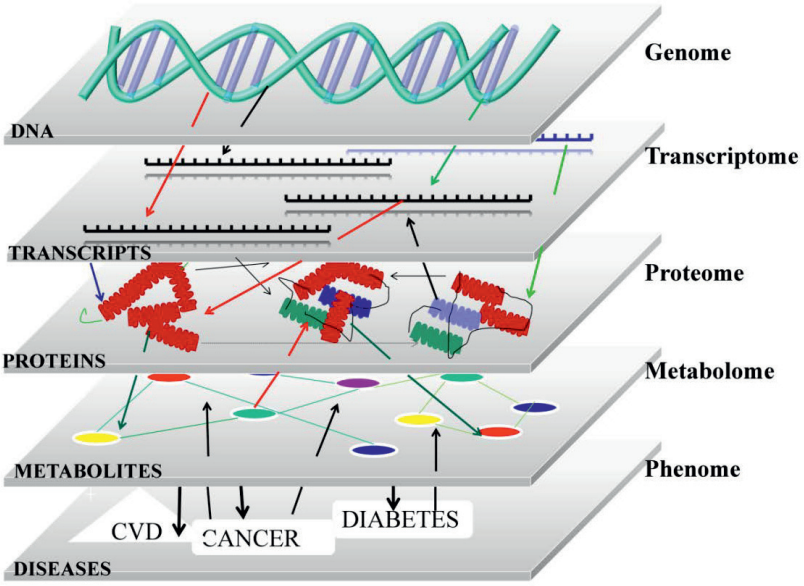

Figure 1.1: Different levels of "omics" cascade. Integrated analysis of quantitative measurements at different levels of the cascade is used in the systems perspective of complex diseases. Metabolites lies close to the phenome and may better represent the dynamic changes in the phenotypes.

Biological mapping and identification of biomarkers using metabolomics is a rapid and objective technology with high clinical relevance within risk screening, patient stratification, and preventive medicine and in treatment monitoring. Further, application of metabolomic techniques can contribute to achieve a biological understanding of complex diseases and can complement the knowledge gained from other molecular techniques. Considering the complexity of most common diseases, a panel of biomarkers which portrays the major aspects of pathophysiology may provide additional information to the clinicians. Clinical decision making based on a panel of biomarkers may address the future goal of personalized medicine by identifying the

individuals who will better benefit from a specific therapy. This thesis is aiming to identify and develop potential biomarkers of healthy and diseased state by applying metabolomics.

1.1.1 Aerobic fitness

Aerobic fitness refers to endurance or the ability to exert for an extended period of time.[5] During aerobic exercise, oxygen is consumed from the atmosphere and is transferred to the muscles via the circulatory system. An individual's aerobic fitness is dependent on age and sex, and can be improved by exercise. The indicator of aerobic fitness level is known as maximal oxygen uptake (VO_{2max}). VO_{2max} is determined by the measurement of oxygen uptake during the performance of maximal work, typically while running on a treadmill or while cycling (Figure 1.2). VO_{2max} is often expressed as the maximum volume of oxygen consumed per minute. It may also be expressed as the absolute volume of oxygen consumed in liters per minute to indicate total work capacity, or volume of oxygen consumed per minute per kilogram body weight.[6]

As with other physiological functions, there are large individual variations in VO_{2max} of subjects of the same age and gender. Some people show high VO_{2max} without regular exercise owing to genetics and other factors, while some other people who exercise regularly do not show high VO_{2max}. Genetics plays a major role in a person's VO_{2max} and heredity can account for up to 25-50% of the variance seen between individuals.[7]

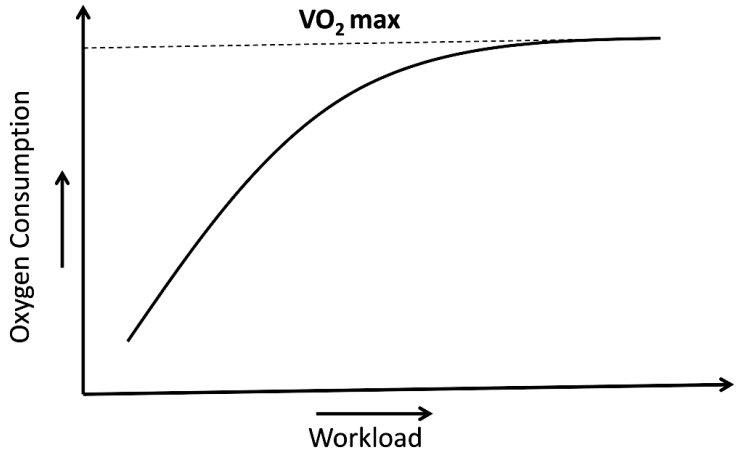

Figure 1.2: Maximal oxygen uptake. The point at which the oxygen consumption saturates is the VO_2 max or the maximum oxygen uptake of an individuals. Adapted from Kent[8]

Several studies report that aerobic fitness level, measured by the VO_{2max}, is the single best predictor of future CVD mortality in healthy people.[9-13] Aerobic power appears to have more of an influence on CVD risk factors than physical activity level.[14] Based on this, more knowledge of the differences between healthy individuals with a large difference in VO_{2max}-level will be of great interest to identify novel biomarkers of low aerobic fitness that may also have a potential as an early biomarker of CVD risk.[13,15-19] Identifying early markers of aerobic fitness may be helpful in actuating preventive strategies in individuals before developing the symptoms of CVD. Furthermore, such markers may be of help in contributing towards the deeper understanding of the complex molecular mechanisms behind fitness and cardiovascular diseases.

1.1.2 Cancer metabolism

Cancer is a disease characterized by uncontrolled proliferation of cells in which the cells acquire genetic changes that allows them to evade the normal growth regulatory signals.

The key biological changes occurring in the development of cancer, the hallmarks of cancer, are sustained proliferative signals, evasion of growth suppressors, resistance to cell death, attaining replicative immortality, induction of angiogenesis and activation of invasion and metastasis.[20] Reprogramming of cellular metabolism and evasion of immune mediated destruction has recently been added as two emerging hallmarks. Metabolic changes occurring in connection with cancer has been noticed almost a century ago by Otto Warburg in his description of a switch in glucose metabolism from oxidative phosphorylation to glycolysis — the Warburg effect.[21] In order to support the high rate of cellular proliferation, cancer cells show a shift in its metabolism towards biosynthesis.

Intrinsic genetic mutations and external responses to the tumor microenvironment in turn control the metabolic phenotypes of tumor cells. Alteration in the cellular growth signaling pathways in cancer cells changes the cellular metabolism to match the need of cell division. Changes occur in the cellular metabolism of cancer cells to provide a continuous and rapid energy supply (ATP synthesis).[22] Beyond the Warburg effect, other changes can occur in the glycolytic pathway (e.g. up regulated M2 isoform of pyruvate kinase) to channel substrates through alternative pathways like the pentose phosphate pathway (PPP) and other pathways so that large quantities of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and other macromolecules are produced.[22,23]

Phospholipids play an important role in proliferating cells as they form an important component of the cell membrane. In eukaryotic cell membrane, phosphatidylcholine

(PtdCho) is the most abundant phospholipid.[24] Phosphocholine (PCho, a precursor and a breakdown product of PtdCho) together with other phospholipids such as phosphatidylethanolamine (PtdEtn) and neutral lipids forms the characteristic bilayer structure of cellular membrane.[25] Changes in the levels of choline containing metabolites are observed in most cancers and have complex links to malignant transformation and oncogenic signaling.[25] This is further complicated by factors like hypoxia and acidic pH in tumor microenvironment which will also contribute to alteration in choline metabolites.[25,26]

Cancer cells are usually under higher oxidative stress compared with normal cells. The production of two of the most abundant antioxidants, reduced NADPH and glutathione (GSH), has been shown to be modulated in cancers. Shunting of glycolysis through PPP results in NADPH production which can act as a crucial anti-oxidant and can fuel macromolecule synthesis.[27] Hence, the metabolic alterations present in cancer cells promote not only ATP resources, but also macromolecular biosynthesis and redox control (Figure 1.3).

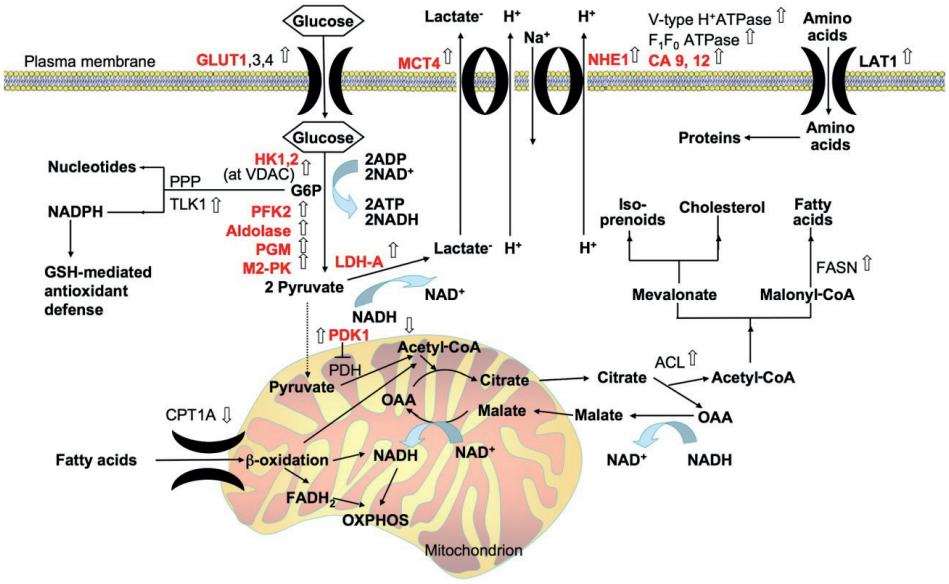

Figure 1.3: The metabolic reprogramming in tumor cells. Small arrows pointing up or down indicate cancer associated upregulation or downregulation of enzymes. Changes indicated in red can be caused by the activation of HIF-1. CA9 and CA12, carbonic anhydrases 9 and 12; CPT, carnitine palmitoyltransferase; GLUT, glucose transporter; GSH, glutathione; HIF, hypoxia-inducible factor; IDO, indoleamine 2,3-dioxygenase; HK, hexokinase; OXPHOS, oxidative phosphorylation; LAT1, L-type amino acid transporter 1; LDHA, lactate dehydrogenase isoform A; MCT, monocarboxylate transporter; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PI3K, phosphatidylinositol 3-kinase; PGM, phosphoglycerate mutase; PKM2, pyruvate kinase isoform M2; PPP, pentose phosphate pathway; SCO2, synthesis of cytochrome c oxidase 2; TLK, transketolase; VDAC, voltage-dependent anion channel. Reprinted with permission from Kroemer et al.[28]

Genetic mutations in the isocitrate dehydrogenase gene (IDH1/2) with the production of an 'oncometabolite' 2-hydroxyglutarate (2HG) have been found in gliomas and leukemias.[29] Specifically this mutation is seen in more than 70% of the secondary gliomas. This finding strengthens the links between mutations in metabolic genes and

common cancers and opens up potential opportunities in exploring metabolic targets in brain cancers and leukemias for diagnosis and prognostication.[30]

1.1.3 Astrocytomas

Astrocytomas are one of the most common primary brain tumors in humans and are subdivided into histological grade II - IV according to the WHO classification (Table 1.1). They arise from the star-shaped cells (astrocytes) that form the supportive tissue of the brain. Apart from astrocytes, other supporting cells in the central nervous system include oligodendrocytes and ependymal cells. Tumors arising from these supporting cells, also known as glial cells are collectively known as gliomas. Noninfiltrating astrocytomas usually grow more slowly than the infiltrating forms. Infiltrating, or diffuse astrocytomas are more common than noninfiltrating astrocytomas.[31] They are generally more common in men and are most common in the cerebral hemispheres of adult patients. Due to the infiltrative growth into adjacent brain tissue a complete surgical removal is not possible. Diffuse astrocytomas tend to recur and lower grades frequently undergo malignant transformation despite advances in radiotherapy and chemotherapy.[32] As there are differences in the management of high and low grade astrocytomas, it is essential to identify molecular and metabolic factors that may stratify these patients with regards to optimal treatment and prognostication.

Table 1.1: WHO Classification (Adapted from Louis et al.[33])

Grade	Prognosis	Examples
I	Excellent prognosis	Juvenile pilocytic astrocytoma Pleiomorhic xanthroastrocytoma
II	Variable Prognosis	Astrocytoma
III	Poor prognosis	Anaplastic astrocytoma
IV	Aggressive tumor	Glioblastoma multiforme

1.1.4 Malignant serous effusions

Serous cavities in the human body are the mesothelial lined potential spaces surrounding lung, heart and abdomen. Normally these cavities are obliterated and contain a very small amount of fluid, which is an ultrafiltrate of plasma. When the production and resorption of this ultrafiltrate are unbalanced, fluid may accumulate, resulting in an effusion. It is believed that the mechanisms underlying malignant effusion accumulation include lymphatic obstruction by metastatic cells impeding the outflow of peritoneal fluid, increased vascular permeability and new blood vessel formation, increased production by lining cells, changes in the peritoneal stroma and fibrin accumulation.[34,35] A major portion of the increase in vascular permeability which contributes to effusion formation is caused by malignancy-induced angiogenesis, resulting in accumulation of protein-rich fluid (a filtrate of whole blood) in the peritoneal cavity. The accumulation of malignant effusions is a common event in clinical practice. The diagnosis of malignant effusion indicates disease progression and is associated with a worse prognosis. Effusions containing tumor cells may accumulate

within the serosal cavities, i.e. the peritoneal, pleural and pericardial cavity in practically every cancer type. In adults, the most common organs of origin are the breast, lung and ovary, with gastrointestinal cancers as an additional relatively common origin, especially in Asian countries.[34] In addition to metastases, the serosal cavities are the site of origin of several cancers, including malignant mesothelioma and primary peritoneal carcinoma, although these are by far outnumbered by metastatic cancer. The finding of cancer cells in effusions is generally a marker of advanced-stage disease and is associated with poor survival in the majority of cases.[34] Often, the patients with malignant effusions are in a critically ill situation, and it is difficult to obtain a tissue sample or metastatic nodule for biopsy. In these patients, the analysis of the malignant fluid may be the only feasible option. Malignant effusions in serosal cavities represent an important source for potential metabolic markers. It may aid in understanding more about the metabolic basis behind malignant effusions, to identify novel biomarkers for diagnosis and treatment and to discover potential targets for therapy.

1.2 Metabolomics

Metabolomics provides a 'top down' integrated view of complex biochemical events occurring in complex organisms by measuring the global, dynamic metabolic responses with a wide array of analytical techniques. Magnetic resonance spectroscopy (MRS) and mass spectrometry (MS) are the most commonly used analytical methods for metabolomic studies.[36] These techniques also help in metabolite identification by providing information on the metabolite structure.[37] Owing to the diversity in metabolites with different physical and chemical composition, it is practically impossible to explore the entire metabolome using a single analytical technique. Even

though there is an intimate connection between genes, proteins and metabolites in a biological system, gene and protein expressions often may not directly correlate to the metabolite concentrations. This emphasise clearly the need for an additional measurement at the metabolite level and the role of metabolomics in studying gene/environment interactions (Fig 1.4).[38]

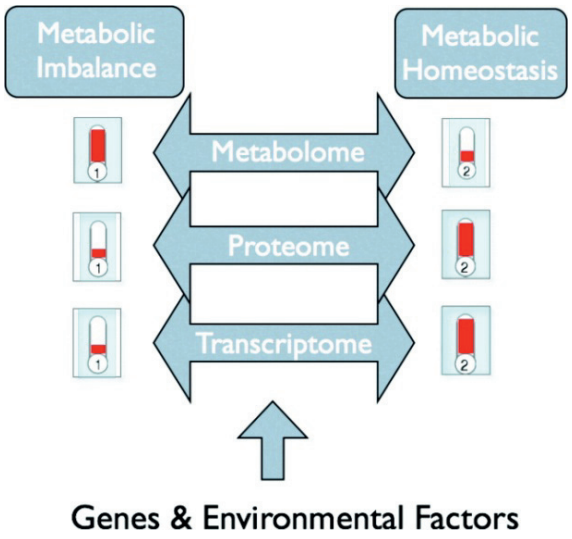

Figure 1.4: Metabolomics to study gene/environment interactions. Metabolism can be affected in two different ways in an extreme scenario. In the first case, a small mutation in the genome can cause minor changes in the transcriptome but a bigger change in the metabolome. Secondly, to maintain the homeostasis of the biological system in response to stressful stimuli, a large change in the transcriptome and proteome can occur with minimal change in the metabolome, hence maintaining the metabolic homeostasis. The figure is reprinted with permission from Manuel Mayr. [38]

Although classic genetics aims to relate the DNA sequences directly to the phenotype, "-omic" technologies allows to move the focus from a specific gene to the actual effect of the gene. Since it is impossible to correlate the gene or protein profiles directly to the

metabolic composition, the importance of measuring small molecular weight metabolites are gaining wider attentions. By "metabolomic profiling", it is now possible to perform quantitative and qualitative measurement of a subset of metabolites in biological samples such as body fluids and tissues. Similar to other "-omic" studies, metabolomics aims for objective and unbiased measurements of metabolite dynamics.[39]

Metabolomic studies offer certain advantages. Being downstream in the traditional biological information cascade from genes, transcripts and proteins, metabolic perturbations will be more close to the phenotype. The metabolome is highly dynamic and changes can occur in short intervals of time (within seconds), and can thus be a rapid indicator of biological changes. Hence metabolic perturbations may have the potential for capturing early changes in clinical systems far ahead of the appearance of disease symptoms and more invasive measures are required.[16]

A typical metabolomic study follows a common workflow.[16,39,40] It starts with a biological question and experiment followed by sample collection. After sample preparation, appropriate analytical experiment(s) are performed to acquire data. The high density metabolic data is then subjected to pre-processing and analysis followed by biological interpretation (Fig. 1.5). The analytical techniques MRS and MS are commonly used for metabolomics studies. Both techniques have their own advantages and disadvantageous. MS is more sensitive than MRS while MRS is more reproducible, needs minimal sample preparation and can be performed in a non-destructive manner. Furthermore, advances in high-field clinical scanners and newer methods for *in vivo*

MRS offers potential for future clinical translation of the *ex vivo* MRS markers to aid *in vivo* diagnostics.

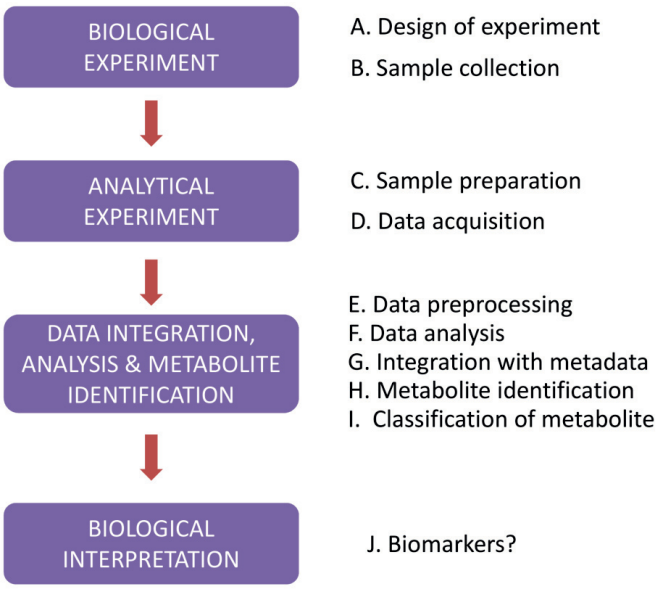

Figure 1.5: Metabolomics Work Flow. Adapted from Mamas M. et al[39]

1.2.1 MR spectroscopy

MR Spectroscopy (MRS) is an analytical technique which can detect and quantify a wide range of biochemical metabolites. All nuclei with non-zero spin have an intrinsic magnetic moment and may be studied by MRS. Spin ½ nuclei that are commonly studied include ^{1}H (the most popular nucleus for NMR studies), ^{13}C, ^{19}F and ^{31}P. In contrast, the abundant isotopes of carbon and oxygen, 12C and 16O, have an even number of both protons and neutrons which form pairs to cancel out the individual spins and hence cannot be studied by MRS. The high natural abundance of protons (^{1}H) in organic compounds and biological samples has made it a common nuclei for magnetic

resonance spectroscopy. Phosphorus MRS (^{31}P) is of particular interest for studies on phospholipid analysis and energy metabolism.[41]

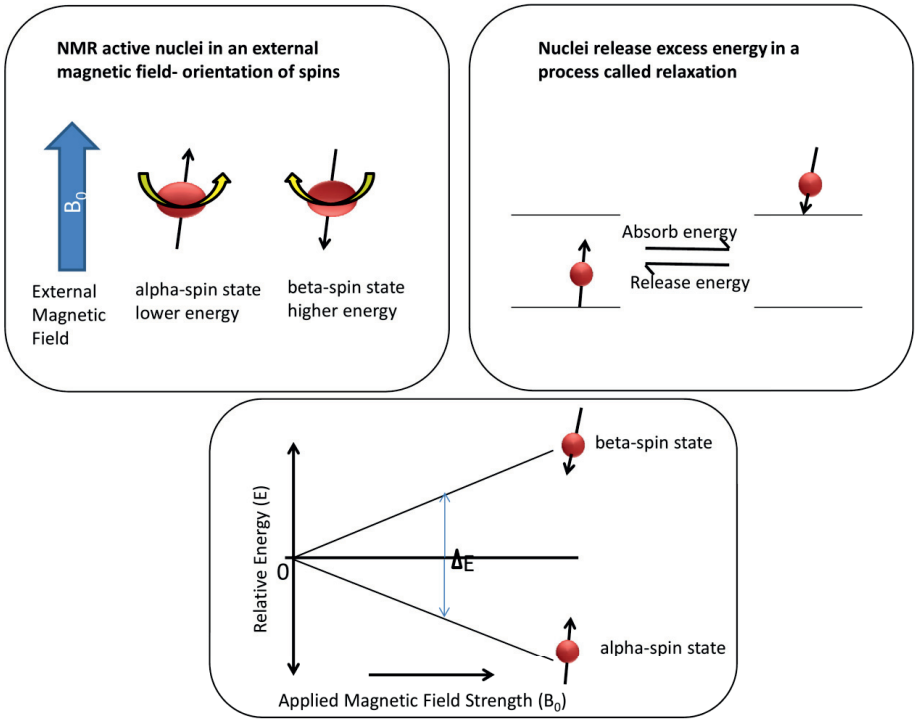

Figure 1.6: Basic principles of magnetic resonance. The figure illustrates the different spin states, energy differences and the field (B_0) frequency relationships. Figure adapted from Shung et al[42]

In the absence of an external or applied magnetic field (B0), the nuclear spins orient randomly. However, when there is an applied magnetic field, the nuclei orient themselves with or against the larger applied field. The spin state which is parallel to the applied field has lower energy than the spin state which is antiparallel to the applied field. The energy difference ΔE between the spin states is proportional to the strength of B0 (Figure 1.6). Spins in the lower energy states can be transferred to a higher energy

state by applying an external radio frequency (RF) pulse. Following an RF pulse, spins return back to their low energy state, emitting the energy back as radio waves. This emitted energy can be detected and forms the basis of the MR signal.

A plot of intensity of MR signal versus the magnetic field frequency is known as the MR spectrum. When the spins returns back to equilibrium, they go through relaxation processes characterized by two time constants called longitudinal (T1) and transverse (T2) relaxation. T1 relaxation depends on the net transfer of the energy to the environment. Larger molecules like proteins and lipids have a relatively short T1 while smaller molecules have a longer T1. The decay of transverse magnetisation (T2) depends on the dephasing of individual magnetic moments. Factors like molecular motion, viscosity, temperature, free water content, presence of paramagnetic atoms and field inhomogeneity can affect the T2. In MR experiments, the T1 and T2 values of molecules are important in setting up the correct acquisition protocols. For obtaining accurate relative signal intensities from a sample, a recycle delay of at least five times the longest T1 has to be used, so that all nuclei can return back from their excited state to equilibrium before the subsequent excitation. Similarly large molecules have a short T2, which can be exploited to filter out the signals from macromolecules like lipids.

The molecular environment around a nucleus is slightly (typically by a few parts per million) modified because of the shielding effect of the electron cloud resulting in small changes in the effective magnetic field experienced by the nucleus. This results in small changes in the resonance frequency of the given nucleus. Being very small, this shift in resonance frequency is expressed in relation to a standard reference frequency and is known as the chemical shift.

Higher magnetic field strength offers well resolved and detailed spectra of small metabolites. For example, overlapping resonances from glycerophosphocholine (GPC), phosphocholine (PCho), and free choline *in vivo*, can be studied separately at higher field strengths with *ex vivo* high-resolution MRS. The greater spectral resolution with increasing magnetic field strength also enhances the quantification precision.

1.2.2 MRS of biofluids

Metabolic profiling of biofluids can provide an extensive view of changes in endogenous metabolites in monitoring cellular responses to normal physiology or perturbations such as diseases and drug treatments.[43-50] Clinical biomarkers of disease conditions are best found in the biofluids which is bathing the most affected organ. For example markers of lung diseases may be present in saliva or breath-condensate, cerebro spinal fluid for neuronal diseases, urine or blood for kidney diseases, and blood for cardiac diseases. Among the biofluids, blood and urine is more widely used for clinical metabolomic studies.[51-56] Analysis of metabolites in biofluids as a diagnostic tool has several advantages such as non-invasive or minimally-invasive sample collection and the possibility of multiple sample collection over a time course thus making it an ideal choice for clinical studies.[57]

1.2.3 HR-MAS MRS

The line width of an MR signal depends strongly on the microscopic environment of the nucleus under study. MR spectra from solid or semi-solid tissue samples present much broader signals compared to liquid samples due to their large dipolar interactions and

chemical shift anisotropy. In liquid samples the rapid isotropic motion of the molecules averages the anisotropic interactions, resulting in an isotropic chemical shift frequency and a disappearance of the line broadening due to dipolar couplings. In solids, the lack of mobility leads to anisotropic broadening and spectra with overlapping signals.[58]

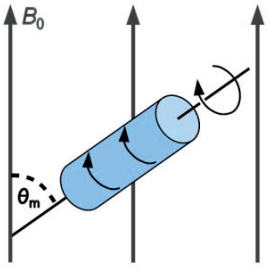

Figure 1.7: Magic-angle spinning: The sample (blue) is rotating with high frequency inside the main magnetic field (B_0). The axis of rotation is tilted by the magic angle θ_m with respect to the direction of B_0. Image credits to Wikimedia Commons.

When the sample is spun at an angle (θ_m) to the magnetic field (Figure 1.7), the dipolar interactions between the nuclei are dependent on the angle and the spin rate. At an angle of 54.7 degree also known as 'magic angle', some of the dipolar interactions are annulled and hence results in improved spectral resolution.[58] This technique has been successfully used to study the metabolic profiles of intact tumor samples from breast, brain, kidney, prostate and lung tumors.[59-64] The technique is non-destructive and requires only minimal sample preparations. The sample remains intact after the MR

experiment and can be used for further analysis like histopathology, proteomics and gene expression studies.

1.2.4 MRS data acquisition

Metabolic data acquisition is typically performed on biofluids or tissue samples (biopsies). Typical proton spectra of malignant effusions associated with carcinoma of breast, ovary and mesothelioma obtained from a high resolution spectrometer (Fig. 1.8) are comprised of sharp signals (narrow line width) from low molecular metabolites such as sugars, amino acids and small metabolites as well as broad signals from different groups of lipids and macromolecules. Most of the biological samples contain a high proportion of water protons and the huge size of the water peak can strongly limit the dynamic range of the metabolite detection and loss of signal from low concentration substances. Hence, suppression of water signal by specialised pulse sequences that use water presaturation or excitation sculpting is commonly used to improve the signal-to-noise ratio for endogenous metabolites.[65] Based on the differences in spin properties of macromolecules and small metabolites, there are several spectral filtering techniques which can selectively enhance or suppress specific groups of metabolites. Macromolecules tend to have shorter T2 relaxation times and smaller diffusion coefficients than those of smaller molecules due to their longer rotational correlation times and limited translational motion. Hence it is possible to filter the MR spectra based on these properties. Smaller molecules such as endogenous metabolites present in biofluids can be observed selectively by applying spin-echo loops (Carr–Purcell–Meiboom–Gill (CPMG)), based on their longer relaxation times, prior to NMR data acquisition. This is known as T2-edited spectroscopy.[66]

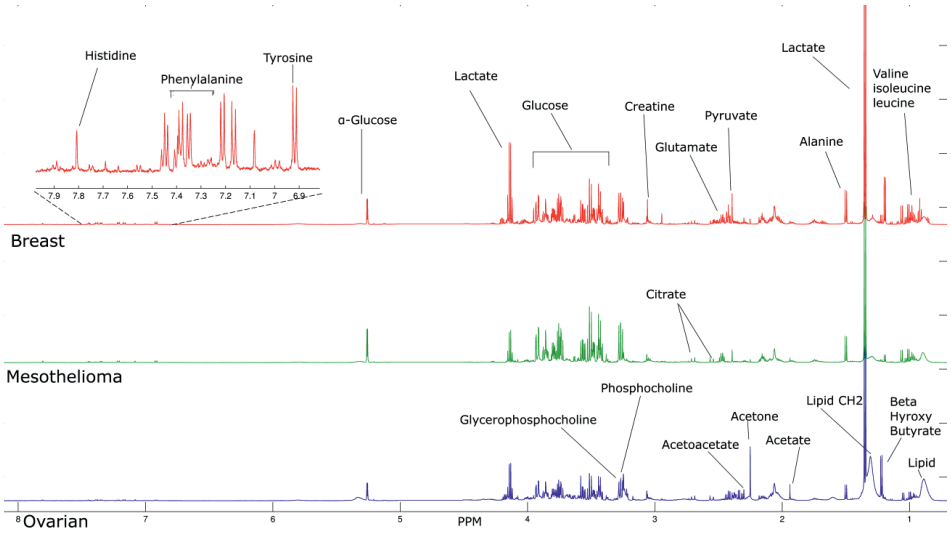

Figure 1.8: Proton magnetic resonance spectra from malignant effusions: Assignments of various metabolites visible in the MR spectra are shown. The region between 6.9 ppm-7.9ppm is scaled up to show the assignments. The red spectrum is from breast carcinoma effusion, the green from mesothelioma and the blue from ovarian carcinoma. Reproduced with permission from Vettukattil et al.[67]

1.3 Data analysis

Metabolomic experiments generate large amounts of data which needs sophisticated and powerful computational tools for proper analysis and interpretation. Multivariate data analysis techniques are able to tackle the colinearities in the MR spectral variables and are commonly used in metabolomic studies. Pattern recognition tools are used to analyse the large multivariate datasets. Both unsupervised and supervised techniques can be used to derive metabolic profiles.[68]

1.3.1 Preprocessing of MR spectra

Prior to multivariate analysis, several preprocessing techniques are applied on the MR spectra to improve the quality of the spectral data for multivariate analysis. Optimal preprocessing techniques are critical in determining the outcome of data analysis.[69] Commonly employed preprocessing routines in MR metabolomic data are baseline correction, scaling, normalisation and peak alignment.

Baseline correction is used to remove the baseline distortions in MR spectra. Baseline distortion can offset the intensity values and result in inaccuracy in peak assignment and quantification. Scaling and normalisation are used to make the data from all samples directly comparable to each other. Special care should be taken during this stage of data analysis to avoid wrong comparison between spectral data.[70] One of the commonly used normalisation technique is to set the total spectral area to a constant sum, known as area normalisation. Area normalisation compensate for the differences in sample weights and concentration. Other commonly used normalisation techniques are range normalisation and normalisation to a "housekeeping" metabolite.[70] The variation in pH, temperature and intermolecular interactions results in misalignment of peaks in MR spectra. Alignment of spectral peaks is an important step before multivariate analysis. Several peak alignment algorithms are used to achieve a properly aligned spectral data.[71] Commonly employed peak alignment techniques for MR spectra includes interval correlated shifting (icoshift)[72], correlation optimized warping (COW)[73] and parametric time warping (PTW).[74]

1.3.2 Principal component analysis (PCA)

PCA is a commonly used non supervised technique for multivariate data exploration.[75] It reduces the dimensionality of the data and reveals the hidden structure within a dataset. The variance structure of the data is explained through linear combinations of the variables called principal components (PCs). The first PCs will be in the direction explaining most of the variance in the data set. In the score plot of the PCA, samples with a similar metabolic profile will cluster, while the corresponding loading profile displays the importance of each variable within the PC.

1.3.3 Partial least squares (PLS)

Partial least squares is a supervised analysis method used to identify the fundamental relations between two matrices, usually the spectral data X and the clinical outcome or some other sample characteristics Y.[75] Similar to PCA, PLS is also a linear decomposition technique while it differs in the optimization problem that is solved to find a projection matrix. PLS finds projection directions for which the covariance between the data matrix or predictor variables, **X**, and the responses, **Y** is maximized. PLS models can be interpreted in a similar way as PCA models using the scores and loadings plots. PLS Discriminant Analysis (PLS-DA) consists of a classical PLS regression where the response variable is a categorical one and expresses the class memberships.

1.3.4 Multilevel analysis

In metabolic studies where the metabolic changes of interest are subtle compared to the between subject variation, it may be difficult to capture the relevant information. If the

interventions are performed on the same patient, it is possible to utilize the multilevel structure of the data to capture within subject variations.[76] Multilevel PLS-DA (MLPLS-DA) is used for paired comparisons of multivariate data. MLPLS-DA can be considered a multivariate extension of a paired t test that generates different multivariate submodels for the between-subject and within-subject variation in the data. This allows to split the variations and hence to analyse without being confounded by the other variation sources.

1.3.5 Validation

Validation of multivariate models is a crucial step to evaluate the performance and stability of the statistical model. This involves several techniques like cross validation, independent test sets and permutation testing. Using a separate independent test set with similar type of samples as used in the calibration set would be the ideal way for validation. However, in most of the real life situations, we have access to only a finite set of samples, usually less than what is wanted. Hence an approach called cross validation is used where the data is split into a training set and a test set. In a full cross validation (leave one out), only one sample is used for testing the model while all other samples are used to build the model. The process is repeated leaving one sample at a time for the whole dataset, and the average of the classification result is estimated. This is a convenient technique for small sample size (n~20) at the expense of overfitting and over optimistic results.[77] Alternate approaches includes using random subsets (a small percentage of the whole data) to test the model.

A permutation test is used to assess the statistical significance of the classification results.[78] During this process the class labels are randomly assigned to the samples. The classification result is calculated after the permutation and after repeating the process several times (typically >1000).[79] The classification error distribution from the model using permuted classes can be compared with the original model to assess the significance of the model.

1.4 Biomarkers

Recently, biomarkers have gained wide attention among the scientific community and clinical practice. Clinicians are always in need of tools to aid in better patient management, and valid biomarkers can significantly help in risk stratification, effective prognostication and to effectively treat patients with diseases. A consensus panel at the National Institutes of Health standardized the definition of a biomarker in 2001 as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other health care intervention'.[80] A biomarker can be measured in biological samples (like serum, tissue or urine), it can be recorded (like blood pressure, Electro cardiogram) or it can be a parameter derived from an imaging test like MR or CT scan.

Potential usefulness of biomarkers can range along the whole spectrum of the disease process. Prior to diagnosis, they can be used for screening and risk assessment. On the other hand, during diagnosis, biomarkers can aid in staging, grading, and selection of

initial therapy. During therapy, they can be useful in monitoring therapy, selecting additional therapies, or in monitoring recurrence.

1.4.1 Characteristics of an ideal biomarker

Optimal patient management is the key expectation out of a biomarker. A biomarker will be of clinical value only if it is accurate, reproducible, acceptable to the patient, easy to interpret by clinicians, and has high sensitivity and high specificity. A new biomarker should prove its ability in multiple studies by showing its ability to explain the outcomes independent of the established predictors in a consistent pattern. There should be validation data to suggest that knowledge of biomarker levels can change the patient management. During validation, performance characteristics like sensitivity, specificity and reproducibility of the biomarker must be evaluated.

The clinical value of a biomarker depends on its accuracy, reproducibility of the measurements, and patient compliance. The intended use of a biomarker may affect the desirable characteristics of a biomarker. For biomarkers indicating disease progression or treatment response, sensitivity or specificity are less important compared to screening biomarkers because the patient serves as his or her own control.[81] Similarly, costs may be less important for prognostic markers because only people with the disease are tested.

The course of biomarker development faces complex challenges and uncertainty. Most of the current biomarkers do not satisfy the required characteristics. Validation and demonstration of clinical utility of new biomarkers needs generation of prospective

data. If a biomarker is to be used as a surrogate endpoint, it needs the highest level of evidence to prove that the marker accurately predicts the clinical endpoint of interest in well-designed studies.[82]

Table 1.2: Biomarker Validation and Qualification

Type of Biomarker	Definition	Purpose
Exploration	Research and development tool	Hypothesis generation
Demonstration	Probable or emerging biomarker	Decision making, supporting evidence with primary clinical evidence
Characterization	Known or established biomarker	Decision making, dose finding, secondary/ tertiary claims
Surrogacy	Biomarker can substitute for a clinical endpoint	Regulatory approval

NOTE: Shown are four categories of biomarkers used for drug development and their intended purpose.
SOURCE: Adapted from Wagner, 2006.[83]

In a complex and heterogeneous diseases like cancers, it is unlikely that a single biomarker can detect all the subtypes and stages of the disease with optimum sensitivity and specificity. Combining several biomarkers could be a way to improve the sensitivity of diagnostic markers. Multiple biomarker panels have been tried in cardiovascular risk assessment and in ovarian carcinomas with improved results.[84,85] The four main categories of biomarkers used for drug development and their intended purposes are shown in Table 1.2.

Recent advances in molecular biology and development of microarrays, proteomics, metabolomics and nanotechnology has opened up new opportunities in the biomarker

development. A system biology based approach with cross collaboration between biologists, clinicians, chemists, computer scientists have greatly improved our ability to retrieve, analyse and characterize huge amount of data generated by the various –omic platforms. These approaches also focus on the multiple components of the deranged regulatory networks and uses multi parametric analyses to detect intricate derangements. Compared to a single biomarker or pathway analysis, this multi-parametric approach may provide better insight in to diagnosis, prognosis and treatment.[86]

1.4.2 Metabolomics in biomarker development

The development and application of novel "omics" technologies are directly related to the recent growth in biomarker discovery. Metabolomics allows simultaneous and parallel assessment of the metabolites present in cells, tissues and biofluids and helps to capture the alteration in the biochemistry associated with pathologies. The process of biomarker discovery involves different phases ranging from preclinical exploration to clinical use and disease control.[87] The first step in metabolic biomarker search often begins with preclinical studies, comparing tumor tissue with non tumor tissue. These are exploratory studies to identify characteristics unique to tumor tissue that might lead to ideas for clinical tests for detecting pathologies like cancer. Key objectives of different phases in biomarker discovery are depicted in Table. 1.3. Most of the work in this thesis involves the first phase in biomarker search.

Table 1.3: Five Phases of biomarker development- from discovery to delivery (adapted from Pepe et al,[87] with permission from Oxford University Press)

Description	Phases	Objective
Preclinical Exploratory	PHASE 1	Identify promising directions & feasibility
Clinical Assay and Validation	PHASE 2	Clinical Assay to Detect established Disease
Retrospective Longitudinal	PHASE 3	Case-control studies using repository specimens
Prospective Screening	PHASE 4	Longitudinal studies to predict disease
Disease Control	PHASE 5	Clinical use

Although metabolomic studies of human diseases in the last decade have discovered a number of novel biomarkers, none have currently made the transition to routine use in clinical practice.[39] Metabolites identified from these early studies will need to form the basis of larger, prospective, externally validated studies in clinical cohorts for their future use in the clinics.

1.4.3 Metabolomics in cancer biomarker development

Biomarkers have a potential role in clinical medicine for prognostic and predictive purpose. Several exploratory studies are done on cell cultures, experimental animals for evaluating metabolic biomarkers for cancer diagnostics followed by their evaluation in tumour tissue or biofluids.[88] Standard metabolomics experiments has shown that many tumors in general have elevated phospholipids, increased glycolytic activity,

channelling of glycolytic carbon for synthetic activity, and high glutaminolysis.[25,89,90] Metabolomics has been successfully used in breast and prostate cancer studies to detect changes in choline metabolites and glycolytic products.[91-93] Similarly, metabolomics studies in brain tumors and ovarian tumors have shown metabolic biomarkers of potential clinical value.[94-96] However, there are still several missing links in the knowledge about the tumor metabolome and the metabolic profiles vary among distinct tumor types making it difficult to generalise the findings among tumor groups.[68]

1.4.4 Metabolomics in cardiovascular health

Prognostic markers of adverse cardiovascular outcomes like low density lipoprotein (LDL) cholesterol are popular even among the general public. Diagnostic markers of acute changes like troponin I and troponin T are widely used to aid the diagnosis of myocardial infarctions. There is always a compelling need for identifying biomarkers of cardiovascular fitness, which can detect the adverse changes at a very early stage of the pathogenesis. Metabolomic studies have been used in experimental and epidemiological studies with the aim of detecting biomarkers of cardiac health. In patients with hypertrophic obstructive cardiomyopathy undergoing "planned myocardial infarction", metabolic profiling of the plasma samples revealed metabolic changes as early as 10 minutes following the procedure.[19] In a normal physiological context, exercise related metabolic changes are detected in the plasma of individuals running on a treadmill.[97] There are several studies which explored the metabolites in blood plasma associated with inflammation, oxidative stress and lipid metabolism, which are key mediators in CVD pathogenesis.[98-102] Although the application of metabolomics in the clinics is still

in its infancy, it is a powerful technique to address complex tasks such as cardio-vascular risk assessment, treatment response monitoring and patient management.

2 Thesis Objectives

The main objective of the research presented in this thesis was to evaluate the use of high resolution MRS together with multivariate analysis based metabolomic pipeline for identifying and characterizing potential biomarkers of health-disease continuum. In more detail:

1. To investigate metabolic differences between healthy individuals with high and low aerobic fitness (VO_{2max}) by MR metabolomics, and further to describe these differences qualitatively and quantitatively.

2. To characterize the metabolic profile of astrocytomas with *ex vivo* HR-MAS MRS and to establish MRS markers to distinguish WHO grade II (A-II) and grade IV astrocytomas (glioblastomas; GBM) at a metabolic level and to assess the correlation between MR spectral profiles, baseline patient characteristics, and preoperative magnetic resonance imaging (MRI)-defined tumor volume.

3. To identify the metabolic differences between ovarian serous carcinoma effusions obtained pre- and post-chemotherapy, as well as to compare ovarian carcinoma effusions with breast carcinoma and malignant mesothelioma specimens.

3 Materials and methods

3.1 Patients and data sets

In paper I, all the subjects were healthy people from the Nord-Trøndelag Health Study (HUNT3) in Norway, which was carried out between 2006 and 2008. Among 50,821 participants in HUNT3, 4631 healthy, adult subjects attended a sub-study called the Fitness Study, designed to measure VO_{2max}.[103] From the Fitness Study-population, 220 individuals between 40 and 59 years were selected pair-wise with one having low and the other high VO_{2max} (selected from top or bottom 15 subjects within each age-year), but otherwise same gender, equal age in years, same physical activity index score (within 15% difference) and equal time since last meal. Subjects were ranged according to VO_{2max} reported as $mL \cdot kg^{-0.75} \cdot min^{-1}$, and maximum five pairs of subjects were matched from each age-year. Two subjects did not provide a blood sample, and the study thus included 218 subjects (45 males and 63 females in the low VO_{2max}-group, and 46 males and 64 females in the high VO_{2max}-group).

The study cohort in paper II was 58 patients (median age, 57 years; range, 27-81 years; 27 female, 31 male patients) with histologically verified supratentorial, diffuse astrocytomas (48 glioblastomas and 10 A-IIs). Preoperative functional status of the patients was evaluated with the Karnofsky Performance Status Scale.[104] Preoperative MRI was used to determine tumor volumes. Tumor volumes were estimated with an ellipsoid volume formula ($\frac{4}{3}\pi r_1 r_2 r_3$) based on the maximum tumor diameters in the perpendicular dimensions as described elsewhere.[105,106] All surgeries were performed

under general anesthesia with an ultrasound-based neuronavigation system. Patient and tumor characteristics are presented in Table 3.1

Table 3.1: Baseline characteristics

Parameter	**GBM**		**A-II**	
Patients[a]	48		10	
Median (range) age (years)	58 (27-81)		47 (29-71)	
Gender(female/male)	25/23		2/8	
Preoperative KPS	KPS 90-100	10%	KPS 90-100	64%
	KPS 70-80	61%	KPS 70-80	27%
	KPS <70	29%	KPS <70	9%
Recurrent Tumor	17[b]		2	

Abbreviations: GBM, glioblastoma; A-II, WHO grade II astrocytoma

Note: Recurrent Tumor denotes that the patient has been operated earlier. For patients with GBM, this means that they have been operated for either an A-II or GBM previously. Four among 17 recurrent GBM has a previous histology of A-II.

[a]Unless otherwise indicated, values are numbers of patients.

[b]Four of the glioblastomas were secondary, i.e. previous histology was A-II.

In paper III, the samples comprised of pleural and peritoneal effusions from 95 patients diagnosed with ovarian carcinoma, 10 with breast carcinomas, and 10 with malignant mesotheliomas. Among the ovarian carcinomas, 8 were paired peritoneal specimens obtained pre- and post-chemotherapy from the same patient. All these specimens were submitted to the Norwegian Radium Hospital, Oslo, Norway from 1999-2012.

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

3.2 Sample handling

For paper I, which is focusing on serum markers of aerobic fitness, venous non-fasting blood samples were collected in serum-tubes with no additives. The blood was centrifuged at 3000 rpm for 10 minutes approximately 1 hour after sample collection. The serum samples were stored at −80°C in the HUNT biobank at Levanger, Norway until being used for metabolic profiling. Before analyses, the serum samples were slowly thawed at 4°C. Aliquots of 150 µL were mixed with equal amounts of buffer solution ($Na_2HPO_4 \times 7H_2O$ (0.075M), 4% NaN_3 in H_2O (5ml, mass % of NaN_3 versus mass % of H_2O), TSP (3-(trimethyl-silyl) propionic acid-d4, 0.4g), D_2O (100 mL), pH adjusted to 7.4 with 1M HCl (1M NaOH), filled up to 500 mL with H_2O) and transferred to high-quality 3 mm MR tubes. The ratio between H_2O and D_2O was 90:10 in all samples.

Paper II deals with tissue samples from brain tumors. During surgery, tumor biopsies for this study were put in cryogenic vials within a median delay of 60 seconds and snap-frozen in liquid nitrogen (-196 ºC). Separate biopsies from the same tumor area were sent for routine histological analysis. All samples to be analysed by HR-MAS were stored in a cryogenic tank to prevent biochemical degradation until analysis.

Samples in paper III were biofluids from pleural and peritoneal effusions. Effusions were submitted for routine diagnostic purposes and were processed immediately after tapping. Effusion specimens were centrifuged, and supernatants were frozen at -70°C. Subsequent treatments were similar to the serum samples in paper I

3.3 MRS protocol

3.3.1 MRS of biofluids

The MR spectra were acquired using a Bruker Avance III 600MHz/54 mm US-Plus (Bruker Biospin, Rheinstetten, Germany) operating at 600 MHz for proton (^{1}H), equipped with a QCI cryoprobe. All spectra were recorded in an automatic fashion using a Bruker SampleJet and the ICON-NMR software (Bruker Biospin, Rheinstetten, Germany). Proton spectra were obtained at a constant temperature of 300 K (27ºC) using a modified Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with presaturation during the relaxation delay (cpmgpr1d; Bruker Biospin pulse sequence library) to achieve water suppression and to facilitate the detection of low molecular weight species by avoiding the large overlapped signals derived from large molecules such as proteins and lipids. The spectra were collected with 64 scans and 4 dummy scans. The acquisition time was 3.067 seconds, measuring the FID via collection of 32K complex data points resulting in a spectral width of 20.0363 ppm. A relaxation delay of 4 seconds was used, during which presaturation at 25 Hz was applied. The receiver gain was kept at a constant value of 90.5 and the effective echo time was 80ms. The FIDs were Fourier transformed after exponential line broadening of 1 Hz. For metabolite quantification, nuclear overhauser effect spectroscopy (“noesy”, Bruker Biospin pulse sequence library: noesygppr1d) spectra were acquired using 32 transients with four

dummy scans, 96 K points per spectrum giving an acquisition time of 2.72 seconds and a mixing time of 10 ms, and apodized using an exponential line broadening parameter of 1Hz, and a 4 seconds recycle delay. Measurement and processing was done in full automation using Bruker Biospin standard automation programs controlled by ICON-NMR (along with TopSpin v3 patchlevel 3, Bruker Biospin, Rheinstetten, Germany).

3.3.2 MRS of tissue samples

The HR-MAS experiments were performed with a Bruker Avance DRX600 spectrometer with a $^{1}H/^{13}C$ HR-MAS probe (Bruker BioSpin GmbH, Rheinstetten, Germany) with magnetic field gradients aligned with the magic angle axis. Before the HR-MAS experiments were performed, tumor tissues were sliced to fit 30-mL leak-proof disposable inserts (Bruker Biospin Corp, Billerica, Massachusetts) filled with phosphate-buffered saline buffer (3 mL of a mixture of TSP [4.5 mmol/L], sodium formate [20 mmol/L] and D_2O-based phosphate buffered saline). Sample preparation was performed on an ice block to maintain a low-temperature working atmosphere. The inserts were further placed in zirconium MAS rotors (4mm). Mean ± SD sample weight was 7.7 ± 3.4 mg. All spectra were acquired at spin rate of 5 kHz and at $4^{o}C$ to minimize tissue degradation. Proton spectra were acquired with a spin-echo Carr-Purcell-Meiboom-Gill sequence (cpmgpr; Bruker Biospin pulse sequence library) with 3-second water suppression before a 90^{o} excitation pulse. T2 filtering to suppress broad resonances from lipids and macromolecules was obtained with an effective echo time of 32 ms. With an acquisition time of 1.64 seconds, 128 transients were collected over a 10-kHz spectral region containing 32K points, giving a repetition time of 4.64 seconds.

Phosphorous HR-MAS experiments were performed with a Bruker Avance III 600 MHz/54 mm US spectrometer with a $^{1}H/^{13}C/^{31}P$ HR-MAS probe (Bruker BioSpin GmbH, Rheinstetten, Germany). ^{31}P HR-MAS spectra were acquired with a spin rate of 5 kHz at 4 ^{o} C with the use of a 1-dimensional power-gated sequence with ^{1}H decoupling (zgpg; Bruker Biospin pulse sequence library). With an acquisition time of 0.67 seconds, 512 transients were collected (repetition time = 2.67 seconds) over a 24-kHz spectral region containing 32K points.

3.4 Data analysis

All multivariate analyses in this thesis were performed with MATLAB (version 7.9.0; The Math Works, Natick, Massachusetts) and PLS_Toolbox version 5.8.3 (Eigenvector Research, Manson, Washington). The spectra were peak aligned with icoshift.[72] PASW Statistics 17.0 (IBM, New York) was used for traditional statistical analyses. All statistical tests were two-sided, and p-values below 0.05 were considered statistical significant. Kolmogorov-Smirnov test was used to test for normality. One-Way ANOVA was used for comparing variables between the high and the low VO_{2max}-groups, and the Kruskal-Wallis test was used in non-parametric analyses. Results are given in mean ± SE. Pearson's correlation was used to study associations between normally distributed variables, and Spearman's correlation was used in non-parametric analyses.

3.5 Clinical diagnostics and Histopathology

Weight and height of subjects in the HUNT study (Paper I) were measured on a combined scale (Model DS-102, Arctic Heating AS, Nøtterøy, Norway), and BMI was

calculated as weight divided by height squared (kg m^{-2}). Blood pressure and resting heart rate were both measured while sitting (Critikon Dinamap 845XT, GE Medical Systems, Little Chalfont, Buckinghamshire, United Kingdom) and followed established guidelines.[107] An individualized protocol was applied to measure VO_{2max} treadmill running to exhaustion.[108] The VO_{2max}-test was performed using a ramp protocol where the speed was constant and the incline was increased with 2% every second minute until VO_{2max} was reached. All clinical-chemical analyses for the paper I was performed on fresh venous non-fasting blood samples at Levanger Hospital, Norway.

For routine histopathological analyses in paper II, resected astrocytoma tissue was fixed in buffered formalin and embedded in paraffin wax, and 5-mm-thick sections were cut and stained with hematoxylin, eosin, and saffron. An experienced neuropathologist examined all sections, and the astrocytomas were graded according to the latest WHO criteria into grades II to IV.[33] After HR-MAS analyses, the sections were examined microscopically to assess the fraction of tumor cells and necrosis. IDH1 expression status was evaluated using immunohistochemistry. Briefly, 5-mm-thick sections were incubated with the primary antibody (antihuman IDH1 R132H; Dianova, Hamburg, Germany; dilution, 1:10, incubation time, 30 minutes at room temperature) after quenching ofendogenous peroxidase activity with 3% hydrogen peroxide and antigen retrieval by pressure cooking. The immunostaining was carried out on a DAKO Autostainer (Dako, Glostrup, Denmark).

For the effusion samples in paper III, cell blocks were prepared using the Thrombin clot method. Diagnoses were established using morphology and immunohistochemistry.

Effusion specimens were centrifuged, and supernatants were frozen at -70°C. Smears and H&E-stained cell block sections were reviewed by a surgical pathologist experienced in cytopathology.

4 Summary of papers

Paper I

Serum levels of choline-containing compounds are associated with aerobic fitness level: the HUNT-study.

Cardiovascular disease (CVD) is a leading cause of death worldwide, and the number of people at risk is continuously growing. New methods for early risk prediction are therefore needed to actuate prevention strategies before the individuals are diagnosed with CVD. Several studies report that aerobic fitness level, measured as maximal oxygen uptake (VO_{2max}), is the single best predictor of future CVD mortality in healthy people. Based on this, we wanted to study differences between healthy individuals with a large difference in VO_{2max} -level to identify new biomarkers of low aerobic fitness that may also have potential as early biomarkers of CVD. Serum samples from 218 healthy individuals with a low VO_{2max} (n = 108, 63 women) or high VO_{2max} (n = 110, 64 women) were analysed with MR metabolomics. In addition, standard clinical-chemical analyses for glucose, lipids, liver enzymes, micro-CRP, and colorimetric analysis on circulating choline were performed. Individuals in the low VO_{2max} -group had increased serum levels of free choline, decreased phosphatidylcholine, increased glucose and decreased unsaturated fatty acids compared to the individuals in the high VO_{2max}-group. Aerobic fitness dependent differences in serum levels of free choline and

phosphatidylcholine are observed. They should be further studied as potential early markers of CVD risk.

Paper II

Differentiating Diffuse World Health Organization Grade II and IV Astrocytomas with Ex Vivo Magnetic Resonance Spectroscopy.

The prognosis and treatment of astrocytomas, which are primary brain tumors, vary depending on the grade of the tumor, necessitating a precise preoperative classification. Magnetic resonance spectroscopy (MRS) provides information about metabolites in tissues and is an emerging non-invasive tool to improve diagnostic accuracy in patients with intracranial neoplasia. This study aims to investigate whether *ex vivo* MRS could differentiate World Health Organization grade II (A-II) and IV astrocytomas (glioblastomas; GBM) and to correlate MR spectral profiles with clinical parameters. Patients with A-II and GBM (n = 58) scheduled for surgical resection were enrolled. Tumor specimens were collected during surgery and stored in liquid nitrogen before being analysed with high-resolution magic angle spinning MRS. The tumors were histopathologically classified according to World Health Organization criteria as GBM (n = 48) and A-II (n = 10). Multivariate analysis of *ex vivo* proton high-resolution magic angle spinning spectra MRS showed differences in the metabolic profiles of different grades of astrocytomas. A-II had higher levels of glycerophosphocholine and myo-inositol than GBM. The latter had more phosphocholine, glycine, and lipids. A significant metabolic difference between recurrent and nonrecurrent GBM ($P < .001$)

was observed. Primary GBM had more phosphocholine than recurrent GBM. A significant correlation ($P < .001$) between lipid and lactate signals and histologically estimated percentage of necrosis was observed in GBM. Spectral profiles were not correlated with age, survival, or magnetic resonance imaging-defined tumor volume. *Ex vivo* MRS can differentiate astrocytomas based on their metabolic profiles.

Paper III

Proton magnetic resonance metabolomic characterization of ovarian serous carcinoma effusions: chemotherapy-related effects and comparison with malignant mesothelioma and breast carcinoma

Malignant serous effusions are a common manifestation of advanced cancer, associated with significant morbidity and mortality. The aim of this study was to identify the metabolic differences between ovarian serous carcinoma effusions obtained pre- and post-chemotherapy, as well as to compare ovarian carcinoma (OC) effusions with breast carcinoma and malignant mesothelioma specimens. The supernatants of 115 effusion samples were analysed by high-resolution magnetic resonance (MR) spectroscopy *in vitro* and multivariate analysis. The samples comprised of pleural and peritoneal effusions from 95 OC, 10 breast carcinomas, and 10 malignant mesotheliomas. Among the OC, 8 were paired peritoneal specimens obtained pre- and post-chemotherapy from the same patient. OC had elevated levels of ketones (aceto-acetate and beta-hydroxybutyrate) and lactate compared to malignant mesotheliomas and breast carcinomas, whereas the latter had more glucose, alanine, and pyruvate. Multivariate analysis of paired effusions in OC showed a significant increase in glucose and lipid levels in the post-treatment spectra ($P=0.039$). MR spectroscopy is a promising

technique for comprehensive and comparative studies of metabolites in malignant serous effusions and our study shows that small metabolites associated with effusions might improve our understanding of tumor biology and disease progression and has diagnostic potential in this differential diagnosis.

5 Discussion

The main goal of this thesis was to evaluate the use of high resolution MR metabolomics for identifying and characterizing potential biomarkers in the health-disease continuum. Subjects in this thesis ranged from healthy volunteers (Paper I) to patients with advanced stage of malignancies (in Paper II and III). In the first study, aerobic fitness dependent differences in serum levels of free choline and phosphatidylcholines in a group of healthy volunteers were observed. In paper II, the possibility of differentiating diffuse World Health Organization Grade II and IV astrocytomas based on their metabolic profiles were showed. Metabolic markers of chemotherapy related changes in ovarian serous carcinoma effusions were identified in Paper III. In addition, the metabolic portraits of ovarian carcinomas were compared with breast carcinoma effusions and mesotheliomas in paper III. Throughout these studies, the utility of using a high resolution MR based metabolomics approach for biomarker discovery has been investigated. This thesis also evaluates the role of advanced multivariate analysis techniques in deciphering the complex and multidimensional spectral data. In paper I and III, the samples were biofluids and its usability in detecting perturbations of endogenous metabolites in normal physiology (aerobic fitness, paper I) and pathology (malignant effusions, paper III) was explored. The extension of the MR metabolomics technique to intact biopsy specimens from brain tumor patients was evaluated in paper II, and potential markers for tumor grading and prognostication were identified. The usefulness of MR metabolomic techniques in capturing molecular signatures of complex pathologies like cancer has been explored (paper II, III). This may contribute further to the scientific understanding of the underlying tumor biology.

5.1 Metabolic profiling for biomarker identification

MRS and mass spectrometry are the two main analytical techniques widely used in the study of metabolites in biological fluids and tissues from biopsy samples.[65,109,110] MRS offers a nondestructive and highly reproducible way for analyzing metabolites with minimal sample preparation. In all the studies included in this thesis, MR based metabolomics were used for metabolic profiling tissue or biofluids. All these studies generated snap shots of metabolic activity ranging from physiological (paper I) to pathological conditions (paper II and III). The complexity of the data was handled by multivariate techniques. To identify the biochemical similarity between samples and to capture the metabolic patterns indicative of a particular physiological or pathological state, linear projection methods like PCA and PLS-DA has been utilized in all papers (I-III). The usefulness of MRS based metabolomic for characterizing the biomarkers of clinical importance and to uncover disease mechanism has been evaluated in these studies.

Most of the works in this thesis are in the discovery or explorative phase of biomarker research. In an exploratory study, it is important to ensure that sample handling and data acquisition is optimized to maintain the stability of metabolites. Sample storage and preparation has to be optimal to avoid sample aging and degradation. In our study, all samples were stored and transported at very low temperatures (−80°C or −196°C) to ensure metabolite stability.[111,112]

The process of biomarker discovery has a long path when it comes to real clinical translation of the findings. The quality of the initial sample set is vital in determining the sensitivity, specificity, accuracy of biomarkers and the success rate of clinical translation. Often the term "differentiating metabolite" is more appropriate than the term biomarker for describing the metabolic markers identified in the exploratory phase of the biomarker discovery.[113] There is always a need for a multistep qualification process with rigorous assessment of precision, accuracy and diagnostic or prognostic value. In addition, it is also beneficial to have additional scientific evidence showing the link between a candidate biomarker and the pathophysiology of interest.

MR metabolomics is a useful technique to identify the biochemical signatures of physiology and pathology. The potential use of metabolomic biomarkers can range widely from screening, prognostication and prediction of disease recurrence to evaluation of treatment related changes. The different studies covered in this thesis have explored the potentials of metabolomic techniques in clinical (paper II and III) and in population based studies (HUNT study, paper I). A summary of potential metabolic biomarkers identified in this thesis are shown in Table 5.1

5.2 Analysis of metabolomic data

In all the papers included in this thesis, multivariate data analysis techniques played an important role in extracting the knowledge from the high dimensional MR spectral data. PCA and PLS-DA were used in paper I and II whereas in paper III, MLPLS-DA was also added to utilise the multilevel structure of the data. Since a single biomarker is often insufficient and nonspecific for a given condition, multivariate methods are of

special importance in metabolomics.[114] One of the advantages of multivariate techniques is the ability to use the whole spectra as an input for analysis, without the need for metabolite quantification. Although metabolite quantification is helpful in detailed understanding of metabolic complexities and for determining characteristic biomarkers, quantitative MRS of biological samples poses several challenges. High degree of overlap in the (peaks obtained from) MR spectra of biological sample makes it difficult to precisely quantify the metabolites. [32,115] The techniques such as peak fitting are more useful in targeted metabolomic studies than non-targeted exploratory studies and are often subjective and time consuming. Absolute metabolic quantification needs a stable reference compound which is added to the sample (external reference) or present inside the sample (internal reference). Using TSP as a reference is limited as it binds to proteins in the tissue samples/biofluids. Using a relative quantification approach based on metabolite ratio is often used. Ratio based approaches can be affected if there exists a positive correlation between the metabolites. In this thesis, being a non-targeted and exploratory phase of biomarker research, only multivariate techniques are used.

By using the whole spectra, multivariate techniques can identify the patterns of metabolites which are linked with the conditions or disease under study. The multivariate models used in this thesis are all linear models and allows the interpretation of the metabolites which are responsible for the discrimination between the study

Table 5.1 Summary of potential metabolic biomarkers identified in this thesis

PAPER	Clinical/Physiological condition		Metabolites(relatively increased)	n	Data analysis	Significance	Sample type	Subjects
I	Low VO2max		Cho, glucose	108	PCA, PLSDA	P <0.001	Biofluid	Healthy Volunteer
	High VO2max		PtdCho, unsaturated fatty acid	110				
II	Astrocytoma Grade II		GPC, myo-inositol	10	PCA, PLSDA	P <0.001	Tissue	Patients with malignancy
	Astrocytoma Grade IV (GBM)		PCho, glycine, Lipids	48				
	Necrosis in GBM		lipids, lactate	17	PCA, Pearson	P <0.001		
	Astrocytoma	Recurrent tumor	GPC, Myo-inositol and creatine	33	PCA, PLSDA	P <0.001		
		Non- recurrent tumor	PCho, Gly	31				
III	Serous effusion -origin	Mesothelioma and breast	glucose, alanine and pyruvate	20	PCA		Biofluid	Patients with malignancy
		Ovarian carcinoma	aceto-acetate, beta-hydroxybutyrate and lactate	95				
	Chemotherapy related changes	Pretreatment	Lactate, beta-hydroxybutyrate	8 pairs	ML-PLSDA	P <0.039		
		Post treatment	Glucose, lipid					

groups. This is important in biomarker studies which are in the exploratory phase so that they can contribute towards improving the biological insights and in possible identification of treatment targets. All PLS-DA models were cross validated to achieve reliable classification results. The classification accuracy was further evaluated using permutation testing to rule out spurious discoveries from random classifications.

The use of multivariate paired data analysis to capture chemotherapy related changes in the metabolic composition of serous effusions was explored in Paper III. In this analysis, the advantage of the multilevel structure of the data has been considered. This method uses the net difference before and after chemotherapy as the input for multivariate analysis so as to examine the treatment related variation. This technique is useful to detect the metabolic changes related to treatment which are more subtle than the larger variation between the patients.

5.3 Serum markers of aerobic fitness

In this study, the metabolic patterns related to aerobic fitness in a group of healthy individuals (218 subjects) using high resolution MR metabolomics of the serum samples were explored. The results showed that individuals with low VO_{2max} had decreased serum levels of phosphatidylcholine, increased choline and decreased unsaturated fatty acids compared to the individuals in the high VO_{2max} group. VO_{2max} being a single best predictor of future cardio-vascular disease related mortality, the indicators of low aerobic fitness may have a potential role in early CVD risk prediction.[9,11-13] Previously, metabolomics studies using plasma and serum samples have been successfully utilized to detect biomarkers associated with clinical conditions such as coronary artery disease

and myocardial infarction.[17,19,116] However, there is a scarcity of evidence on the link between serum metabolites and cardiovascular fitness. In this study, serum samples from a group of healthy volunteers were investigated for markers of aerobic fitness. To account for the age, gender, physical activity and fasting status dependent variations in the metabolites, the participants were thoroughly matched.

Elevated levels of serum free choline have previously been associated with metabolic syndrome with a cluster of CVD risk factors.[117] This may indicate that, elevated levels of cholines in the low VO_{2max} group may have importance in future CVD risk prediction. This needs to be evaluated in future studies. Such early markers of risk prediction may have value in actuating preventive strategies at an earlier stage. As there is a switch in the levels of choline- phosphatidylcholine metabolites between the two groups, there might be differences in the choline biosynthesis or breakdown systems in the body in relation to aerobic fitness. Previously, it was shown that phospholipase D (PLD) activity, an enzyme linked with choline synthesis has been elevated in patients with atherosclerosis, hypertension, oxidative stress and inflammations.[18] PLD catalyses the hydrolysis of PtdCho to phosphatic acid, releasing free choline. A high PLD activity in healthy subjects with low aerobic fitness can be speculated and needs further evaluation. In addition to changes in the levels of choline containing compounds, spectral profiles also indicated a decreased amount of unsaturated fatty acids in subjects with low aerobic capacity. Associations between low serums levels of unsaturated fatty acids have previously been associated with CVD risk.[118]

There were certain limitations in this study. It would have been ideal to get a blood sample after overnight fasting to avoid the effect of chylomicrons from the circulation to have a more detailed overview of the lipids and lipoprotein subclass.[119] However, a longer fasting time is difficult to accomplish in a population-based large-scale study which involves VO_{2max} measurement. Future studies should be conducted on phosphatidylcholine and free choline to validate their potential as early markers of CVD and predictor of VO_{2max}. This may help in actuating preventive strategies like life style modifications at an early stage in the pathogenesis of CVD.

5.4 Characterization of astrocytomas

In paper II, the potential use of MRS in differentiating stage II astrocytomas from stage IV astrocytoma (glioblastoma) has been addressed. Compared to paper I and III, this study involved tissue samples and are therefore analysed by magic angle spinning MR based metabolomics. Multivariate analysis of the spectral data showed higher level of glycerophosphocholine and myo-inositol in grade II astrocytomas and higher levels of glycine, lipids and phosphocholine in grade IV astrocytomas. Further, there were significant metabolic differences between recurrent and non-recurrent gliomas. Histological estimates of necrosis in glioblastomas correlated with the levels of lipids and lactate. Moreover, the oncometabolite 2-hydroxyglutarate was detected in the HR-MAS spectra and was associated with IDH mutation.

Previously, MRS has been used in neurooncology for differentiating brain tumor types, for drug development, response monitoring, and prognosis prediction.[120-126] The total

choline (tCho) signal has been suggested as a biomarker for predicting cancer *in vivo* and is elevated in brain tumors compared to normal brain tissue.[25,123,127] Results from paper II shows that, HR-MAS at higher field strengths provides detailed information on cholines and resolves the tCho signal to GPC, PCho and free choline. The usability of *ex vivo*, high resolution MR metabolomics in identifying and establishing MR biomarkers of tumor grading has been evaluated. The importance of resolving GPC and PCho in discriminating different grades of astrocytomas was confirmed in this study.[128,129]

Abnormal choline metabolism associated with oncogenesis and tumor progression is an emerging metabolic hallmark of cancer.[25] Increased expression of genes encoding choline kinase and choline transporters, and a reduction in phospholipase C gene expression has been observed in high grade gliomas.[129] This may possibly explain the high PCho levels in glioblastomas. The balance between synthesis and degradation of choline metabolites determines the GPC/PCho ratios. Alteration in choline metabolites can also be linked to changes in tumor microenvironment such as hypoxia and acidic pH.[25,26] Noninvasive assessment of choline metabolites by MRS allows discrimination of tumor grades and can have a potential role in assessing treatment response.[130,131] In our study, two different types of astrocytomas were differentiated based on their metabolic profiles. Furthermore, the potential role of phosphorous MRS in improving the detection of GPC and PCho on a pilot basis was explored. These findings may offer a unique translational possibility of using these individual choline metabolites in *in vivo* characterization of diffuse astrocytomas. Advances in MRS techniques and improved field strength can tackle the problems like low sensitivity of phosphorous MRS *in*

vivo.[132-134] The clinical feasibility of detecting phosphorylated brain metabolites has been proved in pilot studies on patients with brain tumors.[134] In addition to tumor grade dependent differences in metabolic profiles, a metabolic difference between recurrent and non recurrent glioblastomas was present. Recurrent glioblastomas had lower PCho levels than non-recurrent glioblastomas, probably reflecting the radiotherapy and/or chemotherapy related changes. This could also be due to difference in the choline metabolism between recurrent and non-recurrent glioblastomas. However, this needs further exploration as the process of glioma progression is a complex event which involves evolution of oncogenes and tumor suppressor genes over a period of time in a changing tumor micro environment.

Recently, IDH mutations are gaining wider attention in the context of gliomas. This is due to the improved life expectancy in glioma patients harboring this mutation.[135,136] A mutant IDH gene results in an altered enzyme which produces 2-hydroxyglutarate (2-HG) from alpha-ketoglutarate.[137] This newly produced 2-HG or oncometabolite is scarce in non-mutants and can be non-invasively detected using MRS. Hence it can be a non-invasive biomarker for sub-classification of glioma by identifying patients with improved prognosis. In our study (paper III), this oncometabolite was detected in the majority of the samples with IDH mutation (5 out of 8). This shows that, probing for MR detectable biomarkers at higher field strength helps to establish MR detectable markers for potential in *vivo* translation with improvements in MR equipments and pulse sequences. Still, it should be kept in mind that sensitivity limitations in MR is often an obstacle in the clinical translation of the findings.

One of the main limitations of this study was the small number of patients. The findings in this study need to be confirmed in a larger patient cohort. As in most of the metabolomic marker research, this study too is in the initial exploratory phase or the phase I of the biomarker development. Astrocytomas are highly heterogenous, especially the glioblastomas, and this can results in overlaps in the multivariate classifier. In this study we were unable to recruit patients with anaplastic astrocytoma (WHO grade III); and future studies including them will aid in a broader comparison among grade II, III and IV. There are certain limitations which are inherent to *ex vivo* studies. Despite the possibility of detecting higher number of metabolites *ex vivo*, studies are vulnerable to metabolic changes which may occur in connection to the biopsy procedure, transportation and experimental process and sample storage. It has been shown that snap freezing can minimize the metabolic changes from sample handling[111], and in this study all samples were snap-frozen. Furthermore, previous studies have shown that high resolution *ex vivo* HR-MAS spectra can be directly related to *in vivo* proton MRS for metabolites like creatine, total choline, N-acetyl aspartate, myo-inositol and lipid signals (1.3 and 0.9 ppm) which are not likely to change within an extended ischemic period during surgery.[112,138] Another potential issue associated with HR-MAS is the sample degradation that may result from sample spinning. To minimize this issue, we kept the sample spinning for less than one hour. There were two samples out of 58 with sub optimal morphology after HR-MAS experiment. However, those samples still had a large number of intact tumor cells for performing histo-pathological examination after HR-MAS. Despite these limitations, this *ex vivo* metabolic profiling of tumor tissue samples provided a wealth of knowledge and insight which might be helpful in the future studies for finding *in vivo* markers that can be

accurate surrogates for pathological classification of astrocytomas in a non-invasive way.

Metabolic profiling using HR-MAS together with multivariate analysis is a useful technique in characterizing and identifying metabolic markers linked with different grades of astrocytomas. The potential role of different MRS technique such as phosphorous MRS in improving the discrimination between GPC and PCho, the most important choline metabolites associated with different grades of astrocytoma, has been explored. A comprehensive approach which incorporates various techniques like ^{31}P MRS, proton MRS, and molecular markers like IDH1 may add a new dimension to classification and management of astrocytomas.

5.5 Metabolic portraits of malignant serous effusions

Metabolic differences between ovarian serous carcinoma effusions obtained pre- and post-chemotherapy has been portrayed in paper III. Metabolic characterization of the malignant serous effusion from ovarian carcinomas was performed and the metabolic profiles were compared to serous effusions in breast carcinoma and malignant mesotheliomas. The use of high resolution MRS of biofluids coupled with multivariate analysis was evaluated. Furthermore, the study demonstrated the power of utilizing the multilevel structure of the data to capture chemotherapy related changes in metabolites.[76]

MRS of effusion fluids has previously been used to differentiate benign and malignant peritoneal effusions.[139] Metabolic profiling of biofluids as a diagnostic tool has several advantages such as noninvasive or minimally invasive sample collection and the possibility of collecting multiple samples over a time course, making it an ideal choice for clinical studies.[57] In paper III, the role of high resolution MRS was evaluated in a rapid, non-targeted manner to identify metabolic biomarkers of diagnostic and therapeutic importance in supernatants of 115 effusion samples. The ovarian carcinomas had elevated levels of ketones (acetoacetate and beta-hydroxybutyrate (BHB)) and lactate compared to malignant mesotheliomas and breast carcinomas whereas the later had more glucose, alanine and pyruvate. Even though the exact biological basis is unclear, elevated levels of acetone, acetoacetate and BHB are observed in serum samples of early stage ovarian cancer and colorectal cancer.[140,141] The growing energy demand by tumor cells can trigger lipolysis which may contribute towards the elevated levels of ketones.[140] Effusions represent an advanced stage of malignancy (stage III and IV) and during the process of metastasis to serous cavities; malignant cells can remain viable while suspended in the effusion fluid. The effusion fluids provide a microenvironment for the tumor cells and helps in the exchange of nutrients and mitogenic factors;[142,143] hence the metabolic composition of the effusion fluid may closely reflect the severity and invasiveness of the metastatic cells. MRS of the effusion fluids help to study the metabolic composition of the serous fluid in a global non-targeted manner. Future targeted studies are needed to further understand the details of the underlying mechanisms of energy transfers and metabolic fluxes in malignant effusions.

Post-chemotherapy samples showed an elevation of glucose and lipids with a reduction in BHB and lactate in the effusions. This may indicate a reduction in the number of live malignant cells or a change in tumor metabolism resulting in reduced glucose uptake from the medium. Treatment related reduction in glucose uptake has been shown in ovarian cancer cell lines (OVCAR-3) in response to cisplatin treatment in previous studies.[144] Future studies targeting glucose uptake by malignant cells in effusion fluids as a tool for chemosensitivity evaluation is warranted. Evaluation of chemotherapy induced changes failed in non-matched samples indicating the importance of multivariate paired data analysis.

The small number of samples involved was also here a limitation to the study. The small number of patient matched samples (8 pairs) precluded the analysis of association between clinical parameters such as treatment response and survival in relation to metabolic profiles. This study looked only at the metabolic profiles of the effusion fluids and a combined metabolic profiling which includes the tumor cells from patient matched ovarian carcinomas from different anatomical sites could be an area of future research.

5.6 Translational perspectives

The most important and ultimate objective of biomarker discovery is the translation of those biomarkers to clinical practice so that the result of the research will be useful for enhancing human health and well-being. With the development in “omics” technologies, there are thousands of scientific publications documenting potential

biomarkers for developing effective therapies and improving patient benefits. However, only a few biomarkers have been successfully validated for routine clinical use.[145,146] There are several practical issues such as logistical and regulatory challenges for large scale validation studies, lack of robustness in analytical technologies and standardisation of protocols used in clinical trials.[146,147] Metabolomics based biomarkers are successfully used in screening neonates for inborn errors of metabolism.[148] However, the number of metabolomic based tests excluding the inborn error of metabolism remains "zero".[149] The changes in the biomarker concentration are often subtle and distributed among several metabolites in many common complex diseases compared to profound metabolic changes in inborn error of metabolism, making it difficult to develop a single, accurate test. There is a need for standardisation of protocols among different laboratories and biomarker discovery should move in to a large collaborative network of multidisciplinary team. There should be more clarity in the description of laboratory techniques and reporting of biomarker research.[149,150] Metabolite reporting should shift from qualitative to quantitative analysis, with explicit description of changes in metabolite concentration than qualitative way, as in many current metabolic studies. The potential of biomarkers in improving patient management and quality of life is greater than several other areas of biomedical research. It is important to tackle these obstacles in translation of biomarker research to a clinically useful product.

6 Conclusions and future prospects

Metabolic profiling of serum samples from healthy volunteers showed that choline containing metabolites are associated with aerobic fitness. Low VO_{2max} was associated with elevated levels of free choline and decreased levels of phosphatidylcholine. We need to do further studies to identify the exact mechanisms behind this shift in choline/phosphatidylcholine profiles between high and low VO_{2max} individuals. They should also be further studied as potential early markers of CVD risk.

The second study (Paper II) showed that HR-MAS based metabolic profiling of intact tissue samples from tumor biopsies could be a potential tool for differentiating different grades of astrocytomas. Resolving GPC and PCho from tCho is important in metabolite based discrimination of astrocytoma grades. No significant correlation was observed between metabolic profiles and baseline patient characteristics. Combined use of different MRS techniques like ^{31}P MRS, ^{1}H MRS together with molecular markers like IDH1 may help in classification and in moving towards a personalized management of astrocytomas. *In vivo* translation of these results still has a way to go. Clinically approved *in vivo* scanners operate at lower field strengths (1.5-3 T) compared with ex vivo spectrometers (in our case, 14 T); thus, the sensitivity is inherently lower. Significant progress is being made toward the use of 7-T MRI in the clinical setting, and the development of technologies requiring shorter scanning time will follow. Hence, clinical translation of our results for in vivo validations should be performed in the near future.

In the third paper, the differences in metabolic profiles of malignant serous effusion from different anatomical sites were detected, and metabolic features related to chemotherapy exposure were identified using MRS. Metabolic characterization by high resolution proton MR spectroscopy could be a promising technique to further understand the mechanisms of effusion development in malignancies and to target clinical intervention.

In summary, all studies in this thesis showed that MRS based metabolomics is a useful technique in characterizing potential biomarkers of physiology and pathology. MR metabolomics allows rapid exploration of physiological and pathologic changes in samples that can be obtained in a minimally invasive manner. The method is therefore suited for early identification of specific metabolites or as patterns within a metabolic profile.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* Mar-Apr 2011;61(2):69-90.

2. Celermajer DS, Chow CK, Marijon E, Anstey NM, Woo KS. Cardiovascular disease in the developing world: prevalences, patterns, and the potential of early disease detection. *J Am Coll Cardiol.* Oct 2 2012;60(14):1207-1216.

3. Kitano H. Computational systems biology. *Nature.* Nov 14 2002;420(6912):206-210.

4. Bory C, Boulieu R, Chantin C, Mathieu M. Diagnosis of alcaptonuria: rapid analysis of homogentisic acid by HPLC. *Clin Chim Acta.* Jul 1990;189(1):7-11.

5. Plowman SA, Smith DL. *Exercise physiology for health, fitness, and performance.* 3rd ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2011.

6. Welch BE, Riendeau RP, Crisp CE, Isenstein RS. Relationship of maximal oxygen consumption to various components of body composition. *J Appl Physiol.* May 1958;12(3):395-398.

7. Bouchard C, Dionne FT, Simoneau JA, Boulay MR. Genetics of aerobic and anaerobic performances. *Exerc Sport Sci Rev.* 1992;20:27-58.

8. Kent M. *The Oxford dictionary of sports science & medicine.* 3rd ed. Oxford ; New York: Oxford University Press; 2006.

9. Farrell SW, Cheng YJ, Blair SN. Prevalence of the metabolic syndrome across cardiorespiratory fitness levels in women. *Obes Res.* May 2004;12(5):824-830.

10. Kavanagh T, Mertens DJ, Hamm LF, et al. Prediction of long-term prognosis in 12 169 men referred for cardiac rehabilitation. *Circulation.* Aug 6 2002;106(6):666-671.

11. LaMonte MJ, Barlow CE, Jurca R, Kampert JB, Church TS, Blair SN. Cardiorespiratory fitness is inversely associated with the incidence of metabolic syndrome: a prospective study of men and women. *Circulation.* Jul 26 2005;112(4):505-512.

12. Maxwell MS, Goslin BR, Gellish RL, et al. Metabolic syndrome status changes with fitness level change: a retrospective analysis. *Metab Syndr Relat Disord.* Mar 2008;6(1):8-14.

13. Myers J, Prakash M, Froelicher V, Do D, Partington S, Atwood JE. Exercise capacity and mortality among men referred for exercise testing. *N Engl J Med.* Mar 14 2002;346(11):793-801.

14. McMurray RG, Ainsworth BE, Harrell JS, Griggs TR, Williams OD. Is physical activity or aerobic power more influential on reducing cardiovascular disease risk factors? *Med Sci Sports Exerc.* Oct 1998;30(10):1521-1529.

15. Sassen B, Cornelissen VA, Kiers H, Wittink H, Kok G, Vanhees L. Physical fitness matters more than physical activity in controlling cardiovascular disease risk factors. *Eur J Cardiovasc Prev Rehabil.* Dec 2009;16(6):677-683.

16. Dunn WB, Broadhurst DI, Atherton HJ, Goodacre R, Griffin JL. Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. *Chem Soc Rev.* Jan 2011;40(1):387-426.

17. Barba I, de Leon G, Martin E, et al. Nuclear magnetic resonance-based metabolomics predicts exercise-induced ischemia in patients with suspected coronary artery disease. *Magn Reson Med.* Jul 2008;60(1):27-32.

18. Tappia PS, Dent MR, Dhalla NS. Oxidative stress and redox regulation of phospholipase D in myocardial disease. *Free Radic Biol Med.* Aug 1 2006;41(3):349-361.

19. Lewis GD, Wei R, Liu E, et al. Metabolite profiling of blood from individuals undergoing planned myocardial infarction reveals early markers of myocardial injury. *J Clin Invest.* Oct 2008;118(10):3503-3512.

20. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* Mar 4 2011;144(5):646-674.

21. Warburg O. On the Origin of Cancer Cells. *Science.* February 24, 1956 1956;123(3191):309-314.

22. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer.* Feb 2011;11(2):85-95.

23. Ponisovskiy MR. Cancer metabolism and the Warburg effect as anabolic process outcomes of oncogene operation. *Crit Rev Eukaryot Gene Expr.* 2010;20(4):325-339.

24. Vance JE, Vance DE. Phospholipid biosynthesis in mammalian cells. *Biochem Cell Biol.* Feb 2004;82(1):113-128.

25. Glunde K, Bhujwalla ZM, Ronen SM. Choline metabolism in malignant transformation. *Nat Rev Cancer.* Dec 2011;11(12):835-848.

26. Gillies RJ, Raghunand N, Karczmar GS, Bhujwalla ZM. MRI of the tumor microenvironment. *J Magn Reson Imaging.* Oct 2002;16(4):430-450.

27. Schiff R, Reddy P, Ahotupa M, et al. Oxidative Stress and AP-1 Activity in Tamoxifen-Resistant Breast Tumors In Vivo. *Journal of the National Cancer Institute.* December 6, 2000 2000;92(23):1926-1934.

28. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. *Cancer Cell.* Jun 2008;13(6):472-482.

29. Dang L, White DW, Gross S, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature.* Jun 17 2010;465(7300):966.

30. Garber K. Oncometabolite? IDH1 discoveries raise possibility of new metabolism targets in brain cancers and leukemia. *J Natl Cancer Inst.* Jul 7 2010;102(13):926-928.

31. Kleihues P, Soylemezoglu F, Schauble B, Scheithauer BW, Burger PC. Histopathology, classification, and grading of gliomas. *Glia.* Nov 1995;15(3):211-221.

32. Hulsebos TJ, Troost D, Leenstra S. Molecular-genetic characterisation of gliomas that recur as same grade or higher grade tumours. *J Neurol Neurosurg Psychiatry.* May 2004;75(5):723-726.

33. Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* Aug 2007;114(2):97-109.

34. Davidson B, Firat P, Michael CW. *Serous Effusions.* New York: Springer; 2011.

35. Garrison RN, Galloway RH, Heuser LS. Mechanisms of malignant ascites production. *J Surg Res.* Feb 1987;42(2):126-132.

36. Nicholson JK, Lindon JC. Systems biology: Metabonomics. *Nature.* Oct 23 2008;455(7216):1054-1056.

37. Saito K, Matsuda F. Metabolomics for functional genomics, systems biology, and biotechnology. *Annu Rev Plant Biol.* 2010;61:463-489.

38. Mayr M. Metabolomics: ready for the prime time? *Circ Cardiovasc Genet.* Oct 2008;1(1):58-65.

39. Mamas M, Dunn WB, Neyses L, Goodacre R. The role of metabolites and metabolomics in clinically applicable biomarkers of disease. *Arch Toxicol.* Jan 2011;85(1):5-17.

40. Brown M, Dunn W, Ellis D, et al. A metabolome pipeline: from concept to data to knowledge. *Metabolomics.* 2005;1(1):39-51.

41. Payne GS, Troy H, Vaidya SJ, Griffiths JR, Leach MO, Chung YL. Evaluation of 31P high-resolution magic angle spinning of intact tissue samples. *NMR Biomed.* Aug 2006;19(5):593-598.

42. Shung KK, Smith MB, Tsui BMW. *Principles of medical imaging.* San Diego: Academic Press; 1992.

43. Enea C, Seguin F, Petitpas-Mulliez J, et al. (1)H NMR-based metabolomics approach for exploring urinary metabolome modifications after acute and chronic physical exercise. *Anal Bioanal Chem.* Feb 2010;396(3):1167-1176.

44. Vallejo M, Garcia A, Tunon J, et al. Plasma fingerprinting with GC-MS in acute coronary syndrome. *Anal Bioanal Chem.* Jul 2009;394(6):1517-1524.

45. Farshidfar F, Weljie AM, Kopciuk K, et al. Serum metabolomic profile as a means to distinguish stage of colorectal cancer. *Genome Med.* 2012;4(5):42.

46. Wei S, Liu L, Zhang J, et al. Metabolomics approach for predicting response to neoadjuvant chemotherapy for breast cancer. *Mol Oncol.* Oct 25 2012.

47. Carrola J, Rocha CM, Barros AS, et al. Metabolic signatures of lung cancer in biofluids: NMR-based metabonomics of urine. *J Proteome Res.* Jan 7 2011;10(1):221-230.

48. O'Connell TM, Watkins PB. The Application of Metabonomics to Predict Drug-Induced Liver Injury. *Clin Pharmacol Ther.* 2010;88(3):394-399.

49. Weiss RH, Kim K. Metabolomics in the study of kidney diseases. *Nat Rev Nephrol.* 2012;8(1):22-33.

50. Calvani R, Miccheli A, Capuani G, et al. Gut microbiome-derived metabolites characterize a peculiar obese urinary metabotype. *Int J Obes.* 2010;34(6):1095-1098.

51. Mutch DM, Fuhrmann JC, Rein D, et al. Metabolite profiling identifies candidate markers reflecting the clinical adaptations associated with Roux-en-Y gastric bypass surgery. *PLoS One.* 2009;4(11):e7905.

52. Bodi V, Sanchis J, Morales JM, et al. Metabolomic profile of human myocardial ischemia by nuclear magnetic resonance spectroscopy of peripheral blood serum: a translational study based on transient coronary occlusion models. *J Am Coll Cardiol.* May 1 2012;59(18):1629-1641.

53. Hasokawa M, Shinohara M, Tsugawa H, et al. Identification of biomarkers of stent restenosis with serum metabolomic profiling using gas chromatography/mass spectrometry. *Circ J.* 2012;76(8):1864-1873.

54. Tenori L, Oakman C, Claudino WM, et al. Exploration of serum metabolomic profiles and outcomes in women with metastatic breast cancer: a pilot study. *Mol Oncol.* Aug 2012;6(4):437-444.

55. Gao X, Chen W, Li R, et al. Systematic variations associated with renal disease uncovered by parallel metabolomics of urine and serum. *BMC Syst Biol.* Jul 16 2012;6 Suppl 1:S14.

56. Nicholson G, Rantalainen M, Maher AD, et al. Human metabolic profiles are stably controlled by genetic and environmental variation. *Mol Syst Biol.* 2011;7:525.

57. Zhang A, Sun H, Wang P, Han Y, Wang X. Recent and potential developments of biofluid analyses in metabolomics. *J Proteomics.* Feb 2 2012;75(4):1079-1088.

58. Andrew ER, Bradbury A, Eades RG. Nuclear Magnetic Resonance Spectra from a Crystal rotated at High Speed. *Nature.* 1958;182(4650):1659-1659.

59. Bathen TF, Sitter B, Sjobakk TE, Tessem MB, Gribbestad IS. Magnetic resonance metabolomics of intact tissue: a biotechnological tool in cancer diagnostics and treatment evaluation. *Cancer Res.* Sep 1 2010;70(17):6692-6696.

60. Righi V, Mucci A, Schenetti L, et al. Ex vivo HR-MAS magnetic resonance spectroscopy of normal and malignant human renal tissues. *Anticancer Res.* Sep-Oct 2007;27(5A):3195-3204.

61. Chen W, Zu Y, Huang Q, et al. Study on metabonomic characteristics of human lung cancer using high resolution magic-angle spinning 1H NMR spectroscopy and multivariate data analysis. *Magn Reson Med.* Dec 2011;66(6):1531-1540.

62. Vettukattil R, Gulati M, Sjobakk TE, et al. Differentiating Diffuse World Health Organization Grade II and IV Astrocytomas With Ex Vivo Magnetic Resonance Spectroscopy. *Neurosurgery.* Feb 2013;72(2):186-195.

63. Sjobakk TE, Vettukattil R, Gulati M, et al. Metabolic profiles of brain metastases. *Int J Mol Sci.* 2013;14(1):2104-2118.

64. Sitter B, Lundgren S, Bathen TF, Halgunset J, Fjosne HE, Gribbestad IS. Comparison of HR MAS MR spectroscopic profiles of breast cancer tissue with clinical parameters. *NMR Biomed.* Feb 2006;19(1):30-40.

65. Beckonert O, Coen M, Keun HC, et al. High-resolution magic-angle-spinning NMR spectroscopy for metabolic profiling of intact tissues. *Nat Protoc.* Jun 2010;5(6):1019-1032.

66. Meiboom S, Gill D. Modified Spin-Echo Method for Measuring Nuclear Relaxation Times. *Review of Scientific Instruments.* 1958;29(8):688-691.

67. Vettukattil R, Hetland TE, Florenes VA, Kaern J, Davidson B, Bathen TF. Proton magnetic resonance metabolomic characterization of ovarian serous carcinoma effusions: chemotherapy-related effects and comparison with malignant mesothelioma and breast carcinoma. *Hum Pathol.* May 6 2013.

68. Griffin JL, Shockcor JP. Metabolic profiles of cancer cells. *Nat Rev Cancer.* Jul 2004;4(7):551-561.

69. van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ. Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics.* 2006;7:142.

70. Craig A, Cloarec O, Holmes E, Nicholson JK, Lindon JC. Scaling and normalization effects in NMR spectroscopic metabonomic data sets. *Anal Chem.* Apr 1 2006;78(7):2262-2267.

71. Giskeodegard GF, Bloemberg TG, Postma G, et al. Alignment of high resolution magic angle spinning magnetic resonance spectra using warping methods. *Anal Chim Acta.* Dec 17 2010;683(1):1-11.

72. Savorani F, Tomasi G, Engelsen SB. icoshift: A versatile tool for the rapid alignment of 1D NMR spectra. *J Magn Reson.* Feb 2010;202(2):190-202.

73. Tomasi G, Berg F, Andersson C. Correlation optimized warping and dynamic time warping as preprocessing methods for chromatographic data. *Journal of Chemometrics.* 2004;18(5):231 - 241.

74. Eilers PHC. Parametric Time Warping. *Anal Chem.* 2004/01/01 2003;76(2):404-411.

75. Martens H, Næs T. *Multivariate calibration.* Chichester [England]: Wiley; 1989.

76. van Velzen EJ, Westerhuis JA, van Duynhoven JP, et al. Multilevel data analysis of a crossover designed human nutritional intervention study. *J Proteome Res.* Oct 2008;7(10):4483-4491.

77. Broadhurst D, Kell D. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics.* 2006/12/01 2006;2(4):171-196.

78. Westerhuis JA, Hoefsloot HC, Smit S, et al. Assessment of PLSDA cross validation. *Metabolomics.* 2008;4(1):81-89.

79. Szymanska E, Saccenti E, Smilde AK, Westerhuis JA. Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics.* Jun 2012;8(Suppl 1):3-16.

80. Biomarkers Definitions Working G. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther.* Mar 2001;69(3):89-95.

81. Vasan RS. Biomarkers of cardiovascular disease: molecular basis and practical considerations. *Circulation.* May 16 2006;113(19):2335-2362.

82. Srivastava S, Wagner JA. Surrogate endpoints in medicine. *Disease Markers.* 2002;18(2):39-40.

83. Wagner PD, Verma M, Srivastava S. Challenges for biomarkers in cancer detection. *Ann N Y Acad Sci.* Jun 2004;1022:9-16.

84. Yurkovetsky ZR, Linkov FY, D EM, Lokshin AE. Multiple biomarker panels for early detection of ovarian cancer. *Future Oncol.* Dec 2006;2(6):733-741.

85. de Lemos JA, Lloyd-Jones DM. Multiple biomarker panels for cardiovascular risk assessment. *N Engl J Med.* May 15 2008;358(20):2172-2174.

86. Marko-Varga G, Lindberg H, Lofdahl CG, et al. Discovery of biomarker candidates within disease by protein profiling: principles and concepts. *J Proteome Res.* Jul-Aug 2005;4(4):1200-1212.

87. Pepe MS, Etzioni R, Feng Z, et al. Phases of Biomarker Development for Early Detection of Cancer. *Journal of the National Cancer Institute.* July 18, 2001 2001;93(14):1054-1061.

88. Spratlin JL, Serkova NJ, Eckhardt SG. Clinical applications of metabolomics in oncology: a review. *Clin Cancer Res.* Jan 15 2009;15(2):431-440.

89. Ackerstaff E, Glunde K, Bhujwalla ZM. Choline phospholipid metabolism: a target in cancer cells? *J Cell Biochem.* Oct 15 2003;90(3):525-533.

90. Mazurek S, Eigenbrodt E. The tumor metabolome. *Anticancer Res.* Mar-Apr 2003;23(2A):1149-1154.

91. Swanson MG, Zektzer AS, Tabatabai ZL, et al. Quantitative analysis of prostate metabolites using 1H HR-MAS spectroscopy. *Magn Reson Med.* Jun 2006;55(6):1257-1264.

92. Cheng LL, Wu C, Smith MR, Gonzalez RG. Non-destructive quantitation of spermine in human prostate tissue samples using HRMAS 1H NMR spectroscopy at 9.4 T. *FEBS Lett.* Apr 6 2001;494(1-2):112-116.

93. Bathen TF, Jensen LR, Sitter B, et al. MR-determined metabolic phenotype of breast cancer in prediction of lymphatic spread, grade, and hormone status. *Breast Cancer Res Treat.* Aug 2007;104(2):181-189.

94. Griffin JL, Kauppinen RA. A metabolomics perspective of human brain tumours. *FEBS J.* Mar 2007;274(5):1132-1139.

95. Petrik V, Loosemore A, Howe FA, Bell BA, Papadopoulos MC. OMICS and brain tumour biomarkers. *Br J Neurosurg.* Oct 2006;20(5):275-280.

96. Odunsi K, Wollman RM, Ambrosone CB, et al. Detection of epithelial ovarian cancer using 1H-NMR-based metabonomics. *Int J Cancer.* Feb 20 2005;113(5):782-788.

97. Lewis GD, Farrell L, Wood MJ, et al. Metabolic signatures of exercise in human plasma. *Sci Transl Med.* May 26 2010;2(33):33ra37.

98. Rasmiena AA, Ng TW, Meikle PJ. Metabolomics and ischaemic heart disease. *Clin Sci (Lond).* Mar 2013;124(5):289-306.

99. Dzeletovic S, Breuer O, Lund E, Diczfalusy U. Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. *Anal Biochem.* Feb 10 1995;225(1):73-80.

100. Chen X, Liu L, Palacios G, et al. Plasma metabolomics reveals biomarkers of the atherosclerosis. *J Sep Sci.* Sep 2010;33(17-18):2776-2783.

101. Teul J, Ruperez FJ, Garcia A, et al. Improving metabolite knowledge in stable atherosclerosis patients by association and correlation of GC-MS and 1H NMR fingerprints. *J Proteome Res.* Dec 2009;8(12):5580-5589.

102. Turer AT, Stevens RD, Bain JR, et al. Metabolomic profiling reveals distinct patterns of myocardial substrate use in humans with coronary artery disease or left ventricular dysfunction during surgical ischemia/reperfusion. *Circulation.* Apr 7 2009;119(13):1736-1746.

103. Aspenes ST, Nauman J, Nilsen TI, Vatten LJ, Wisloff U. Physical activity as a long-term predictor of peak oxygen uptake: the HUNT Study. *Med Sci Sports Exerc.* Sep 2011;43(9):1675-1679.

104. Schag CC, Heinrich RL, Ganz PA. Karnofsky performance status revisited: reliability, validity, and guidelines. *J Clin Oncol.* Mar 1984;2(3):187-193.

105. Gulati S, Jakola AS, Nerland US, Weber C, Solheim O. The risk of getting worse: surgically acquired deficits, perioperative complications, and functional outcomes after primary resection of glioblastoma. *World Neurosurg.* Dec 2011;76(6):572-579.

106. Stummer W, Pichlmeier U, Meinel T, et al. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol.* May 2006;7(5):392-401.

107. Mancia G, De Backer G, Dominiczak A, et al. 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens.* Jun 2007;25(6):1105-1187.

108. Rognmo O, Hetland E, Helgerud J, Hoff J, Slordahl SA. High intensity aerobic interval exercise is superior to moderate intensity exercise for increasing aerobic capacity in patients with coronary artery disease. *Eur J Cardiovasc Prev Rehabil.* Jun 2004;11(3):216-222.

109. Beckonert O, Keun HC, Ebbels TM, et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc.* 2007;2(11):2692-2703.

110. Lindon JC, Nicholson JK. Spectroscopic and statistical techniques for information recovery in metabonomics and metabolomics. *Annu Rev Anal Chem (Palo Alto Calif).* 2008;1:45-69.

111. Garrod S, Humpfer E, Spraul M, et al. High-resolution magic angle spinning 1H NMR spectroscopic studies on intact rat renal cortex and medulla. *Magn Reson Med.* Jun 1999;41(6):1108-1118.

112. Opstad KS, Wright AJ, Bell BA, Griffiths JR, Howe FA. Correlations between in vivo (1)H MRS and ex vivo (1)H HRMAS metabolite measurements in adult human gliomas. *J Magn Reson Imaging.* Feb 2010;31(2):289-297.

113. Koulman A, Lane GA, Harrison SJ, Volmer DA. From differentiating metabolites to biomarkers. *Anal Bioanal Chem.* Jun 2009;394(3):663-670.

114. Madsen R, Lundstedt T, Trygg J. Chemometrics in metabolomics--a review in human disease diagnosis. *Anal Chim Acta.* Feb 5 2010;659(1-2):23-33.

115. Martineau E, Tea I, Akoka S, Giraudeau P. Absolute quantification of metabolites in breast cancer cell extracts by quantitative 2D (1) H INADEQUATE NMR. *NMR Biomed.* Aug 2012;25(8):985-992.

116. Sabatine MS, Liu E, Morrow DA, et al. Metabolomic identification of novel biomarkers of myocardial ischemia. *Circulation.* Dec 20 2005;112(25):3868-3875.

117. Konstantinova SV, Tell GS, Vollset SE, Nygard O, Bleie O, Ueland PM. Divergent associations of plasma choline and betaine with components of metabolic syndrome in middle age and elderly men and women. *J Nutr.* May 2008;138(5):914-920.

118. Miettinen TA, Naukkarinen V, Huttunen JK, Mattila S, Kumlin T. Fatty-acid composition of serum lipids predicts myocardial infarction. *Br Med J (Clin Res Ed).* Oct 9 1982;285(6347):993-996.

119. Soininen P, Kangas AJ, Wurtz P, et al. High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism. *Analyst.* Sep 2009;134(9):1781-1785.

120. Yamasaki F, Kurisu K, Kajiwara Y, et al. Magnetic resonance spectroscopic detection of lactate is predictive of a poor prognosis in patients with diffuse intrinsic pontine glioma. *Neuro Oncol.* Jul 2011;13(7):791-801.

121. Venkatesh HS, Chaumeil MM, Ward CS, Haas-Kogan DA, James CD, Ronen SM. Reduced phosphocholine and hyperpolarized lactate provide magnetic resonance biomarkers of PI3K/Akt/mTOR inhibition in glioblastoma. *Neuro Oncol.* Mar 2012;14(3):315-325.

122. Srinivasan R, Phillips JJ, Vandenberg SR, et al. Ex vivo MR spectroscopic measure differentiates tumor from treatment effects in GBM. *Neuro Oncol.* Nov 2010;12(11):1152-1161.

123. Howe FA, Opstad KS. 1H MR spectroscopy of brain tumours and masses. *NMR Biomed.* May 2003;16(3):123-131.

124. Howe FA, Barton SJ, Cudlip SA, et al. Metabolic profiles of human brain tumors using quantitative in vivo 1H magnetic resonance spectroscopy. *Magn Reson Med.* Feb 2003;49(2):223-232.

125. Hattingen E, Jurcoane A, Bahr O, et al. Bevacizumab impairs oxidative energy metabolism and shows antitumoral effects in recurrent glioblastomas: a 31P/1H MRSI and quantitative magnetic resonance imaging study. *Neuro Oncol.* Dec 2011;13(12):1349-1363.

126. Chawla S, Oleaga L, Wang S, et al. Role of proton magnetic resonance spectroscopy in differentiating oligodendrogliomas from astrocytomas. *J Neuroimaging.* Jan 2010;20(1):3-8.

127. Horska A, Barker PB. Imaging of brain tumors: MR spectroscopy and metabolic imaging. *Neuroimaging Clin N Am.* Aug 2010;20(3):293-310.

128. Wright AJ, Fellows GA, Griffiths JR, Wilson M, Bell BA, Howe FA. Ex-vivo HRMAS of adult brain tumours: metabolite quantification and assignment of tumour biomarkers. *Mol Cancer.* 2010;9:66.

129. Righi V, Roda JM, Paz J, et al. 1H HR-MAS and genomic analysis of human tumor biopsies discriminate between high and low grade astrocytomas. *NMR Biomed.* Jul 2009;22(6):629-637.

130. Aboagye EO. Phosphatidylcholine metabolic transformation and progression signature as a pharmacodynamic biomarker. *Oncotarget.* Jul 2010;1(3):163-166.

131. Kato Y, Holm DA, Okollie B, Artemov D. Noninvasive detection of temozolomide in brain tumor xenografts by magnetic resonance spectroscopy. *Neuro Oncol.* Jan 2010;12(1):71-79.

132. Gonen O, Mohebbi A, Stoyanova R, Brown TR. In vivo phosphorus polarization transfer and decoupling from protons in three-dimensional localized nuclear magnetic resonance spectroscopy of human brain. *Magn Reson Med.* Feb 1997;37(2):301-306.

133. Klomp DW, Wijnen JP, Scheenen TW, Heerschap A. Efficient 1H to 31P polarization transfer on a clinical 3T MR system. *Magn Reson Med.* Dec 2008;60(6):1298-1305.

134. Wijnen JP, Scheenen TW, Klomp DW, Heerschap A. 31P magnetic resonance spectroscopic imaging with polarisation transfer of phosphomono- and diesters at 3 T in the human brain: relation with age and spatial differences. *NMR Biomed.* Oct 2010;23(8):968-976.

135. Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science.* Sep 26 2008;321(5897):1807-1812.

136. Yan H, Parsons DW, Jin G, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med.* Feb 19 2009;360(8):765-773.

137. Elkhaled A, Jalbert LE, Phillips JJ, et al. Magnetic resonance of 2-hydroxyglutarate in IDH1-mutated low-grade gliomas. *Sci Transl Med.* Jan 11 2012;4(116):116ra115.

138. Barton SJ, Howe FA, Tomlins AM, et al. Comparison of in vivo 1H MRS of human brain tumours with 1H HR-MAS spectroscopy of intact biopsy samples in vitro. *MAGMA.* May 1999;8(2):121-128.

139. Bala L, Sharma A, Yellapa RK, Roy R, Choudhuri G, Khetrapal CL. (1)H NMR spectroscopy of ascitic fluid: discrimination between malignant and benign ascites and comparison of the results with conventional methods. *NMR Biomed.* Jul 2008;21(6):606-614.

140. Ludwig C, Ward DG, Martin A, et al. Fast targeted multidimensional NMR metabolomics of colorectal cancer. *Magn Reson Chem.* Dec 2009;47 Suppl 1:S68-73.

141. Garcia E, Andrews C, Hua J, et al. Diagnosis of early stage ovarian cancer by 1H NMR metabonomics of serum explored by use of a microflow NMR probe. *J Proteome Res.* Apr 1 2011;10(4):1765-1771.

142. Martinez-Outschoorn UE, Pestell RG, Howell A, et al. Energy transfer in "parasitic" cancer metabolism: mitochondria are the powerhouse and Achilles' heel of tumor cells. *Cell Cycle.* Dec 15 2011;10(24):4208-4216.

143. Kassis J, Klominek J, Kohn EC. Tumor microenvironment: What can effusions teach us? *Diagnostic Cytopathology.* 2005;33(5):316-319.

144. Egawa-Takata T, Endo H, Fujita M, et al. Early reduction of glucose uptake after cisplatin treatment is a marker of cisplatin sensitivity in ovarian cancer. *Cancer Sci.* Oct 2010;101(10):2171-2178.

145. Poste G. Bring on the biomarkers. *Nature.* Jan 13 2011;469(7329):156-157.

146. Drucker E, Krapfenbauer K. Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalised medicine. *EPMA J.* 2013;4(1):7.

147. Sabroe I, Dockrell DH, Vogel SN, Renshaw SA, Whyte MK, Dower SK. Identifying and hurdling obstacles to translational research. *Nat Rev Immunol.* Jan 2007;7(1):77-82.

148. Chace DH. Mass spectrometry in the clinical laboratory. *Chem Rev.* Feb 2001;101(2):445-477.

149. Xia J, Broadhurst DI, Wilson M, Wishart DS. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. *Metabolomics.* Apr 2013;9(2):280-299.

150. Rifai N, Annesley TM, Berg JP, et al. An appeal to medical journal editors: the need for a full description of laboratory methods and specimen handling in clinical study reports. *Clin Chem Lab Med.* Mar 2012;50(3):411-413.

Paper I

OPEN ACCESS Freely available online

PLoS one

Serum Levels of Choline-Containing Compounds Are Associated with Aerobic Fitness Level: The HUNT-Study

Anja Bye[1,2☯], Riyas Vettukattil[1*☯], Stian T. Aspenes[1,2], Guro F. Giskeødegård[1], Ingrid S. Gribbestad[1], Ulrik Wisløff[1,2], Tone F. Bathen[1]

1 Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trondheim, Norway, **2** K. G. Jebsen Center of Exercise in Medicine, Trondheim, Norway

Abstract

Background: Cardiovascular disease (CVD) is a leading cause of death worldwide, and the number of people at risk is continuously growing. New methods for early risk prediction are therefore needed to actuate prevention strategies *before* the individuals are diagnosed with CVD. Several studies report that aerobic fitness level, measured as maximal oxygen uptake (VO_{2max}), is the single best predictor of future CVD mortality in healthy people. Based on this, we wanted to study differences between healthy individuals with a large difference in VO_{2max}-level to identify new biomarkers of low aerobic fitness that may also have potential as early biomarkers of CVD risk.

Methodology/Principal Findings: Serum samples from 218 healthy individuals with a low VO_{2max} (n = 108, 63 women) or high VO_{2max} (n = 110, 64 women) were analysed with MR metabolomics. In addition, standard clinical-chemical analyses for glucose, lipids, liver enzymes, micro-CRP, and colorimetric analysis on circulating choline were performed. Individuals in the low VO_{2max}-group had increased serum levels of free choline, decreased phosphatidylcholine, increased glucoşe and decreased unsaturated fatty acids compared to the individuals in the high VO_{2max}–group.

Conclusions/Significance: Aerobic fitness dependent differences in serum levels of free choline and phosphatidylcholine are observed. They should be further studied as potential early markers of CVD risk.

Citation: Bye A, Vettukattil R, Aspenes ST, Giskeødegård GF, Gribbestad IS, et al. (2012) Serum Levels of Choline-Containing Compounds Are Associated with Aerobic Fitness Level: The HUNT-Study. PLoS ONE 7(7): e42330. doi:10.1371/journal.pone.0042330

Editor: Daniel Monleon, Instituto de Investigación Sanitaria INCLIVA, Spain

Received March 14, 2012; **Accepted** July 3, 2012; **Published** July 30, 2012

Funding: The study was supported by grants from the K.G. Jebsen Foundation, the Norwegian Council on Cardiovascular Disease, St. Olavs Hospital and the Liaison Committee between the St. Olavs Hospital and the Faculty of Medicine, Norwegian University of Science and Technology (NTNU), the Norwegian Research Council Funding for Outstanding Young Investigators, the Royal Norwegian Society of Sciences and Letters (DKNVS) and the Foundation for Cardiovascular Research at St. Olavs Hospital and NTNU. There are no disclosures to report. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: muhammad.r.vettukattil@ntnu.no.

☯ These authors contributed equally to this work.

Introduction

Cardiovascular disease (CVD) is a leading cause of death worldwide, and the number of people at risk is continuously growing [1]. New methods for early risk prediction are therefore needed to actuate prevention strategies *before* the individuals are diagnosed with CVD. Aerobic fitness level, measured as maximal oxygen uptake (VO_{2max}), is a strong marker for cardiac health. Large-scale epidemiological studies have demonstrated that low VO_{2max} is the single best predictor of future CVD mortality both in healthy individuals and in patients with CVD [2–6]. Based on this, more knowledge of the differences between healthy individuals with a large difference in VO_{2max}-level will be of great interest to identify new biomarkers of low aerobic fitness that may also have a potential as an early biomarker of CVD risk.

Emerging metabolite profiling technologies have recently made it possible to acquire "snapshots" of the metabolic processes at a given point in time [7,8]. This methodology, termed metabolomics, involves a high throughput analysis of small-molecular metabolites that are downstream products of preceding gene expressions and protein activity. Within systems biology, magnetic resonance (MR) metabolomics has become one of the key platforms, allowing rapid analysis of samples with minimal sample preparation. The acquired metabolic profiles can be useful for a better understanding of the metabolic perturbations associated with health and disease.

Previously, serum and plasma MR metabolomics have been successfully used in the detection of biomarkers associated with various clinical conditions such as coronary artery disease and myocardial infarction [9–11]. Serum metabolites such as citric acid, threonine, and choline have previously been associated with the incidence of CVD, but so far the evidence is sparse [9–11]. To our knowledge, no previous study has searched for serum metabolites associated with aerobic fitness level in a healthy population. The aim of the present study was to investigate metabolic differences between healthy individuals with high and low VO_{2max} by MR metabolomics, and further to describe these differences qualitatively and quantitatively.

Results

The high and the low VO_{2max}-groups, which were matched for age, fasting time and level of self-reported physical activity, had significantly different body weight, waist circumference, waist-to-hip-ratio, body mass index (BMI), mean arterial blood pressure, resting heart rate, non-fasting glucose, triglycerides, micro C-reactive protein (CRP), alanine aminotransferase (ALAT) and gamma glutamyl transferase (Gamma-GT), but not total cholesterol (Table 1). Data from questionnaires revealed that the own-reported health status was significantly better in the high VO_{2max}-group compared to the low VO_{2max}-group (Table 2). Dietary questionnaires indicated only differences in fruit and berry intake between the two groups (Table 2).

MR spectra indicated differences between the metabolic profiles of the high and low VO_{2max}-groups (Figure 1A). Exploration of the corresponding loading profiles (Figure 1B) and MR spectra (Figure 2) showed that low VO_{2max}-subjects had higher levels of lipid methylene (-CH2-) protons (peak at 1.3 ppm), indicating decreased amounts of unsaturated fatty acids in serum from the low VO_{2max}-subjects. The low VO_{2max}-subjects also had lower levels of phosphatidylcholine (PtdCho) ($-N(CH_3)_3^+$, peak at 3.24 ppm) (Figure 2). A permutation test showed that the differences in the metabolic profiles between high and low VO_{2max}-subjects were highly significant ($p<0.001$). The model created by the MR metabolomics analysis could predict whether a subject has a low or high VO_{2max} with a sensitivity and specificity of 63% and 65%, respectively.

A subsequent colorimetric analysis to further study the differences in choline-containing compounds showed that the levels of free choline were significantly higher in the low VO_{2max}-group compared to the high VO_{2max}-group (14.57 ± 1.55 vs. 10.13 ± 0.91 μM, $p = 0.017$). The serum choline levels seemed to correlate with the serum triglycerides levels (high VO_{2max}-group, $r = 0.50$, $p<0.005$ and low VO_{2max}-group, $r = 0.74$, $p<0.0001$). There was no correlation between free choline levels and fasting status.

Replicate ^{1}H MR spectra of serum with assignments of the main metabolites are illustrated in Figure 3. The score plot (Figure 3) clearly displays a larger inter subject variance compared to intra subject variance, which indicates excellent reproducibility. To further assess the agreement between metabolite levels obtained by laboratory assays and MR, the glucose concentration obtained by standard methodology were correlated with the MR signal intensities (relative quantification by peak integration 3.90–3.94 ppm) for glucose. The data showed strong correlations ($R^2 = 0.83$).

Discussion

The main findings of this study were that the subjects with low VO_{2max} had increased serum levels of free choline and decreased serum levels of phosphatidylcholine (PtdCho) compared to subjects with high VO_{2max}. In human cells, the majority of choline is taken up by the cells and conversed into PtdCho. Since the ratio of choline/PtdCho is switched between the subjects with high and low VO_{2max}, there might be difference in one of the enzymes of the plasma membrane, phospholipase D (PLD). PLD catalyzes the hydrolysis of PtdCho to phosphatic acid (PA), releasing soluble choline. High PLD activity and increased level of PA has previously been associated with oxidative stress, hypoxia, inflammation, atherosclerosis and hypertension (reviewed in [12]). In the

Table 1. A statistical overview of the participants in this study.

Variable	Low VO_{2max}-group			High VO_{2max}-group			p-value
	n	mean	CI	n	mean	CI	
Age	108	49.5	48.4–50.6	110	49.5	48.4–50.6	–
VO_{2max} ($mL \cdot kg^{-0.75} \cdot min^{-1}$)	108	93.9	90.9–96.9	110	138.0	133.4–142.7	–
Physical activity index score	108	3.7	3.4–4.0	110	3.7	3.4–4.0	–
Waist (cm)	108	93.6	91.5–95.7	110	86.3	84.6–88.1	0.0004**
Hip (cm)	108	103.8	102.4–105.3	110	100.1	99.2–101.1	0.0004**
Arm circumference (cm)	108	30.3	29.7–30.9	110	28.7	28.3–29.2	0.0004**
Weight (kg)	108	80.7	77.9–83.5	110	73.5	71.4–75.6	0.0004**
Waist-to-hip-ratio	108	0.90	0.88–0.91	110	0.86	0.85–0.87	0.0004**
BMI	108	27.5	26.8–28.3	110	24.8	24.3–25.2	0.0004**
Heart rate at rest	103	62.3	60.2–64.4	102	55.8	53.9–57.7	0.0004**
Systolic blood pressure (mmhg)	108	128.5	125.6–131.5	110	124.9	122.2–127.5	0.070
Diastolic blood pressure (mmhg)	108	75.2	73.2–77.1	110	72.5	70.7–74.3	0.051
Mean arterial pressure	108	93.0	90.9–95.1	110	90.0	88.0–91.9	0.038*
Alanine aminotransferase (U/L)	13	40.3	20.7–59.9	25	25.3	21.3–29.4	0.038*
Gamma glutamyl transferase (U/L)	13	52.8	20.2–85.4	25	27.8	18.8–36.8	0.049*
Non-fasting glucose (mmol/L)	103	5.7	5.4–6.0	104	5.2	5.0–5.3	0.004**
Cholesterol (mmol/L)	103	5.6	5.4–5.7	104	5.5	5.3–5.6	0.447
HDL-cholesterol (mmol/L)	103	1.4	1.3–1.4	104	1.5	1.4–1–5	0.074
Triglycerides (mmol/L)	77	1.7	1.5–1.9	90	1.3	1.1–1.4	0.002**
Serum micro C-reactive protein (mg/L)	76	2.2	1.3–3.2	90	1.2	0.9–1.6	0.040*

VO_{2max}: Maximal oxygen uptake, CI: Confidence Interval, BMI: Body Mass Index, HDL: High Density Lipoprotein. P-values below 0.05 are flagged. **$p<0.005$, *$p<0.05$.
doi:10.1371/journal.pone.0042330.t001

Table 2. Data from questionnaires.

Variable	Low VO_{2max}-group			High VO_{2max}-group			p-value
	n	mean	CI	n	mean	CI	
Own reported health status (scale 1–4)	104	3.0	2.8–3.1	107	3.3	3.2–3.4	0.001**
Vegetables intake (scale 1–5)	108	3.5	3.3–3.6	110	3.6	3.4–3.7	0.293
Fruit and berry intake (scale 1–5)	108	3.4	3.2–3.7	110	3.8	3.6–4.0	0.029*
Sausage and hamburger intake (scale 1–5)	108	1.3	1.3–1.5	108	1.2	1.2–1.4	0.102
High-fat fish intake (scale 1–5)	108	1.5	1.5–1.8	108	1.5	1.4–1.7	0.730

Food intake: 1 = 0–3 times a month, 2 = 1–3 times a week, 3 = 4–6 times a week, 4 = Once a day, 5 = 2 times or more each day. VO_{2max}: Maximal oxygen uptake, CI: Confidence interval. P-values below 0.05 are flagged. **p<0.01, *p<0.05.
doi:10.1371/journal.pone.0042330.t002

heart, high PLD activity and increased level of PA are suggested to be involved in the signaling cascade promoting pathological cardiac hypertrophy [13]. If our assumptions are correct, even healthy individuals with a low aerobic fitness may have a high PLD activity, which may link low aerobic fitness to the future development of CVD.

Other explanation for the high levels of serum choline in the low VO_{2max}-group may be release of choline from damaged organs, impaired tissue uptake or choline-rich diet [14,15]. In patients with severe repetitive arrhythmias and hemodynamic compromises, choline has been shown to leak from ischemic tissues into the blood stream [16–18]. However, since the participants in the current study were healthy it seems unlikely that the increased levels of choline arise from myocardial release. Regarding diet, a previous study indicated a weak inverse association between serum choline and time since last meal [19]. In the current study the groups were matched on fasting status, and no important differences were found in diet. Thus, the observed differences in free choline levels are not likely to be caused by differences in food intake.

High serum levels of free choline have previously been associated with an increased prevalence of the metabolic syndrome (a cluster of risk factors of CVD) [19]. Since the subjects in the low VO_{2max}-group not fulfilled the criteria for the metabolic syndrome, our results may indicate that serum levels of choline may have prognostic value for future CVD even among healthy subjects. Free choline levels should therefore be assessed in a large healthy cohort to prospectively study the prognostic value for later cardiovascular events. Furthermore, elevated levels of choline have recently been recognized as a novel biomarker for early risk stratification in patients with suspected acute coronary syndrome [16–18]. To our knowledge, no previous study has reported associations between VO_{2max} level and free choline.

In addition to the differences in choline-containing compounds, the MR spectra also indicated that the subjects with low VO_{2max} had decreased amounts of unsaturated fatty acids. Decreased serum levels of unsaturated fatty acids have previously been associated with increased risk of CVD [20].

The differences in weight, waist circumference, waist-to-hip-ratio, BMI, mean arterial blood pressure, resting heart rate, non-

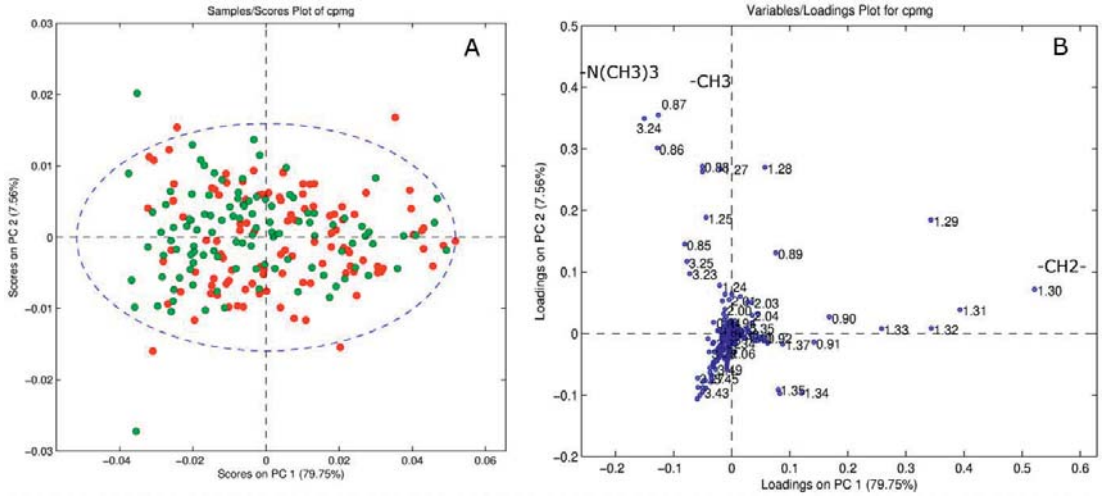

Figure 1. Principal Component Analysis (PCA) of the serum 1H MR spectra. (A) In the score plot, high VO_{2max} subjects are shown in green (higher density in upper left part) and low VO_{2max} subjects are shown in red. (B) The loadings plot visualizes the differences in metabolites between the two groups. The signals originating from within the core of the serum lipoprotein particles (-CH_3 at 0.86 ppm, -CH_2- at 1.3 ppm) and choline-containing compounds (-N $(CH_3)_3^+$, at 3.24 ppm) are mainly responsible for the clustering. VO_{2max}: Maximal oxygen uptake.
doi:10.1371/journal.pone.0042330.g001

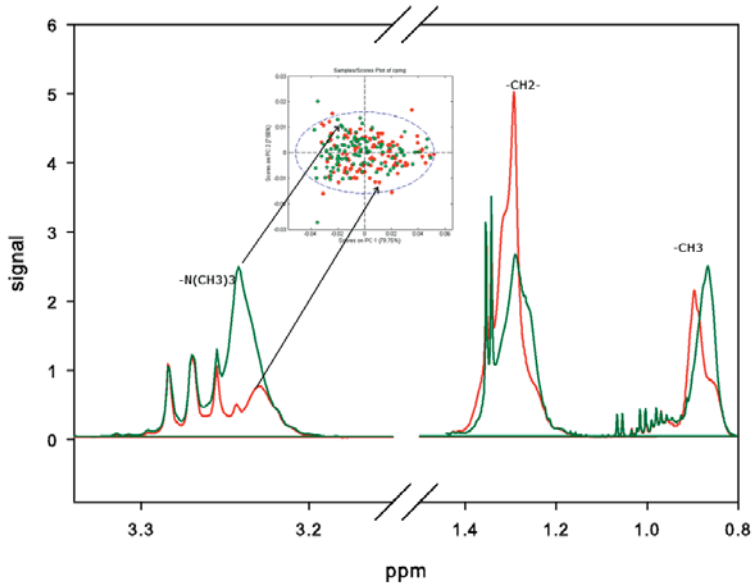

Figure 2. Visualization of the metabolic differences in MR spectra. The green spectrum is from a high VO_{2max} subject (green dots in PCA score plot) and the red spectrum is from the low VO_{2max} (red dots in PCA score plot). VO_{2max}: Maximal oxygen uptake.
doi:10.1371/journal.pone.0042330.g002

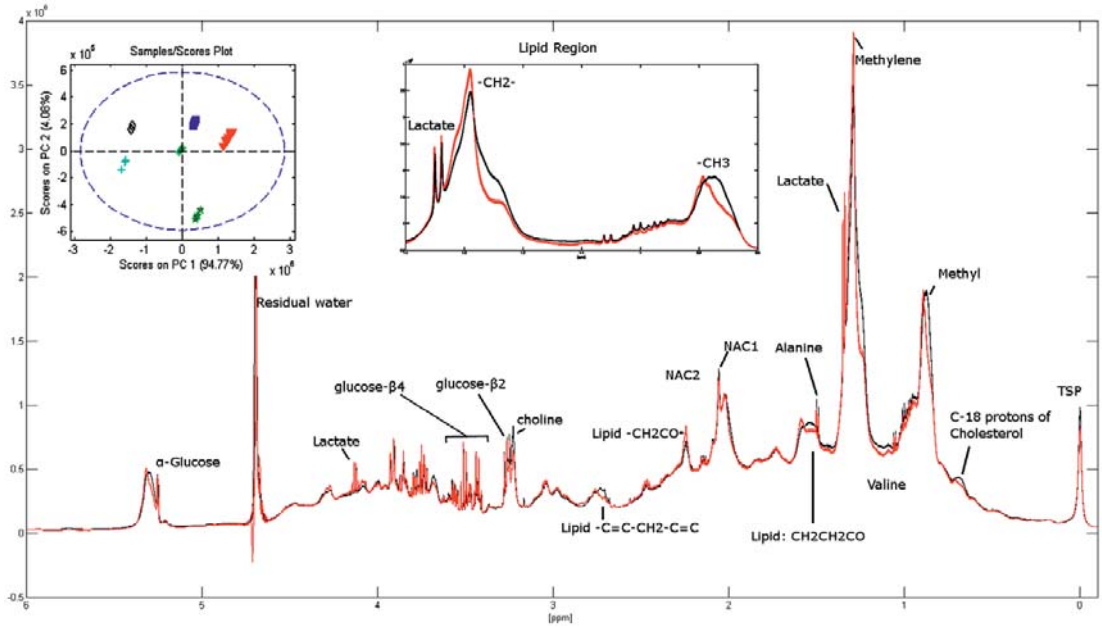

Figure 3. Representative ^{1}H NOESYGPPR 1D spectra with assignments of the main metabolites. The spectra in red (and black) consist of 5 spectra (superimposing) from two of the subjects. The reproducibility was evaluated by PCA and the score plot shows the spectra from all 6 subjects, clearly depicting larger inter subject variance compared to intra subject variance. NACl and NAC2 refer to composite acetyl signals from α1-acid glycoprotein. PCA: Principal Component Analysis.
doi:10.1371/journal.pone.0042330.g003

fasting glucose, triglycerides, micro-CRP between the high and low VO_{2max}-groups are supported by previous findings [21–23]. In addition, the increased levels of circulating liver enzymes (ALAT and Gamma-GT) in the low VO_{2max}-group may reflect more liver fat and increased insulin resistance [24]. Previous results from a study of rats with genetically low aerobic capacity indicate that low VO_{2max} impairs the hepatic oxidative capacity and therefore contributes to increased amounts of liver fat [25]. Elevated levels of Gamma-GT, even within the normal range, have previously been associated with the presence of CVD risk factors, metabolic syndrome, and type 2-diabetes [26,27].

Ideally, obtaining blood samples after overnight fasting would be preferable due to the elimination of chylomicrones from the circulation further enabling a more detailed overview of lipids and lipoprotein sub-classes [28]. However, fasting for several hours is neither easy to accomplish in a population-based large-scale study, nor preferable when performing a VO_{2max}-test. In addition to a possible influence on the lipid metabolites in the MR spectra, it is also possible that the lack of fasting may have interfered with the results on glucose and total cholesterol levels. In this study there was no difference in total cholesterol levels between the high and low VO_{2max}-group. Previous studies have reported inverse correlation between VO_{2max} and total cholesterol [29,30].

In conclusion, low VO_{2max} is associated with elevated levels of free choline and decreased levels of phosphatidylcholine, even in a cohort of healthy individuals. The precise reason for the shift in the choline/phosphatidylcholine ratio between subjects in the high versus the low VO_{2max}-group is unclear, but might be associated with phospholipase activity, or differences in cardiac or hepatic release. Further studies should be conducted on free choline and phosphatidylcholine to validate their potential as early risk-markers of CVD and predictors of VO_{2max}.

Materials and Methods

Study Participants

The third wave of the Nord-Trøndelag Health Study (HUNT3) in Norway was carried out between 2006 and 2008 and the results reported in the present publication stems from this part of the large HUNT study. Among 50,821 participants in HUNT3, 4631 healthy, adult subjects attended a sub-study designed to measure VO_{2max}, called the Fitness Study [31]. Participants in the Fitness Study reported to be free from heart- or lung-disease (details previously described [31]). From the Fitness Study-population, 220 individuals between 40 and 59 years were selected pair-wise with one having low and the other high VO_{2max} (selected from top or bottom 15 subjects within each age-year), but otherwise same gender, equal age in years, same physical activity index score (within 15% difference) and equal time since last meal. Subjects were ranged according to VO_{2max} reported as $mL{\cdot}kg^{-0.75}{\cdot}min^{-1}$, and maximum five pairs of subjects were matched from each age-year. Two subjects did not provide a blood sample, and the study thus included 218 subjects (45 males and 63 females in the low VO_{2max}-group, and 46 males and 64 females in the high VO_{2max}-group).

The study was approved by the Regional Committee for Medical Research Ethics, the Norwegian Data Inspectorate, and by the National Directorate of Health. The study is in conformity with Norwegian laws and the Helsinki declaration, and all participants signed a document of consent.

Clinical Measurements

Weight and height were measured on a combined scale (Model DS-102, Arctic Heating AS, Nøtterøy, Norway), and BMI was calculated as weight divided by height squared ($kg\ m^{-2}$). Blood pressure and resting heart rate were both measured while sitting (Critikon Dinamap 845XT, GE Medical Systems, Little Chalfont, Buckinghamshire, United Kingdom) and followed established guidelines [32].

An individualized protocol was applied to measure VO_{2max} treadmill running to exhaustion [33]. The VO_{2max}-test was performed using a ramp protocol where the speed was constant and the incline was increased with 2% every second minute until VO_{2max} was reached. Oxygen uptake kinetics were measured directly by a portable mixing chamber gas-analyzer (Cortex MetaMax II, Cortex, Leipzig, Germany) with the participants wearing a tight face mask (Hans Rudolph, Kansas City, USA) connected to the MetaMax II. The system has previously been found valid [34]. Heart rate was measured by radio telemetry (Polar S610i, Polar Electro Oy, Kempele, Finland). From the warm-up pace, the load was regularly increased when oxygen uptake kinetics flattened. Along with a respiratory quotient of 1.05 or higher, a maximal test was considered achieved when the oxygen uptake did not increase more than 2 $mL{\cdot}kg^{-1}{\cdot}min^{-1}$ at the highest effort or before the participant disembarked the treadmill [35]. VO_{2max} was measured as litres of oxygen per minute ($L{\cdot}min^{-1}$), and subsequently calculated as VO_{2max} relative to body mass ($mL{\cdot}kg^{-1}{\cdot}min^{-1}$) and VO_{2max} scaled ($mL{\cdot}kg^{-0.75}{\cdot}min^{-1}$).

Blood Analysis

All clinical-chemical analyses were performed on fresh venous non-fasting blood samples at Levanger Hospital, Norway. Non-fasting glucose (mmol/L) was analysed by Hexokinase/G-G-PDH methodology (reagent kit 3L82-20/3L82-40 Glucose, Abbot, Clinical Chemistry, USA). HDL-cholesterol (mmol/L) was analysed by Accelerator selective detergent methodology (reagent kit 3K33-20 Ultra HDL, Abbot, Clinical Chemistry, USA). Triglycerides (mmol/L) were analysed by Glycerol Phosphate Oxidase methodology (reagent kit; 7D74 Triglyceride, Abbot, Clinical Chemistry, USA). Creatinine (mg/dl) was analysed by Alkaline Picrate methodology (reagent kit; 7D65-20 Creatinine, Abbot, Clinical Chemistry, USA). Alanine aminotransferase (U/L) was analysed by NADH (with P-5′-P) methodology (reagent kit; 8D36-30 Alanine aminotransferase activated, Abbot, Clinical Chemistry, USA). Measurements below the instrument range were recorded as 9 U/L. Gamma glutamyl transferase (Gamma-GT) (U/L) was analysed by L-Gamma-glytamyl-3-carboxy-4-nitroanilide substrate methodology (reagent kit; 7D65-20 Gamma-glutamyl transferase, Abbot, Clinical Chemistry, USA). Measurements below and above instrument range were recorded as 3 U/L and 1544 U/L, respectively. Serum micro C-reactive protein (mg/L) was analysed by Areoset CRP Vario kit (Abbot, Clinical Chemistry, USA). Measurements below instrument range are recorded as 0.

Questionnaire-based Information

Physical activity was registered based on the responses to a self-administered questionnaire applied (http://www.ntnu.edu/hunt/data/que) [36]. The questionnaires included three questions: Question 1: "How frequently do you exercise?", with the response options "Never" (0), "Less than once a week" (0), "Once a week" (1), "2–3 times per week" (2.5) and "Almost every day" (5). Question 2: "If you exercise as frequently as once or more times a week: How hard do you push yourself?" with the response options: "I take it easy without breaking a sweat or losing my breath" (1), "I push myself so hard that I lose my breath and break into sweat" (2) and "I push myself to near exhaustion" (3). Question 3: "How

long does each session last?", with the response options: "Less than 15 minutes" (0.1), "16–30 minutes" (0.38), "30 minutes to 1 hour" (0.75) and "More than 1 hour" (1.0). Each participant's response to the above mentioned three questions (i.e. numbers in brackets) were multiplied to calculate a physical activity index score [36]. As the second and third question only addressed people who exercised at least once a week, both "Never" and "Less than once a week" yielded an index score of zero. Participants with a zero score were categorized as inactive.

Dietary habits were self-reported in a questionnaire. For "Fruit and berries", "Vegetables", "Sausages/hamburgers" and "High-fat fish" the possible response-options were "0–3 times a month" (1), "1–3 times a week" (2), "4–6 times a week" (3), "once a day" (4), and "twice or more a day" (5). Health-status was also self-reported and the options were "bad" (1), "not quite good" (2), "good" (3), or "very good" (4). The mean values were calculated from the answers from all the participants in each group (i.e. numbers in brackets).

Metabolic Profiling

Venous non-fasting blood samples were collected in serum-tubes with no additives. The blood was centrifuged at 3000 rpm for 10 minutes approximately 1 hour after sample collection. The serum samples were stored at −80°C in the biobank until being used for metabolic profiling. The serum samples were slowly thawed at 4°C. Aliquots of 150 µL were mixed with equal amounts of buffer solution (Na_2HPO_4 ×7H_2O (0.075M), 4% NaN_3 in H_2O (5ml, mass % of NaN_3 versus mass % of H_2O), TSP (3-(trimethyl-silyl) propionic acid-d4, 0.4g), D_2O (100 mL), pH adjusted to 7.4 with 1M HCl (1M NaOH), filled up to 500 mL with H_2O) and transferred to high-quality 3 mm MR tubes. The ratio between H_2O and D_2O was 90:10 in all samples. In order to assess the reproducibility of sample preparation and spectral acquisition, our daily protocol included a set of five samples individually prepared from a single healthy individual every day.

MR Experiments

The MR spectra were acquired using a Bruker Avance II (Bruker Biospin, Rheinstetten, Germany) with digital receiver unit (DRU) operating at 600 MHz for proton (1H). The probe was a TCI 1H-13C/15N/D with z-gradient and automated tuning and matching unit. All spectra were recorded in an automatic fashion using a BACS-60 sample changer and the ICON-NMR software (Bruker Biospin). Proton spectra were obtained at a constant temperature of 310 K using a modified Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with presaturation during the relaxation delay (Bruker: cpmgpr1d) to achieve water suppression and to facilitate the detection of low molecular weight species by avoiding the large overlapped signals derived from proteins and large molecules. The spectra were collected with 64 scans and 4 dummy scans. The acquisition time is set to 3.067 sec, measuring the FID via collection of 36864 complex data point resulting in a sweep width of 20.0363 ppm. A relaxation delay of 4 seconds was used, during which a presaturation of 25 Hz was applied. Effective echo time was 80ms and data acquisition starts at maximum of last echo. An exponential apodization of 1Hz was applied prior to Fourier transform. Measurement and processing was done in full automation using Bruker standard automation programs controlled by ICON-NMR (along with TopSpin v2.1 patchlevel 6). Chemical shift was calibrated to the middle of the alanine peaks at 1.50 ppm. The reproducibility spectra were acquired using nuclear Overhauser effect spectroscopy (NOESY, Bruker: noesygppr1d) with the same parameters as CPMG with the exception of 32 scans. The assignments of chemical shifts were done on the basis of previously published data [37].

Data Processing and Multivariate Analysis

Data analysis was performed with MATLAB (Version 7.9.0; The Math Works, Natick, MA, USA). The spectra were divided into 850 segments, each 0.01 ppm wide for a spectral window ranging from 0.5 to 9.0 ppm to reduce minor chemical shift alterations [38]. The segments between 4.5–5.0 ppm were excluded to remove variation in water suppression efficiency. Spectra were finally normalized by setting the total spectral area to a constant value (= 1) for all spectra to minimize possible differences in serum concentration between the samples.

Unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA) were performed using PLS_Toolbox v5.8.3 (Eigenvector Research, Manson, WA, USA). PCA reduces the dimensionality of the data and summarizes the structure of the multiple MR spectra visualized in score plots and loading profiles. The variance structure of the data is explained through linear combinations of the variables called principal components (PCs). The first PCs will be in the direction explaining most of the variance in the data set. In the score plot of the PCs, samples with a similar metabolic profile will cluster, while the corresponding loading profile displays the importance of each variable within the PC. PLS-DA is a supervised classification method which uses the class information to detect variables generating maximum separation between the classes (high and low aerobic capacity). All statistical models were cross-validated with a single 10-fold Venetian blind cross validation; in each run 10% of the data were left out of the training and used to test the model. The optimal model contains the number of latent variables yielding the lowest percentage of misclassification. A permutation test was performed (10000 permutations) to evaluate the significance of the difference between the classes [39].

To evaluate the reproducibility of the sample preparation and metabolomics analyses, PCA of the replicate spectra from single subjects (in total 30 sample preparations from 6 subjects) were performed for comparison of the inter versus intra subject variance.

Colorimetric Analysis of Free Choline

In a sub-cohort of 39 participants (20 women and 19 men) from the low VO_{2max}-group and 38 participants (21 women and 17 men) from the high VO_{2max}-group, the level of free choline was measured in serum. The groups were matched on fasting status, age and physical activity index score. The serum was analysed with the Choline/Acetylcholine Quantification Kit according to the manufacturer's instructions (Abnova, Taipei City, Taiwan).

Statistical Analyses

PASW Statistics 17.0 (IBM, New York, USA) was used for traditional statistical analyses. All statistical tests were two-sided, and p-values below 0.05 were considered statistical significant. Kolmogorov-Smirnov test was used to test for normality. One-Way ANOVA was used for comparing variables between the high and the low VO_{2max}-groups, and Kruskal-Wallis test was used in non-parametric analyses. Results are given in mean ± SE. Pearson's correlation was used to study associations between normally distributed variables, and Spearman's correlation was used in non-parametric analyses. The correlation analyses were performed separately for the high and low VO_{2max}-group.

Acknowledgments

Nord-Trøndelag Health Study (The HUNT Study) is a collaboration between HUNT Research Centre (The Faculty of Medicine, Norwegian University of Science and Technology NTNU), Helse Midt-Norge, Nord-Trøndelag County Council and The Norwegian Institute of Public Health.

Author Contributions

Conceived and designed the experiments: TFB UW ISG RV AB. Performed the experiments: RV AB. Analyzed the data: RV AB GFG STA. Wrote the paper: RV AB.

References

1. WHO (1999) Report of a WHO consultation: definition of the metabolic syndrome, diagnosis, and classification of diabetes mellitus and its complications. I. Diagnosis and classification of diabetes mellitus. 1–59.
2. Maxwell MS, Goslin BR, Gellish RL, Hightower KR, Olson RE, et al. (2008) Metabolic syndrome status changes with fitness level change: a retrospective analysis. Metab Syndr Relat Disord 6: 8–14.
3. LaMonte MJ, Barlow CE, Jurca R, Kampert JB, Church TS, et al. (2005) Cardiorespiratory fitness is inversely associated with the incidence of metabolic syndrome: a prospective study of men and women. Circulation 112: 505–512.
4. Farrell SW, Cheng YJ, Blair SN (2004) Prevalence of the metabolic syndrome across cardiorespiratory fitness levels in women. Obes Res 12: 824–830.
5. Kavanagh T, Mertens DJ, Hamm LF, Beyene J, Kennedy J, et al. (2002) Prediction of long-term prognosis in 12 169 men referred for cardiac rehabilitation. Circulation 106: 666–671.
6. Myers J, Prakash M, Froelicher V, Do D, Partington S, et al. (2002) Exercise Capacity and Mortality among Men Referred for Exercise Testing. New England Journal of Medicine 346: 793–801.
7. Nicholson JK, Wilson ID (2003) Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. Nat Rev Drug Discov 2: 668–676.
8. Idle JR, Gonzalez FJ (2007) Metabolomics. Cell Metab 6: 348–351.
9. Sabatine MS, Liu E, Morrow DA, Heller E, McCarroll R, et al. (2005) Metabolomic identification of novel biomarkers of myocardial ischemia. Circulation 112: 3868–3875.
10. Lewis GD, Wei R, Liu E, Yang E, Shi X, et al. (2008) Metabolite profiling of blood from individuals undergoing planned myocardial infarction reveals early markers of myocardial injury. J Clin Invest 118: 3503–3512.
11. Barba I, de Leon G, Martin E, Cuevas A, Aguade S, et al. (2008) Nuclear magnetic resonance-based metabolomics predicts exercise-induced ischemia in patients with suspected coronary artery disease. Magn Reson Med 60: 27–32.
12. Tappia PS, Dent MR, Dhalla NS (2006) Oxidative stress and redox regulation of phospholipase D in myocardial disease. Free Radic Biol Med 41: 349–361.
13. Dhalla NS, Xu YJ, Sheu SS, Tappia PS, Panagia V (1997) Phosphatidic acid: a potential signal transducer for cardiac hypertrophy. J Mol Cell Cardiol 29: 2865–2871.
14. Zeisel SH, Blusztajn JK (1994) Choline and human nutrition. Annu Rev Nutr 14: 269–296.
15. Buchman AL, Jenden D, Roch M (1999) Plasma free, phospholipid-bound and urinary free choline all decrease during a marathon run and may be associated with impaired performance. J Am Coll Nutr 18: 598–601.
16. Danne O, Mockel M (2010) Choline in acute coronary syndrome: an emerging biomarker with implications for the integrated assessment of plaque vulnerability. Expert Rev Mol Diagn 10: 159–171.
17. Danne O, Lueders C, Storm C, Frei U, Mockel M (2007) Whole blood choline and plasma choline in acute coronary syndromes: prognostic and pathophysiological implications. Clin Chim Acta 383: 103–109.
18. Danne O, Mockel M, Lueders C, Mugge C, Zschunke GA, et al. (2003) Prognostic implications of elevated whole blood choline levels in acute coronary syndromes. Am J Cardiol 91: 1060–1067.
19. Konstantinova SV, Tell GS, Vollset SE, Nygard O, Bleie O, et al. (2008) Divergent associations of plasma choline and betaine with components of metabolic syndrome in middle age and elderly men and women. J Nutr 138: 914–920.
20. Miettinen TA, Naukkarinen V, Huttunen JK, Mattila S, Kumlin T (1982) Fatty-acid composition of serum lipids predicts myocardial infarction. British Medical Journal (Clinical research ed) 285: 993–996.
21. Kuo HK, Yen CJ, Chen JH, Yu YH, Bean JF (2007) Association of cardiorespiratory fitness and levels of C-reactive protein: data from the National Health and Nutrition Examination Survey 1999–2002. Int J Cardiol 114: 28–33.
22. Sassen B, Cornelissen VA, Kiers H, Wittink H, Kok G, et al. (2009) Physical fitness matters more than physical activity in controlling cardiovascular disease risk factors. Eur J Cardiovasc Prev Rehabil 16: 677–683.
23. Laukkanen JA, Lakka TA, Rauramaa R, Kuhanen R, Venalainen JM, et al. (2001) Cardiovascular fitness as a predictor of mortality in men. Arch Intern Med 161: 825–831.
24. Messier V, Karelis AD, Robillard ME, Bellefeuille P, Brochu M, et al. (2010) Metabolically healthy but obese individuals: relationship with hepatic enzymes. Metabolism 59: 20–24.
25. Thyfault JP, Rector RS, Uptergrove GM, Borengasser SJ, Morris EM, et al. (2009) Rats selectively bred for low aerobic capacity have reduced hepatic mitochondrial oxidative capacity and susceptibility to hepatic steatosis and injury. J Physiol 587: 1805–1816.
26. Bougle D, Zunquin G, Sesboue B, Sabatier JP (2010) Relationships of cardiorespiratory fitness with metabolic risk factors, inflammation, and liver transaminases in overweight youths. Int J Pediatr 2010: 580897.
27. Kim DJ, Noh JH, Cho NH, Lee BW, Choi YH, et al. (2005) Serum gamma-glutamyltransferase within its normal concentration range is related to the presence of diabetes and cardiovascular risk factors. Diabet Med 22: 1134–1140.
28. Soininen P, Kangas AJ, Wurtz P, Tukiainen T, Tynkkynen T, et al. (2009) High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism. Analyst 134: 1781–1785.
29. Carnethon MR, Gulati M, Greenland P (2005) Prevalence and cardiovascular disease correlates of low cardiorespiratory fitness in adolescents and adults. Jama 294: 2981–2988.
30. Dvorak RV, Tchernof A, Starling RD, Ades PA, DiPietro L, et al. (2000) Respiratory fitness, free living physical activity, and cardiovascular disease risk in older individuals: a doubly labeled water study. J Clin Endocrinol Metab 85: 957–963.
31. Aspenes ST, Nauman J, Nilsen TI, Vatten L, Wisloff U (2011) Physical Activity as a Long Term Predictor of Peak Oxygen Uptake: The HUNT-Study. Med Sci Sports Exerc 43: 1675–1679.
32. Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, et al. (2007) 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). J Hypertens 25: 1105–1187.
33. Rognmo Ø, Hetland E, Helgerud J, Hoff J, Slørdahl SA (2004) High intensity aerobic interval exercise is superior to moderate intensity exercise for increasing aerobic capacity in patients with coronary artery disease. Eur J Cardiovasc Prev Rehabil 11: 216–222.
34. Meyer T, Davison RC, Kindermann W (2005) Ambulatory gas exchange measurements–current status and future options. International Journal of Sports Medicine 26 Suppl 1: S19–27.
35. Shephard RJ (2000) Maximal Oxygen Uptake. In: Shephard RJ, Åstrand PO, editors. Endurance in Sport. Second ed. Cornwall: Blackwell Science. 301–310.
36. Kurtze N, Rangul V, Hustvedt BE, Flanders WD (2008) Reliability and validity of self-reported physical activity in the Nord-Trøndelag Health Study: HUNT 1. Scandinavian Journal of Public Health 36: 52–61.
37. Nicholson JK, Foxall PJ, Spraul M, Farrant RD, Lindon JC (1995) 750 MHz 1H and 1H-13C NMR spectroscopy of human blood plasma. Anal Chem 67: 793–811.
38. Barton RH, Nicholson JK, Elliott P, Holmes E (2008) High-throughput 1H NMR-based metabolic analysis of human serum and urine for large-scale epidemiological studies: validation study. International Journal of Epidemiology 37: i31–i40.
39. Westerhuis J, Hoefsloot H, Smit S, Vis D, Smilde A, et al. (2008) Assessment of PLSDA cross validation. Metabolomics 4: 81–89.

Paper II

Is not included due to copyright

Paper III

Is not included due to copyright