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Applications of High-Resolution Magic Angle Spinning MRS in Biomedical Studies I -- Cell Lines and Animal Models

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Summary

High-resolution magic angle spinning (HRMAS) magnetic resonance spectroscopy (MRS) allows for direct measurements of non-liquid tissue and cell specimens to present valuable insights of the cellular metabolisms of physiological and pathological processes. HRMAS produces high resolution spectra comparable to those obtained from solutions of specimen extracts but without complex metabolite extraction processes, and preserves the tissue cellular structure suitable for pathological examinations following spectroscopic analysis. The technique has been applied in a wide variety of biomedical and biochemical studies and become one of the major platforms of metabolomic studies. By quantifying single metabolites, metabolite ratios or metabolic profiles in their entirety, HRMAS presents promising possibilities for diagnosis and prediction of clinical outcomes for various diseases, as well as deciphering of metabolic changes resulting from drug therapies or xenobiotic interactions. In this review, we evaluate HRMAS MRS results on animal models and cell lines reported in literature, and present the diverse applications of the method for the understanding of pathological processes, the effectiveness of therapies, the developments of disease animal models, and the new progresses in the HRMAS methodology.

Graphical Abstract

High resolution magic angle spinning (HRMAS) magnetic resonance spectroscopy (MRS) is a method which enhances resolution for non-liquid biological samples. Since its introduction in 1996 (19), HRMAS has been used in a wide variety of biomedical and biochemical studies and is one of the major techniques used in metabolomic analysis. In this review, HRMAS results on animal models and cell lines are evaluated, with discussions of applications for understanding pathological processes, therapy effectiveness, disease animal model development, and methodology progress.

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Keywords

metabolomics; high resolution magic angle spinning (HRMAS); magnetic resonance spectroscopy (MRS); cell lines; animal model

Introduction

Magnetic resonance spectroscopy (MRS) has been widely used as analytical method in biochemical and biomedical studies to elucidate molecular structures of new compounds (1,2), and investigate cellular metabolisms in tissue specimens (3) and cultured cells (4). MRS has further found broad implementations in investigations of new diagnostic, prognostic and therapeutic evaluation tools for a wide range of diseases (5–8).

Metabolites are the signatures of biochemical pathways, providing insight into cellular carbohydrate, protein, lipid, amino acid and xenobiotic metabolism. The ability of MRS to explore the metabolic statuses within healthy or diseased cells can help in the understanding of the physiological and pathological transformations accompanying various diseases and provide detailed insights into the metabolic mechanisms at cellular levels (9–11). Moreover, MRS enables the evaluation of cellular metabolic responses towards the developments of new and the monitoring of established drug therapies (12,13). Furthermore, complementary to genomics, transcriptomics and proteomics, the field of metabolomics, currently investigated by MRS alongside with mass spectrometry, is an emerging innovative tool for drug developments (14–16), biomarker discoveries (17) and for understanding the underlying molecular mechanisms of diseases (10,11). Thus, MRS offers the opportunity to

identify and quantify metabolites within cells and tissue specimens, and assists in the understanding and construction of metabolic profiles of benign or diseased tissues or cell lines to visualize the overall metabolic changes within the metabolome (18).

High-resolution magic angle spinning (HRMAS) proton magnetic resonance spectroscopy (^1H MRS) is a MRS technique introduced in 1996 to enhance the resolution of MRS for non-liquid biological samples (19). It can significantly increase the spectral resolution of non-liquid samples by averaging both the residual dipolar interactions and the isotropic bulk magnetic susceptibility existing in these samples (20). In comparison with solution MRS approaches assisted by chemical extractions of these non-liquid cell and tissue samples, HRMAS method allows a direct metabolic analysis at high spectral resolution comparable to that achieved with extract solutions, while preserves intact cellular structures (21,22). Therefore, not only the sample preparation time is significantly shortened, and procedures simplified, but the native morphological structures of these samples are preserved. Thus, HRMAS allows the analyzed tissue to be further studied including the regular histopathological evaluations and molecular biology analyses, such as IHC, PCR etc. (23,24). This benefit, for the first time, permitted histological and high resolution metabolic information to be collected from the exact same samples.

Here, studies employing HRMAS in cell line and animal models are reviewed and discussed. The review is organized in sections including studies aiming at providing a better understanding of disease mechanisms; utilizations for the assessment of new animal models; evaluations of therapy responses; and new developments and improvements in HRMAS methodology.

Understanding disease mechanisms

A variety of research reports have been focused on providing better understandings of the underlying biochemical processes for a wide range of diseases through studies of bodily cells and tissues by employing HRMAS.

In this section, first we discuss studies demonstrating new opportunities in diagnostic analyses, whether by providing new (lipid) biomarkers for certain diseases (25–27) or enabling new approaches in genetic diagnostics (28,29) and cancer subtype discrimination (30). Further, evaluations focusing on metabolic changes due to noxae (31–38) as well as, the HRMAS-assisted elucidation of molecular bacterial structures (39–41) will be reviewed.

Lipid biomarkers—As lipids and their metabolism as well as its regulation are essential in the context of cellular biology, alterations in cellular homeostasis are likely to be reflected in a change in lipid profile. Thus, lipid biomarkers play an important role within the metabolic evaluation of numerous diseases and are being discussed in a variety of publications.

In this context a study published in 2012 quantified lipids in nervous system cancer cell lines of both murine and human origin via HRMAS, finding a specific profile for each cell line as depicted in Fig. 1 (42). It was found that MRS visible lipid signals were likely derived only from lipid droplets within the cells, and the detected lipid signals were mostly attributed to unsaturated and saturated fatty acids. The study further showed that the detected signal was

dependent on the type of cell as well as on the size of the droplets, and lipid droplets with a diameter less than 0.34 μ m induced only weak signals. This information is crucial for the assessment of many other studies, which often discuss signals from unsaturated and saturated lipids as diagnostic tool, especially in association with malignant diseases (12,43,44). In a second study from 2013, the group investigated the same nervous system cancer cell lines with respect to the differences in lipid composition of isolated lipid droplets and whole cell lipid extraction. They revealed that lipid signals from isolated lipid droplets compared with those from whole cell extraction were similar but also showed substantial differences concerning the distribution of the lipid components, such that the whole cell lipid extraction contains lipids from lipid droplets, as well as cell and organelle membranes, which dominate the signal. (45). The study also confirmed that NMR of isolated lipid droplets compares to HRMAS of whole intact cells, suggesting that HRMAS lipid signals are from lipid droplets. Therefore, the possibly confounding influence that cell and organelle membrane lipids might have on the acquired lipid signal should be considered when performing HRMAS on the lipid fraction of whole cell lipids and their extracts. Thus, HRMAS may help identify malignant cells by evaluating their lipid profiles and furthermore, help specify these lipid signals as an analytical chemistry technique regarding the derivation of the respective lipid signals.

Lipids are also considered to be useful biomarkers for conditions other than cancer. Studies investigated the viability of intramyocellular lipids (IMCL) and other molecules as biomarkers in burn trauma and infections caused by *Pseudomonas aeruginosa*, an opportunistic pathogen commonly found in soil and water, that typically infects the human airway, urinary tract, burns, and wounds (25,27). In these studies, IMCLs showed significant increases with severity of the condition and could serve as biomarkers for grading the progression of a *Pseudomonas aeruginosa* infection as well as for the therapeutic effectiveness in burn trauma patients. Further, correlations between IMCL concentrations and down-regulations of various genes such as IRS1, PGC-1 β were reported, indicating alterations in insulin signaling and glucose metabolism, as well as mitochondrial dysfunction, all assisting understanding of molecular and metabolic processes relevant to burn trauma and *Pseudomonas aeruginosa* infection (25,27). Hence, by applying HRMAS, the authors were able to identify possible lipid biomarkers, and also obtained an understanding of molecular and metabolic processes attendant to burn trauma and *Pseudomonas aeruginosa* infection.

Moreover, studies indicate that in the context of lipid detection HRMAS resolves the issue of the known dependability of the measurable lipid intensities on the specific chemical extraction procedure employed. This phenomenon has been reported for other metabolic alterations resulted from non-cancer diseases. For instance, a study of intact mouse brain tissue revealed a new marker, N-acetyl, for the lysosomal storage disorder Sandhoff's disease (26). The detected N-acetyl resonance was not visible in both HPLC and mass spectrometry measurements of tissue extracts, thus emphasizing the usefulness of HRMAS intact tissue studies by detecting signals that might otherwise be lost in the process of chemical extraction.

Genetic diagnostics—Research explored the potential of HRMAS for genetic diagnostics by evaluating metabolic profiles to detect and classify genetic diseases (28,46). A murine pancreatic cell model study investigated a mutation in the tumor suppressor gene, MEN1, responsible for the hereditary multiple endocrine neoplasia syndrome (46). The study showed that four metabolites (glycerol, glutathione, γ -amino-N-butyrate and inosine) are able to significantly discriminate between wild type and MEN1 variants. Until now, while genetic testing can identify variants in the genome, it often fails to classify the impact of a certain mutation on cell functions. Nevertheless, this gap may be filled by metabolic observations and consequent interpretations of the exact impact of certain gene alterations. Thus, the metabolic phenotyping of genomic mutations by HRMAS may provide the possibility to carry out functional tests of yet unclassified metabolic effects associated with genomic mutations.

In this context HRMAS has been applied in order to identify potential therapeutic targets for genetically different types of neuroblastoma based on metabolic profiles of patient-derived MYCN-amplified and non-amplified neuroblastoma cells (28). In this study, the HRMAS-assisted establishment of the different molecular signatures which follow the various amplification of the oncogene MYCN first served to distinguish between different cancer subtypes and subsequently helped to reveal potential therapeutic targets in the choline pathway. Significant increases in phosphocholine (PC)/total choline (tCho) and taurine/tCho ratios and a decrease in glycerophosphocholine (GPC)/tCho were observed in MYCN amplified cells. Further, the study established that GPC was significantly lowered with amplification and might be a better marker and target than the increase in PC (28).

In this context, HRMAS was applied to investigate the metabolic changes due to genetic alterations in association with mechanisms underlying cancer metastasis. Here the metabolic information was not used for the diagnosis of a certain disease or cancer subtype but rather, to understand the mechanism of metastasis in an animal breast cancer model (29). It was demonstrated before that CXCR4, an alpha chemokine receptor, is up-regulated in many different cancer types (47). The truncation of the receptor's C-terminal end (34-CXCR4) has been linked to an enhanced invasiveness and metastasis in human breast cancer as recently reviewed by Muller et al. (31). In this context, HRMAS successfully characterized the metabolic signature of the described CXCR4-gene mutation and also a significant decrease in fatty acids was observed in the truncated 34-CXCR4 cells indicating marked metabolic changes due to the genetic alteration (29). Thus, HRMAS may help to evaluate the metastatic potential within cancer diagnostics in the future, although further studies are needed in this context.

Discrimination of cancer subtypes—Another interesting HRMAS-approach was pursued to examine the possibility of diagnosing and distinguishing glioma subtypes by provoking metabolic challenges (e.g. hyperglycemia under moderate hypothermia or normothermia) in glioma bearing mice. The study used HRMAS to assist MRS imaging (MRSI) for the detection of possible differences in glucose levels between healthy and tumor bearing animals (Fig. 2). Alongside the perception of an existing spatial heterogeneity in glioma concerning the response to hyperglycemia, an increase in glucose up to 6.6 fold in the tumor center was observed under hypothermia. Thus, the authors suggest that the MRSI,

assisted by HRMAS, could be employed in future studies characterizing different brain tumor subtypes *in vivo* (30). Nevertheless further studies are needed in order to confirm the ability of HRMAS-assisted MRSI to discriminate different tumor types and grades and its implementation in future studies of other animal models of human brain tumors.

Noxae impacts—A number of reports investigated the effects of various noxae using animal models with the assistance of HRMAS. Among other things, the effects of alcohol on murine brains were studied (32–35). Results from brain tissue studies show that ethanol exposure evokes significant metabolic changes within different parts of the brain, indicating neuronal degeneration and dysfunction as a consequence of alcohol consumption. These metabolic alterations were reported to be independent of the length of ethanol exposure and the age of the animal. Most alterations were observed in the frontal cortex; however, one study investigated fetal ethanol syndrome and localized the most prominent metabolic changes in the cerebellum and striatum of neonatal rat brains including deficits in glutamate, N-acetylaspartate (NAA) and creatine (35). In adult mice, a decrease in NAA and taurine and an increase in GABA (34,35), as well as changes in phospholipid metabolism and myo-inositol, were detected (32). Worth mentioning is a study conducted in 2012, in which the investigators compared *ex vivo* HRMAS and *in vivo* 1H MRS data in order to eradicate overlapped peaks seen in the lower resolution *in vivo* MRS (32). Such a comparison allowed differentiating choline-containing metabolite peaks overlapped in *in vivo* spectra and achieved a more accurate characterization of metabolic changes following ethanol exposure. The choline-containing resonance observed in the *in vivo* spectra could clearly be divided into separate peaks, including free Cho, PC, and GPC.

In summary, all reports agreed that the described metabolic alterations might eventually aid quantitative diagnosis, the development of therapeutic drugs, or serve as biomarkers for understanding of alcohol consumption.

Besides the effects of ethanol, another study focused on the impact of neurotoxin kainic acid on rat hippocampi to understand changes in neurochemistry due to microglia activation. Kainic acid is a widely used excitotoxic agent which can lead to a variety of neuropathological events and facilitate the experimental simulation of many neurological conditions. The HRMAS work successfully demonstrated the possibility to measure changes in GABA, glutamate, and myo-inositol due to neurotoxicity, apoptosis, epileptic seizures, and brain injuries, as well as other mechanisms related to excitatory amino acid transmission (36).

HRMAS was further employed to study metabolic alterations caused by such toxic industrial solvents as ethylene glycol monomethyl ether (EGME) and toluene (37,38). The effects of EGME on rat male reproductive organs were investigated and presented distinct metabolic changes in testes and capita epididymides, indicating a disturbance in energy supply processes and oxidative stress due to EGME exposure. The dominant metabolic changes in the reproductive organs entailed an increase in lactate, acetate, and amino acid levels and a decrease of creatine, glutathione, and PC, as well as a decrease in lipids in the caput epididymides region (37).

Toluene is used in the production of spray paints, gasoline and paint thinners and is frequently misused for its euphoric effects, mostly by adolescents. Toluene HRMAS study investigated metabolic changes due to binge toluene exposure in adolescent rat brains and reported brain region and toluene concentration dependent effects on glutamate and GABA. The initially observed increase in metabolic activities was followed by a dose-dependent decrease in the activities. Some metabolic effects endured for more than one week after the exposure. The study concluded that age-dependent neurochemical responses to toluene may contribute to unique behavioral patterns associated with drug abuse among adolescents (38). The aforementioned studies indicate the ability of HRMAS to reveal and decipher pathological processes arising not only from diseases but also from intoxications.

HRMAS has also shown utility as a useful tool for the development of new toxicological screening methods: a spheroid rat liver cell model and the liver toxin D-galactosamine were used to distinguish toxic treatment effects from untreated cell cultures. Metabolic alterations such as elevations in free cholesterol, cholesteroles, saturated and unsaturated triglycerides, as well as a decrease in other saturated triglyceride resonances, were able to separate treated samples from untreated controls (48).

Besides the investigation of the impact of chemical noxae, the impact of ultraviolet B (UVB) radiation on rat lenses as a probable cause for cataract development was systematically studied by using HRMAS (49–51). Between 2004 and 2006, a research group pursued different approaches to understand the correlation between UVB radiation and the formation of cataract. Starting with the investigation of a possible dose dependent relationship between UVB exposure and cataract, the research group was able to detect metabolic changes following UVB radiation of the rat lens and rule out a dose dependency (49). In all experiments, changes in water soluble metabolites were observed, namely decreases in e.g. taurine, PC, betaine and GSH, possibly indicating membrane damage caused by UVB irradiation. HRMAS results were cross-measured by lens forward light-scattering, which confirmed the lens damage occurred due to UVB exposure. Moreover, the group tried to elucidate the protective and repair mechanisms following the UVB radiation of the lens. Through utilization of HRMAS they came to the conclusion that all provoked metabolic changes returned to normal levels after 26 days, indicating a reversibility of the damage if no repeated UVB radiation was applied within the first month after exposure (Fig. 3) (50). Finally the group examined changes in different compartments of the murine lens after a high-dose UVB radiation exposure (51). Interestingly, they concluded that metabolic differences between the compartments within unexposed lenses are more distinct than in exposed lenses. UVB radiation seemed to generally reduce metabolic activity in certain compartments of the lens and to have triggered several biochemical mechanisms such as oxidation, osmotic changes, membrane dysfunction and problems with cell transportation.

HRMAS of bacteria

HRMAS has also been employed in order to investigate bacterial strains of *Campylobacter jejuni* (*C. jejuni*) for various purposes (39–41). *C. jejuni* colonizes poultry intestines and is a common source for human enteritis (39). Thus, researchers have been focused on the elucidation of its capsular polysaccharide structure (CPS), which plays a crucial role in its

pathogenic potential. By characterizing defined CPS variants and mutants, HRMAS was utilized to evaluate the role of CPS modifications in *C. jejuni* phage recognition and infectivity as well as in the development of phage resistance *in vivo*. With this knowledge, the authors hope to promote phage therapy against human pathogens, including *C. jejuni*, by targeting components within the CPS (39).

In addition, a study was implemented to understand the involvement of CPS, particularly of O-methyl phosphoramidate (MeOPN), in the process of infection and cellular interaction (41). With the aid of HRMAS, serum resistance assays and other methods, the authors were able to prove that the presence of MeOPN on the cell surface of *Campylobacter jejuni* provides significant protection against the humoral immune system, proving MeOPN to be a vital part of *C. jejuni*'s virulence.

Moreover, studies of the mechanism of colonization of poultry with focus on the *in vivo* biosynthesis of N-linked glycans in *C. jejuni*, an important player in infections, were also conducted (40). In this study, by employing HRMAS, the authors were able to characterize the bacterial N-linked protein glycolisation pathway for the first time. According to the authors, this information can be exploited for the purposes of glycoengineering, and hence, help to optimize the creation of antibodies with enhanced immune cell recruitment abilities (40).

Developing new animal models

HRMAS has been used to assess the suitability of metabolic phenotyping of new animal models for human diseases, for which there is still a lack of reliable models in the context of research and clinic.

One example is the HRMAS-assisted characterization of murine models for non-alcoholic fatty liver disease (NAFLD). NAFLD stages are classified by environmental, genetic, and histopathological features with different metabolic profiles measurable via HRMAS. The metabolic profiles showed an increase in the total lipid to water ratio, a decrease in polyunsaturated fatty acids as well as a decrease in choline compounds with increasing severity of disease. Thus, normal liver tissue and different stages of steatosis can be distinguished accurately by these metabolic profiles (52).

A different approach for evaluating potential animal models via HRMAS is to directly compare metabolic profiles of potential models with profiles obtained from human tissue to confirm the suitability of these models. Such an approach has been demonstrated in acquiring metabolic abnormalities in guanidinoacetate methyltransferase (GAMT) knockout mice successfully simulating GAMT deficiency (53); and in investigating metabolomic alongside transcriptomic characteristics of a panel of human breast cancer xenografts (54) (Fig. 4).

Diagnosis of neuropsychiatric disorders represents another area in which HRMAS murine model investigations provided insight into the complex pathophysiology and etiology of diseases, acknowledging that the lack of a comprehensive understanding of neuropsychiatric diseases has presented challenges in developing suitable animal models. Nonetheless,

several studies have successfully applied HRMAS measurements in the testing and design of murine models that display similar neurochemical patterns as those observed in human patients, such as the proposed NMDA hypofunction model of schizophrenia. In this context, two HRMAS studies assessed the effects of NMDA glutamate receptor blockage on neurochemical profiles to evaluate whether the observed metabolic abnormalities are consistent with clinical ¹H MRS findings of patients with schizophrenia and may be used as chemical murine models of the disease. A ketamine induced increase in glutamate levels in rat prefrontal cortex detected with HRMAS is found to be consistent with the hyperglutamatergic state seen in early stages of schizophrenia (55); and prolonged exposure to a low dose of the NMDA glutamate receptor antagonist phencyclidine produces different neurochemical profiles for different rat brain regions, including the frontal cortex, Ncl. accumbens, striatum, amygdala and hippocampus (56). These results are consistent with the observed decreases in glutamate seen in chronically ill patients (55), most likely due to the neuronal damage caused by the early excitotoxic state induced by the activation of glutamatergic neurotransmission. Supporting the hypothesis that the observed neurochemical changes are the results of the disease itself rather than a treatment effect caused by psychotropic drugs, an earlier study demonstrated that no changes in neurochemical profiles occurred after long term treatment with haloperidol, a common antipsychotic drug (57). Murine models for schizophrenia have been further investigated with HRMAS by comparing metabolic profiles of murine hippocampus and frontal cortex tissue exposed to single and early maternal deprivation with described neurochemical changes seen in patients of schizophrenia (58–60). Their results also indicate a reduction in glutamatergic signaling shown by decreases in glutamine and glutamate, similar to the observations made in chronically ill patients (55).

Furthermore, HRMAS has also been used for neuropsychiatric evaluation of a single prolonged stress (SPS) protocol as a model for post-traumatic stress disorder (PTSD) in mice (61). In contrast to the maternal deprivation protocol, biochemical changes in neurochemicals critical for energy metabolism (creatine and lactate), excitatory (glutamate and glutamine) and inhibitory (GABA) neurotransmission, and neuronal integrity (NAA) in the medial prefrontal cortex, amygdala complex, and hippocampus observed in SPS mice only agree with metabolic alterations seen in the prefrontal cortex of patients with PTSD, but did not agree with the observed neurochemical patterns in the other human brain regions, such as hippocampus and amygdala. Therefore, HRMAS studies caution the SPS murine model for PTSD may only be used to investigate certain aspects of the disease.

In summary, HRMAS provides the opportunity to control the functionality of already established models as well as to create reliable new animal models for all kinds of pathologies, including the establishment of models for psychiatric diseases, which still present a very challenging and partially uncharted field within medical research.

Evaluating therapy responses

The evaluations of treatment responses and drug interactions within the human body have always been an essential part of medical research. MRS has been utilized to develop new or assess known chemotherapeutics drugs. In addition to oncology-oriented research, HRMAS

has also been applied by other clinical disciplines in the search for new treatment possibilities (62–64).

Evaluation of cancer and stem cell treatment—While numerous HRMAS-based studies investigated well established chemotherapeutics, such as Cisplatin, Methotrexat (MTX), Doxorubicin, Tamoxifen, tyrosine kinase inhibitors and Bevacizumab (12,13,65–70) – mostly focused on providing a more detailed understanding of the drugs' mechanisms of action and intracellular processes triggered by those drugs, such as apoptosis - others explored the possibilities of new agents (14–16,63,64,71–76).

In the following section, first studies investigating known chemotherapeutics will be reviewed, followed by a presentation of new therapy approaches. Finally, stem cell treatments are discussed.

A number of studies tested the alkylating drug Cisplatin on different cell lines and with varied goals, but all attempted to discover viable biomarkers for monitoring treatment outcome (Fig. 5) (12,67,68). These studies include the investigation of glioma cells exposed to either Cisplatin or starvation to measure common and distinct metabolite changes following these two different origins of cell death (68); and comparing metabolic changes due to Cisplatin treatment vs. treatments of Doxorubicin and Methotrexate in human osteosarcoma cells (67) to discover differentiating biomarkers as well as the synergism among different drug effects. Metabolic changes following Cisplatin treatment are summarized in Table 1. This table reveals that except for valine and leucine, most amino acids consistently tend to decrease after treatments with Cisplatin (68). These metabolic changes may be interpreted as the consequence of changes in biochemical pathways, including activated anti-oxidative protection (67), increased activity of the Krebs cycle and oxidative phosphorylation (12,67), and increased catabolism in general (12). It is worth noting that all studies measured significant alterations in the lipid signals after the administration of Cisplatin, likely reflecting cell cycle arrest and PCD (43,77–79). Differences in the observed choline-containing metabolites were also discussed, but were considered to be in need of further investigations for conclusive statements. It also has to be kept in mind, that these are *in vitro* results that do not necessarily translate into an *in vivo* setting.

In comparison to Cisplatin, other drugs such as MTX, Doxorubicin, and Chloroethyl-nitrosurea showed very different metabolic changes regardless of their similar therapeutic effects through interaction with DNA in cells to reach eventual cell death (Table 2). Unsaturated fatty acids and GPC increases are seen in all studies presented in Table 2, indicating the involvement of membrane degradation in the process of PCD. However, regarding amino acids, unlike Cisplatin treatment under which almost all amino acids decrease, there is a clear tendency for increase in the cells treated with MTX and Chloroethylnitrosurea. A study investigating Chloroethylnitrosurea treatment of lung carcinoma and melanoma tissue reported an accumulation of glutamine, glycine and aspartate, which was interpreted to be consequent to a down-regulation in nucleotide triphosphate and deoxynucleotide triphosphate synthesis (13), and further being suggested

that glutamine, behaving as a PCD suppressor (80), was likely accumulated through treatment to protect the tumor cells.

HRMAS-assisted drug studies have also been controlled by and compared to *in vivo* measurements. Such an example is the investigation of the efficacy of two drugs targeting the VEGF pathway, monoclonal antibody Bevacizumab and VEGFR tyrosine kinase inhibitor SU1498, in cultured glioma cells (70). Bevacizumab, a VEGF-antibody established in 2004, has been utilized to treat various types of solid cancer diseases including colon and breast cancer (81) and was approved for glioblastoma treatment by the FDA in 2009 (82,83), however, it was rejected by the European Medical Agency (EMA) due to lack of evidence (84). Appositely to this discordance, the HRMAS cell results reviewed here show that, although a treatment effect of Bevacizumab was observed *in vivo* in glioblastoma patients (82), there are no significant metabolic changes taking place in *in vitro* experiments. In contrast, cells treated with SU1498 showed distinctive changes in lipids and GPC, hence proving the PCD effect of SU1498 *in vitro*. Results concerning the *in vivo* effectiveness of SU1498 are widely unknown. Remarkably, it was reported that HRMAS analysis could demonstrate the metabolic changes prior to visible evaluations that could be conducted by conventional cell viability assays, indicating the ability of HRMAS to detect early changes in cell metabolism following drug therapies. However, one should challenge the eligibility of a cell culture model, if the investigated drug knowingly exerts its main effect on blood vessels. As the authors discuss, using a primary glioblastoma cell culture model can only investigate the direct antibody effect on glioma cells, which often do express VEGF and VEGFR. Nonetheless, the effect on the vasculature may not be fully examined. A potential off-target effect by the small molecule inhibitor SU1498 should also be considered.

While the above study presented contradictory results concerning *in vivo* and *in vitro* experiments, there are also publications which report significant correlations between *in vivo* and *in vitro/ex vivo* results in the context of drug studies.

In 2006, the effectiveness of the vascular disrupting agent ZD6126 used for treatment of fibrosarcoma was analyzed *in vivo* and *ex vivo* in mice and correlating results of reductions in PC and GPC levels with therapy were reported (74). Similarly, *in vivo* 1H MRS and *ex vivo* HRMAS comparisons of the treatment responses of murine xenograft breast cancer models to Docetaxel were reported in 2009 (69). Both studies revealed significant decreases in choline, indicating changes in the vascular dynamics and proliferation as well as in the metabolic profile of the tumors. From these comparisons, it was suggested that both 1H MRS and HRMAS are useful methods for studying treatment responses in *in vivo* and *ex vivo/in vitro* (animal) studies, respectively. Although especially HRMAS studies of cultured cells are considered to be a valid and potentially powerful model to understand and simulate tumor treatment and drug response *in vivo*, the metabolic results observed in *in vivo/ex vivo* and *in vivo/in vitro* comparisons *respectively*, need to be treated with caution and evaluated critically and carefully. Hence, when comparing *in vivo* experiments with *ex vivo* tissue investigations, confounding factors like overlapping metabolite signals *in vivo* as well as ischemia time and commencing autolysis of the *ex vivo* tissue have to be reflected; when using *in vitro* cell culture models, the lacking existence of a (tumor) microenvironment and the differences in drug concentrations achieved at target should be considered.

In addition, to utilize HRMAS measurements to understand the therapeutic effect of chemotherapies, the method has also been used to examine the adverse effects of hormonal therapeutic Tamoxifen treatment in endometrial cancer cells (85). Tamoxifen, a selective estrogen receptor modulator, acts as an estrogen receptor antagonist in breast tissue and is, therefore, used to treat hormone-receptor positive breast cancer. However, Tamoxifen, as an estrogen receptor agonist in endometrial tissue, increases the risk of endometrial cancer in breast cancer patients (86,87). HRMAS results showed that cultured endometrial cells treated with Tamoxifen presented significant metabolic changes in nucleotides and choline-containing metabolites seemingly indicating proliferation and cell growth. Beyond that, the changes in nucleotides, namely an increase in uridine, adenosine, and their derivatives might reflect the drug's effect on RNA transcription for proliferation and thereby explaining further the mechanism of action of Tamoxifen in endometrial cells. However, since the reported measurements were conducted with Ishikawa cells, derived from a well-differentiated adenocarcinoma of the endometrium, they are likely to react differently to the drug than benign endometrial cells. Nevertheless, HRMAS may represent a powerful tool to further understand these adverse effects in the context of anti-hormonal treatment of breast cancer.

As mentioned above, HRMAS has also been used to study and develop new anti-cancer agents and investigate their therapeutic efficacy against different cancer types (14–16,63,64,71–76,85).

One new therapeutic approach in oncology is presented by the application of glycolipid derivatives or glycosides in glioma treatment. Although glycosides exhibit antimitotic activity on different glioma cell lines (88), it is still unclear as how the drugs exert their activity on the molecular level. Studies of cultured glioma cells treated with glycosides have reported a significant increase in total choline levels (15,71). A significant decrease in Coenzyme A (CoA) metabolites accounts for either an inhibition of the *de novo* synthesis of fatty acids or an increase in β -oxidation (71). Since the described alterations in phospholipid and fatty acid metabolism have been frequently associated with cell death, these studies reinforced the proposed cytostatic abilities of glycosides.

Another example for a new cancer treatment option is the investigation of rat glioma treated with a thymidine kinase–ganciclovir gene therapy regimen (73,75,85). With lipid resonances showing prominent presence in tumors (89), MRS visible lipids were investigated and characterized during programmed cell death (PCD) caused by gene therapy. Changes in polyunsaturated fatty acids (PUFA), namely $\text{CH}=\text{CH}$ and $\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}$, became evident as the most characteristic for PCD following a time-dependent increase (85). Furthermore, results show that the detected lipids possessed a high degree of rotational freedom, and therefore are unlikely to be membrane-bound. This observation is consistent with the idea that lipid resonances detected with HRMAS can only arise from intracellular droplets, i.e. lipid bodies or droplets (43), as previously discussed. Results also indicated the extensive increases in saturated and polyunsaturated lipids during PCD, and presented them as the most informative markers for PCD (73). Moreover, changes in other metabolites, including increases in PC, GPC and taurine were observed (75). Thus, the association of

lipid bodies with a progression of PCD can prospectively enable the monitoring of thymidine kinase-ganciclovir gene therapy and other apoptosis-inducing drugs.

The potential of HRMAS for monitoring cellular differentiation processes including stem cell therapy also has been evaluated. Mouse liposarcoma cells' response to the PPAR γ -ligands treatment, Thiazolidinedione, was investigated by HRMAS. Thiazolidinedione, a medication traditionally used in the treatment of diabetes mellitus type 2 (72), is believed to induce terminal adipocyte differentiation in liposarcoma cells and, thereby, making the cancer cells resume the process of maturation. HRMAS results suggested that the increases in PTC:PC ratio, myo-inositol, and glycerol might be possible biomarkers for the monitoring of the differentiation treatment with Thiazolidinedione. Similarly, metabolic profiling for differentiation of murine mesenchymal stem cells was monitored by HRMAS (62). Mesenchymal stem cells present a considerable potential for clinical applications within cell replacement therapies but cannot be monitored or tracked well once transplanted. The reported HRMAS study presented differentiated cells of showing significant decreases in choline, creatine, glutamate and myo-inositol, as well as increases in fatty acids, and suggested them, particularly those of fatty acids as biomarker for mesenchymal stem cell differentiation may be monitored *in vivo* during stem cell therapies.

Evaluation of neurological therapies—HRMAS has been frequently used to investigate new pharmacological interventions for Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). A study using a transgenic mouse model of ALS showed a significant decrease in both glutamate and glutamine levels in murine brain tissue and an extended lifespan following methionine sulfoximine (MSO) treatment, a glutamine synthetase inhibitor (64). Glutamate toxicity has been discussed as a mechanism for the degeneration of motor neurons in a variety of neurodegenerative diseases (90). By employing HRMAS measurements of intact mouse brain tissue, this study successfully proved that the *in vivo* treatment with MSO reduced glutamate and glutamine brain levels by inhibiting *in vivo* glutamate synthesis. As the disease manifests in both brain and spinal cord, the reported results emphasized the need for further investigations using metabolic profiling to determine the disease-specific response to MSO treatment for different parts of the central nerve system.

Many treatment approaches for AD focus on decreasing the amyloid plaque load, one of the currently known hallmarks of AD. In this context, HRMAS was successfully utilized to study the effects of different pharmacological agents inhibiting the formation of A β oligomers and, thus the onset of the β -amyloid pathology seen in AD.

Based on the known ability of cyclohexanols, such as scyllo-inositol (*s*-Ino), in inhibiting the formation of oligomers *in vitro* (91), a study evaluated the ability of HRMAS to quantify *s*-Ino levels in cortical and hippocampal tissues of two different transgenic AD mouse models following *s*-Ino administration *in vivo* (92). The intact tissue HRMAS results show that dietary supplementation of *s*-Ino led to an increase of *s*-Ino levels in cortex and hippocampus of AD mice (Fig. 6) and suggest HRMAS as a useful tool for the evaluation of future inositol treatment trials. The observed metabolic differences in glutamate, NAA, and

taurine levels in transgenic AD mouse models and wild type animals also support this conception.

A more recent study investigated methylene blue (MB) as another promising pharmacological agent for AD control. Behavioral tests evaluated the effect of MB on cognitive impairment in a transgenic AD mouse model. The results showed a positive effect of MB application on cognitive impairment, as well as a decreased β -amyloid deposition. To gain a more comprehensive understanding of the biochemical mechanisms involved in β -amyloid deposition, HRMAS was used to define several metabolites that enable a differentiation between AD and wild type animals (63). The results are in agreement with a decrease in NAA and GABA accompanying AD as described in the literature (93). However, no significant differences between taurine and inositol levels were observed. The authors attributed these differences to the variable age of the mice, resulting in different plaque loads, a factor that has to be considered when conducting mouse brain experiments. Following MB treatment, two of seven defined metabolites measured through HRMAS, alanine and lactate, were restored. Both alanine and lactate are classic markers of mitochondrial dysfunction, which has been shown to be a part of AD's pathophysiology by several studies (94,95).

Improving methodologies

Although HRMAS has been utilized as a research tool for almost twenty years, continuing improvements of the methodology have been achieved. Varieties of proposals published within the last ten years testify to the interest in this method and the broad spectrum of possible applications. The foci of those studies range from investigations of the impact of sample preparation, data acquisition, to new approaches in data processing and interpretation.

Data acquisition—A number of studies have been devoted to different sample preparation procedures and their possibly confounding impacts on the measured results. All of these studies aimed at better and more accurate presentations of cellular metabolites based on HRMAS measurements of intact tissues and cells.

Considering that data acquisition times can be long, sample spinning rates may be high, and biologically intact samples can still undergo various metabolic reactions, microwave radiation of samples was proposed to enhance their metabolic stability for measurement. Heating tissue samples rapidly to 80–90 degrees Celsius for 1–2 s with pulsed microwaves inactivates enzyme activity within the tissue specimens and “freezes” the tissue's metabolic state for HRMAS analysis. No significant change was seen both in the metabolic profile by HRMAS measuring of samples at 37°C and in tissue histology following microwave radiation (96,97). The impact of sample ischemia, prolonged spinning times (98) and freeze-thawing of samples (99) also have been studied. Three hours of spinning at 5kHz and 4°C resulted in more significant metabolic changes in rat brain tissue than the affect of a three-hour period of ischemia in a 20°C environment. The metabolic changes due to spinning entail increases in acetate, alanine, GABA, lactate and glycine, and decreases in GPC, tCho and glutamine.

Significant changes in rat kidney tissue before and after freeze-thawing were reported with more metabolites observed after freeze-thawing, probably due to a release of metabolites out of their macromolecule compounds (99). The information presented by this study is critical for all studies investigating changes in the metabolic profile as a consequence of a disease or therapeutic intervention, as studies have to make certain that any observed changes are effectively derived from a pathology or drug treatment, not due to experimental conditions. When discussing the role of data acquisition conditions, another study investigating the effect of the acquisition temperature on the metabolic pattern should be noted (100). In the respective study, reversible temperature-dependent changes mostly affecting mobile lipids and choline-containing compounds resonances were observed. Mobile lipid signals showed increased intensity at 37°C when compared to 0°C, presumably due to phase change in the neutral lipids contained in the lipid droplets. Despite the fact that the study's findings were ascertained in human brain tissue, they can be adapted to an animal *ex vivo* setting. Following from this, the many possible confounders occurring during data acquisition should be evaluated carefully before planning and conducting HRMAS experiments.

Shortly after the introduction of HRMAS, a study conducted in 1998 determined the benefit of spinning cell pellets in comparison to static conditions demonstrated that HRMAS allows for the identification of a large number of metabolites that were invisible under non-spinning conditions (20). Similar studies have been undertaken in more recent years (101,102), and accomplished comparisons between metabolic profiles through HRMAS of intact tissue samples and static MRS tissue extract experiments in animal brain tissues. While some results showed no significant difference between HRMAS and extract solution MRS experiments except a weaker Cho resonance seen in the HRMAS experiment that was absent in the extract measurement (101), the others concluded that some metabolite peaks could only be accurately identified through metabolite extraction (102). Nevertheless, all of these reports considered HRMAS method as a valuable alternative to extract solution MRS with the advantage of shorter preparation times and enhanced resolution when compared to *in vivo* or unspun *ex vivo* tissue investigations.

Work on new pulse programs for HRMAS data acquisition also has been introduced to identify and potentially quantify metabolites within tissue and cell lines (103,104). A two-dimensional HRMAS ¹H MRS program for quantitative metabolite analysis was proposed by using human breast and prostate cancer cells (103), as well as a new algorithm, TARQUIN (totally automatic robust quantitation in NMR), enabling the quantification of metabolites in any HRMAS data set was introduced (104). Both studies state that the essential information obtainable by quantitative HRMAS analysis would improve the validity of the method in functional genomic testing as well as drug response investigations. In addition to metabolic quantifications, an HRMAS method that facilitates the measurement of ¹H relaxation times in biological samples has been suggested (105). The utility of relaxation time information in the measurements of i.e. drug response was emphasized, together with its critical role in quantification analyses of metabolites.

Use of different pulse sequences represents one method by which more accurate metabolite measurements are achieved. Pulse and acquire sequences allow concentrations to be obtained free from the effects of T2 decay and free from the modulation of lipid and

macromolecule resonances. Where lipids are large, however, such as in breast cancer tissue, the large size and overlapping nature of metabolite peaks makes difficult quantification of small molecule resonances. To solve this issue, a Carr-Purcell-Meiboom-Gill (cpmg) sequence with an echo time (TE) of about 270 ms can be employed, which attenuates the lipids and essentially removes macromolecules. This change allows easier quantification of metabolites, but concentrations are no longer accurate due to the different T2 values of the metabolites.

Concentration standards can also complicate data acquisition, such as the fact that the common standard trimethylsilylpropanoic acid (TSP) binds to proteins. As an alternative, the Electronic Reference To access In vivo Concentrations (ERETIC) method has been explored, which inputs an electronically-generated NMR signal into the spectrum as a quantification reference point (106,107).

Proper metabolite assessment also can be complicated if repetition time (TR) between pulses is not long enough. If TR is too short, signal intensity for each metabolite will vary inversely with T1 relaxation time for each rather than actually representing metabolite concentration (108).

While the advantages of HRMAS in *ex vivo* analyses of intact tissues and cells are evident from the above discussions, *in vivo* application of the concept is difficult and challenging. Nevertheless, efforts in applying magic angle spinning (MAS) for *in vivo* profiling are noted (109–113). Studies examining the link between innate immune-deficiency, trauma in aging and insulin resistance found the fruit fly *Drosophila melanogaster* present a useful model for the investigation of genetics and physiology (Fig. 7). The fruit flies' metabolic profiles, obtained under 2 kHz spinning and 4°C of living animals, were analyzed to understand pathophysiological occurrences inside cells, such as mitochondrial dysfunction that accompanies insulin resistance and muscle wasting that occurs in trauma, as well as aging and immune deficiencies leading to higher susceptibility for infection. The studies compared and related the observed metabolic changes to the human pathophysiology and evaluated *Drosophila melanogaster* as an accurate model for the MRS investigation of genetic and metabolic diseases (109–111).

Studies further investigated a living mammal with phase-corrected magic angle turning (PHORMAT) (112). The ultra-slow spinning (1Hz) of a live mouse showed an enhanced spectral resolution compared with static *in vivo* MRS studies (Fig. 8). Recently, a new method, localized magic angle turning (LOCMAT), that extended PHORMAT, was also proposed. The approach allows acquisition of spectra from particular volumes of interest (VOI), instead of acquiring a spectrum from the entire body (113). Therefore, LOCMAT has the potential to open up new possibilities within research of metabolic activity, molecular diagnostics, therapy response evaluations, and drug testing in live animals. Furthermore, the LOCMAT technique may be used in a future clinical setting, where not the patient but the magnetic field could be spun.

Although HRMAS has overcome a number of confounding physical factors hampering 1H NMR spectroscopy, such as chemical shift anisotropy, dipolar couplings and bulk magnetic

susceptibility, assignments and quantifications of metabolites can be difficult when co-resonances occur. A number of studies have employed diffusion ordered spectroscopy (DOSY) in order to separate co-resonant signals, which particularly pose a problem in tissues with high lipid content (114,115).

Data processing—Various HRMAS data processing schemes have been reported (116–119). In a 2012 study conducted by Maria et al., a filter diagonalization method, similar to fast Fourier transformation (FFT), was suggested by fitting MRS time-domain signals and presented a viable alternative for processing heterogeneous biological materials without the need of water suppression and T_2 filtering (116). Other suggestions included detection and analyses of magnetization transfers between water and protons in phospholipids and proteins. By assessing the magnetization transfer through mapping of the magnetization transfer network, variables such as water relaxation and the amount of bound and free metabolites can be calculated to determine pulse sequences for adequate water suppression based on the information on interaction between water and cellular metabolites (117).

Data interpretation—Methods for understanding and interpretation of HRMAS spectra in terms of magnetic susceptibility and cell volume changes due to spinning and the impacts of these factors on the observed spectral results have been discussed (120,121). Magnetic susceptibility variation can be responsible for significant changes in the measured spectra, including the proposed interpretation of the apparent two water peaks seen in measurements of spinning cell suspensions (120). Contrary to previous assumptions (72,122), it is assumed that the two peaks don't derive from intra- and extracellular water resonances, but as a result of the formation of two water phases within the spinning rotor, where almost no exchange of water occurs between these two phases. A study has also reported that cell volume seemed to be independent of spinning rate or pressure from spinning, and no volume changes result due to fast rotation (121).

HRMAS has also been exerted as means to improve the interpretation of MR images. Investigations of the cell uptake of specific contrast agents commonly used in clinical radiology (Gd-BOPTA, Gd-DTPA, and Gd-DOTA), and the analogous complexes obtained by replacing Gd(III) with Dy(III), Nd(III), and Tb(III) were reported (122). Based on the consideration that the centrifugal force occurring in HRMAS separates cells from the extracellular water within the rotor, the intracellular and extracellular water may be investigated separately, and water exchange across erythrocyte plasma membrane was studied (123). The results showed very little water transport across the plasma membrane of erythrocytes. In respect of the fact that contrast agents either target the intracellular or the extracellular compartment, this information is valuable for properly interpreting contrast agent assisted MR images.

Overall observations

Since the introduction of HRMAS 20 years ago, its utilization in medical research has increased significantly, particularly during the last 10 years. Although the investigation of cancer diseases is still the number one area for applications of HRMAS, a wide range of

other fields, particularly in the investigation of neurological and psychiatric diseases, has also been witnessed (Fig. 9).

Conclusion

We reviewed a diverse and broad implementation of HRMAS MRS spectroscopy in biomedical research. This volume of work emphasizes the tremendous potential that HRMAS can contribute to medical sciences. This review demonstrates the diverse and broad implementation of HRMAS MRS spectroscopy in biomedical research and emphasizes the tremendous potential that lies within this method. Although the methodology itself is still being modified and improved, HRMAS has been implemented in a wide range of studies, including the investigation of the biochemical and molecular mechanisms of diseases or drug responses. Furthermore, HRMAS has also been utilized to augment and verify results of lower resolution *in vivo* MRS studies (69,74,124) for its ability to detect low concentration metabolites. Regrettably, HRMAS has not yet been used in clinical settings. The technique of HRMAS cannot be performed on human subjects. Among others, impeding factors for its clinical implementation include specialized expertise and access to high field MR spectrometers since most experimental studies are conducted with high magnetic field spectrometers of 7T or more, while clinically used magnets have maximum magnetic field strengths of 1.5–3T. Although high magnetic field spectrometers have the advantage of higher resolution, studies conducted at lower resolution at 3T or less would be more compatible with the existing equipment in most clinics. These circumstances might also explain why metabolic approaches have not yet substituted conventional pathological diagnostic approaches, such as histology or gene and protein expression analyses. Above all, the most important factor for the implementation of a technique like HRMAS in clinical practice is the establishment of the necessary synergy between the ones who have the medical questions, the clinicians, and the ones who have the technical means, the scientists. Nevertheless, considering the increase in synergic developments seen in laboratories around the world, HRMAS holds many possibilities in terms of diagnosis and prediction of clinical outcomes for various diseases and its potential clinical applications should be further explored.

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Abbreviations

¹H MRS	proton magnetic resonance
AD	Alzheimer's disease
Ado	adenosine
Ala	alanine

ALS	amyotrophic lateral sclerosis
Cho	choline
cpmg sequence	Carr-Purcell-Meiboom-Gill sequence
CPS	capsular polysaccharide
EGME	ethylene glycole monoethyl ether
ERETIC	Electronic Reference To access In vivo Concentrations
f.a	fatty acid
FFT	fast Fourier transformation
GAMT	guanidinoacetate methyltransferase
Glu	glucose
GPC	glycerophosphocholine
GSH	glutathione
HRMAS	high-resolution magic angle spinning
IMCL	intramyocellular lipid
L	lipid
Leu	leucine
LOCMAT	localized magic angle turning
Lys	lysine
MB	methylene blue
MRSI	magnetic resonance spectroscopy imaging
MSO	methionine sulfoximine
MTX	methotrexat
NAA	n-acetylaspartate
NAFLD	non-alcoholic fatty liver disease
PC	phosphocholine
PCD	programmed cell death
PE	phosphoethanolamine
Phe	phenylalanine
PHORMAT	phase-corrected magic angle turning

Pro	proline
PTC	phosphatidylcholine
PTSD	post-traumatic stress disorder
PUFA	polyunsaturated fatty acids
sIns	scyllo-inositol
SPS	single prolonged stress
TARQUIN	totally automatic robust quantification in NMR
Tau	taurine
tCho	total choline
TE	echo time
TG	triacylglyceride
TR	repetition time
Tyr	tyrosine
T1	spin-lattice relaxation time
T2	spin-spin relaxation time
UDP	uridine diphosphate
UDP-GalNaC	uridine diphosphate galactosamine
UDP-GlcNaC	uridine diphosphate glucosamine
UFA	unsaturated fatty acids
Urd	uridine
UTP	uridine triphosphate
UVB	ultraviolet B radiation
Val	valine
VOI	volumes of interest

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Figure 1. HRMAS spectra of five brain and nervous system tumor cell lines. The assignment of lipid signals is as follows: 1-CH₃, 2-(CH₂)_n-, 3-CH₂-CH₂-C=O, 4-CH₂-CH=, 5-CH₂-CH₂-C=O, 6=CH-CH₂-CH=, 7-CH=CH- (42).

Figure 2.

(A) MRSI time-course spectral patterns in hyperglycemic, hypothermic animals collected from position that best describes the center of the tumor mass displayed on the image at: euglycemia (black), 22 min hyperglycemia (blue), 44 min hyperglycemia (green) and 66 min hyperglycemia (red). (B) Ex vivo HRMAS water-suppressed spectra (LB = 12.0 Hz) obtained from euglycemic and hypothermic (1) and hyperglycemic and hypothermic (2) groups ((1) straight lines and (2) dotted lines), shown overlaid; spectra corresponding to samples collected from the tumor center. Right Column, enlarged views of the highlighted grey regions shown in the left column spectra (30).

Figure 3.

Relative differences in metabolite concentrations between exposed and non-exposed rat lenses 5 (n = 6), 25 (n = 6), 125 (n = 7), and 625 (n = 6) hr after UVB irradiation. Data were calculated as (exposed lens – control lens)/control lens. The bars represent 95% confidence intervals for the mean differences (50).

Figure 4. Mean HRMAS spectra of different breast cancer subtypes. Spectra of a) basal-like (n = 19), b) luminal-B (n = 6), c) ER negative (n = 27) and d) ER positive (n = 6) xenograft samples, and of e) ER negative (n = 13), and f) ER positive (n = 37) breast cancer tissue samples from patients (54).

Figure 5.
HRMAS NMR spectra of (a) control and (b) cisplatin-treated cells, at 48 h (12).

Figure 6. Four HRMAS spectra from the hippocampus of two triple transgene mice with and two without dietary scyllo-inositol supplementation. There was little overlap in the scyllo-inositol values between the two groups of mice ($p < 0.001$) (92).

Figure 7.

Experimental set up of in vivo HRMAS for the investigation of live *Drosophila* at 14.1 T. External standard trimethylsilyl-propionic-2,2,3,3-d₄ acid in deuterated water (TSP/D₂O). In the square: rotor position at the magic angle in HRMAS probe (109).

Figure 8.

The mouse-MAS NMR probe. a: An 70-day-old anesthetized female BALBc mouse placed in one-half of the mold. Also shown is the second half containing the mouse-shaped cavity. When the mold is closed the tail of the mouse is folded under its body. The area between the dotted lines indicates the part of the body generating the NMR signal in the PHORMAT experiment. b: Top part of the probe with the mouse and the mold mounted in place. The area between the arrows is the NMR-sensitive area of the NMR coil (112).

HRMAS - Fields of application

Figure 9. Fields of Application. Others: Regenerative medicine (1), Dermatology (2), Biochemistry (1), Traumatology (1), Food industry (2), Pulmonology (1).

Table 1

Selected metabolic changes due to Cisplatin treatment from (12,67,68).

	Duarte et al. ((12))	Mirbahai et al. ((68))	Lamego et al. ((67))
Lipids and Choline-containing metabolites	GPC↑ PC↓ UFA↑ TG↑	GPC↑ PC↓	GPC↑ PC↑ UFA↑
Amino Acids	Glu↓ Ala↓ Lys↓ Phe↓ Pro↓ Tyr↓	Glu↓ Ala↓ Lys↓ Phe↓ Pro↓ Tyr↓ Val↑ Leu↑	Glu↓Ala↓ Gly↓ Tau↓
Nucleotides	UDP-GlcNac↑ UDP-GalNac↑ Niaciamide↓	n/a	UDP-GlcNac↑ UTP↑ UDP↑ Inosine↓ Adenosine↓

Table 2

Metabolic changes due to classic chemotherapeutics from (12,67,68) for Cisplatin; (67) for Doxorubicin and Methotrexate; (66) and (13) for Chloroethyl-nitrosurea.

	Cisplatin	Doxorubicin	Methotrexate	Chloroethyl-nitrosurea	Chloroethyl-nitrosurea
Cell line/Tissue	Cell line	Cell line	Cell line	Cell line	Tissue
Cell type					
	Glioma(G)/Lung(L)/Osteosarcoma(O)	Osteosarcoma	Osteosarcoma	Melanoma	Melanoma (M)/Lung(L)
Lipids and Choline-containing metabolites					
	GPC↑ PC↓(G,L)/↑(O) UFA↑ - -	GPC↑* PC↓* UFA↑* Cho↑* -	GPC↑* PC↓ UFA↑* Cho↑* -	GPC↑ PC↓ - Cho↔ PE↑	GPC↑(M)/GPC↓(L) PC↑(M)/PC↑(L) UFA↑(L) Cho↑(L) PE↑
Amino Acids					
	Tyr↑(G,L) - Glu↓ Ala↓ Tau↓(O) Lys↓(G,L) Phe↓(G,L) Pro↓(G,L) Val↑(G) Leu↑(G)	Tyr↑ Gly↓ - - - - - - - -	Tyr↑* Gly↑* Glu↓* - - - Phe↑ - Val↑* -	- Gly? - - - - - - - -	- Gly↑(M) Glu↑ Ala↑(M) Tau↑(M) Lys↑ - Pro↑(M)/Pro↓(L) - Leu↑
Nucleotides					
	UDP-GlcNac↑(L,O) UTP↑(L,O) UDP↑(L,O) -	UDP-GlcNac↑ UTP↑* UDP↑* Uridine↑*	- UTP↓* UDP↓* Uridine↑*	- - - -	- - - -
Other compounds					
	Sorbitol↑ - - - - - - -	GSH↓* Creatine↑* M-inositol↓ S-inositol↓ - - -	- GSH↓* Creatine↓* - - - -	- - - - - -	- GSH↔(M) Creatine↑ - - Glucose↑ Succinate↓(M) Acetate↑(L) Formate↑(L)

* p>0.05