e3927, which has been published in final form at https://doi.org/10.1002/nbm.3927. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

NMR based metabolomics of biofluids in cancer

Guro F. Giskeødegård[1], Torfinn S. Madssen[1], Leslie R. Euceda[1], May-Britt Tessem[1], Siver A. Moestue[2,3], Tone F. Bathen[1].

1: Dept. of Circulation and Medical Imaging, Norwegian University of Science and Technology- NTNU, Trondheim, Norway; 2: Dept. of Clinical and Molecular Medicine, Norwegian University of Science and Technology-NTNU, Trondheim, Norway; 3: Dept. of Health Science, Nord University, Bodø, Norway.

Word count: 5904

Running head: Cancer metabolomics of biofluids

Keywords: biomarker, metabolism, urine, serum, diagnosis, monitoring.

Abbreviations used: AML: acute myeloid leukemia, AUC: area under the curve, BCa: breast cancer, BE: Barretts' esophagus, BPH: benign prostatic hyperplasia, CIN: cervical intraepithelial neoplasia, COPD: chronic obstructive pulmonary disease, CPMG: Carr-Purcell-Meiboom-Gill sequence, CRC: colorectal cancer, ER: estrogen receptor, FEC: fluorouracil, epirubicin, and cyclophosphamide, GERD: gastroesophageal reflux disease, HCC: hepatocellular carcinoma, HER2: human epidermal growth factor receptor 2, HPV: human papillomavirus, LC: liquid chromatography, MS: mass spectrometry, NAC: N-acetylated glycoproteins, NOESY: nuclear Overhauser effect spectroscopy, OCa: ovarian cancer, OPLS-DA: orthogonalized partial least squares discriminant analysis, OS: overall survival, PCa: prostate cancer, PSA: prostate specific antigen, ROC: receiver operating characteristic, TTP: time to progression,

Abstract

Metabolomics is the branch of “omics” technologies that involves high-throughput identification and quantification of small molecule metabolites in the metabolome. NMR-based spectroscopy of biofluids represents a potential method for non-invasive characterization of cancer. While the metabolism of cancer cells is altered compared to normal non-proliferating cells, the metabolome of several biofluids (e.g. blood and urine) reflects the metabolism of the entire organism. This review provides an update on the current status of NMR metabolomics analysis of biofluids with respect to 1) cancer risk assessment, 2) cancer detection, 3) disease characterization and prognosis and 4) treatment monitoring. We conclude that many studies show impressive associations between biofluid metabolomics and cancer progression, and suggest that NMR metabolomics can be used to provide information with prognostic or predictive value. However, translation of these findings to clinical practice is currently hindered by a lack of validation, difficulties in biological interpretation and non-standardized analytical procedures.

Introduction

The metabolic characteristics of cancer cells change during disease progression, and this will be reflected in their metabolic profiles.[1-3] Typical changes include deregulated uptake of glucose and amino acids, increased demand for nitrogen, and increased flux through anabolic metabolic pathways. This metabolic reprogramming can be used to discriminate between tumors of different phenotype, grade, or stage *ex vivo.*[4-6] Obtaining such information from biofluids would reduce the need for invasive collection of tissue biopsies. Blood and urine are easily accessible biofluids, and several attempts have been made to identify signatures associated with cancer risk, presence, and prognosis using nuclear magnetic resonance (NMR) as the analytical platform. However, metabolic profiles of biofluids reflect the metabolic state of the entire organism – and not only that of the tumor (Figure 1). NMR metabolomics can therefore potentially provide information about the overall health status of individuals.[7,8] As metabolite concentrations are regulated by numerous homeostatic mechanisms in the body, it may be challenging to relate metabolic profiles directly to cancer cell metabolism. As an example, serum lactate levels do not scale directly with tumor burden despite the fact that most tumors excrete this metabolite in large amounts.[9-11] Nevertheless, several recent studies suggest that biofluid metabolomics can provide information about cancer phenotype, grade and stage, and has potential as a clinical tool for cancer diagnostics, prognostics, and treatment monitoring.

Metabolomics is the branch of "omics" technologies primarily concerned with high-throughput identification and quantification of small molecule metabolites in the metabolome. In the serum[12] and urine metabolome[13] databases, detailed information is given on more than 4600 and 3100 human metabolites, respectively. These metabolites are downstream products of genome and proteome-wide interactions, and the metabolome thus provides a sensitive measure of the phenotype. Blood passes through every organ in the body, serving as transport for all molecules secreted or excreted by different tissues in response to physiological needs or stresses, while urine contains water-soluble products eliminated by renal filtration. The diagnostic potential of these biofluids is evident by their widespread use in clinical chemistry through history. [14,15]

The most commonly used platforms for metabolomics studies are NMR and mass spectrometry (MS), each associated with their inherent advantages and disadvantages. In general, MS methods are more sensitive than NMR (picomolar versus micromolar range), while NMR is highly reproducible and can provide quantitative measures without the need for labour-intensive sample preparation and fractionation.

However, the methodologies should be considered complementary, and the advantages of NMR based metabolomics are thoroughly covered in a recent review.[16] Detailed protocols for biofluid sample preparation are described by Bernini et al.[17] One-dimensional (1D) proton (^{1}H) NMR experiments are the most commonly applied sequences for NMR metabolomics,[18,19] usually including a NOESY sequence for serum and urine spectra and an additional CPMG sequence for filtering out macromolecule signals in serum samples (Figure 2). Two-dimensional (2D) NMR experiments are indispensable tools for metabolite identification in NMR-based metabolomics studies,[16,19-21] and also allows for metabolite quantification.[22] Further, there are now automatic platforms offering detailed quantification of lipoprotein subclasses, their lipid concentrations, and apolipoprotein A-I and B levels in serum,[23,24] which expands the use of NMR-based metabolomics towards more lipidomics related approaches.

In this review we will give an update on the current status of NMR metabolic profiling of biofluids for detecting and characterizing cancer (Figure 3).

Prediction of cancer development and detection of precursor lesions

For many cancers, curative treatment depends on early detection. However, cancers can be asymptomatic for months or years, during which they could be treated with minimal intervention, avoiding more invasive treatment and possibly increasing survival. Most approaches have so far focused on early detection of already present tumors, and include interventions such as mammogram screening for breast cancer (BCa). Several models for assessing the risk for various cancers based on clinical characteristics have been developed. Although they may show significant results at the population level, they generally have low predictive value for individual patients.[25] NMR-analyses of biofluids allows high-throughput, global, and unbiased metabolic analyses, which could be used for this purpose. Serum metabolic profiles can be predictive of several diseases, including cardiovascular disease,[26] diabetes,[27] and preeclampsia,[28] and several risk factors for cancer, such as smoking, obesity and diet, affect the serum metabolome.[29-31] It is therefore plausible that metabolomic analyses of biofluids could reveal metabolic phenotypes associated with increased risk of cancer.

Few studies have been performed linking NMR of biofluids prospectively to cancer risk, while a larger number of studies have assessed cancer risk by metabolic profiling using MS.[32-35] In 2015, Bro et al. published a study predicting whether Danish women (n=838) would develop BCa within 5 years based on a combination of NMR-analysis of non-fasting serum and lifestyle factors.[36] Using this approach, they

achieved a prediction accuracy of 82% in an independent validation set of 129 samples, approaching the diagnostic accuracy of mammogram screening.[37] This was later reproduced using a larger sample size from the same cohort, resulting in an area under the curve (AUC) for the receiver operating characteristic (ROC) curve of 0.86 for prediction of BCa development within 5 years.[38] However, in the same study, no relationship was found for prediction of future colon cancer. In a study on hepatocellular carcinoma (HCC), Fages et al. showed in a subgroup analysis with 22 cases and 43 controls that addition of NMR metabolic data improved the ability of current biomarkers to predict development of HCC within 2 years.[39] Development of HCC was associated with perturbations in fatty acid oxidation and amino acid-, lipid-, and carbohydrate metabolism two years prior to diagnosis, and adding metabolomic analysis to liver function and alpha-fetoprotein scores increased prediction sensitivity. While these findings must be reproduced in a representative and relevant target population before they can be considered for clinical use, they suggest that NMR of serum could have clinical utility in risk assessment for cancer.

A small number of studies have shown an association between NMR metabolic profiles in blood and urine and the presence of precancerous lesions. In a cohort of 31 controls and 31 patients with Barretts' esophagus (BE), Davis et al. showed that urinary metabolomic profiles could discriminate the two groups with an AUC of 0.96.[40] Hasim et al. showed that cervical intraepithelial neoplasia (CIN) could be discriminated from healthy women with a sensitivity and specificity of more than 90% based on NMR analysis of plasma samples.[41] Wang et al.[42] showed in a Canadian cohort that urine NMR could detect colonic polyps with a sensitivity of 83% and a specificity of 51%, which was confirmed by Deng et al.[43] for a Chinese cohort. This outperforms fecal immunochemical tests for detecting colorectal polyps, which have ranged in sensitivity from 9% for proximal polyps and 23% for distal polyps.[44]

It is important to consider whether these associations are reflective of the direct metabolic contributions of the precancerous lesion, the host response, the metabolic effects of associated factors, or a combination of the three. Several risk factors for cancer may affect serum metabolic profiles, including age, smoking, BMI, and levels of androgens and other growth hormones. Relating metabolic changes to the tumor is therefore challenging. However, given the high specificities reported in the literature, and the matched case-control designs used, it seems unlikely that these associations are explained merely by differences in known risk factors. CIN is caused by malignant transformation in response to persistent infection by human papillomavirus (HPV). Since HPV-infection is highly prevalent in the healthy population,[45] with most cases never progressing to CIN, the findings of Hasim et al.[41] may suggest that

failure to control HPV-replication induces systemic metabolic changes in the host. Similar reasoning could be followed for the findings of Davis et al.[40] on BE, which is caused by gastroesophageal reflux. In the Western adult population, the prevalence of symptomatic gastroesophageal reflux disease (GERD) is estimated to be 10-20%,[46] but most patients with GERD do not have BE.[47] It could therefore be hypothesized that progression from normal to precancerous tissue is associated with systemic metabolic changes, although no pattern of metabolic changes is consistently observed in these studies.

Cancer detection and diagnosis

Detecting cancer by metabolic profiling of biofluids would facilitate easy and minimally-invasive diagnostics, and allow for screening. Prostate cancer (PCa) has similar clinical symptoms as benign prostatic hyperplasia (BPH), and prostate specific antigens (PSA), often measured in men with suspicion of PCa, does not reliably separate these two conditions.[48] Thus new and easily accessible biomarkers would be highly beneficial. A pilot-study by Giskeødegård et al. showed significant differences in serum metabolism by combined NMR and MS analyses, separating PCa from BPH with a classification accuracy of 78%.[49] Increased dimethylsulfone was the NMR measured metabolite with highest discriminatory power (AUC 0.74); however using a panel of NMR and MS metabolites provided the best classification. Further, Kumar et al. demonstrated the possibility to separate PCa from healthy controls,[50] and PCa from both BPH and healthy controls by NMR analysis of filtered serum,[51] with classification accuracies ranging from 82-94%. In their cohort, dimethylsulfone was not quantified and they detected increased levels of sarcosine in PCa patients compared to both BPH and healthy controls. Urinary and serum sarcosine levels have previously been suggested as potential PCa biomarkers,[52,53] however the role of sarcosine in PCa has been debated as preliminary results could not be validated.[54] By NMR, sarcosine levels in urine and unfiltered serum might be too low to be reliably measured.

Several studies on small cohorts have searched for PCa biomarkers in semen or expressed prostatic secretions, showing decreased citrate levels in PCa compared to healthy controls.[55-57] This could however not be confirmed in a larger study performing metabolic profiling of seminal plasma from 151 high-risk participants, of which 98 had proven PCa.[58] In this cohort, citrate was not a predictor for PCa, and the significant metabolites choline and leucine did not improve diagnosis compared to PSA measurements. However, Perez-Rambla et al. have recently demonstrated significant metabolic differences in urine samples from PCa patients and BPH, without prior prostate massage.[59] The predictive value of this

classification model was moderate and only significant after variable selection, and thus requires validation in independent cohorts.

For BCa, the most common malignancy in women, NMR metabolic profiling of urine showed significant differences between BCa and controls (n=120 participants),[60] but with modest classification accuracy. Here, 28/30 quantified metabolites were significantly lower in urine samples from BCa patients, with formate, succinate, and uracil among the most important for separation. A study on a small cohort (n=57) further demonstrated the possibility to separate BCa from healthy controls using serum samples,[61] with higher lactate and a tendency of lower glucose and taurine in BCa patients. No further studies have validated these findings using NMR. However, Shen et al.[62] described 78 MS-measured metabolites differently expressed in plasma from BCa patients compared to controls where neither lactate, glucose, nor taurine were among the significant metabolites. Thus, there seems to be no consistency in which metabolic changes are characteristic of BCa, and the potential of detecting BCa by NMR of biofluids needs further investigation.

Pancreatic cancer has high mortality rates, and there is no reliable method for early diagnosis. Two small-scale studies have shown promising results for detecting pancreatic cancer by NMR metabolic profiling of urine samples with high classification accuracies.[63,64] Further, OuYang et al. showed that NMR metabolic profiles of serum from 17 patients and 23 healthy controls could be separated using principal component analysis.[65] Among the discriminatory metabolites were decreased levels of 3-hydroxybutyrate and lactate, which were also detected in a study comparing plasma from pancreatic cancer patients with chronic pancreatitis patients and healthy controls.[66] Further, Bathe et al. demonstrated the possibility to distinguish pancreatic cancer from benign hepatobiliary disease by NMR metabolic profiling of serum from 99 participants with high accuracy. A recent meta-analysis has been performed to assess the diagnostic potential of literature-curated NMR and MS metabolite markers using MS profiling of serum samples from pancreatic cancer patients (n = 59) and three clinically relevant control groups (colorectal cancer (CRC) patients, type 2 diabetes patients, and healthy controls).[67] The resulting panel of 10 metabolites (increased lactate, lyso-PC, alanine, choline, threonine, asparginine, tyrosine, lysine, and decreased palmitate and 3-hydroxybutyrate in cancer) showed high classification accuracy (AUC=0.99) for separating pancreatic cancer patients from non-cancers. However, the specificity for pancreatic cancer was low as the AUC remained high when comparing CRC with healthy controls. This shows that the identified metabolic pattern might be indicative of cancer, while not being cancer-type specific.

Detection of bladder cancer from urine is an interesting approach as urine is in direct contact with the bladder, and the potential for detection of bladder cancer has been examined in several studies by MS approaches.[68] NMR has also demonstrated potential of detecting bladder cancer from urine in a pilot study of 33 bladder cancer patients and 70 controls, where increased taurine levels in urine were detected in patients.[69] Moreover, two studies have demonstrated the potential of NMR profiling of serum for detecting bladder cancer. Cao et al. detected significantly increased levels of acetoacetate and glucose, and lower levels of citrate, glycine, lactate, tyrosine, and phenylalanine in cancer patients (n=39) compared to healthy controls (n=25).[70] Bansal et al. further demonstrated how serum metabolomics could significantly separate cancer patients (n=67) from healthy controls (n=32), and high from low grade bladder cancer with high sensitivity and specificity.[71] In contrast to the study by Cao et al., this study found increased lactate levels in serum from cancer patients; thus serum metabolic alterations related to bladder cancer development are not yet established.

Lung cancer is often at an advanced stage when detected and have poor survival rates, making earlier detection highly warranted. Both early and advanced lung cancer (n=77) could be separated from chronic obstructive pulmonary disease (COPD) (n=22) by serum NMR profiling with high classification accuracy.[72] This may be clinically relevant as both diseases are associated with tobacco use and chronic inflammation. Presence of lung cancer was characterized by reduced acetate, citrate, and methanol, and increased N-acetylated glycoproteins (NAC)-1, leucine, lysine, mannose, choline, and lipids. In the same study, early and late cancer could also be successfully separated, with patients with advanced cancer having further increases in NAC1.[72] Additionally, in a study by Puchades-Carrasco et al.[73] lung cancer cases could be separated from heathy controls when examining serum from a large patient cohort (n = 296), allowing for validation in an independent set of samples with high sensitivity and specificity.[73] Lung cancer patients had increased levels of lactate and glutamate, and decreased glutamine and histidine compared to healthy controls. Increased lactate and glutamate levels in cancer patients were also detected in a small study (n=35) by Zhang et al.[74] including only non-smokers. An earlier study including 85 lung cancer patients and 78 healthy controls also detected highly significant metabolic changes in plasma from cancer patients;[75] however in this study all lung cancer patients were smokers whereas more than half the control group were non-smokers or ex-smokers. Smoking status is a major confounder in these studies, as smoking has a significant effect on serum metabolism,[29] and there is a need for larger studies in representative cohorts to assess the clinical potential of metabolomics based detection of lung cancer.

Ovarian cancer (OCa) has the highest mortality rates among gynecologic cancers, and early stages are difficult to detect. Slupsky et al. demonstrated significant differences in NMR measured urine metabolic profiles between OCa patients (n=50) and healthy controls (n=72).[60] Surprisingly, the levels of all 30 quantified metabolites were significantly lower in OCa patients compared to controls. Significant metabolic differences have also been detected in serum samples between OCa patients and controls,[76] with validation of an independent set of samples yielding a sensitivity and specificity of 95% and 68%, respectively. The resulting model was also tested on a cohort of patients with renal cancer carcinoma. These patients were not incorrectly classified as OCa patients, indicating that the serum metabolic profiling bears potential for cancer type-specific diagnosis. OCa patients displayed significantly lower levels of alanine, valine, choline, creatinine/creatine, and lipids, and higher levels of acetoacetate, acetone, and β-hydroxybutyrate. NMR metabolic profiles of ovarian cyst fluid has been shown to contain information to separate benign from malignant cysts,[77,78] with both studies showing increased levels of lactate, isoleucine, valine, methionine, and alanine. More recently, Kyriakides et al. demonstrated significant metabolic differences in fluid from benign, borderline, and malignant ovarian cysts. Here malignant cysts were characterized by lower citrate levels and increased levels of lysine compared to benign and borderline cysts, respectively.[79] Interestingly, non- significantly increased levels of alanine, valine, lactate, and choline in malignant cysts were observed in agreement with previous findings.[78] Thus, metabolic profiling of several biofluids shows potential for detection of ovarian cancer.

The potential for cancer detection from NMR metabolic profiling of serum has also been demonstrated in small cohorts in other cancers, such as oral squamous cell carcinoma,[80-82] esophageal adenocarcinoma,[83] HCC,[84] upper urinary tract urothelial carcinoma,[85] and colorectal cancer (CRC)[86] with moderate to high classification accuracies. Another interesting approach for detection of CRC is examining fecal water as this in principle will reflect the full length of the colorectum. Metabolic profiling of fecal water extracts for detection of CRC in a study of 111 CRC patients and 412 controls resulted in a sensitivity and specificity comparable to that of colonoscopy.[87] Similar results were obtained in two smaller studies,[88,89] and as a consensus all studies showed low levels of butyrate as a potential marker for CRC, while there was less consistency between other metabolites. Common for NMR metabolomics of biofluids as a tool for detecting cancer is the lack of consistency between significant metabolites, and the need for validation in large, representative cohorts.

Disease characterization and prediction of prognosis

As discussed above, NMR metabolomics can be applied to various biofluids for detection of cancer in a screening setting and discrimination between malignant and benign conditions in symptomatic individuals. However, there is also a medical need for methods that can provide prognostic and predictive information and contribute to selection of the most appropriate therapeutic strategy for individual patients.

In BCa, it has been reported that metabolic profiling by MS of plasma can discriminate between human epidermal growth factor receptor 2 (HER2)-positive and –negative tumors, and between estrogen receptor (ER)-positive and –negative tumors.[90] However, this study illustrates the challenges of biological interpretation of metabolomics data. Relatively high levels of lactate and low levels of glucose are seen in HER2-positive subjects, in line with the metabolic characteristics of HER2-positive cell lines.[91,92] The interpretation of this finding is that higher aerobic glycolysis in HER2-positive tumors is reflected in the plasma metabolite levels. However, several studies have shown that ER-positive breast tumor tissues are associated with high lactate production.[4,93] A reasonable expectation would therefore be that plasma from patients with ER-positive tumors should have higher lactate and lower glucose levels than plasma from patients with ER-negative tumors. However, high glucose levels were found in plasma from ER-positive patients whereas no difference in lactate levels were observed. These results demonstrate how the plasma metabolome reflects more than just the metabolic input and output of cancer cells. Despite the unresolved questions related to pathobiological interpretation, NMR analysis of serum and/or urine can provide prognostic information.

In BCa, for example, the plasma metabolome has been found to discriminate between early stage and metastatic disease.[10] Among the most important discriminant metabolites were lactate, glucose, and pyruvate. Interestingly, there was a negative correlation between plasma lactate level and disease stage, emphasizing the complex tumor-host relationship in cancer. In a similar experiment, Jobard et al.[94] discriminated early BCa from metastatic BCa with 90% sensitivity and 79% specificity. Here, an OPLS-DA model was built using data from 85 patients and validated in a cohort of 112 patients. A metabolic profile consisting of 9 discriminant metabolites was established, but this profile was remarkably different from the one described by Richard et al..[10] The two studies had only one discriminant metabolite in common (pyruvate), but were in disagreement with respect to the level of pyruvate in metastatic versus early disease.

The ability of serum or urine NMR metabolomics to discriminate between different grades of disease has also been demonstrated in lung cancer,[73] oral cancer,[80] renal cancer,[95] pancreatic cancer,[63] bladder cancer,[71] and PCa.[50] In some cases, the diagnostic performance is good; Bansal et al.[71] compared metabolic signatures from patients with low-grade and high-grade bladder cancer with correct classification >95% in the validation cohort, whereas Kumar et al.[50] reported >90% correct classification in a study of low-grade versus high-grade PCa.

Another opportunity for clinical application of NMR metabolomics is to use metabolic information in biofluids to predict patient outcome. Using only pre-treatment serum samples, Bertini et al.[96] used metabolic profiles from patients with metastatic CRC to construct a classifier that could predict overall survival. Applying this predictor to a validation cohort, patients with short overall survival were identified with a hazard ratio of 3.4. The NMR-based metabolic profile was an independent predictor of overall survival, with higher predictive power than traditional predictive criteria (ECOG-Performance status and clinical chemistry biomarkers).

Several studies have used serum metabolomics to identify patients at risk of recurrence following curative BCa surgery. Asiago et al.[97] combined NMR and MS-based techniques in a longitudinal study of 56 patients, where 20 developed recurrent disease during the study. Using a supervised approach, patients with recurrent disease could be identified with a diagnostic accuracy of 85%. Interestingly, recurrence could be predicted from serum samples collected 13 months prior to recurrence. Similar findings were published by Tenori et al.[98], who studied a cohort of 80 patients with ER-negative BCa. Using a serum sample collected post-surgery, recurrence could be predicted with a diagnostic accuracy of 75%. Patients with recurrence displayed higher levels of glucose and lower levels of histidine than patients with no relapse. These results were recently reproduced in a multicenter trial including 699 patients.[99] Here, a training set of serum samples collected pre-surgery from 285 patients with early BCa and 109 patients with metastatic disease was used to develop a classification model that subsequently predicted recurrence with an accuracy of 71%, independently of age, tumor size, grade, or lymph node status. Collectively, these studies indicate that NMR metabolomics may be useful for stratification of patients to receive tailored treatment based on their individual risk profile.

Leukemias represent a special case, where serum represents the matrix in which the cancer cells reside. It could be speculated that this would result in a more direct relationship between the metabolic activity

of the cancer cells and the metabolic composition of serum. In a cohort of 415 subjects, Wang et al.[100] could discriminate between patients with acute myeloid leukemia (AML) and healthy controls, using a model where 22 metabolites significantly contributed to the classification. Furthermore, dividing the patients into groups with good and less favorable prognosis, the serum metabolome could contribute with prognostic information. Consistent with a report from Musharraf et al.,[101] lactate concentrations were paradoxically lower in AML patients than in healthy subjects, and patients with poor prognosis had lower lactate levels than patients with medium prognosis.

Treatment monitoring

Among external environmental factors exerting modifications in metabolic profiles is medication. Following the administration of a certain therapy, changes in the metabolome occur as a result of the biological activity. Pinpointing specific drug-related alterations in metabolite levels and pathways as an effect of a specific therapy can provide information related to mechanism of action and/or resistance, toxicity, interactions with other drugs, or even predict clinical outcome.

In a study by Wei et al.[102] serum metabolic profiles from a small cohort of BCa patients (n=28) who later received neoadjuvant chemotherapy were acquired using both NMR spectroscopy and liquid chromatography (LC)-MS. A multivariate prediction model built with information from three NMR detected amino acids; isoleucine, glutamine and threonine, and the MS detected fatty acid linolenic acid could discriminate pathological complete responders from stable disease with 100% specificity and 80% sensitivity. The study showed potential for combined NMR and MS metabolomics in predicting BCa response to neoadjuvant chemotherapy before treatment start. However, this required validation as the low number of samples with leave-one-out cross validation may give over-optimistic results.

In a study by Tenori et al.[103] an NMR metabolomics approach was employed on serum from 579 BCa patients from an international clinical trial. The patients were all treated with the chemotherapeutic agent paclitaxel, while they were randomized to additionally receive either the anti-HER2 drug lapitinib or a placebo. The authors focused on investigating metabolic profiles from serum at three different time points: at baseline and at the on-treatment weeks 9 and 21. When comparing samples from each time point separately, no meaningful associations of the metabolic profiles with their clinical endpoints of time to progression, overall survival, and toxicity were found. The same was the case when examining each treatment arm individually within each time point. Only when they included only HER2-positive patients

receiving anti-HER2 treatment (n=49), could they detect clinical associations. When further dividing this patient group into three groups based on time to progression (TTP), extreme TTP groups, i.e. TTP range: 62–175 days (n=11) vs TTP range: 367–821 days (n=11) could be discriminated based on NMR metabolic profiles with an accuracy of 89.6%. Similarly, when dividing HER2-positive patients receiving anti-HER2 treatment (n=34) into three groups based on overall survival (OS), OS range: 105-470 days (n=9) and OS range: 664-843 days (n=7) could be discriminated based on NMR metabolic profiles with an accuracy of 78%. However, like in the previously mentioned study, the small number of patients did not allow for a proper validation on an independent test set of samples with similar biological characteristics. Still, univariate Wilcoxon tests showed significantly higher glucose and lower glutamate and phenylalanine ($p<0.05$) for patients in the upper TTP extreme compared to the lower TTP extreme patients.

Other studies have focused on investigating the effect of BCa treatment on serum metabolic signatures in the context of high adiposity. When studying serum of 21 post menopausal women receiving chemotherapy in the form of fluorouracil, epirubicin, and cyclophosphamide (FEC) sampled before and during treatment, Keun et al.[104] found that pre-treatment levels of lactate and alanine could individually predict >1.5 kg weight gain (AUC >0.77; $p < 0.05$). Of these two metabolites, lactate seemed to be the most interesting, showing the highest change (up to 75% increase) on average as an effect of chemotherapy. Stebbing et al.[105] on the other hand, investigated correlations between metabolic syndrome, NMR metabolic profiles, and treatment response in BCa patients. Although the association between metabolic syndrome and metabolites was not directly evaluated, a significant trend ($p=0.003$) of increased incidence of metabolic syndrome with poorer treatment response was observed. Likewise, multivariate logistic regression with variable selection revealed high glucose and lactate, and low alanine to be significantly associated with poorer response, i.e. progressive disease, independently of whether patients received FEC or taxane-based chemotherapy.

Renal cell carcinomas that become metastatic are typically resistant to a broad range of therapies including chemotherapy and radiotherapy.[106] Targeted therapies that act upon the VEGF and mTOR pathways have become promising treatment alternatives for metastatic renal cell carcinoma. The French clinical trial TORAVA assessed the effectiveness of combining the VEGF and mTOR inhibitors bevacizumab and temsirolimus, respectively, for this patient group, compared to that of two different standard treatments: sunitinib and interferon-α+bevacizumab.[107] Pre-treatment and serial on-treatment serum samples from 121 patients included in the trial were examined by Jobard et al.[108] using NMR spectroscopy

to identify metabolic signatures associated with targeted therapies. For the targeted bevacizumab+temsirolimus arm, significant metabolic changes were detected already at the second week of treatment, while no changes were detected for the standard treatment arms. This effect was characterized by a significant increase in lipids, lipoproteins (VLDL and LDL), glucose, and N-acetylglycoproteins. While metabolic differences between treatment arms were not significant after multiple testing correction, trends indicated higher serum concentrations of lipids, lipoproteins (VLDL and LDL), cholesterol, glucose, the end-products of *β*-oxidation (acetone, acetoacetate, and 3-hydroxybutyrate), *N*-acetylglycoproteins, branch-chained amino acids (valine, leucine, isoleucine), alanine, and acetate in the patients given bevacizumab+temsirolimus compared to those receiving sunitinib.

Biofluid studies in cancer using NMR spectroscopy have not been limited to the hydrogen nucleus. In a multiple myeloma study, phosphorus (^{31}P) NMR spectroscopy was used to study the effect of chemotherapy on serum phospholipids.[109] At baseline, patients (n=20) exhibited significantly lower levels of phosphatidylcholine and phosphatidylethanolamine + sphingomyelin relative to inorganic phosphate compared to healthy controls (n=22). After therapy, the serum levels for these compounds returned to a more normal state for responders, while this was not the case for non-responders. This could be potentially useful for monitoring of treatment response. A similar effect was observed in another multiple myeloma study using ^{1}H NMR spectroscopy on serum,[110] in which chemotherapy-treated patients (n=27) who achieved complete remission showed reversed trends for some metabolites to resemble those observed in healthy individuals (n=31). These trends included higher levels of lipids in healthy controls compared to patients at the time of diagnosis, similarly as for the aforementioned study using ^{31}P NMR. Healthy controls additionally exhibited higher levels of glutamine, cholesterol, lipids, lysine, and choline, and lower levels of glucose. Similarly, in another ^{1}H NMR study of myeloma patients (n=32),[111] higher choline and glucose in serum was associated with the time point of remission after chemotherapy compared to the diagnosis time point. However, long term follow-up showed that patients who relapsed after 3 years or more exhibited higher levels of choline than those who remained in remission. Interestingly, when comparing active disease samples (diagnosis and long-term relapse) with non-active disease samples, i.e. from patients in remission, carnitine and acetylcarnitine were found to be significantly decreased in the former. Urine from the same patients was also examined, where higher phenylalanine and alanine and lower betaine, glycine, hippurate and trimethylamine N-oxide were among the trends associated with diagnosis compared to remission after chemotherapy.

NMR metabolomics of serum for the assessment of treatment toxicity could help avoiding treatment that causes more harm than benefit, which is especially important for patients where the goal is palliative. care. In a study by Backshall et al.,[112] a higher toxicity grade of the single-agent capecitabine as treatment for inoperable CRC (n=52 patients) was associated with higher low-density lipoprotein-derived lipids, including polyunsaturated fatty acids and choline phospholipids in serum prior to treatment. This suggests potential for prediction of toxicity based on minimally-invasively accessible serum.

Challenges and future aspects

In several of the above studies, significant associations between the biofluid metabolome and cancer risk, presence, prognosis, and treatment effect have been described. However, in most cases there is no clear association between metabolic biomarkers of disease observed in cancer cells/tumors and the metabolic biomarkers identified in serum and urine. Also considering the relatively low sensitivity of NMR, this indicates that the metabolic information from biofluids measured by NMR is largely related to tumor-host interaction (Figure 1). At the stage where a malignant tumor is detected, it is likely that it will affect functions of the organ of origin to a certain degree, and that the altered organ function and associated immune response is reflected in the biofluid metabolome. It is also known that tumors can cause systemic effects through release of various endocrine mediators.[113,114] Combined, these effects can modulate the metabolic state of many other organs,[115] resulting in an altered global metabolic phenotype. This explains why for example inflammation[116] and cachexia[117] can be detected by metabolomics.

Biological interpretations is also difficult due to the heterogeneous findings in different studies. There is little consistency regarding which metabolites are important for different cancer types, and in several cases metabolite trends are contradictory between different studies investigating the same cancer type and aspects. One explanation for this is that so far, most studies have been performed in small patient cohorts. There is a need for large clinical studies in representative and relevant target populations, allowing for proper validation, before results can be considered for clinical use. In order to implement metabolic findings in clinical practice, randomized prospective clinical studies would be necessary to validate the value of using metabolic profiling in screening, cancer detection, prognostic evaluation and/or treatment monitoring. Of high clinical value would also be patient monitoring for early detection of recurring disease. Such studies should aim to demonstrate diagnostic superiority of metabolic profiling on a patient-by-patient basis compared to existing diagnostic tools, and must be rigorously designed with

respect to collection of samples, NMR analysis and processing of the metabolic data. Furthermore, controlled preclinical experiments are necessary to provide mechanistic explanations for the changes in metabolic profiles at different stages of the disease. However, if a model is validated in a sufficiently large, representative and relevant population, it can be argued that the model can have clinical implications even if the metabolic differences are not mechanistically understood. Several machine learning methods, such as neural networks and random forest classifiers, focus on predictive performance, and do not yield easily interpretable models. However, such approaches pose important challenges, including possibly stagnating the generation of biomedical knowledge, and not knowing whether the model bases its decisions on statistically associated "red herrings", as opposed to relevant clinical factors.[118]

While standardization of the NMR experiment itself is important for comparing results between metabolomics studies, there is also a need for standardized procedures when handling the resulting spectral data. It is well-known that different types of normalization emphasize different metabolites, and may in addition affect statistical classification accuracy.[119] Another challenge with NMR is that different metabolites are quantified in different studies, and in several cases there is disagreement on the identification of peaks at the same ppm region, despite the fact that one of the strengths with NMR is the possibility to do safe metabolite identification.[16] NMR-spectra from biofluids may contain hundreds (serum) to thousands (urine) of signals, and severe overlap pose major difficulties in quantification, even with developments in instrumentation such as higher magnetic field strengths and cryogenically cooled probes. Consequently, much of the NMR-based metabolomics literature only report semi-quantitative metabolite levels. A more standardized solution for this would be more extensive use of validated quantification softwares (Chenomx, AMIX etc) and algorithms such as BATMAN[120] and Urine Shift Predictor.[121]

Possible confounders affecting the metabolic profiles must also be considered, such as drugs. As shown in Figure 2, paracetamol intake will highly affect the NMR metabolic profiles of urine.[122] If the disease state being investigated causes discomfort and pain, it is likely that the patients will be taking more of these drugs than the control group, and it is important to make sure that the metabolic differences measured between the groups are not a result of drug intake. Also for diseases that are highly correlated to external factors, such as lung cancer and smoking, it is important to be aware that exposure to these factors might affect the measured biofluid metabolism.

Collectively, the body of data suggests that it may be possible to develop biomarkers for cancer risk assessment, detection, diagnostics, and treatment monitoring using NMR metabolomics, as evidenced by the high classification accuracies of several of the above studies. Since biofluids in general mirrors the metabolic activity of the entire organism, mechanistic studies under controlled conditions (i.e. in relevant preclinical disease models) are needed if we are to understand the biological underpinnings of such metabolic signatures. Furthermore, it must be stressed that the metabolic signatures associated with disease progression probably depend on cancer type as well as other biological variables. This, combined with the effect of the statistical tools used to analyze spectroscopy data, emphasizes the need for rigorous validation of all proposed metabolic biomarkers.

Figures legends

Figure 1: The metabolome of several biofluids (e.g. blood and urine) reflects the metabolic activity of the entire organism, which is influenced by a host of endogenous and exogenous factors. A selection of factors potentially affecting systemic metabolism is presented here.

Figure 2: Representative NMR spectra of urine (A-B) and serum (C). Spectra shown in A and C were acquired from urine and serum, respectively, belonging to the same patient. The influence of medication on the urine NMR metabolic profile is exemplified in B, where large paracetamol-derived metabolite peaks can be observed. The urine spectra (A-B) are acquired using the NOESY sequence, while the serum spectrum (C) is acquired using CPMG. 1: 3-hydroxyisobutyrate; 2: 3-hydroxyisovalerate; 3: threonine/lactate; 4: 2-alfa-hydroxyisobutyrate; 5: alanine; 6: p-cresol sulfate; 7: citrate; 8: dimethylamine; 9: creatine; 10: creatinine; 11: trimethylamine-N-oxide (TMAO); 12: glycine; 13: hippurate; 14: trigonelline; 15: water; 16: 4-hydroxyphenylacetate; 17: phenylacetylglutamine; 18: 3-indoxyl sulfate; 19: paracetamol-derived metabolites, 20: leucine; 21: valine; 22: isoleucine; 23: lipid; 24: lactate; 25: lysine; 26: acetate; 27: N-acetyl cysteine (NAC); 28: glutamate/glutamine; 29: pyruvate; 30: glutamine; 31: glucose; 32: tyrosine; 33: histidine; 34: phenylalanine.

Figure 3: NMR metabolic profiling of biofluids may reflect several phases of cancer development, making it applicable for different purposes, from assessing the risk of future cancer development to detecting and characterizing existing cancer. The minimally-invasive nature makes metabolic profiling of biofluids a suitable tool for treatment monitoring.

References

1. Aboagye EO, Bhujwalla ZM. Malignant Transformation Alters Membrane Choline Phospholipid Metabolism of Human Mammary Epithelial Cells. *Cancer Res.* 1999;59(1):80-84.
2. Teahan O, Bevan CL, Waxman J, Keun HC. Metabolic signatures of malignant progression in prostate epithelial cells. *Int J Biochem Cell Biol.* 2011;43(7):1002-1009.
3. Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab.* 2016;23(1):27-47.
4. Giskeødegård GF, Grinde MT, Sitter B, et al. Multivariate Modeling and Prediction of Breast Cancer Prognostic Factors Using MR Metabolomics. *J Proteome Res.* 2010;9(2):972-979.
5. 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.* 2006;19(1):30-40.
6. Tian Y, Xu T, Huang J, et al. Tissue Metabonomic Phenotyping for Diagnosis and Prognosis of Human Colorectal Cancer. *Sci Rep.* 2016;6:20790.
7. Wijeyesekera A, Selman C, Barton RH, Holmes E, Nicholson JK, Withers DJ. Metabotyping of long-lived mice using 1H NMR spectroscopy. *J Proteome Res.* 2012;11(4):2224-2235.
8. Fischer K, Kettunen J, Wurtz P, et al. Biomarker profiling by nuclear magnetic resonance spectroscopy for the prediction of all-cause mortality: an observational study of 17,345 persons. *PLoS medicine.* 2014;11(2):e1001606.
9. Duarte IF, Gil AM. Metabolic signatures of cancer unveiled by NMR spectroscopy of human biofluids. *Prog Nucl Magn Reson Spectrosc.* 2012;62:51-74.
10. Richard V, Conotte R, Mayne D, Colet JM. Does the 1H-NMR plasma metabolome reflect the host-tumor interactions in human breast cancer? *Oncotarget.* 2017;8(30):49915-49930.
11. Field M, Block JB, Levin R, Rall DP. Significance of blood lactate elevations among patients with acute leukemia and other neoplastic proliferative disorders. *The American Journal of Medicine.* 1966;40(4):528-547.
12. Psychogios N, Hau DD, Peng J, et al. The human serum metabolome. *PLoS One.* 2011;6(2):e16957.
13. Bouatra S, Aziat F, Mandal R, et al. The human urine metabolome. *PLoS One.* 2013;8(9):e73076.
14. Berger D. A brief history of medical diagnosis and the birth of the clinical laboratory. Part 1--Ancient times through the 19th century. *MLO Med Lab Obs.* 1999;31(7):28-30, 32, 34-40.

15. Berger D. A brief history of medical diagnosis and the birth of the clinical laboratory. Part 2--Laboratory science and professional certification in the 20th century. *MLO Med Lab Obs.* 1999;31(8):32-34, 36, 38.
16. Markley JL, Bruschweiler R, Edison AS, et al. The future of NMR-based metabolomics. *Curr Opin Biotechnol.* 2017;43:34-40.
17. Bernini P, Bertini I, Luchinat C, Nincheri P, Staderini S, Turano P. Standard operating procedures for pre-analytical handling of blood and urine for metabolomic studies and biobanks. *J Biomol NMR.* 2011;49(3-4):231-243.
18. Gebregiworgis T, Powers R. Application of NMR metabolomics to search for human disease biomarkers. *Combinatorial chemistry & high throughput screening.* 2012;15(8):595-610.
19. Beckonert O, Keun HC, Ebbels TMD, et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protocols.* 2007;2(11):2692-2703.
20. Bingol K, Bruschweiler-Li L, Li DW, Bruschweiler R. Customized metabolomics database for the analysis of NMR (1)H-(1)H TOCSY and (1)(3)C-(1)H HSQC-TOCSY spectra of complex mixtures. *Anal Chem.* 2014;86(11):5494-5501.
21. Bingol K, Li DW, Bruschweiler-Li L, et al. Unified and isomer-specific NMR metabolomics database for the accurate analysis of (13)C-(1)H HSQC spectra. *ACS chemical biology.* 2015;10(2):452-459.
22. Hu K, Ellinger JJ, Chylla RA, Markley JL. Measurement of absolute concentrations of individual compounds in metabolite mixtures by gradient-selective time-zero 1H-13C HSQC with two concentration references and fast maximum likelihood reconstruction analysis. *Anal Chem.* 2011;83(24):9352-9360.
23. Monsonis Centelles S, Hoefsloot HCJ, Khakimov B, et al. Toward Reliable Lipoprotein Particle Predictions from NMR Spectra of Human Blood: An Interlaboratory Ring Test. *Anal Chem.* 2017;89(15):8004-8012.
24. Soininen P, Kangas AJ, Wurtz P, Suna T, Ala-Korpela M. Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. *Circulation Cardiovascular genetics.* 2015;8(1):192-206.
25. Thrift AP, Whiteman DC. Can we really predict risk of cancer? *Cancer epidemiology.* 2013;37(4):349-352.

26. Würtz P, Havulinna AS, Soininen P, et al. Metabolite Profiling and Cardiovascular Event Risk: A Prospective Study of Three Population-Based Cohorts. *Circulation.* 2015.
27. Liu J, Semiz S, van der Lee SJ, et al. Metabolomics based markers predict type 2 diabetes in a 14-year follow-up study. *Metabolomics.* 2017;13(9):104.
28. Austdal M, Tangeras LH, Skrastad RB, et al. First Trimester Urine and Serum Metabolomics for Prediction of Preeclampsia and Gestational Hypertension: A Prospective Screening Study. *International journal of molecular sciences.* 2015;16(9):21520-21538.
29. Xu T, Holzapfel C, Dong X, et al. Effects of smoking and smoking cessation on human serum metabolite profile: results from the KORA cohort study. *BMC medicine.* 2013;11:60.
30. Playdon MC, Sampson JN, Cross AJ, et al. Comparing metabolite profiles of habitual diet in serum and urine. *Am J Clin Nutr.* 2016;104(3):776-789.
31. Kim HJ, Kim JH, Noh S, et al. Metabolomic analysis of livers and serum from high-fat diet induced obese mice. *J Proteome Res.* 2011;10(2):722-731.
32. Schmidt JA, Fensom GK, Rinaldi S, et al. Pre-diagnostic metabolite concentrations and prostate cancer risk in 1077 cases and 1077 matched controls in the European Prospective Investigation into Cancer and Nutrition. *BMC medicine.* 2017;15:122.
33. Cross AJ, Moore SC, Boca S, et al. A prospective study of serum metabolites and colorectal cancer risk. *Cancer.* 2014;120(19):3049-3057.
34. Kühn T, Floegel A, Sookthai D, et al. Higher plasma levels of lysophosphatidylcholine 18:0 are related to a lower risk of common cancers in a prospective metabolomics study. *BMC medicine.* 2016;14:13.
35. Mondul AM, Moore SC, Weinstein SJ, Karoly ED, Sampson JN, Albanes D. Metabolomic analysis of prostate cancer risk in a prospective cohort: The alpha-tocopherol, beta-carotene cancer prevention (ATBC) study. *Int J Cancer.* 2015;137(9):2124-2132.
36. Bro R, Kamstrup-Nielsen MH, Engelsen SB, et al. Forecasting individual breast cancer risk using plasma metabolomics and biocontours. *Metabolomics.* 2015;11(5):1376-1380.
37. Hofvind S, Geller BM, Skelly J, Vacek PM. Sensitivity and specificity of mammographic screening as practised in Vermont and Norway. *The British Journal of Radiology.* 2012;85(1020):e1226-e1232.
38. Acar E, Gurdeniz G, Savorani F, et al. Forecasting Chronic Diseases Using Data Fusion. *J Proteome Res.* 2017;16(7):2435-2444.

39. Fages A, Duarte-Salles T, Stepien M, et al. Metabolomic profiles of hepatocellular carcinoma in a European prospective cohort. *BMC medicine.* 2015;13:242.
40. Davis VW, Schiller DE, Eurich D, Sawyer MB. Urinary metabolomic signature of esophageal cancer and Barrett's esophagus. *World journal of surgical oncology.* 2012;10:271.
41. Hasim A, Ali M, Mamtimin B, Ma J-Q, Li Q-Z, Abudula A. Metabonomic signature analysis of cervical carcinoma and precancerous lesions in women by (1)H NMR spectroscopy. *Experimental and Therapeutic Medicine.* 2012;3(6):945-951.
42. Wang H, Tso V, Wong C, Sadowski D, Fedorak RN. Development and Validation of a Highly Sensitive Urine-Based Test to Identify Patients with Colonic Adenomatous Polyps. *Clinical and Translational Gastroenterology.* 2014;5(3):e54.
43. Deng L, Fang H, Tso VK, et al. Clinical validation of a novel urine-based metabolomic test for the detection of colonic polyps on Chinese population. *Int J Colorectal Dis.* 2017;32(5):741-743.
44. Levy BT, Bay C, Xu Y, et al. Test Characteristics of Fecal Immunochemical Tests (FIT) Compared with Optical Colonoscopy Revised JMS-14-003.R2. *J Med Screen.* 2014;21(3):133-143.
45. de Sanjose S, Diaz M, Castellsague X, et al. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. *The Lancet Infectious diseases.* 2007;7(7):453-459.
46. Dent J, El-Serag HB, Wallander MA, Johansson S. Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut.* 2005;54(5):710-717.
47. Winters C, Jr., Spurling TJ, Chobanian SJ, et al. Barrett's esophagus. A prevalent, occult complication of gastroesophageal reflux disease. *Gastroenterology.* 1987;92(1):118-124.
48. Meigs JB, Barry MJ, Oesterling JE, Jacobsen SJ. Interpreting results of prostate-specific antigen testing for early detection of prostate cancer. *J Gen Intern Med.* 1996;11(9):505-512.
49. Giskeødegård GF, Hansen AF, Bertilsson H, et al. Metabolic markers in blood can separate prostate cancer from benign prostatic hyperplasia. *Br J Cancer.* 2015;113(12):1712-1719.
50. Kumar D, Gupta A, Mandhani A, Sankhwar SN. Metabolomics-derived prostate cancer biomarkers: fact or fiction? *J Proteome Res.* 2015;14(3):1455-1464.
51. Kumar D, Gupta A, Mandhani A, Sankhwar SN. NMR spectroscopy of filtered serum of prostate cancer: A new frontier in metabolomics. *The Prostate.* 2016;76(12):1106-1119.
52. Sreekumar A, Poisson LM, Rajendiran TM, et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature.* 2009;457(7231):910-914.

53. Lucarelli G, Fanelli M, Larocca AM, et al. Serum sarcosine increases the accuracy of prostate cancer detection in patients with total serum PSA less than 4.0 ng/ml. *Prostate.* 2012;72(15):1611-1621.
54. Jentzmik F, Stephan C, Miller K, et al. Sarcosine in Urine after Digital Rectal Examination Fails as a Marker in Prostate Cancer Detection and Identification of Aggressive Tumours. *Eur Urol.* 2010;58(1):12-18.
55. Averna TA, Kline EE, Smith AY, Sillerud LO. A decrease in 1H nuclear magnetic resonance spectroscopically determined citrate in human seminal fluid accompanies the development of prostate adenocarcinoma. *J Urol.* 2005;173(2):433-438.
56. Kline EE, Treat EG, Averna TA, Davis MS, Smith AY, Sillerud LO. Citrate concentrations in human seminal fluid and expressed prostatic fluid determined via 1H nuclear magnetic resonance spectroscopy outperform prostate specific antigen in prostate cancer detection. *J Urol.* 2006;176(5):2274-2279.
57. Serkova NJ, Gamito EJ, Jones RH, et al. The metabolites citrate, myo-inositol, and spermine are potential age-independent markers of prostate cancer in human expressed prostatic secretions. *The Prostate.* 2008;68(6):620-628.
58. Roberts MJ, Richards RS, Chow CWK, et al. Seminal plasma enables selection and monitoring of active surveillance candidates using nuclear magnetic resonance-based metabolomics: A preliminary investigation. *Prostate international.* 2017;5(4):149-157.
59. Perez-Rambla C, Puchades-Carrasco L, Garcia-Flores M, Rubio-Briones J, Lopez-Guerrero JA, Pineda-Lucena A. Non-invasive urinary metabolomic profiling discriminates prostate cancer from benign prostatic hyperplasia. *Metabolomics.* 2017;13(5):52.
60. Slupsky CM, Steed H, Wells TH, et al. Urine Metabolite Analysis Offers Potential Early Diagnosis of Ovarian and Breast Cancers. *Clin Cancer Res.* 2010;16(23):5835-5841.
61. Gu H, Pan Z, Xi B, Asiago V, Musselman B, Raftery D. Principal component directed partial least squares analysis for combining nuclear magnetic resonance and mass spectrometry data in metabolomics: Application to the detection of breast cancer. *Anal Chim Acta.* 2011;686(1-2):57-63.
62. Shen J, Yan L, Liu S, Ambrosone CB, Zhao H. Plasma metabolomic profiles in breast cancer patients and healthy controls: by race and tumor receptor subtypes. *Translational oncology.* 2013;6(6):757-765.

63. Napoli C, Sperandio N, Lawlor RT, Scarpa A, Molinari H, Assfalg M. Urine metabolic signature of pancreatic ductal adenocarcinoma by (1)h nuclear magnetic resonance: identification, mapping, and evolution. *J Proteome Res.* 2012;11(2):1274-1283.

64. Davis VW, Schiller DE, Eurich D, Bathe OF, Sawyer MB. Pancreatic ductal adenocarcinoma is associated with a distinct urinary metabolomic signature. *Ann Surg Oncol.* 2013;20 Suppl 3:S415-423.

65. OuYang D, Xu J, Huang H, Chen Z. Metabolomic Profiling of Serum from Human Pancreatic Cancer Patients Using 1H NMR Spectroscopy and Principal Component Analysis. *Appl Biochem Biotechnol.* 2011;165(1):148-154.

66. Zhang L, Jin H, Guo X, et al. Distinguishing pancreatic cancer from chronic pancreatitis and healthy individuals by (1)H nuclear magnetic resonance-based metabonomic profiles. *Clin Biochem.* 2012;45(13-14):1064-1069.

67. Mehta KY, Wu HJ, Menon SS, et al. Metabolomic biomarkers of pancreatic cancer: a meta-analysis study. *Oncotarget.* 2017;8(40):68899-68915.

68. Rodrigues D, Jeronimo C, Henrique R, et al. Biomarkers in bladder cancer: A metabolomic approach using in vitro and ex vivo model systems. *Int J Cancer.* 2016;139(2):256-268.

69. Srivastava S, Roy R, Singh S, et al. Taurine - a possible fingerprint biomarker in non-muscle invasive bladder cancer: A pilot study by 1H NMR spectroscopy. *Cancer Biomark.* 2010;6(1):11-20.

70. Cao M, Zhao L, Chen H, Xue W, Lin D. NMR-based Metabolomic Analysis of Human Bladder Cancer. *Analytical Sciences.* 2012;28(5):451-456.

71. Bansal N, Gupta A, Mitash N, et al. Low- and High-Grade Bladder Cancer Determination via Human Serum-Based Metabolomics Approach. *J Proteome Res.* 2013;12(12):5839-5850.

72. Deja S, Porebska I, Kowal A, et al. Metabolomics provide new insights on lung cancer staging and discrimination from chronic obstructive pulmonary disease. *J Pharm Biomed Anal.* 2014;100(Supplement C):369-380.

73. Puchades-Carrasco L, Jantus-Lewintre E, Perez-Rambla C, et al. Serum metabolomic profiling facilitates the non-invasive identification of metabolic biomarkers associated with the onset and progression of non-small cell lung cancer. *Oncotarget.* 2016;7(11):12904-12916.

74. Zhang X, Zhu X, Wang C, Zhang H, Cai Z. Non-targeted and targeted metabolomics approaches to diagnosing lung cancer and predicting patient prognosis. *Oncotarget.* 2016;7(39):63437-63448.

75. Rocha CM, Carrola J, Barros AS, et al. Metabolic signatures of lung cancer in biofluids: NMR-based metabonomics of blood plasma. *J Proteome Res.* 2011:null-null.
76. 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.* 2011;10(4):1765-1771.
77. Massuger LF, van Vierzen PB, Engelke U, Heerschap A, Wevers R. 1H-magnetic resonance spectroscopy: a new technique to discriminate benign from malignant ovarian tumors. *Cancer.* 1998;82(9):1726-1730.
78. Boss EA, Moolenaar SH, Massuger LF, et al. High-resolution proton nuclear magnetic resonance spectroscopy of ovarian cyst fluid. *NMR Biomed.* 2000;13(5):297-305.
79. Kyriakides M, Rama N, Sidhu J, Gabra H, Keun HC, El-Bahrawy M. Metabonomic analysis of ovarian tumour cyst fluid by proton nuclear magnetic resonance spectroscopy. *Oncotarget.* 2016;7(6):7216-7226.
80. Tiziani S, Lopes V, Gunther UL. Early stage diagnosis of oral cancer using 1H NMR-based metabolomics. *Neoplasia.* 2009;11(3):269-276.
81. Gupta A, Gupta S, Mahdi AA. (1)H NMR-derived serum metabolomics of leukoplakia and squamous cell carcinoma. *Clin Chim Acta.* 2015;441:47-55.
82. Jain NS, Dürr UHN, Ramamoorthy A. Bioanalytical methods for metabolomic profiling: Detection of head and neck cancer, including oral cancer. *Chinese Chemical Letters.* 2015;26(4):407-415.
83. Zhang J, Liu L, Wei S, et al. Metabolomics study of esophageal adenocarcinoma. *J Thorac Cardiovasc Surg.* 2011;141(2):469-475, 475.e461-464.
84. Nahon P, Amathieu R, Triba MN, et al. Identification of serum proton NMR metabolomic fingerprints associated with hepatocellular carcinoma in patients with alcoholic cirrhosis. *Clin Cancer Res.* 2012;18(24):6714-6722.
85. Li P, Tao J, Wei D, et al. Serum metabolomic analysis of human upper urinary tract urothelial carcinoma. *Tumour Biol.* 2015;36(10):7531-7537.
86. Deng L, Gu H, Zhu J, et al. Combining NMR and LC/MS Using Backward Variable Elimination: Metabolomics Analysis of Colorectal Cancer, Polyps, and Healthy Controls. *Anal Chem.* 2016;88(16):7975-7983.
87. Bezabeh T, Somorjai RL, Smith IC. MR metabolomics of fecal extracts: applications in the study of bowel diseases. *Magnetic resonance in chemistry : MRC.* 2009;47 Suppl 1:S54-61.

88. Monleon D, Morales JM, Barrasa A, Lopez JA, Vazquez C, Celda B. Metabolite profiling of fecal water extracts from human colorectal cancer. *NMR Biomed.* 2009;22(3):342-348.
89. Lin Y, Ma C, Liu C, et al. NMR-based fecal metabolomics fingerprinting as predictors of earlier diagnosis in patients with colorectal cancer. *Oncotarget.* 2016;7(20):29454-29464.
90. Fan Y, Zhou X, Xia TS, et al. Human plasma metabolomics for identifying differential metabolites and predicting molecular subtypes of breast cancer. *Oncotarget.* 2016;7(9):9925-9938.
91. Cheyne RW, Trembleau L, McLaughlin A, Smith TA. Changes in 2-fluoro-2-deoxy-D-glucose incorporation, hexokinase activity and lactate production by breast cancer cells responding to treatment with the anti-HER-2 antibody trastuzumab. *Nucl Med Biol.* 2011;38(3):339-346.
92. Walsh AJ, Cook RS, Manning HC, et al. Optical metabolic imaging identifies glycolytic levels, subtypes, and early-treatment response in breast cancer. *Cancer Res.* 2013;73(20):6164-6174.
93. Tang X, Lin CC, Spasojevic I, Iversen ES, Chi JT, Marks JR. A joint analysis of metabolomics and genetics of breast cancer. *Breast Cancer Res.* 2014;16(4):415.
94. Jobard E, Pontoizeau C, Blaise BJ, Bachelot T, Elena-Herrmann B, Tredan O. A serum nuclear magnetic resonance-based metabolomic signature of advanced metastatic human breast cancer. *Cancer Lett.* 2014;343(1):33-41.
95. Falegan OS, Ball MW, Shaykhutdinov RA, et al. Urine and Serum Metabolomics Analyses May Distinguish between Stages of Renal Cell Carcinoma. *Metabolites.* 2017;7(1).
96. Bertini I, Cacciatore S, Jensen BV, et al. Metabolomic NMR fingerprinting to identify and predict survival of patients with metastatic colorectal cancer. *Cancer Res.* 2012;72(1):356-364.
97. Asiago VM, Alvarado LZ, Shanaiah N, et al. Early Detection of Recurrent Breast Cancer Using Metabolite Profiling. *Cancer Res.* 2010;70(21):8309-8318.
98. Tenori L, Oakman C, Morris PG, et al. Serum metabolomic profiles evaluated after surgery may identify patients with oestrogen receptor negative early breast cancer at increased risk of disease recurrence. Results from a retrospective study. *Mol Oncol.* 2015;9(1):128-139.
99. Hart CD, Vignoli A, Tenori L, et al. Serum Metabolomic Profiles Identify ER-Positive Early Breast Cancer Patients at Increased Risk of Disease Recurrence in a Multicenter Population. *Clin Cancer Res.* 2017;23(6):1422-1431.
100. Wang Y, Zhang L, Chen WL, et al. Rapid diagnosis and prognosis of de novo acute myeloid leukemia by serum metabonomic analysis. *J Proteome Res.* 2013;12(10):4393-4401.
101. Musharraf SG, Siddiqui AJ, Shamsi T, Choudhary MI, Rahman AU. Serum metabonomics of acute leukemia using nuclear magnetic resonance spectroscopy. *Sci Rep.* 2016;6:30693.

102. Wei S, Liu L, Zhang J, et al. Metabolomics approach for predicting response to neoadjuvant chemotherapy for breast cancer. *Molecular Oncology.* 2013;7(3):297-307.
103. 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.* 2012;6(4):437-444.
104. Keun HC, Sidhu J, Pchejetski D, et al. Serum Molecular Signatures of Weight Change during Early Breast Cancer Chemotherapy. *Clin Cancer Res.* 2009;15(21):6716-6723.
105. Stebbing J, Sharma A, North B, et al. A metabolic phenotyping approach to understanding relationships between metabolic syndrome and breast tumour responses to chemotherapy. *Ann Oncol.* 2012;23(4):860-866.
106. Lin L, Huang Z, Gao Y, Yan X, Xing J, Hang W. LC-MS based serum metabonomic analysis for renal cell carcinoma diagnosis, staging, and biomarker discovery. *J Proteome Res.* 2011;10(3):1396-1405.
107. Negrier S, Gravis G, Perol D, et al. Temsirolimus and bevacizumab, or sunitinib, or interferon alfa and bevacizumab for patients with advanced renal cell carcinoma (TORAVA): a randomised phase 2 trial. *Lancet Oncol.* 2011;12(7):673-680.
108. Jobard E, Blanc E, Negrier S, et al. A serum metabolomic fingerprint of bevacizumab and temsirolimus combination as first-line treatment of metastatic renal cell carcinoma. *Br J Cancer.* 2015;113(8):1148-1157.
109. Kuliszkiewicz-Janus M, Baczynski S. Chemotherapy-associated changes in 31P MRS spectra of sera from patients with multiple myeloma. *NMR Biomed.* 1995;8(3):127-132.
110. Puchades-Carrasco L, Lecumberri R, Martínez-López J, et al. Multiple Myeloma Patients Have a Specific Serum Metabolomic Profile That Changes after Achieving Complete Remission. *Clin Cancer Res.* 2013;19(17):4770.
111. Lodi A, Tiziani S, Khanim FL, et al. Proton NMR-based metabolite analyses of archived serial paired serum and urine samples from myeloma patients at different stages of disease activity identifies acetylcarnitine as a novel marker of active disease. *PLoS One.* 2013;8(2):e56422.
112. Backshall A, Sharma R, Clarke SJ, Keun HC. Pharmacometabonomic profiling as a predictor of toxicity in patients with inoperable colorectal cancer treated with capecitabine. *Clin Cancer Res.* 2011;17(9):3019-3028.
113. Roxburgh CS, McMillan DC. Cancer and systemic inflammation: treat the tumour and treat the host. *Br J Cancer.* 2014;110(6):1409-1412.

114. Pettersen K, Andersen S, Degen S, et al. Cancer cachexia associates with a systemic autophagy-inducing activity mimicked by cancer cell-derived IL-6 trans-signaling. *Sci Rep.* 2017;7(1):2046.
115. Xu S, Tian Y, Hu Y, et al. Tumor growth affects the metabonomic phenotypes of multiple mouse non-involved organs in an A549 lung cancer xenograft model. *Sci Rep.* 2016;6:28057.
116. Weljie AM, Dowlatabadi R, Miller BJ, Vogel HJ, Jirik FR. An inflammatory arthritis-associated metabolite biomarker pattern revealed by 1H NMR spectroscopy. *J Proteome Res.* 2007;6(9):3456-3464.
117. Winnard PT, Jr., Bharti SK, Penet MF, et al. Detection of Pancreatic Cancer-Induced Cachexia Using a Fluorescent Myoblast Reporter System and Analysis of Metabolite Abundance. *Cancer Res.* 2016;76(6):1441-1450.
118. Castelvecchi D. Can we open the black box of AI? *Nature.* 2016;538(7623):20-23.
119. Saccenti E. Correlation Patterns in Experimental Data Are Affected by Normalization Procedures: Consequences for Data Analysis and Network Inference. *J Proteome Res.* 2017;16(2):619-634.
120. Hao J, Liebeke M, Astle W, De Iorio M, Bundy JG, Ebbels TM. Bayesian deconvolution and quantification of metabolites in complex 1D NMR spectra using BATMAN. *Nature protocols.* 2014;9(6):1416-1427.
121. Takis PG, Schafer H, Spraul M, Luchinat C. Deconvoluting interrelationships between concentrations and chemical shifts in urine provides a powerful analysis tool. *Nature communications.* 2017;8(1):1662.
122. Bales JR, Sadler PJ, Nicholson JK, Timbrell JA. Urinary excretion of acetaminophen and its metabolites as studied by proton NMR spectroscopy. *Clin Chem.* 1984;30(10):1631-1636.