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Review

Bioactive Sphingolipids as Biomarkers Predictive of Disease Severity and Treatment Response in Cancer: Current Status and Translational Challenges

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Abstract. *Recent translational studies in cancer have produced a wealth of evidence to support an association between sphingolipid metabolism and clinical outcomes, which underscores the clinical importance of sphingolipid-related biomarkers in cancer diagnosis and prognosis. Importantly, circulating levels of bioactive sphingolipids were demonstrated to correlate with patient survival and treatment response in different tumour types, which could provide novel non-invasive cancer biomarkers. Here, we give a comprehensive overview of recent findings on bioactive sphingolipid species and protein regulators of their metabolism and signalling as novel potential biomarkers for risk assessment, prevention and prediction of treatment response in several types of solid cancers, including prostate, liver, pancreatic, breast and colon cancer, head and neck squamous cell carcinoma and gliomas. Finally, we critically discuss current issues in clinical translation of sphingolipid biomarkers and give our perspective on how these problems could be handled to facilitate implementation of sphingolipid-based diagnostics into clinical practice.*

An Introduction to Sphingolipid Structure and Metabolism

Sphingolipids play an essential role as regulators of eukaryotic cell viability. Due to their amphiphilic properties, most sphingolipids are structural components of cell membranes. However, they can also be found in biological fluids such as in blood as constituents of circulating lipoprotein particles, or can be carried by other proteins (*e.g.* serum albumin). Sphingolipids are comprised of a long-chain sphingoid base (18 carbon amino-alcohol backbone) and a fatty acid or head group attached to the base. The most common sphingoid bases are sphingosine, dihydrosphingosine (sphinganine) and phytosphingosine (Figure 1) (1). Addition of fatty acids of different chain lengths or diverse head groups determines the biological functions of sphingolipids. Numerous variations in sphingoid bases, *N*-acyl-linked fatty acids and head groups contribute to the extreme structural diversity and complexity of this lipid class. Ceramide, a bioactive sphingolipid composed of sphingoid base and a fatty acid attached to the C2 position (Figure 1), serves as a precursor for the formation of all complex sphingolipids through addition of different head groups at C1 position of ceramide chain. For example, sphingomyelins, the most abundant sphingolipids, are formed by the addition of phosphocholine head group. Glycosphingolipids differ in the sugar residue attached to their base. Thus, glucosphingolipids contain glucose attached to the C1 hydroxyl position, whereas galactosphingolipids are formed by the addition of galactose to the base (Figure 1) (2).

Ceramides can be produced by either *de novo* synthesis or hydrolysis of complex sphingolipids (Figure 2). *De novo* synthesis of ceramide begins with the formation of 3-ketodihydrosphingosine on the cytoplasmic side of the endoplasmic reticulum. Following 3-ketodihydrosphingosine reduction to dihydrosphingosine, the addition of different chain length fatty acids takes place to produce dihydroceramide. The latter reaction is called *N*-acylation and is catalysed by the family of enzymes known as ceramide synthases (CERS, formerly known as longevity assurance genes). There are six different CERS with distinct tissue expression, and each of them exhibits high specificity for the acyl CoA chain length for *N*-acylation, which determines the fatty acid composition of ceramides (Figure 3).

The formation of complex sphingolipids proceeds *via* three distinct routes of ceramide conversion to produce either

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glucosylceramide and sphingomyelin in the Golgi apparatus (3), or galactosylceramide in the endoplasmic reticulum (Figure 2) (4). While the formation of most abundant sphingolipids in the human body predominantly occurs in the *trans*-Golgi, synthesis of glucosylceramide takes place in the *cis*-Golgi where glucosylceramide synthase (GCS) catalyses the addition of UDP-glucose to a ceramide chain to form glucosylceramide (3).

Ceramide can also be used as a precursor for the formation of other sphingolipid species such as ceramide-1-phosphate (C1P) by ceramide kinase, mainly in the *trans*-Golgi but also in the plasma membrane. Ceramide can be hydrolysed to sphingosine and free fatty acid that leave the lysosome and enter salvage pathways serving as building blocks for other ceramide synthesis performed by CERS (5). Sphingosine can be further phosphorylated to sphingosine-1-phosphate (S1P) by the enzymes sphingosine kinase 1 and 2 (SPHK1 and -2), which can be degraded by either lipid phosphate phosphatases or by S1P lyase. The final degradation of ceramide is catalysed by ceramidases (acid, neutral and alkaline), a family of enzymes that have specific localization within the cell and therefore have specific affinity towards ceramides with particular fatty acid chain lengths.

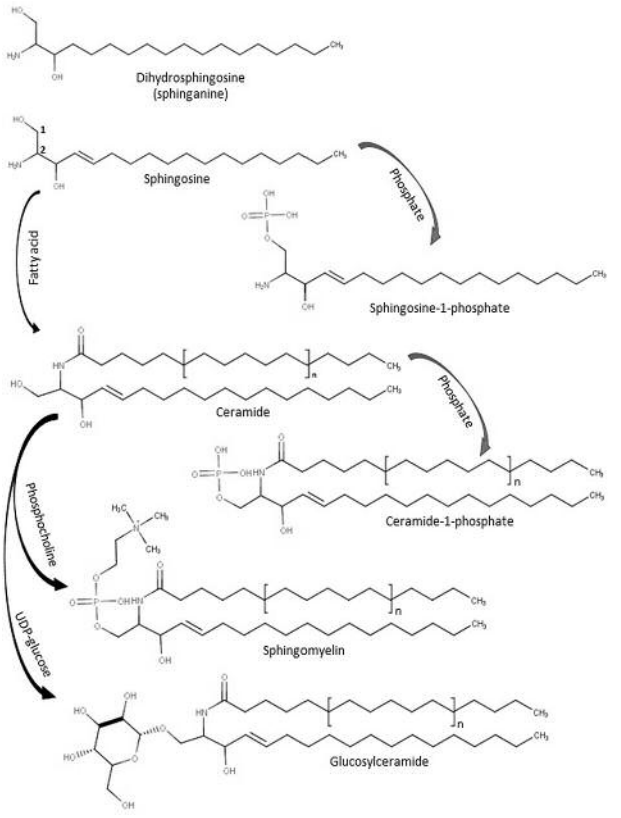

Figure 1. *Structures of some simple and complex sphingolipids. The fatty acid moiety in the structures is indicated in brackets, where n denotes variations in the fatty acid chain length.*

Simple Sphingolipids and Their Metabolic Enzymes in Cancer Formation and Progression

Sphingolipid rheostat is defined as the balance between bioactive sphingolipids, in particular pro-apoptotic ceramide and sphingosine, and pro-proliferative ceramide-1-phosphate (C1P) and S1P. Maintaining this balance is important for normal functioning of the cell, and the enzymes responsible for ceramide production and its conversion to S1P have the main role in this process (Figure 3). In the development of cancer, overexpression of enzymes that steer ceramide metabolism towards the production of proliferative sphingolipid species is common. Thus, an up-regulation of ceramide kinase, ceramidases (in particular acid ceramidase) and SPHK1 and -2 have been reported in many cancer types, which opens novel avenues for therapeutic intervention in cancer.

SPHK1 has a demonstrated role in promoting tumour cell survival, proliferation, growth, differentiation and angiogenesis. Knockdown of *SPHK1* leads to a decrease in the S1P level, increased amount of ceramide and inhibition of cancer cell survival, proliferation, invasion and migration of different cancer cells *in vitro* including colorectal (6), non-small cell lung (7), ovarian (8), acute myeloid leukaemia (9), prostate (10) and breast (11) cancer cells as well as glioblastoma (12) cells. Importantly, SPHK1 was recognized as a molecular regulator of interactions between tumour cells and the surrounding stroma *via* production of S1P, which is exported to the tumour microenvironment and signals through its cell-surface G-protein-coupled receptors, especially sphingosine-1-phosphate receptors (S1PR1 to -3), (so-called 'inside-out' signalling) to promote cancer progression (13). Study in melanoma cells has shown that conditioned medium from SPHK1-expressing melanoma cells promotes differentiation of dermal fibroblasts into myofibroblasts, increases the production of matrix metalloproteinase-2 and -9 involved in melanoma cell invasion, and increases SPHK1 expression and activity in fibroblasts. On the other hand, S1P released from SPHK1-overexpressing fibroblasts stimulates melanoma cell migration through binding to S1PR3 on the surface of melanoma cells. *In vivo* experiments in mice showed that co-injection of melanoma cells with wild-type skin fibroblasts markedly promoted tumour growth and development of metastasis in comparison with mice co-injected with *SPHK1*$^{-/-}$ fibroblasts (14). Altogether, these findings support the role of SPHK1 as a regulator of the cross-talk between dermal fibroblasts and melanoma cells, which is deemed critical for metastatic spread.

Although some studies have demonstrated the ability of SPHK2 to promote tumour progression, others have shown that knockdown or pharmacological inhibition of SPHK2

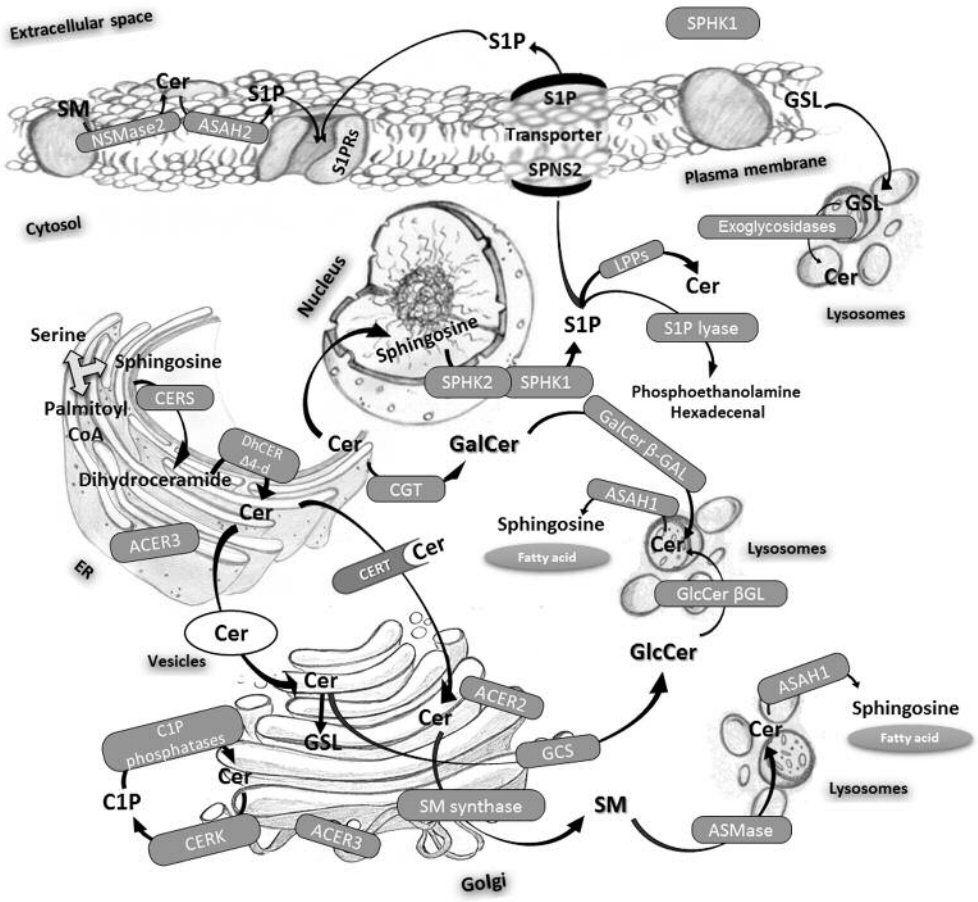

Figure 2. *An overview of sphingolipid metabolic pathways. Cer: Ceramide; CERS: ceramide synthase; DhCER Δ4-d: dihydroceramide Δ4-desaturase; ACER: alkaline ceramidase; ASAH1: acid ceramidase; ASAH2: neutral ceramidase; CERK: ceramide kinase; SM: sphingomyelin; ASMase: acid sphingomyelinase; NSMase: neutral sphingomyelinase; GSL: glycosphingolipids; CGT: ceramide galactosyltransferase; GCS: glucosylceramide synthase; GalCer: galactosylceramide; GlcCer: glucosylceramide; C1P: ceramide-1-phosphate; S1P: sphingosine-1-phosphate; S1PRs: sphingosine-1-phosphate receptors; SPHK: sphingosine kinase; LPPs: lipoproteins; CERT: ceramide transport protein; GalCer β-GAL: galactosylceramide beta galactosidase; GlcCer βGL: glucosylceramide beta glucosidase; SPNS2: protein spinster homolog 2.*

reduces proliferation, inhibits growth, induces apoptosis and enhances sensitivity to anticancer drugs in different cancer types, including non-small cell lung (15), colorectal (16), bladder (17), prostate (18), ovarian (19) and hepatocellular (20) carcinoma. Moreover, depletion of SPHK2 expression has more potent antiproliferative, anti-migratory and anti-invasive effects in cancer cells than suppression of SPHK1 (21). Interestingly, the biological function of SPHK2 in cancer cells seems to be determined by its cellular expression level. Thus, high-level SPHK2 overexpression reduces cell proliferation and survival, while low-level SPHK2 overexpression facilitates cell survival and proliferation, induces oncogenic signalling and neoplastic transformation *in vitro*, and drives tumour formation *in vivo* (22).

Endogenous ceramides have been shown to exert opposing roles in the regulation of cancer cell growth and survival depending on their fatty-acid chain length, which brings into focus the importance of CERS in cancer pathogenesis (Figure 3). Studies in head and neck squamous cell carcinoma (HNSCC) have indicated that C16 ceramide produced by CERS6 promotes tumour proliferation, whereas CERS1-generated C18 ceramide inhibits tumour growth (23). Similarly, an overexpression of CERS4 and CERS6 in human breast and colon cancer cells induced production of short-chain ceramides (C16:0, C18:0 and C20:0 ceramides), attenuated cell proliferation and promoted apoptosis, whereas up-regulation of CERS2 facilitated cell proliferation (24). Furthermore, co-expression of CERS with CERS4 and CERS6 in colon cancer cells reversed the suppression of cell proliferation and induction of apoptosis driven by long-chain ceramides (25). These and similar studies come the conclusion that a balance between very long- and long-chain ceramides govern cell

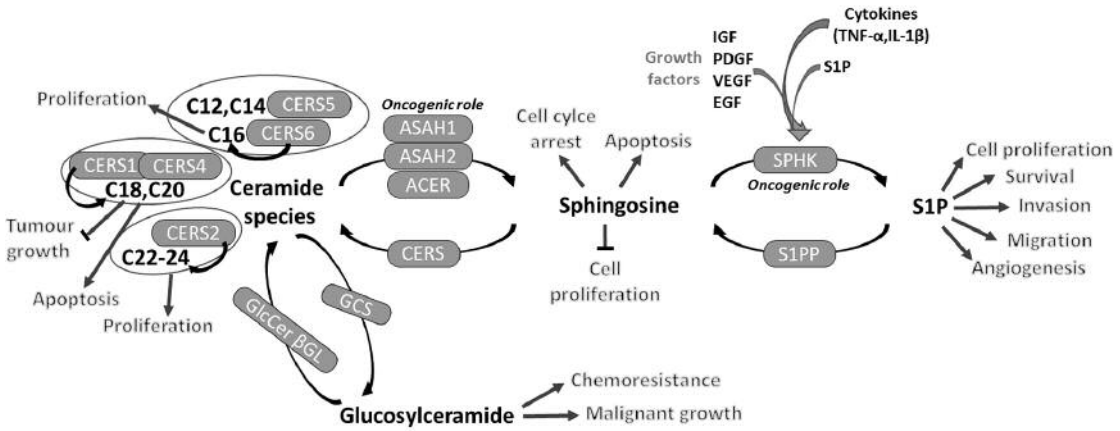

Figure 3. *Roles of simple sphingolipids and their metabolic regulators in cancer cell behaviour and fate. SPHK: Sphingosine kinase; S1P: sphingosine-1-phosphate; S1PP: sphingosine-1-phosphate phosphatase; IGF: insulin-like growth factor; VEGF: vascular endothelial growth factor; EGF: epidermal growth factor; PDGF: platelet-derived growth factor; TNF-α: tumor necrosis factor-α; IL-1β: interleukin 1β; ASAH1: acid ceramidase; ASAH2: neutral ceramidase; ACER: alkaline ceramidase; CERS: ceramide synthase; GlcCer: glucosylceramide; GlcCer βGL: glucosylceramide beta glucosidase.*

behaviour and that dysregulation of the levels of distinct ceramide species contributes to tumorigenesis.

Aberrant regulation of ceramide metabolic enzymes is associated with tumorigenesis. For example, accumulation of acid ceramidase (ASAH1) was detected in different tumours, which suggests pro-proliferative and pro-survival role of this enzyme. Thus, targeting ASAH1 in prostate (26), hepatocellular (27), HNSCC (28), breast (29) and colorectal (30) carcinoma, melanoma cells (31), and leukaemia (32) is considered a promising strategy in fighting these malignancies.

Ceramide glycosylation catalysed by the enzyme GCS to produce glucosylceramide is a rate-limiting step in the biosynthesis of glycosphingolipids. The balance between ceramide and glucosylceramide or some other glycosphingolipids controls cellular behaviour and growth; GCS tips this balance in favour of the production of metabolites that promote malignant growth. Continuous ceramide glycosylation resulting from GCS overexpression drives cancer progression *via* regulation of the expression of genes associated with tumour metastasis, maintaining the stemness of cancer stem cells and acquisition of a drug-resistant phenotype (33). Indeed, excessive GCS was recognised as a cause of acquired drug resistance in different cancer cells including breast, ovarian, colonic and cervical cancer, leukaemia, and glioblastoma (34). Genetic or pharmacological inhibition of GCS increases the sensitivity of drug-resistant cancer cells including head and neck cancer (34), non-small cell lung cancer and malignant pleural mesothelioma (35), pancreatic cancer (36), and hepatocellular carcinoma (37) to different anticancer drugs *in vitro* and *in vivo*, which clearly indicates that targeting of GCS might be an efficient strategy to counter drug resistance in cancer.

Sphingolipid Biomarkers in Cancer Prognosis and Stratification

Prostate cancer. Prostate cancer (PCa) is the second most common cancer in men worldwide. Currently, screening for PCa is based on measuring the blood level of prostate-specific antigen (PSA), but this approach suffers a lack of sensitivity and specificity. There is a wealth of evidence to support the role of S1P and SPHK1 as potential biomarkers for PCa diagnosis and prognosis (38, 39). Thus, it has been demonstrated that circulating levels of S1P decrease during early PCa progression due to down-regulation of erythrocyte SPHK1 activity, and were correlated with the clinical stage and grade. In addition, the finding that plasma S1P significantly correlates with the mortality of patients with PCa puts forward the idea of using circulating S1P as a predictive biomarker for PCa outcome (38). Further supporting a role of SPHK1 in prostate cancer-risk assessment, the enzymatic activity of SPHK1 was reported to be significantly increased in fresh tissues from patients with PCa in comparison with their non-tumorous counterparts, and to correlate with adverse clinical features (39). The finding that SPHK1 activity was correlated with odds ratios for treatment failure brought further evidence that SPHK1 holds the potential to be a clinical indicator of treatment efficacy in PCa.

Another enzyme of sphingolipid metabolism that has garnered attention as a novel potential PCa biomarker is ASAH1, whose increased expression at the gene level was detected in primary prostate tumour tissues when compared with matched normal tissues (40). The same study also

showed that an upsurge in *ASAH1* gene expression was more common in grade 7 than grade 6 prostate tumours. Similar results were obtained at the protein level as well, showing that high histological grade prostate tumour samples have increased expression of ASAH1 when compared to patient-matched normal tissues (41). The correlation between ASAH1 expression and PCa progression was also corroborated by Camacho *et al*. (42), who carried out immunohistochemical analysis of primary PCa samples and found that higher levels of ASAH1 were associated with more advanced stages of PCa. All these studies clearly indicate that ASAH1 expression is linked to PCa severity, which may have further implications for the development of risk stratification strategies for patients with PCa.

ASAH1 expression may also indicate the efficacy of treatment approach for PCa, as demonstrated by Cheng *et al*. (43), who found significantly higher expression levels of ASAH1 in human PCa tissues after radiation therapy failure in comparison with therapy-naive PCa, prostatic intraepithelial neoplasia, or benign prostate tissues. The same study also reported increased ASAH1 expression in PCa tissues after unsuccessful radiotherapy in comparison with patient-matched PCa tissues prior to radiation therapy. These findings suggest that ASAH1 expression might be a suitable biomarker for the evaluation of patient response to radiation therapy for PCa and the propensity for relapse. In addition, findings from preclinical studies showing enhanced response to radiotherapy and prevention of relapse in PCa induced by ASAH1 inhibition (43) encourage further investigation into targeting ASAH1 as adjuvant to radiotherapy in the clinical setting.

Liver cancer. Liver cancer has become the most common primary liver malignancy and the third leading cause of cancer-related deaths worldwide, and develops predominantly in patients with underlying chronic liver disease and cirrhosis (44). The clue that simple sphingolipids and their metabolic enzymes may have prognostic potential in liver cancer was provided by recent studies reporting that the expression of SPHK1 and SPHK2 is increased in tissues from patients with hepatocellular carcinoma (HCC) in comparison with matched adjacent non-cancerous tissues at the mRNA and protein levels, and was correlated with tumour size, stage, degree of tumour differentiation and microvascular invasion (45, 46). Furthermore, patients with HCC with high SPHK1 expression had shorter survival rates in comparison with those having low SPHK1 expression, which suggests that up-regulation of SPHK1 might be an indicator for poor prognosis in HCC. A similar study reported that a higher level of *SPHK2* mRNA was a risk factor for intra- and extra-hepatic recurrence (46).

In spite of overexpression of both isoforms of sphingosine kinases in HCC tissues, the level of their phosphorylation product S1P was reported to be reduced in HCC tissues relative to non-tumorous tissues (46). This unexpected finding might be explained by increased degradation of S1P as deduced from elevated mRNA levels of S1P lyase in HCC tissues compared with non-HCC tissues. On the contrary, the levels of S1P and its precursor sphingosine in serum from patients with HCC were found to be elevated in comparison with patients with cirrhosis (47-49), which points to the diagnostic utility of long-chain sphingoid bases in HCC surveillance.

A similar distribution pattern was also observed for ceramides in HCC. Thus, the levels of different ceramide species were reported to be lower in HCC tissues in comparison with adjacent non-tumorous tissues (50). In contrast, a significant rise in the serum levels of different long-chain and very long-chain ceramides (C16-C24) was observed in patients with HCC compared to those with cirrhosis, among which C16 ceramide correlated with markers of hepatocellular injury (47). Similarly, Krautbauer *et al*. provided the evidence for the association between the ratio long-/very long-chain ceramides and the extent of liver injury (51). Importantly, both serum C16 ceramide and S1P had a high diagnostic accuracy in discriminating between patients with HCC and those with liver cirrhosis, and even outperformed the current clinical biomarker for HCC, namely alpha-fetoprotein, in HCC diagnosis (47). A similar study also showed S1P to have good sensitivity and specificity in discriminating between patients with HCC and those affected by hepatitis C-associated cirrhosis (49). Collectively, all these findings highlight the potential of C16 ceramide and S1P as novel diagnostic markers for detection of HCC in patients with liver diseases.

Pancreatic cancer. Pancreatic cancer remains the fourth cause of cancer-related deaths worldwide (52). Due to occurrence of specific clinical symptoms only when disease reaches the late stage, pancreatic cancer is characterised with a high mortality rate. The only clinically approved blood-based biomarker for the management of pancreatic cancer, namely carbohydrate antigen 19-9, has limited sensitivity and specificity, and fails to accurately differentiate patients with pancreatic cancer from healthy individuals and from those diagnosed with some other non-pancreatic neoplastic condition or benign pancreatic disease (53).

Several previous studies lend support to the idea that ceramides and their metabolites are implicated in pathogenesis and that altered regulation of their metabolism is associated with a more aggressive phenotype in pancreatic cancer. Specifically, the levels of C16:0 and C24:1 ceramides are significantly higher in pancreatic tissues from patients with positive regional lymph node metastasis of pancreatic cancer than in patients without such metastases and patients with pancreatitis (54). Although no significant difference was observed in pancreatic tissue levels of S1P and dihydrosphingosine-1-phosphate, these two

long-chain bases were found to be markedly increased in the plasma from patients with metastasis of pancreatic cancer compared to patients with non-metastatic pancreatic cancer and those with pancreatitis (54). Another study provided additional evidence that sphingoid bases may prove useful in diagnosis of pancreatic cancer. Thus, it has been found that D-sphingosine is significantly up-regulated in the serum of patients with pancreatic cancer in comparison with healthy controls (55). Importantly, the same study showed good sensitivity and specificity for sphingosine in differentiating between patients and controls. Further studies are warranted to explore the clinical utility of specific ceramide species and sphingoid bases as possible discriminatory biomarkers indicative of early onset of pancreatic cancer and to investigate whether their combination with carbohydrate antigen 19-9 holds potential for improving diagnosis.

Colon cancer. Colon cancer represents the third most common type of malignancy and the fourth major cause of cancer-associated deaths in the world (56), and poses great challenges in terms of timely detection and clinical management. Although genetic and epigenetic events underlying adenoma to carcinoma progression in colorectal cancer have been extensively studied, its detection, particularly in early stages, is hampered due to the heterogeneous nature of this disease and the absence of non-invasive clinical biomarkers with an adequate diagnostic performance that would be used as a stand-alone diagnostic tool (57). This is exactly the area where sphingolipids and the enzymes regulating sphingolipid metabolism may come into play since their roles in colon carcinogenesis have been propounded by numerous studies. Among these, SPHK1 stands out as a promising candidate biomarker since its expression was shown to be increased in colon cancer tissues compared to matched adjacent normal mucosa (58-60). SPHK1 expression in adenocarcinomas was found to be significantly up-regulated compared to both normal mucosa and adenomas (59), which implies that SPHK1 plays a central role in colon cancer progression. Importantly, SPHK1 might be considered a promising biomarker for detection and risk assessment of metastatic colorectal cancer because its expression in metastatic colon cancer tissues is markedly increased compared to colon cancer without metastasis (59). Similar studies also set a role for SPHK1 in malignant progression in colon cancer by demonstrating that overexpression of SPHK1 in colorectal cancer tissues significantly correlates with lymph node metastasis, liver metastasis, and advanced tumour stages (61, 62). The finding that SPHK1 is a significant independent predictor of mortality in patients with colon cancer (62) raises the possibility that SPHK1 expression might be of clinical interest for improving risk stratification for death from metastatic colorectal cancer in order to guide decisions about best intervention options for this group of patients. In addition to its prognostic significance, SPHK1 holds potential in predicting treatment efficacy in patients with colon cancer as demonstrated in a study by Rosa *et al.* (63), who found that high tumour tissue levels of SPHK1 significantly correlated with poor response to cetuximab-based therapy in patients with colorectal cancer with wild-type Kirsten rat sarcoma viral oncogene homolog (*KRAS*).

Other evidence to support a predictive value of the SPHK/S1P axis in colorectal cancer was provided by Lin *et al.* (64). They investigated the expression and prognostic significance of S1PR1 at the protein level in patients with colorectal cancer and showed it to be significantly up-regulated in lesions compared to adjacent non-tumorous tissues. A clinically relevant finding arising from this study was that increased intratumoural S1PR1 expression was associated with metachronous liver metastasis and poor overall survival, and was identified as an independent prognostic predictor for worse outcome in colorectal cancer. Similarly, increased expression of S1PR1 was found in tumour tissues from patients with colorectal cancer compared with normal tissues in another study (60). On the contrary, the same authors found that the expression of S1PR2 was markedly decreased at the gene and protein levels in tumour in comparison with normal tissues and demonstrated that pharmacological and genetic inhibition of S1PR2 facilitated the migration and invasion of colon cancer cells *in vitro*.

Aberrant regulation of the key regulatory nodes in sphingolipid metabolism creates an imbalance between pro- and anti-apoptotic sphingolipid species, which endows colon cancer cells with an invasive and metastatic phenotype. Distinct sphingolipid signatures could, thus, serve as an indicator for colorectal cancer progression stage. Indeed, a recent retrospective pilot study showed a significant increase in the serum levels of C16-, C18-, C18:1- and C24:1-ceramides, as well as those of sphingosine, in patients with metastatic stage IV colorectal cancer in comparison with controls (65). Importantly, this study demonstrated that ceramides were significantly associated with stage IV CRC. Deregulation of ceramide metabolism was also observed in tumour tissues from patients with colorectal cancer (among which 2%, 45%, 42% and 11% had tumour stages I, II, III and IV, respectively), where significantly increased levels of C16:0, C24:0 and C24:1 ceramide were detected, whereas the levels of C18 and C20 ceramides were decreased in comparison with adjacent non-cancerous tissues (66). Among these, very long-chain ceramides seem to be associated with metastatic and aggressive behaviour of colorectal cancer, as up-regulation of C24:0 ceramide positively correlated with lymph node invasion, and a higher level of C24:1 was observed in metastatic tissues. Partial discrepancies between the levels of particular ceramide species measured in serum and tissue samples in the two independent studies described

above could, at least to some extent, be explained by the fact that patients enrolled in these studies had different tumour stages of colorectal cancer, which again indicates that each step in the progression of colorectal cancer is associated with specific perturbations in sphingolipid metabolism. Other factors that might potentially contribute to observed differences include heterogeneity within tumours not reflected in the human plasma samples and difficulty in selecting and enrolling adequate controls (67). Observed alterations in ceramide content in colon cancer might be attributed to aberrant regulation of the ceramide metabolic pathway. Indeed, CERS1, -2, -5 and -6 were shown to be overexpressed in tissues from patients with colorectal cancer in comparison with patient-matched adjacent normal mucosa (66, 68, 69). Importantly, CERS5 correlated with poor patient survival and was an independent predictor of 5-year overall survival and disease recurrence in patients with colorectal cancer (69).

Besides their potential use in surveillance of colorectal cancer progression, ceramides may also prove useful in stratifying patients according to treatment response, which could facilitate the development of tailored approaches to increase the efficacy of treatment. The feasibility of this concept was demonstrated by Dubois *et al*. (70), who investigated the potential of plasma ceramide as a predictor of response to hypofractionated stereotactic body radiation therapy in combination with irinotecan chemotherapy in patients with metastases derived from primary colorectal carcinomas. Their study revealed a significant rise in the plasma levels of total ceramide and specific ceramide species including C16:0, C18:0, C20:0, C22:0, C22:1, C24:0, and C24:1 ceramide in objective responders compared with non-responders early during treatment. Importantly, clustering analysis of patients according to plasma levels of total ceramide was able to clearly distinguish between responders and non-responders, which suggests that plasma ceramide levels might be considered predictors of response to stereotactic body radiation therapy combined with irinotecan in patients with colorectal cancer.

Head and neck squamous cell carcinoma. Squamous cell carcinoma makes up more than 90% of cancers of the head and neck and develops in the mucous membranes of the upper aerodigestive regions including oral cavity, oropharynx, nasal cavity and paranasal sinuses, nasopharynx, larynx and hypopharynx. HNSCC represents the seventh most frequent malignancy worldwide accounting for more than half a million new cases each year (71). In spite of improvements in surgery, chemotherapy and radiation, the 5-year survival rate of patients with advanced and recurrent disease remains poor. One of the reasons for unsuccessful management of patients with HNSCC is the high genomic complexity and high mutation rate in comparison to other malignancies (72). Thus, identifying multiple molecular biomarkers for predicting clinical outcome and response to therapy in HNSCC patients could move the field forward.

A wealth of evidence supports the notion of dysregulated sphingolipid metabolism, in particular of ceramide, in HNSCC. Loss of ceramide is indicative of HNSCC progression since tissue ceramide progressively was found to diminish from normal laryngeal mucosa *via* laryngeal precancerous lesions to laryngeal squamous cell carcinoma (73, 74). Similarly, Young *et al*. reported that premalignant oral lesions and oral squamous cell carcinoma had lower ceramide levels in comparison with normal keratinocytes (75). The decrease in ceramide level observed in these studies can be attributed to either its breakdown or its metabolic conversion to more complex sphingolipids. Indeed, ceramide-degrading enzyme ASAH1 was found to be up-regulated at both the protein and mRNA levels in the majority of studied HNSCC tumour tissues compared with paired normal tissues (28, 41, 76). Moreover, an increase in the levels of particular neutral glycosphingolipids, including glucosylceramide and galactosylceramide, was also detected in HNSCC tumour tissues compared to normal tissues (77), which substantiates that the observed reduction in ceramide pools may be partially ascribed to metabolic conversion to specific glycosphingolipid species that propel neoplastic growth and malignant transformation in HNSCC.

In contrast to these findings, another study reported that the total ceramide levels were higher in HNSCC tumour tissues than in their matched adjacent non-cancerous tissues (78). However, the analysis of individual ceramide species further revealed that only C18 ceramide (both C18:0 and C18:1 ceramides) was selectively significantly reduced in the HNSCC tumour tissues when compared to their adjacent non-tumorous tissues (78, 79). Thus, alterations in the level of a specific ceramide rather than total ceramides might serve to estimate clinical rate of progression of disease in patients with HNSCC. Additional evidence to support the diagnostic importance of individual ceramide species, in particular of C18 ceramide, was provided by Karahatay *et al*. (79). They reported that lower levels of C18 ceramide in HNSCC tumour tissues *vs*. normal tissues were significantly correlated with higher incidences of lymphovascular invasion and nodal metastasis. Thus, a decline in tissue level of C18 ceramide should be taken into consideration as a potential indicator for the diagnosis of advanced HNSCC. Still, additional studies investigating the relationship between C18 ceramide levels and the overall survival of the patients with HNSCC are warranted to ascertain prognostic significance of C18 ceramide. Further effort to evaluate the potential clinical utility of C18 ceramide in the management of HNSCC was undertaken in a phase II clinical trial reported by Saddoughi *et al*. (80). In this clinical study, patients with recurrent or metastatic HNSCC showing non-responsiveness

to first-line platinum-based therapy had been receiving gemcitabine and doxorubicin, and their serum levels of ceramides (C12 to C26 ceramides), C16-dihydro-ceramide, sphingosine, and S1P were measured every two treatment cycles (6 weeks). Among the measured sphingolipids, only serum C18 ceramide proved to be clinically relevant, as statistically significant differences were observed in the patterns of increase of C18 ceramide in patients with complete response, partial response and stable disease *vs.* patients with progressive disease after two treatment cycles (80). This finding strongly suggests that serum C18 ceramide represents a promising candidate biomarker of response to chemotherapy in patients with HNSCC.

There is ample evidence that enzymes regulating sphingolipid metabolism also hold great potential for diagnostic applications in HNSCC. In particular, attention has been focused on SPHK1, whose expression was shown to be up-regulated in HNSCC tumour tissues in comparison with matched normal tissues (81-85). Several lines of evidence demonstrate that increased expression levels of SPHK1 correlate with advanced tumour stage, locoregional recurrence and distant metastasis (83, 86). A study by Li *et al.* revealed that patients with nasopharyngeal carcinoma with high expression levels of SPHK1 had markedly shorter overall survival, and that SPHK1 was an independent prognostic indicator for nasopharyngeal carcinoma (83). The prognostic relevance of SPHK1 expression was also corroborated in oral squamous cell carcinoma in a study by Facchinetti *et al.* (82), who found that positive expression of SPHK1 was associated with a worse outcome.

Gliomas. Gliomas are tumours that arise from the glial cells. The most common type of glioma in adults and children are astrocytomas, which develop from astrocytes, brain cells that support neurones. Astrocytomas can be slow (low grade) or fast growing (high grade), or grades from I to IV. Among these, the most aggressive and malignant form of astrocytoma is glioblastoma multiforme, the highest grade glioma (grade IV) tumour. Glioblastoma is distinguished from lower-grade glioma by the presence of necrosis and angiogenesis, high mitotic index and rapid infiltration into adjacent healthy brain tissue, which represents a formidable hurdle to successful treatment, resulting in an average 5-year survival rate of less than 5% and a median survival time of 14.6 months (87).

A wealth of evidence supports the notion that identification of specific aberrations in the sphingolipid content and signalling might add new pieces to the puzzle of the complex pathophysiology of gliomas to improve diagnosis, prognosis and prediction of treatment response. Findings from different studies converge to indicate that deregulation of ceramide metabolism is associated with neoplastic transformation and malignant progression in human astrocytoma. It has been reported that ceramide levels are markedly lower in astrocytoma than in normal brain tissue, and that a significant decrease in the amount of ceramide was observed with a progressively increasing malignancy grade (88, 89). These observations clearly point to an inverse correlation between ceramide levels and the malignant potential of astrocytoma. Reduction in ceramide content detected in glioma tissues was mainly ascribed to depletion of C18 ceramide (C18:1 and C18:0 ceramide) (88). Importantly, ceramide levels were shown to be significantly associated with the survival of patients with glioma. The patients with glioma with the shortest survival time had the lowest ceramide level, which implies that ceramide levels might be a predictor of outcome (89).

A possible explanation for the low levels of ceramide especially in high-grade gliomas may reside in deregulation of the molecular mechanisms that control ceramide homeostasis, in particular ceramide breakdown. Indeed, the expression of ASAH1 was detected in adult and paediatric gliomas (88, 90). Moreover, increased expression of ASAH1 was associated with shorter progression-free survival and lower overall survival (91). This enzyme also plays an important role in radioresistance of glioblastoma (90, 92). Thus, Doan *et al.* demonstrated up-regulation of *ASAH1* in recurrent glioblastoma tissues from patients after radiotherapy compared to patient-matched glioblastoma tissues before radiation (92). Another molecular factor contributing to loss of ceramide in gliomas includes B-cell lymphoma 2 like 13 (BCL2L13), a CERS inhibitor which is up-regulated in glioblastoma (93). BCL2L13 binds to pro-apoptotic CERS2) and CERS6, leading to the inhibition of CERS2/6 complex activity. Thus, suppression of BCL2L13 might be an efficient strategy to restore cellular levels of ceramide in glioblastoma and improve clinical outcome in these patients.

Differential analysis of sphingolipid metabolites between human astrocytoma tissues and normal grey matter tissue also revealed an increase in the levels of S1P in glioblastoma tissues compared with normal grey matter. Importantly, the level of S1P rose with increasing glioma grade (88). Accumulation of S1P in glioblastoma tissues may result either from its excessive production or from inhibition of its de-phosphorylation and cleavage. Indeed, several studies have reported that the expression of SPHK1 is higher in glioblastoma tumour tissues than in normal brain (88, 94-96). Furthermore, the expression of S1P phosphatase 2 (SGPP2) which dephosphorylates S1P to produce sphingosine, was found to be reduced in glioblastoma tumour tissues in comparison with normal grey matter. Importantly, increased tumour levels of S1P significantly correlated with increased SPHK1 and reduced SGPP2 expression levels (88).

SPHK1 was recognised as a potential prognostic indicator for patients with glioblastoma since its expression level correlates

with worse outcome in these patients (12, 95). Thus, patients with glioblastoma with high levels of SPHK1 expression had a significantly shorter survival time in comparison with those with low levels of SPHK1 expression (12, 95). Results from different studies reinforce the role of SPHK1 as a critical regulator of malignant progression to high-grade gliomas (94, 95). It has been demonstrated that SPHK1 expression progressively increases from primary up to recurrent and to secondary glioblastoma tumour tissues in comparison with non-tumorous brain tissues (94). Similar studies demonstrated a significant correlation between the expression of SPHK1 and the histopathological staging of astrocytomas by showing that the degree of SPHK1 overexpression increases with progressively increasing grades of astrocytoma (88, 95). In light of these findings, analysis of SPHK1 expression may have further clinical implications in the stratification of patients according to glioma aggressiveness.

S1P can also be produced by SPHK2, although there seems to be an opposing regulation of SPHK1 and SPHK2 levels in human glioma. Unlike SPHK1, which is critically involved in late stages of glioma progression, SPHK2 seems to be important for early stages of glioma carcinogenesis. SPHK2 was detected in glioblastoma specimens, with highest levels observed in primary tumours compared to non-tumorous brain tissues (12, 94). Similar results were obtained by Abuhusain *et al*., who found that SPHK2 expression was down-regulated in glioblastoma tissues relative to normal grey matter, and that lower glioma grade had higher *SPHK2* expression levels (88).

S1P exerts its cellular effects through specific receptors, S1PRs, located on the plasma membrane to regulate glioblastoma cell proliferation, motility and invasiveness (97). Human glioblastoma tissues overexpress several receptors for S1P including S1PR1, S1PR2, S1PR3 and S1PR5 compared to normal non-cancerous brain tissue, and their expression gradually increases from primary to recurrent tumours and reaches a peak level in secondary glioblastoma tissues compared to normal brain tissue (12, 94, 98). Bien-Möller *et al*. also found significantly increased expression levels of S1PR1, S1PR2 and S1PR3 in glioblastoma tissues in comparison with non-malignant brain, but unlike previous reports, they revealed a trend towards a decrease in the S1PR5 expression level in glioblastoma tissues. Moreover, these authors did not detect significant differences in the expression of S1PRs between patients with primary tumours and those with relapse (96).

Interestingly, Yoshida *et al*. also reported that human glioma expressed S1PR1, S1PR2, S1PR3 and S1PR5, but their findings partially stand in contrast to similar studies by showing a significant decline in the expression level of S1PR1 in glioblastoma tissues in comparison with normal brain tissues (99). These authors also showed that the expression levels of S1PR1 were significantly reduced with increasing histological tumour grade in human astrocytoma tissues (99, 100). Such variations in the expression levels of S1PRs in glioblastoma tumour tissues between different studies might be potentially associated with a marked heterogeneity of glioblastoma at the cellular, molecular, genetic, epigenetic and metabolic levels (101).

It has been suggested that the expression level of S1PR1 might be a prognostic factor for glioblastoma since low expression of S1PR1 significantly correlated with poor survival of patients with glioblastoma (96, 99, 100). In contrast, the expression of S1PR2 and S1PR5 negatively correlated with the survival time of patients with glioblastoma. Thus, patients with glioblastoma with high expressions of S1PR2 and S1PR5 had a significantly shorter survival time in comparison with those with low expression of these receptors (94, 96). Inverse expression levels of S1PRs in relation to prognosis of patients with glioblastoma might be at least partially attributed to the propensity of individual receptor subtypes to regulate distinct aspects of glioma cell behaviour as well as to intricate nature of interactions between these receptors and other tumour-promoting factors (96, 102).

Breast cancer. Breast cancer is a heterogeneous disease with four intrinsic molecular subtypes having distinct clinical outcomes that differ in the expression of oestrogen receptor (ER), progesterone receptor (PR), and overexpression of human epidermal growth factor receptor 2 (HER2/neu). These subtypes are designated luminal A, luminal B, HER2 overexpressing, and basal-like breast cancer. Luminal A tumours represent those that are ER-/PR-positive and HER2 negative, whereas luminal B are ER-/PR-positive and HER2-positive. Luminal B tumours are often of higher grade and have worse prognosis than the luminal A subtype (103).

Observations from different studies indicate that bioactive sphingolipids are markedly elevated in tumour tissues from patients with breast cancer in comparison with normal breast tissues. In particular, a significant increase in the levels of sphingoid bases including sphingosine, dihydrosphingosine, S1P and dihydro-S1P was detected in tumour tissues compared to patient-matched non-cancerous breast tissues (104). The same study found a correlation in the levels of S1P between the tumour and patient-matched normal breast tissues, suggesting that S1P mediates the interaction between tumour cells and the surrounding normal breast tissue. Elevated levels of sphingosine, dihydrosphingosine and S1P but not dihydro-S1P were also found in interstitial fluid from human breast tumour tissue compared to that from normal breast tissue (105), which adds another layer of evidence to support the role of S1P signalling in the tumour microenvironment that could be important for breast cancer invasion and metastasis.

Additional data argue that S1P is critically involved in breast cancer metastatic progression, as markedly increased

levels of S1P were found in the tumour tissues from patients with lymph node metastasis of breast cancer compared to those with negative nodes (106). Similarly, a marked rise in the serum level of S1P was documented in stage IIIA breast cancer with lymph node metastasis in comparison with age/ethnicity-matched healthy volunteers (107). Findings from these studies bear out the role of S1P levels as a potential biomarker of lymphatic metastasis in breast cancer.

S1P levels are also associated with specific molecular subtypes of breast cancer. In particular, elevated tumour tissue levels of S1P were found in luminal A and B as well as in triple-negative breast cancer subtypes (106). Furthermore, patients with HER2 overexpression had lower levels of S1P in comparison with those with HER2-negative breast cancer. Of note, the correlation between breast cancer tissue levels of S1P and ER or PR expression was not found in this study.

The presumption that increased S1P levels in breast cancer arise from the up-regulation of *SPHK1* holds true, as demonstrated by Tsuchida *et al*. (106) who confirmed the association by showing that breast cancer tissues with increased SPHK1 expression had markedly higher levels of S1P. Overexpression of SPHK1 seems to be an inherent feature of breast cancer as supported by several studies reporting that breast cancer tissues have higher expression levels of SPHK1 than patient-matched adjacent normal breast tissues which increased with advanced stages (108-111). High SPHK1 expression significantly correlated with higher histological grading, lymphatic and distant metastasis, lower survival and poor prognosis in patients with breast cancer (108, 109).

The expression of SPHK1 was reported to vary between different molecular subtypes of breast cancer, which points to an intricate pattern of cross-regulation between sphingolipid metabolism and hormone- and growth factor-mediated signalling in breast cancer. For example, ER-negative tumours were found to have higher SPHK1 expression than ER-positive tumours, and its high tumour expression correlated with worse outcome regardless of the ER status (109, 112-114). Similarly, higher expression of SPHK1 was also detected in HER2-negative tumours than in HER2-positive tumours (111), and tumours with HER2 overexpression/amplification exhibited supressed expression of SPHK1 (106). Interestingly, high tumour expression of SPHK1 was shown to be markedly associated with shorter survival of patients with HER2-positive breast cancer in comparison to those with HER2-positive tumours having low tumour expression of SPHK1 (112), although the clinical prognostic relevance of this finding should be additionally investigated in larger patient cohort. Confounding data were reported on the regulation of SPHK1 expression in triple-negative breast cancer. Some authors found that triple-negative tumours had a higher expression of *SPHK1* mRNA in comparison with other breast tumours, which correlated with poor patient survival (109, 111). On the contrary, another study based on immunohistochemical analysis showed that triple-negative breast tumour tissues had lower tumour expression of phospho-SPHK1 compared to tumours from luminal-type breast cancer (106). Discrepancy between the results obtained in these studies might be attributed to differences in methodology used to examine SPHK1 expression producing different types of molecular information, which hampers their comparison. Another reason may be a poor correlation between mRNA and protein abundances in cells as established in previous studies (115).

There is accumulating evidence that the expression levels of SPHK1 and S1PRs in combination with the ER status might be novel predictive indicators of therapy response and survival in breast cancer patients. In patients with ER-positive breast cancer who underwent tamoxifen treatment, it was shown that high membrane and cytoplasmic S1PR1 expression in tumours was significantly associated with shorter time to recurrence on tamoxifen (116). Similarly, high cytoplasmic S1PR3 expression levels correlated with reduced survival in these patients. Importantly, high nuclear and cytoplasmic expression levels of SPHK1 in tumours were shown to be significantly correlated with increased resistance to tamoxifen and poor survival in those with ER-positive breast cancer in comparison with patients with low tumour SPHK1 levels (116, 117). Opposing findings were obtained by Ruckhäberle *et al*., who found that patients with luminal ER-positive breast cancer with high tumour SPHK1 expression had a significantly higher pathological complete response rate after neoadjuvant treatment based on tamoxifen and doxorubicin in combination with either docetaxel or cyclophosphamide than those with low SPHK1 expression. (112) . Improved clinical outcome in this study in spite of high SPHK1 expression might, at least partially, be attributed to synergistic effects of the administered drug combinations.

Ceramide plays an important role in breast cancer pathogenesis as its levels were shown to be significantly increased in malignant breast cancer tissues compared to normal breast tissues (104, 118). In particular, a significant increase in the levels of C16:0, C18:0, C20:0, C22:0, C24:1, C24:0 and C26:1 ceramides was detected in breast cancer tissues relative to normal tissues (104, 119). This can be ascribed to increased mRNA levels of specific CERS including *CERS2, CERS4* and *CERS6* in breast cancer tissues in comparison with paired normal tissues (118, 119). Among the identified ceramide species, C16:0 ceramide stands out as the most promising prognostic biomarker since a significant increase in its levels was detected in tumour tissues from patients with a positive lymph node status relative to tumours with a negative lymph node status (118). The same authors

also found that ER regulated the levels of specific ceramide species including C18:0 and C20:0 ceramides, as only these ceramides were markedly increased in ER-positive malignant breast cancer tissues in comparison with ER-negative tissues. Specific regulation of ceramide metabolism depending on the ER status was also demonstrated by several other studies. For example, increased expression of CERS4 and CERS6 was found in ER-positive tumours. In addition, ASAH1 expression was also shown to be higher in ER-positive than ER-negative tumours and correlate with improved prognosis of patients with ER-positive tumours (113). Prognostic significance of ASAH1 in breast cancer was also ascertained by Sänger *et al*., who reported that high expression of ASAH1 was associated with better prognosis in invasive breast cancer independently of the type of adjuvant treatment, and might be also considered a predictor for improved outcome in non-invasive ductal carcinoma *in situ* (29). The finding that ER-positive breast cancer has pronounced induction of ceramide synthesis and breakdown also points to a complex interplay between sphingolipid metabolic enzymes in the regulation of cancer cell behaviour. In support of this hypothesis, a recent study in colon cancer cells showed that increased expression of CERS6 transcriptionally activated ASAH1, resulting in increased mRNA, protein expression and activity of this enzyme (120).

Conclusion and Perspective

Identification of reliable and sensitive biomarkers for patient stratification remains a cornerstone for early cancer detection, prevention and achievement of the optimal treatment outcome. In spite of the rapid development of systems and integrative biology, the role of sphingolipids as cancer biomarkers has often been overlooked. Fortunately, this trend is about to change, as demonstrated by an increasing number of studies conducted in clinical settings which clearly showed diagnostic, prognostic and predictive potentials of simple sphingolipid species and the enzymes responsible for their synthesis and turnover in the management of patients with cancer. Discovery of novel sphingolipid-related biomarkers has been greatly facilitated by the advent of mass spectrometry-based sphingolipidomics that enables the analysis of large numbers of lipid species with a high degree of structural accuracy and sensitivity. Mass spectrometric analysis of human blood sphingolipids holds promise in delivering novel, non-invasive diagnostic biomarkers for cancer. However, it would be necessary first to determine reference (normal) concentration intervals and identify traits that induce normal perturbations in sphingolipid levels, such as age, sex, and ethnicity. Study on a large group of European population samples identified genetic variants that strongIy influence the circulating sphingolipid concentrations in the general population, which provides a rationale for further testing variants in these loci for their role in the development of cancer (121).

Clinical translation of sphingolipid biomarkers relies on studies in patients with cancer that often suffer from inconsistency. One of the reasons for this discrepancy between different studies performed on the same cancer type lies in tumour heterogeneity, histological type and anatomical sites of the tumour studied. In addition, the use of different analytical methods for the analysis of enzymes and receptors associated with sphingolipid metabolism and signalling (*e.g.* immunohistochemistry and real-time polymerase chain reaction) limits direct comparison of data obtained across multiple studies. Another issue that should not be overlooked is that many studies included rather small numbers of tumour samples, which does not fully represent the complexity of the underlying biology. Multi-centric studies in large and well-defined clinical cohorts, availability of good quality clinical samples, multi-region sampling of primary and metastatic tumours, and standardized analytical protocols for the measurement of sphingolipids and their metabolic enzymes could provide more uniform and reproducible data to facilitate translation from sphingolipid biomarker discovery to clinical utility.

A better understanding of the molecular mechanisms that regulate the enzymes of sphingolipid metabolism and their products in cancer, mapping of sphingolipid–protein interactions and deciphering biological roles of distinct sphingolipid species in cancer biology still remain challenging. Knowing that sphingolipid metabolism is intertwined with signalling networks that control cancer cell behaviour and fate, the use of sphingolipid-related biomarkers should be combined with the key molecular features characteristic for each cancer type (*e.g.* the expression of specific growth factor receptor, oncogene expression and mutational status, and hormone receptor status) in order to improve prognostic accuracy and specificity. In addition, the integration of validated sphingolipid biomarkers with current screening modalities would further increase diagnostic performance and help identify the best therapeutic approaches in patients with cancer.

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Ethics Approval

This article does not contain any studies with human participants or animals performed by any of the Authors.

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