From The Department Of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

ON THE ROLE OF BIOACTIVE SPHINGOLIPIDS AND THEIR METABOLIZING ENZYMES IN CANCER

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Karolinska Institutet

Stockholm 2017

Published by Karolinska Institutet.
Printed by AJ E-print AB 2017

ISBN 978-91-7676-398-8

ON THE ROLE OF BIOACTIVE SPHINGOLIPIDS AND THEIR METABOLIZING ENZYMES IN CANCER

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Friday, April 7, 2017 at 9:30 a.m.

Sit down before a fact as a little child, be prepared to give up every preconceived notion. Follow humbly wherever and to whatever abysses nature leads, or you shall learn nothing.

Thomas H. Huxley (1860)

TO MY FAMILY

ABSTRACT

Ceramide is a family of closely related molecules, which are presumed to be in the center of sphingolipid metabolism. Among sphingolipid metabolites, several ceramide subspecies and sphingosine induce apoptosis, cell cycle arrest and death, whereas sphingosine 1-phosphate (S1P) mediates cell proliferation, invasion, angiogenesis and metastasis. Cell fate is largely dependent on the balance of ceramide and sphingosine versus S1P. The sphingosine kinases (SKs) are responsible for maintaining this balance, which leans towards S1P in many cancers. Thereby SKs have been suggested as targets for cancer therapy. The overall aim of this thesis was to target SKs in order to enhance the effects of anti-cancer agents in hepatocellular carcinoma (HCC) and bladder cancer cells.

In *study I*, we have treated HCC cell lines with selenite in combination with pharmacological inhibitors of sphingolipid-metabolizing enzymes. Selenite treatment induced the activity of neutral sphingomyelinase and increased the levels of long chain ceramides. Moreover, the sphingosine kinase 1 inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole (SKI-II) sensitized HCC cells to selenite treatment by increasing the levels of ceramide subspecies, inducing reactive oxygen species formation and apoptosis, and inhibiting cell cycle progression and cell viability.

In *study II*, HCC cell lines were co-treated with the multi-tyrosine kinase inhibitor sorafenib and the sphingosine agonist FTY720 in order to improve the efficacy of sorafenib treatment. We have shown that a marginally toxic dose of FTY720 synergistically increased the cytotoxicity of sorafenib towards the Huh7 and HepG2 cell lines. Combined treatment with FTY720 and sorafenib mediated cell cycle arrest, caspase-dependent and –independent apoptosis, autophagy blockage and cell death in Huh7 cells.

In *study III*, the treatment effects of supernatant from Bacillus Calmette-Guérin -activated macrophages (SupBCG) and SKI-II on murine bladder cancer cell lines were studied. Combined treatment with SKI-II and SupBCG mediated a decrease in cell viability compared to SupBCG treatment alone. There was a transient increase in Sphk1 mRNA level following SupBCG treatment, but the SK1 protein level was unaffected. SupBCG and SKI-II individually induced PARP-cleavage. The level of dihydro C16-ceramide was increased following SKI-II treatment alone, and was further enhanced after combined treatment with SupBCG.

In our studies we have observed that inhibition of SKs enhanced the cytotoxicity of treatment with selenite or sorafenib in HCC cell lines, and of BCG in bladder cancer cell lines. Therefore, we conclude that targeting these enzymes could potentiate other treatment effects in HCC and bladder cancer cells.

LIST OF SCIENTIFIC PAPERS

I. V. Chatzakos*, A. K. Rundlöf*, **D. Ahmed,** P. J. de Verdier, J. Flygare. Inhibition of sphingosine kinase 1 enhances cytotoxicity, ceramide levels and ROS formation in liver cancer cells treated with selenite. Biochemical Pharmacology, 84 (2012) 712–721.
*The authors contributed equally to this article.

II. **Dilruba Ahmed,** Petra J. de Verdier, Charlotta Ryk, Oscar Lunqe, Per Stål & Jenny Flygare. FTY720 (Fingolimod) sensitizes hepatocellular carcinoma cells to sorafenib-mediated cytotoxicity. Pharma Res Per, 3(5), 2015, e00171,
doi: 10.1002/prp2.171

III. **Dilruba Ahmed,** Petra J. de Verdier and Jenny Flygare. Effects of treatment with Bacillus Calmette-Guérin and an inhibitor of sphingosine kinase 1 in urothelial bladder-cancer cells (Manuscript).

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LIST OF ABBREVIATIONS

SKs	Sphingosine kinases
S1P	Sphingosine 1-phosphate
SKI-II	2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole
SM	Sphingomyelin
CerS	Ceramide synthases
ASMase	Acid sphingomyelinase
nSMase	Neutral sphingomyelinase
Des	Dihydroceramide desaturase
GCS	Glucosyl ceramide synthase
HCC	Hepatocellular carcinoma
HBV	Hepatitis B virus
HCV	Hepatitis C virus
NAFLD	Non-alcoholic fatty liver disease
TERT	Telomerase reverse transcriptase
PDGF	Platelet derived growth factor
LC3	Microtubule-associated protein 1 light chain 3
ERK	Extracellular signal-regulated kinase
eFF1A	Elongation factor 1 alpha
NF-κB	Nuclear factor kappa light chain enhance of activated B cells
TNF	Tumor necrosis factor
VEGFR	Vascular endothelial growth factor receptors
BC	Bladder cancer
BCG	Bacillus Calmette Guérin

CIS	Carcinoma in situ
SupBCG	Supernatant from BCG-activated macrophages
SupCtr	Supernatant from untreated macrophages
TNM	Tumor-node-metastasis
TUR	Transurethral resection
NMIBC	Non-muscle-invasive bladder cancer
MIBC	Muscle-invasive bladder cancer
MAPK	Mitogen-activated protein kinase
AKT	Serine/threonine-protein kinases
PI3K	Phosphatidylinositol 3-kinase
XTT	Sodium 3'-[1-[(phenylamino)-carbonyl]-3, 4-tetrazolium]-bis (4-methoxy-6- nitro) benzene-sulfonic acid hydrate assay
PARP	Poly (ADP-ribose) polymerase
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase
MYC	V-Myc avian myelocytomatosis viral oncogene homolog
TRAF	Tumor necrosis factor receptor-associated factor
RTK	Receptor tyrosine kinase

1 INTRODUCTION

1.1 SPHINGOLIPIDS

Sphingolipids were named after the Greek mythological creature 'Sphinx' because of their enigmatic nature. They were first described by the neurochemist J. L. W. Thudicum in 1880s when he discovered and named 'sphingosine' meaning 'bind tight' as a compound of brain lipids [1, 2]. Sphingolipids, e.g. ceramides, sphingosine, sphingomyelins (SM), sulfatides and gangliosides, are compounds that possess a long chain amino alcohol backbone and free-fatty bases. Sphingolipids are ubiquitous; they constitute about 30% of the total lipid of the plasma membranes. Sphingomyelin and glycosphingolipids (GLSs) are the major sphingolipids in the eukaryotic biological membranes and contribute to the regulation of membrane fluidity and sub-domain structure, and cell–cell contact. Various intermediates of the sphingolipid metabolism e.g. sphingosine, sphingosine 1-phosphate (S1P), ceramide (Cer) and ceramide 1-phosphate (C1P) function as intra-or extracellular messengers. Sphingolipids play vital roles in development, cell growth, adhesion, death, migration, senescence, immune cell trafficking, behavior of proteins and receptors and vascular and endothelial integrity. They are crucial regulators in normal physiology as well as in inflammatory diseases and cancer [2-8].

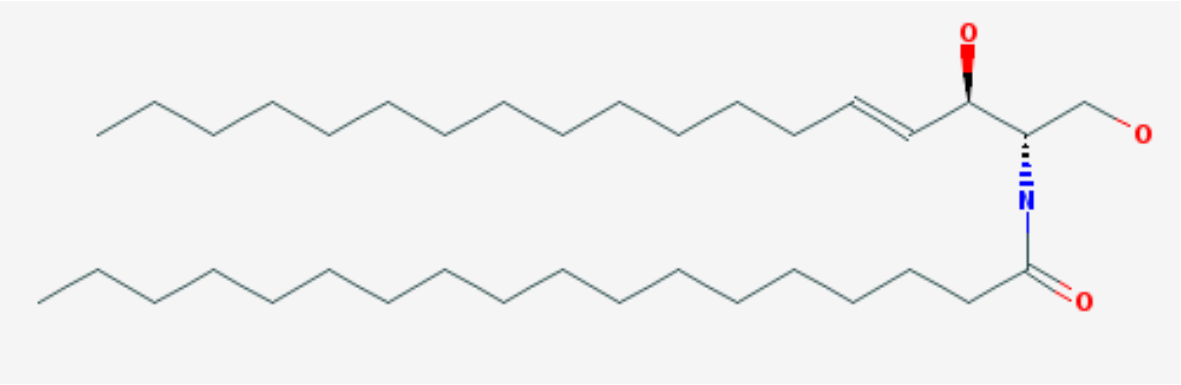

Figure 1 Structure of C18- ceramide
Image collected from National Center for Biotechnology Information, PubChem Compound Database [9].

1.1.1 Sphingolipid metabolism

The central metabolite of sphingolipid metabolism is considered to be ceramide. Ceramide is a class of molecules rather than a single molecule, which serves as the structural and metabolic precursor for complex sphingolipids and contains amide-linked acyl chains of different lengths [6] Fig 1. More than 200 subspecies of mammalian ceramides are the result of combinatorial synthesis from collaboration of several enzymes [10]. Ceramides of different chain lengths may have opposing effects in different tissues [11]. They can be synthesized *de novo* in the endoplasmic reticulum by the condensation of serine and palmitoyl-Co A by the action of serine palmitoyltransferase, followed by the sequential act of 3-ketodihydrosphingosine reductase, (dihydro) ceramide synthases (CerS) and dihydroceramide desaturase (Des) (Fig 2) [12]. Six different CerS (CerS1-6) were identified in mammals. CerS have different tissue- and substrate specificity; substrate specificity is

largely dependent on acyl-CoA chain lengths, which leads to the generation of various chain length ceramides. They show a number of overlapping substrate specificities, e.g. CerS1 can produce C18/ C20-Cer; CerS2 produces C20- to C26-Cer; CerS3 produces C18- to C32-Cer; CerS4 produces C18/ C20-Cer; CerS5 produces C16-Cer; and CerS6 produces C14/ C16/ C18-Cer [13-15]. The last step of the *de novo* ceramide synthesis pathway is the oxidation of dihydroceramide (dhCer) to ceramide by dihydroceramide desaturase 1 [16]. Physiological and pathological functions of ceramide are well known but it was believed for a long time that dhCer species are physiologically inactive. In recent years it was shown that dhCer accumulated following treatment with fenretinide, resveratrol, curcumin, celecoxib, and following hypoxia. Chemical and genetic suppression of Des led to dhCer accumulation, which led to apoptosis, cell cycle arrest, and autophagy [16-18]. Ceramides may have tumor promoting or suppressing effects depending on their fatty acid chain lengths; e.g. in head and neck squamous cell carcinoma C16- ceramide induced tumor growth whereas C18- ceramide inhibited tumor growth [11, 19]. The *de novo* pathway can be activated by heat shock [20], chemotherapeutic agents [21], and cannabinoids [22]. Ceramide can also be formed in the endo/ lyso-some by the hydrolysis of sphingomyelin by the action of either acid or neutral sphingomyelinases (SMases) [23]. *In vitro* this pathway of ceramide generation can be activated by treatment with TNF- α, Fas ligands or oxidative stress [24]. The third way of ceramide generation, which is called salvage pathway or sphingolipid-recycling pathway is more complex and exploits a variety of enzymes. In the plasma membrane and lysosomes complex sphingolipids are broken down into sphingosine, which can be recycled back by CerS to ceramide [25]. Possible enzymes taking part in this pathway are ceramidases, dihydro ceramide synthases and SMases. Selection of the pathway activation to generate ceramide might be dependent on different stimuli [25]. Ceramides have many biological functions including apoptosis [26], cellular trafficking and growth arrest [25].

Ceramides can be glycosylated by glucosylceramide synthase (GCS) to produce glucosylceramide, which is linked to multi-drug resistance or deacylated by ceramidase to form sphingosine, which has tumor suppressive effects by inducing cell cycle arrest and apoptosis [27]. Metabolism of ceramide to sphingomyelin and to the majority of complex glycosphingolipids occurs in the golgi. Transportation of ceramide from endoplasmic reticulum (ER) to golgi is mediated by the ceramide transfer (CerT) protein during sphingomyelin synthesis and by vesicular transportation during glycospingolipid synthesis [2]. Sphingosine can further be phosphorylated to S1P by the catalytic activity of sphingosine kinases. S1P promotes angiogenesis, cancer cell proliferation and metastasis [28]. Another complex sphingolipid galactosylceramide can be synthesized by the enzyme ceramide galactosyltransferase (CGT), which exploits UDP-galactose and ceramide in the process. CGT has limited tissue distribution (e.g. oligodendrocytes, kidneys, intestines and testis), and galactosylceramide is the precursor of sulfatides which are extremely important for the function of oligodendrocytes e.g. for myelination. Ceramides can also be phosphorylated in the trans-golgi and possibly in the plasma membrane to produce ceramide 1-phosphate by ceramide kinase (CK). CK has higher specificity towards ceramides containing acyl chains

longer than 12 carbons. In the golgi, C1P can function as a docking site for cytosolic phospholipase A2, and there by facilitates release of arachidonic acid [6]. Sphingolipids have rapid turnover and their levels are maintained by the balance between synthesis and degradation in different compartments [10].

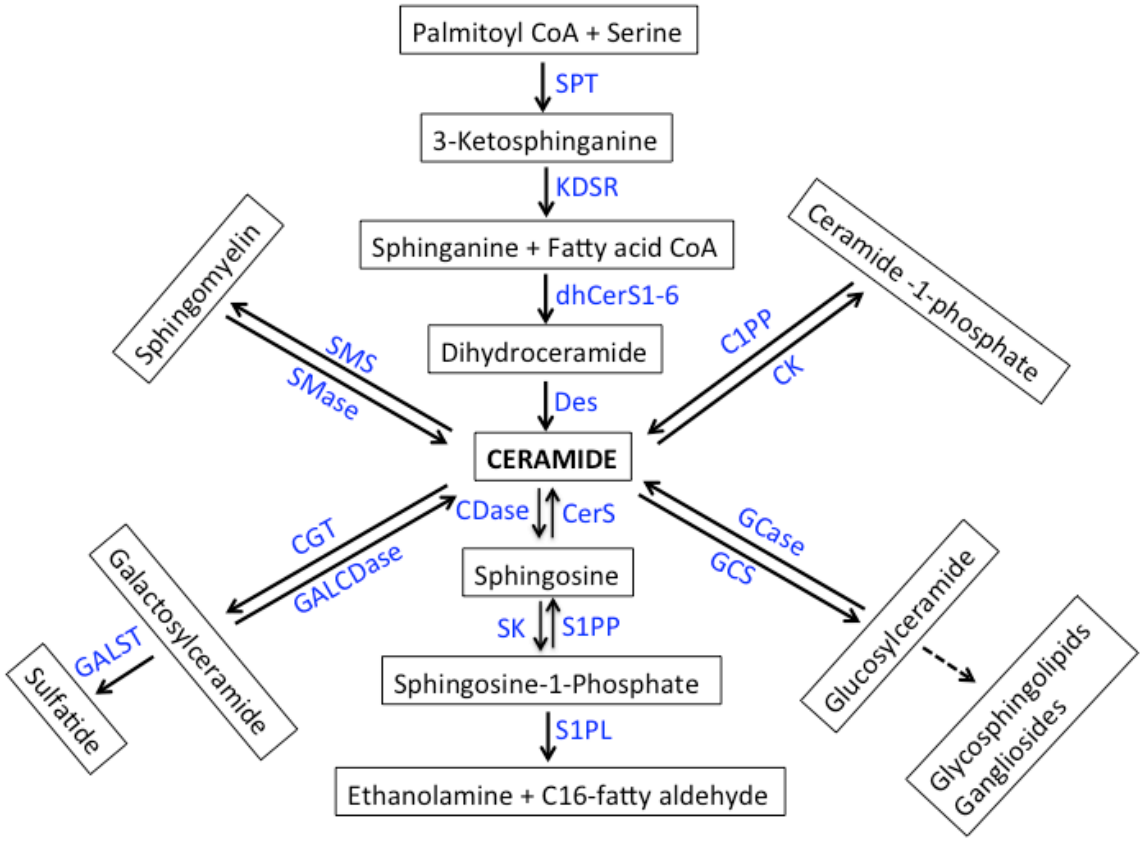

Figure 2 Sphingolipid metabolism pathways

Sphingolipids are inter-convertible and ceramide is considered to be the central molecule of sphingolipid metabolism. It can be synthesized *de novo* by the condensation of serine and palmitoyl CoA or from the hydrolysis of sphingomyelin or cerebrosides (Glucosylceramide, Galactosylceramide). Ceramide can be further phosphorylated to ceramide-1-phosphate by ceramide kinase or can serve as a substrate for the synthesis of glycolipids and sphingomyelin. Ceramide can be metabolized to sphingosine by ceramidases, and sphingosine in turn can be phosphorylated to produce S1P by the action of sphingosine kinases. S1P can be cleaved to ethanolamine 1- phosphate and C_{16}-fatty aldehyde by S1P lyase and to exit from the sphingolipid pathway. SPT– Serine palmitoyl transferase; KDSR– 3-keto dihydro sphinganine reductasse; dhCerS1-6– dihydro ceramide synthase 1-6; Des– dihydroceramide desaturase; CerS– Ceramide synthase; CDase– Ceramidase; SK– Sphingosine kinase; S1PP– Sphingosine -1- phosphate phosphatase; S1PL– S1P lyase; CGT– Ceramide galactosyl transferase; GALCDase– Galactosyl ceramidase; GALST– Galactosylceramide sulfotransferase; SMS– sphingomyelin synthase; SMase– Sphingomyelinase; C1PP– C1P phosphatase; CK– Cearmide kinase; GCase– Glucosyl ceramidase; GCS– Glucosyl ceramide synthase.

1.1.2 Sphingosine kinases

Sphingosine kinases are evolutionary conserved enzymes and are expressed in humans, mice, yeast, plants, worms and flies. They are located mainly in the cytosol but can translocate to the biological membranes upon phosphorylation. Two distinct isoforms of sphingosine kinases were isolated and characterized in mammals that are SK1 and SK2 [29, 30]. These two iso-enzymes can have compensatory, overlapping or different functions depending on

cell or tissue expression. Knocking out of either of them in mice does not affect development or survival. However, double knock out is embryonic lethal due to lack of S1P [31, 32]. The enzymes arise from the Sphk1 and Sphk2 genes, each generating a number of splice variants. Both of the SKs possess very similar amino acid sequences and five evolutionarily conserved domains. SK2 differs from SK1 in its amino terminus and central region, where SK2 possesses a proline-rich polypeptide insert [29, 30, 33]. Their size, cellular localization, expression and catalytic properties vary from each other. The chromosomal locations of human SK1 and SK2 are 17q25.2 and 19q13.1 respectively. They share similarities with large diacylglycerol (DAG) kinase family regarding their catalytic domain and diverse structure and tissue distribution [29, 30]. Sphingosine kinases phosphorylate non-polar sphingosine to polar S1P. The level of S1P is tightly regulated inside and outside of the cell by controlling its synthesis mediated by SKs, its irreversible conversion to ethanolamide by S1P lyase and its reversible dephosphorylation to sphingosine by S1P phosphatase. The biological activities of SKs seem to be keeping the balance between their substrate sphingosine and product S1P. S1P is a bioactive polar lipid that is a ligand to S1P receptors (S1PR1-5), which are members of the endothelial differentiation gene (EDG) family of G protein-coupled receptors. S1P functions extracellularly via the S1P receptors to regulate many important cellular processes e.g. cell survival, motility, angiogenesis, immunity and cytoskeletal rearrangement (Fig 3). Studies have reported that S1P may also have intracellular functions regulating calcium homeostasis, cell growth and suppression of apoptosis [33, 34]. S1P generated by SK1 can bind intracellularly to TRAF2 protein to regulate NF-κB function. S1P formed by nuclear SK2 directly targets histone deacetylases 1 and 2 (HDAC1/2) activity which links S1P to epigenetic regulation of gene expression, and regulate transcription of p21 and c-Fos genes, and also binds to prohibitin 2 (PHB2) in the mitochondria to regulate cytochrome c oxidase (Cox-2) [35, 36].

Functions of SKs depend on the level of expression of the enzymes and external stimuli and, or protein-protein interactions. Interaction with ERK1/2, Lyn/Fyn [37, 38], δ-catenin [39], and elongation factor 1 alpha (eFF1A) [40] proteins may increase the activity of SK1 whereas, PP2A [41], four and a half LIM domains protein 2 (FHL-2) [42], platelet and endothelial cell adhesion molecule 1 (PECAM-1) [43] and aminoacylase 1 [44] may decrease the catalytic activity of SK1 as reviewed by Pitson [45]. A number of factors and stimuli including growth factors, cytokines, oncogenes and even S1P can activate SK1 [45, 46]. Activation of SK1 includes its phosphorylation on Ser225 by extracellular signal-regulated kinase (ERK) 1/ 2 which does not affect its affinity towards sphingosine or ATP but increases its catalytic activity by 14-fold. Conversely SK1 undergoes dephosphorylation by protein phosphatase 2A (PP2A) [41]. SK1 activity includes not only its phosphorylation but also its translocation to the cell membrane. Phosphorylation-dependent translocation of SK1 to the plasma membrane is essential for its oncogenic potential, which is mediated by calcium- and integrin-binding protein 1 (CIB1) [47-51]. Recombinant human SK1 produced in bacteria was active implying that catalytic activity of SK1 is not dependent on its post-translational modification [52]. Post-translational modification of SK1 by acetylation leading to protein

stabilization was reported [53]. Research about SK2 activation and its function in normal- and disease conditions is increasing but a little is known to date. SK2 has been shown to promote colorectal cancer cell proliferation and invasion by enhancing MYC expression [54]. Sarah Spiegel and co-workers have shown activation of SK2 by epidermal growth factor (EGR) and phorbol ester, which is dependent on extracellular signal–regulated kinase (ERK1) signaling in a breast cancer cell line model [55].

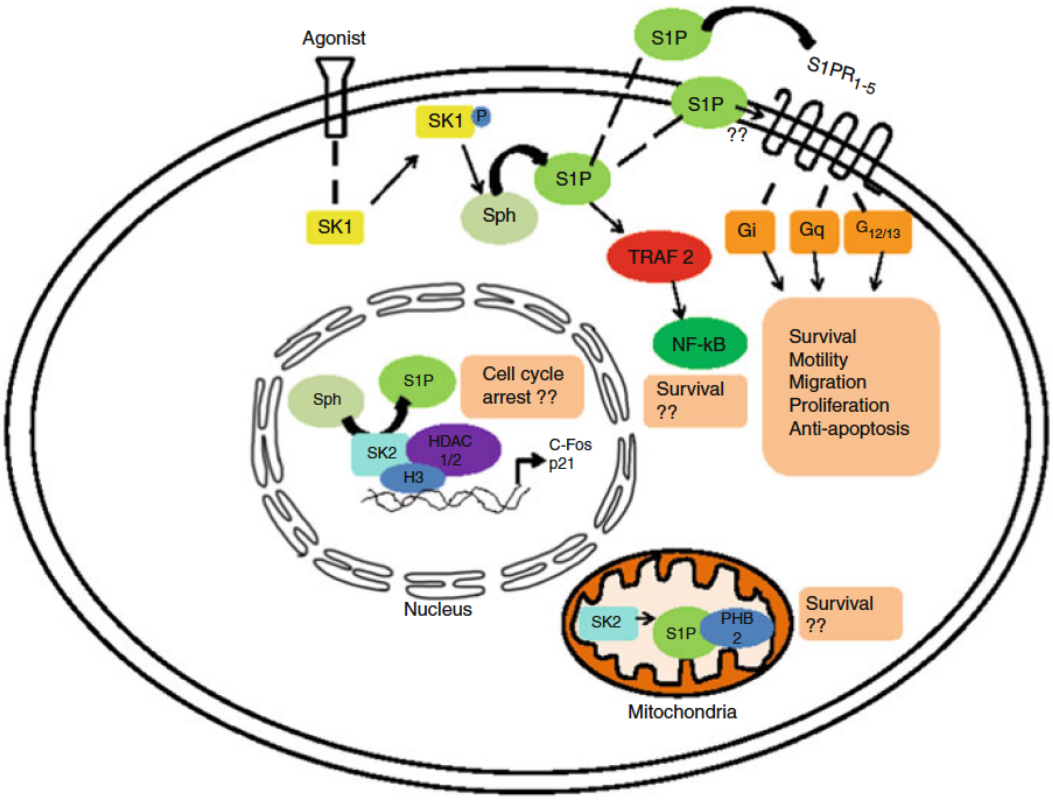

Figure 3 SK1/ S1P signaling and intracellular targets of S1P
The SK/S1P signaling pathway includes the activation of SK1 by agonist-mediated receptor action. Activated SK1 translocates to the inner leaflet of the plasma membrane to phosphorylate sphingosine to S1P. Generated S1P is then transported outside the cell by S1P transporters and engage in an autocrine or paracrine fashion to S1P receptors to induce an array of downstream mechanisms. S1P generated by SK1 can also bind intracellularly to TRAF2 protein to regulate NF-κB function. Moreover, S1P generated by SK2 interacts with HDAC1/2 in the nucleus to regulate transcription of p21 and c-Fos genes, and also binds to prohibitin 2 (PHB2) in the mitochondria to regulate cytochrome c oxidase (Cox-2). HDAC, histone deacetylases; PHB2, prohibitin 2. Image was adopted with permission from REF [36].

1.1.3 Sphingolipids in cancer

As a result of the opposing functions of ceramide and sphingosine compared to S1P, it was assumed that cell fate is dependent on the balance between these two pools. Therefore the 'sphingolipid rheostat' theory was proposed in 1996 and has been well recognized afterwards [19, 56, 57]. Being the regulator of the 'sphingolipid rheostat' SK1 was recognized to be one of the key determinant of cell viability or death. Years of studies to understand how the 'rheostat' functions and what are the implications eventually led to the findings that S1P and ceramide play vital roles in the etiology of several cancers. Additionally, the complexity of the interplay between ceramide, sphingosine, and S1P and how they regulate cellular

responses were investigated [19]. Ceramide is generally regarded as a tumor suppressor, which affects cell cycle progression and promotes apoptosis and autophagic cell death. It hereby inhibits cell growth, and enzymes regulating ceramide levels in the cells are dysregulated in cancers [58-60]. Sphingolipid-metabolizing enzymes and receptors are subject to modification in human cancers, which affects cellular signaling e.g. through transactivation of tyrosine kinase receptors (RTKs), complex formation or amplification of regulatory loops [61-64]. SK1 and its phosphorylated product S1P are potential therapeutic targets because of their involvement in cancer pathogenesis. SK1 expression in cells has protective effects against TNF-α and Fas ligand-mediated apoptosis [65] and mediates cell survival under starvation and stress [66]. SK1 has been attributed to have oncogenic functions, and shorter survival time and poor prognosis have been observed in glioblastoma multiforme patients with higher tumor SK1 levels compared to those with lower levels [67]. Increased expression of SK1 mRNA and/or protein levels have been reported in several cancers including stomach, lung, colon, kidney, breast [68], brain [67], hepatocellular carcinoma (HCC) [69, 70] and in non-Hodgkin lymphoma [71]. Knockdown of SK1 in the MCF-7 breast cancer cells resulted in cell death and apoptosis [72], and SK1 inhibition has shown promising results in many xenograft models of cancer [73] and in several genetically or chemically induced mouse models of carcinogenesis [74]. High expression of S1PR1 and S1PR3 along with SK1, and ERK1/2 has been shown to be associated with tamoxifen-resistance in breast cancer patients [75]. S1P and SK1 were proposed as novel biomarkers for clinical prognosis in breast, prostate and hematological cancers [76]. Inhibition of SK2 has been shown to induce apoptosis and suppression of tumor growth in multiple myeloma cells as well as in mouse xenograft model by down regulating cMYC and Mcl-1 [77]. Several sphingolipids including S1P were reported to be increased in breast tumors compared to normal tissues in patients [78]. Sphingosine 1 phosphate lyase (S1PL), functioning at the last step of irreversible sphingolipid catabolism, appeared to be dysregulated in colon carcinoma [79] and HCC [70]. The glucosylceramide synthesis pathway is considered to have an important role in drug resistance in different types of cancer. The levels of glucosylceramide synthase and glucosylceramide were increased in drug-resistance breast cancer tissue [80, 81], epidermoid carcinoma and ovarian adenocarcinoma cell lines [82] . Moreover, inhibition of GCS in HCC cells reverted resistance to sorafenib treatment [83]. Inhibition of acid ceramidase, which was increased in head and neck cancer tissue samples and correlated with cisplatin-resistance, sensitized cells to cisplatin treatment [84]. Moreover, C1P was shown to be associated with cell migration and invasion of human pancreatic cells [85].

1.1.4 Sphingolipids in inflammation

Although numerous pathophysiological conditions such as asthma, inflammatory bowel disease and autoimmune diseases (e.g. multiple sclerosis and rheumatoid arthritis) can be developed from infections or inflammation, sphingolipid metabolism pathways are crucial role players at different stages of the development of these disorders [2]. Ceramide, S1P, C1P, lactosylceramides, and GM3 gangliosides have been implicated in the inflammatory processes [86-89]. Ceramides are considered to be pro-inflammatory, C1P can both pro-and

anti-inflammatory depending on the cell types and the role of S1P in inflammation is unclear as reviewed in [90]. Nonetheless, among all sphingolipids S1P is considered to be the most potent intercellular signaling molecule. It has been implicated in inflammatory processes through regulation of inflammation-related genes [91] or by recruiting and activating inflammatory cells in response to different stimuli, including the release of cytokines [92-94]. Increased level of SK1 was observed in nonalcoholic fatty liver disease in human and in a mouse model with activation of NF-κB, increased cytokine production and infiltration of immune cells suggesting a role of SK1 in inflammation in this disease [95]. Initiation and progression of ulcerative colitis and inflammatory bowel disease in patient and mouse models are correlated with increased activity of SK/ S1P [96].

1.1.5 Pharmacological inhibitors of the SKs/ S1P axis

In cancer the therapeutic aim is to increase the level of pro-apoptotic ceramide by lowering anti-apoptotic and angiogenic S1P. Years of research in this area generated several inhibitors that target SK1 or SK2 as well as molecules or antibodies targeting S1P or its receptors [60]. French et al. screened several non-lipid compounds in order to identify effective inhibitors of SK in 2003. Among them SKI-II was found to be highly potent and most selective SK inhibitor, and did neither inhibit other recombinant kinases nor compete for the ATP-binding site. SKI-II has also shown anti-proliferative and anti-tumor activities in cell lines and in an animal model [68, 97, 98]. SKI-II can induce proteasomal degradation of SK1, and it has recently been shown to also inhibit dihydroceramide desaturase [99, 100], which led to the induction of growth arrest in prostate cancer cells [101]. Combined treatment with SKI-II and temozolomide has shown to induce glioblastoma cell death by accumulating dihydroceramide and dihydrosphingosine, endoplasmic reticulum stress and autophagy [102]. ABC294640, an inhibitor of SK2, was shown to be promising in combination with sorafenib to treat mouse models of HCC, kidney carcinoma and pancreatic adenocarcinoma [103, 104]. FTY720 (Fingolimod, Gilenya®) is in clinical use for treating relapsing forms of multiple sclerosis has also shown promising anti-tumor activity in HCC [73]. It is a structural analogue of sphingosine, which is synthesized from the fungal metabolite myriocin, and substrate of SK2. Its phosphorylated form FTY720-P induces internalization and degradation of the SIPR1 receptor, and therefore inhibits lymphocyte egress from secondary lymphoid tissues [105]. FTY720 can have a number of different biological effects including antitumor activity which are not dependent on its phosphorylation and S1PR-interactions [106]. FTY720 is a competitive inhibitor of SK1 and induce proteasomal degradation of the enzyme in breast and prostate cancer cells [107, 108]. Moreover, it inhibits PI3K/AKT, adapter protein 14-3-3 and cytosolic phospholipase A_2 ($cPLA_2$), whereas it activates protein phosphatase 2A (PP2A) and caspases [105, 106]. FTY720 has also been shown to inhibit CerS and thereby interfere with *de novo* synthesis of ceramide [109-111]. It has been shown to enhance chemo-sensitivity of colon cancer cells to doxorubicin [112] and radiation sensitivity of breast cancer cells [113]. Moreover, there are ongoing studies to inhibit S1P by using antibodies for treating metastatic renal cell carcinoma [114, 115].

1.2 CANCER

Cancer is the name given to a collection of more than 100 related diseases with diverse risk factors. It can originate from most cell types and organs in human, sustain proliferation and metastasize to other distant organs. Cancer is an evolutionary process, which develops from continuous alteration of inheritable genes in cells followed by natural selection processes acting on the acquired phenotypic diversity [116]. According to the list of National Cancer Institute, there are around 200 different cancer types based on organ location [117]. Normal cells have tight regulation over production and release of cell cycle and growth signals, survival, and energy metabolism to maintain tissue homeostasis to keep normal tissue functions and architecture. In the process of carcinogenesis, cells deregulate these signals and energy metabolism to become master of their own destiny. Cancer cells can sustain proliferation in a number of different alternative ways: by producing growth factor ligands for autocrine growth signals, by stimulating normal cells in the supporting tumor-stroma, by elevating receptor proteins rendering these cells hyper-responsive to the growth factors, and by averting growth factor dependency by constitutive stimulation of components of signaling pathways operating downstream of these receptors [118-120]. In 2000 Hanahan and Weinberg proposed six biological capabilities termed as 'hallmarks of cancer' in order to better understand the complexities of this disease. The hallmarks are sustaining proliferative signaling, evading growth suppression, resisting cell death, limitless replicative potential, sustaining angiogenesis, and tissue invasion and metastasis [121]. Two more hallmarks were added to this list in 2011, which are reprogramming of energy metabolism and escaping immune destruction [120]. Cancer heterogeneity, underlying complexities, adoptability to treatments, evasiveness have been addressed during the last 40 years of research, yet persistent disease free responses are rare, and cures even rarer for most forms of cancer [122].

1.2.1 HEPATOCELLULAR CARCINOMA

Primary liver cancer is the sixth most common cancer worldwide and is the third most common source of cancer-related death globally [123, 124]. Geographically, the incidence of HCC is not evenly distributed worldwide; Sub-Saharan Africa and East Asia have the highest incidence of HCC. Where as, United States and European countries have relatively low incidences [125]. According to the annual update report of the United States on cancer, 1975-2012, overall cancer incidence and death has decreased with the exception of liver cancer. Death and incidence of liver cancer has increased greatly in both sexes. The incidence rate was twice as high in men than in women and the increase in the incidence is related to the age for both sexes [126]. European countries have a slightly higher incidence of HCC relative to United States. Central European countries, where male versus female ratio is more than 4:1, have reported the highest gender difference in HCC incidence [125]. In 2008 the incidence rate of HCC in Sweden was 3.2 in male and 1.4 in female; the incidence rate was adjusted to age and calculated per 100,000 liver cancer cases in Europe [127]. Underlying liver diseases, resistance to the existing chemotherapy, and late diagnosis due to insufficient surveillance of risk groups such as, patients with cirrhosis are the important reasons for the lethality of liver cancer. Liver cancer is composed of histologically discrete primary hepatic neoplasms

including hepatocellular carcinoma (HCC), intrahepatic bile duct carcinoma or cholangiocarcinoma, bile duct cystadenocarcinoma, hepatoblastoma, haemangiosarcoma, and epitheliod haemangioendothelioma [128]. Approximately 90% of all cases of primary liver cancers are HCC. The main risk factors for developing HCC in China is hepatitis B virus (HBV) infection; in Japan is infection with hepatitis C virus (HCV); in U.S.A and Europe are infection with HCV, non-alcoholic fatty liver disease (NAFLD), and alcoholic liver disease (ALD). Other risk factors include liver cirrhosis, fungal metabolite aflatoxin B1 ingestion, tobacco smoking and diabetes mellitus [125, 129, 130].

1.2.2 Signaling pathways in HCC

Development of HCC is a multi-step, complex process that results from significant accumulation of genetic and epigenetic modifications. Each HCC consists of an average of 40 genomic alterations; among them a few are considered to be the driver mutations. Genetic alterations in HCC are linked to the underlying risk factors and co-operate with each other in the process of carcinogenesis. Common mutations in HCC affect telomere maintenance (mutations in telomere reverse transcriptase (TERT)) [131], chromatin remodeling [132, 133], p53 signaling [134, 135], WNT–β-catenin pathway activation [136, 137], VEGF, PDGF, EGF, RAS–RAF–MAPK and PI3K/AKT signaling pathway activation [138, 139], oxidative stress pathways activation [136], and mammalian target of rapamycin (mTOR) signaling pathway (Fig 4). Cumulative effects of these pathway alteration results in increased angiogenesis, cell proliferation, metastasis, inflammation, escape from apoptosis and drug resistance [130, 139, 140].

Additionally, p62 and autophagy play important role in the process of developing HCC. Accumulation of p62 has been reported in HCC patient samples [141], which is supposed to be cleared from the cells by a functional autophagy. p62 is involved in the process of carcinogenesis by preventing cell death during oxidative damage and other stress conditions. It acts as a signaling hub, which harbors functional motifs for several important signaling molecules including microtubule-associated protein 1 light chain 3 (LC3) interacting region (LIR), tumor necrosis factor receptor-associated factor (TRAF) 6 binding site (TB). p62 activates mechanistic targets of mTOR complex 1, nuclear factor erythroid 2-related factor (Nrf2), and nuclear factor kappa-B (NF–κβ). These activated pathways are responsible for nutrient sensing (by mTORC1), induction of antioxidant transcription (by Nrf2) and inflammatory responses (by NF–κβ) [142-147].

Alteration of sphingolipid metabolism has been shown to be associated with many different kinds of cancer. In HCC patients with underlying liver disease increased serum levels of C16-ceramide and S1P were reported. This may serve as novel diagnostic markers for the identification of HCC in patients with liver diseases [148]. Studies have shown that increased mRNA expression of Sphk1 and S1PL in HCC is related to poor differentiation and micro-vascular invasion [69, 70]. On the other hand, higher level of SK2 mRNA expression was associated with intra-and extra-hepatic recurrence [70]. Increased level of GCS was reported in sorafenib-resistant cell lines. Levels of GCS, acid sphingomyelinase (ASMase) and Sphk1

mRNA were increased following sorafenib treatment in HCC cell lines. These cell lines together with a xenograft mouse model were sensitized to sorafenib treatment after inhibition of GCS [83]. Recombinant human acid sphingomyelinase was also studied in combination with sorafenib in experimental HCC model in order to increase sorafenib-mediated treatment efficacy [149].

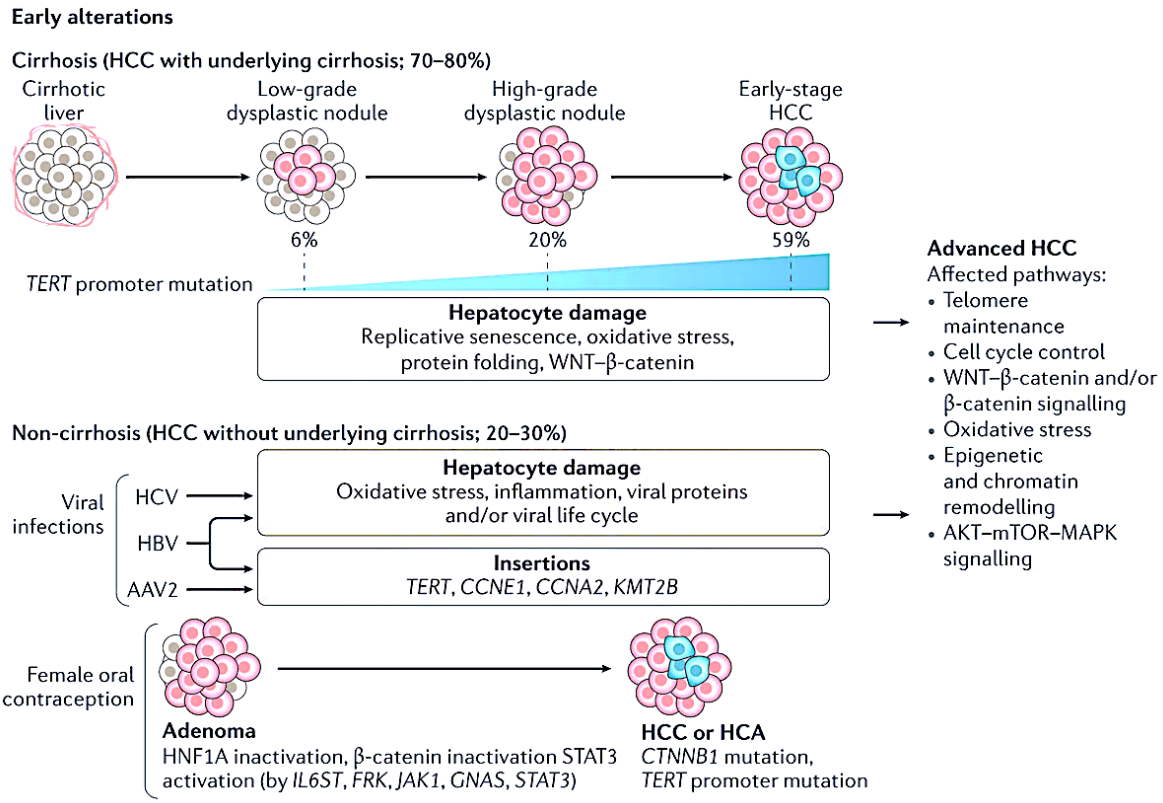

Figure 4 HCC progression and driver genes

Major recurrent molecular defects that are observed early in liver carcinogenesis. TERT promoter mutations are common early events that are identified in most cases of HCC. Other mechanisms are specifically related to risk factors; for example, HBV and adeno-associated virus 2 (AAV2) infections. Moreover, hepatocellular adenoma (HCA), a rare benign liver tumor occurring most frequently in women who take oral contraception, can transform into HCC. CCN, cyclin; FRK, fyn-related Src family tyrosine kinase; GNAS, GNAS complex locus; HNF1A, hepatocyte nuclear factor 1α; IL6ST, interleukin 6 signal transducer; JAK1, Janus kinase 1; KMT2B, lysine (K)-specific methyltransferase 2B. Image was adapted with permission from REF [130].

1.2.3 Treatment options for HCC

Molecular classification of HCC is not established yet, and Barcelona Clinic Liver Cancer (BCLC) staging classification (Fig 5) is the set of criteria that is used to guide management of patients with hepatocellular carcinoma. Vaccination against HBV has been demonstrated as primary prevention of HCC. In patients with chronic infections, antiviral therapies for HBV and HCV that offer prolonged virological responses are effective in reducing HCC incidence [127, 130]. According to BCLC guidelines, treatments for very early stages HCC are resection, liver transplantation, and local ablation with radiofrequency, microwave or percutaneous ethanol injection; intermediate stage HCC is trans-catheter chemoembolization (TACE); advanced stage HCC is systemic therapy with sorafenib; whereas end stage HCC

patients are given palliative support including pain management, nutrition and psychological care [127].

Sorafenib is a standard systemic treatment, which received FDA approval in 2006 for treating advanced HCC with preserved liver function, i.e. BCLC stage C. Yet there is no available clinical or biological biomarker to identify best responders to sorafenib treatment and there is no second-line therapy for non-responders to sorafenib treatment [127]. Sorafenib is a multi-kinase inhibitor, which targets serine/threonine and receptor tyrosine kinases, predominantly vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR), c-Kit and RET to decrease tumor growth and angiogenesis. VEGFR, PDGFR, fibroblasts derived growth factor receptor (FGFR), and insulin growth factor receptor (IGFR) are all perturbed in the process of HCC development [150-152]. Side effects related to sorafenib treatment include diarrhea, hand and foot reaction and fatigue. Treatment efficacy of sorafenib-targeted therapy is limited to a median of 3 months increase of overall survival. Limitations of sorafenib treatment are limited survival benefit, necessary exclusion of patients with impaired liver function and inefficiency of using it in the adjuvant settings. There are numerous phase II and III trials investigating first-line or second-line therapies with other compounds but most of them failed to produce any successful results. Limited success in treating HCC is predicted to be due to tumor heterogeneity, diverse mutations and perturbations in the signaling pathways and underlying liver diseases [130, 151, 153]. Studies with several targeted therapy, e.g. Brivanib [154] and sunitinib were promising but failed to prove better efficacy over sorafenib or exhibited severe side effects, respectively. Somewhat more promising outcomes have emerged from ramucirumab, a recombinant monoclonal IgG antibody with specificity towards VEGFR2 and regorafinib, a multikinase inhibitor that are currently undergoing phase III clinical trial as a second-line treatment option for a subgroup of patients [130, 151, 152]. Most of the phase III drugs currently being tested are anti-angiogenic agents, immune checkpoint inhibitors, cell-cycle blockers or receptor tyrosine kinase inhibitors. Based on the genomic studies performed during the last decade, HCC has been classified to have either proliferative (HBV related) or non-proliferative (HCV and alcohol related) genotypes, comprising 50% of patients in each group [138]. Lack of knowledge about main driver mutations and corresponding pathways, and biomarker stratification are assumed to be the reasons for being failure in the drug trials [130, 151]. Since sorafenib treatment is the standard of care, it is suggested from the American Association for the Studies of Liver Diseases and Journal of the National Cancer Institute to combine new molecules tested in the first-line settings with sorafenib, to demonstrate superiority [151, 155].

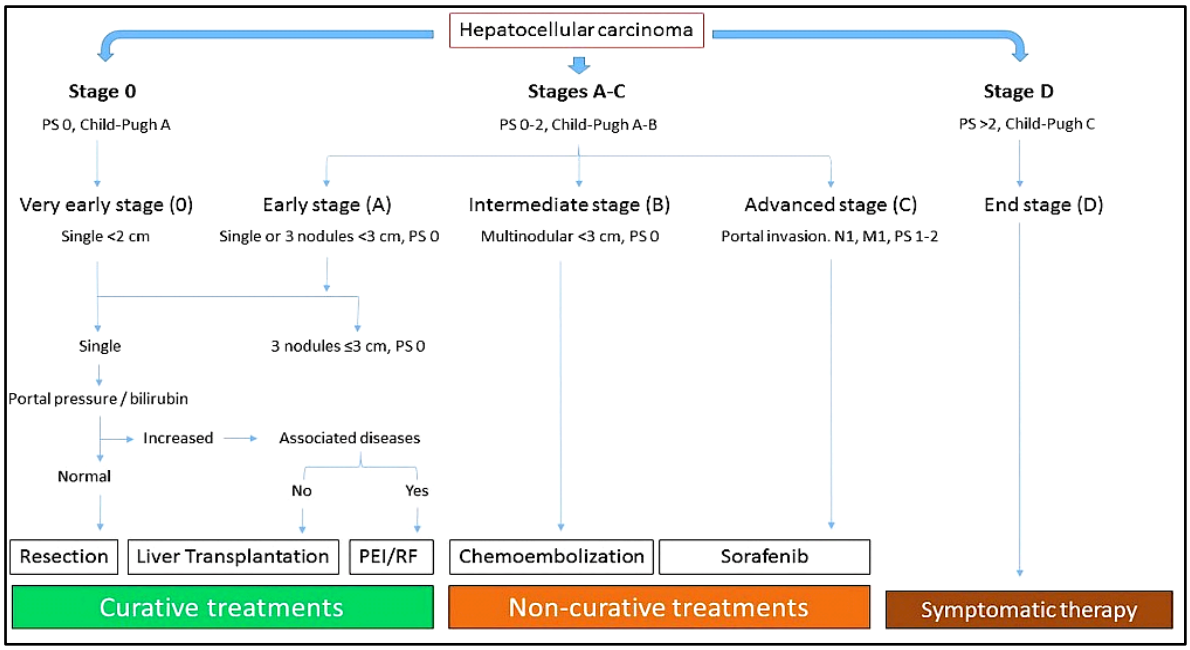

Figure 5 Management of HCC according to Barcelona Clinic Liver Cancer
There are five stages of HCC in BCLC classification in order to choose the best therapies for the best matched candidates. Stages 0–A patients are with asymptomatic early tumors; stage B patients are with multi-nodular HCC; stage C patients are at advanced stage, symptomatic HCC with portal vein invasion; stage D, which is the end stage with poor prognosis. PS, performance status. Case courtesy of Dr Matt A. Morgan, Radiopaedia.org, rID: 34365.

1.2.4 BLADDER CANCER

The ninth most common cancer in the world is bladder cancer (BC), which is also the most common cancer of the urinary tract [156, 157]. According to GLOBOCAN estimate, there were 430 000 new cases and 165 000 death from BC occurred worldwide in 2012. BC is more prevalent in males than females. Geographically it is predominant in Southern and Western Europe, North America, and certain countries in Northern Africa and Western Asia. In Europe the highest incidence was in Southern Europe (Spain and Italy) followed by the Nordic countries (e. g, Denmark: Age Standardized Rate = 27.4 per 100 000) [157]. In Sweden 2793 cases of urinary bladder cancer were diagnosed in 2011, among them 2049 were in men and 744 were in women. This constitutes 6.8% of all cancers in male and 2.7% of all cancer cases in women [158]. The most significant risk factor of BC is age and the median age at diagnosis is around 70 years. Environmental risk factors play a vital role in the development of bladder cancer. Among which tobacco smoking (>50% of bladder cancer) [159, 160], occupational exposure to aromatic amines and other chemicals (paints, dye, rubber, or aluminum industries workers) [160], chronic urinary tract infection, and Schistosomiasis (prevalent in Egypt) are the important risk factors [161, 162]. Genetic risk factors include polymorphisms in two carcinogen-detoxifying genes, N-acetyltransferase-2 (NAT2) and glutathione S-transferase-µ1 (GSTM1). Single-nucleotide polymorphisms (SNPs) that are associated with moderate risk of bladder cancer are close to the genes MYC, TP63, prostate stem cell antigen (PSCA), telomerase reverse transcription (TERT),

fibroblasts growth factor receptor 3 (FGFR3), cyclin E1 (CCNE1), and UDP glucoronosyltransferase 1 family and polypeptide A complex (UGT1A) [159, 160, 163].

Histologically >90% of the bladder cancers are transitional cell (urothelial) carcinomas (TCC), ~5% are squamous cell carcinomas, and <2% are adenocarcinomas [164]. These tumors are classified based on World Health Organization (WHO) grading in 1973 and 2004, which is based on the cellular characteristics, and staging is based on the tumor-node-metastasis (TNM) system (Tis-T4) (Fig 6). Grossly bladder cancer can be divided into non-muscle-invasive (NMIBC) and muscle invasive bladder cancer (MIBC). The majority of the patients (~75%) with BC present with a disease confined to the mucosa (stage Ta, carcinoma in situ (CIS)) or submucosa (stage T1). Due to long-term survival and lower risk of cancer specific mortality compared to T2-4 tumors, they have a higher incidence. The majority of the TCCs are low-grade papillary tumors (Ta) at the time of diagnosis. Stage T1 tumors which have invaded lamina propria but not inner muscles are high grade as are MIBC. 50-70% of NMIBCs recur but invasion to muscle is not very common (10-15%) [163]. Carcinomas *in situ* (CIS/ Tis) are flat, high-grade tumors that are confined to the mucosa. CIS and T1 lesions have shown high malignancy potential in both clinical practice and molecular biology techniques. About 54% of the patients with CIS develop MIBC within 5 years span if untreated [164, 165].

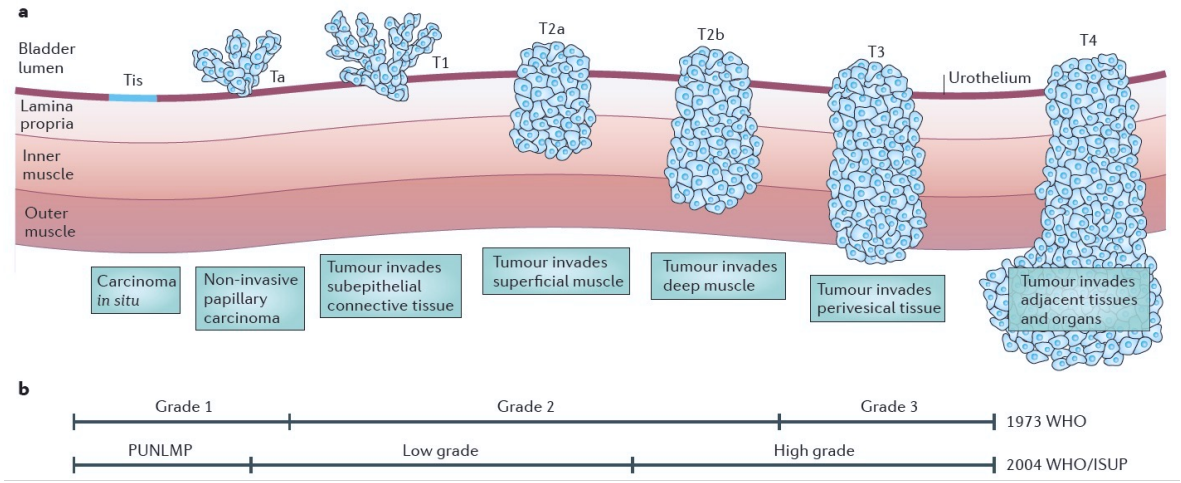

Figure 6 Staging and grading of bladder cancer

a. Bladder cancer staging according to the Tumor-Node-Metastasis (TNM) system. b. Grading according to the 1973 WHO and 2004 WHO/ International Society of Urological Pathology (ISUP) criteria. PUNLMP = papillary urothelial neoplasm of low malignant potential; Image was adopted with permission from REF [163]

Hematuria is the most common symptom in patients, which is recommended to follow up by physical examination, cystoscopy, urinary cytology, and biopsy [166]. Transurethral resection (TUR) is a treatment option for NMIBC. To delay disease recurrence, surgery may be followed by instillation of chemotherapeutic agents (e.g. mitomycin C). Thereafter, patients' urine sediments are followed up by cytological examination and periodic cystoscopy is performed [163, 165]. Intravesical Bacillus Calmette-Guérin (BCG) is the standard treatment for high-risk, NMIBC including CIS, high-grade papillary tumors (stage Ta), and lamina-propria-invasive tumors (Stage T1). Treatment with BCG is associated with a lower risk of

disease recurrence compared to TUR alone and BCG can also minimize the risk of progression to invasive disease [167].

1.2.5 BCG treatment in bladder cancer

In 1921 Albert Calmette and Camille Guerin succeeded in attenuating the cow tuberculosis bacillus, *Mycobacterium bovis* at the Pasteur Institute of France [168]. BCG eventually became an effective vaccine for preventing human tuberculosis regardless of the less virulence capacity [169]. The idea of using BCG in treating cancer was triggered by an autopsy study by the biologist Raymond Pearl that patients with tuberculosis had seemingly lower incidence of cancer [170]. Holmgren first reported the use of BCG inoculation as an anti-cancer treatment option in 1935 [171]. Numerous research have been done on the use of BCG in different types of cancer albeit its use in bladder cancer was stemmed out after Coe and Feldman's findings that bladder is an immuno-competent organ and responsive to topical BCG [172]. Morales et al first described successful intravesical BCG treatment in recurrent BC in 1976 [173]. After Morales success in 1980 Lamm et al showed in a randomized study that BC recurrences dropped from 42% to 22% when BCG instillation was combined with endoscopic management compared to endoscopic management alone [174]. Since then BCG instillation has been established as the standard treatment following TUR of high-risk NMIBC. Most clinical practice guidelines recommend maintenance therapy with BCG for 1–3 years [175]. BCG is administered through a urethral catheter and patients are suggested to retain it for 1-2 hours [165]. The exact mechanisms of action of the antitumor effects of BCG is yet unknown.

Antitumor activity of BCG depends on adequate stimulation of patient's immune system, live BCG and contact of BCG with bladder cancer cells [176, 177]. Following BCG instillations their attachment to the urothelial wall is facilitated by fibronectin [178]. Thereby BCG is internalized by urothelial and dendritic cells resulting in the release of a number of cytokines, including interleukin 6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Consequently macrophages, granulocytes and leucocytes are recruited locally. Cell death occurs from direct cytotoxicity of BCG and the release of different apoptotic factors [179, 180]. Macrophages are believed to play a crucial role in BCG mediated antitumor activity. Increased number of macrophages and cytokines were found in the voided urine of BC patients following BCG treatment. After BCG treatment macrophages can also be found in the bladder wall of patients [181-183]. BCG stimulated macrophages have shown to induce cytotoxicity in several bladder cancer cells *in vitro* [184-187]. Many of the cytokines secreted in the urine of BC patients after BCG treatment are found to be secreted by BCG stimulated macrophages e.g. IL-1β, IL-6, IL-8, IL-10, IL-12, interferon-γ, and TNF-α [188-190]. Even though immunotherapy with BCG is the golden standard adjuvant treatment for high-risk NMIBC, more than 30% of the patients fail the treatment [191] and the disease recurs in around 20% of the patients [192, 193]. A number of BC patients are intolerant to BCG treatment because of side effects ranging from cystitis, irritation during voiding to uncommon but life-threatening BCG sepsis [194, 195].

1.2.6 Signaling pathways involved in bladder cancer

There is a need for molecular biomarkers for diagnosis and development of effective treatment of bladder cancer. Point mutation of the TERT promoter region and mutation of FGFR3 are the common genetic alterations discovered till now [196-198]. *In vitro* FGFR3 mutation activates RAS-MAPK pathway and phospholipase Cγ leading to survival and proliferation of urothelial cells [163]. Disruption of p53 signaling and activation of RTK signaling followed by activation of PI3K/AKT/mTOR pathways have been correlated to poor clinical outcomes, progression to invasive BC and resistance to therapy [199-201]. To date, not much is known about sphingolipid metabolism status in bladder cancer. In 2014 it was shown for the first time that BC tissues express a high level of SK1 mRNA and protein. High level of SK1 was strongly associated with histologic grade, tumor stage and reduced overall 5-year survival rates. The study suggested that SK1 is an independent prognostic factor for poor disease outcome [202]. Moreover, gangliosides were found to be accumulated in superficial papillary tumors compared to the invasive tumors [203]. Overexpression of ganglioside reduced cell proliferation, motility, and invasion and induced apoptosis of murine bladder cancer (MBT2) cells and reduced tumor growth in a xenograft mouse model [204]. Additionally, local or exogenous administration of GM3 ganglioside inhibited both invasion and growth in xenograft mouse model [203, 205].

2 AIMS

The aims of this thesis were to target sphingolipid-metabolizing enzymes in order to study the effect of cytotoxic treatment on the levels of a subset of sphingolipids, to investigate whether treatment with inhibitors of SKs could enhance the cytotoxicity, and to elucidate mechanisms behind possible effects of combined treatment in HCC and bladder cancer cells.

Study specific aims were:

Paper I: to investigate the cytotoxic effects of selenite treatment in the presence of inhibitors of sphingolipid-metabolizing enzymes in a hepatocellular carcinoma cell line.

Paper II: to sensitize hepatocellular carcinoma cell lines to sorafenib-mediated cytotoxicity by cotreatment with the sphingosine analogue FTY720.

Paper III: to study the treatment effects of BCG in combination with an inhibitor of SKs in urothelial bladder cancer cell lines.

3 MATERIALS AND METHODS

This section will focus on general discussions about some of the models and methods used in this thesis. More detailed and study-specific protocols are described in the individual papers.

3.1 Cell lines

Cell lines are widely used models in *in vitro* research due to accessibility to large pool of samples, ease of handling, relative low cost and flexibility to test different therapeutic agents. Several immortalized cell lines were used as research model in our studies. In paper I and II human hepatocellular carcinoma cell lines Huh7 and HepG2, and the close to normal immortalized liver cell line MIHA were used. The Huh7 cell line is derived from well-differentiated hepatocellular carcinoma containing mutated p53 [206]. Acknowledging the extreme heterogeneity of advanced HCC we have also used the HepG2 cell line that has wild-type p53 phenotype [207]. In paper III, we have used the murine bladder cancer cell line MBT2, the human bladder cancer cell line T24 and the murine macrophage cell line RAW 264.7. In this study it was important to mimic the *in vivo* response to BCG treatment *in vitro* by provoking an immune response by stimulating macrophages with BCG before treating bladder cancer cells with supernatant from the BCG-treated macrophages. As human macrophage cell lines respond poorly to BCG, we were limited to use murine cell lines in this model system.

Wilding and Bodmer have reviewed pros and cons of using different *in vitro* and *in vivo* models. Some crucial disadvantages of using cell lines as research model particularly in drug development are: genetic changes during cell culturing, lack of tumor heterogeneity present in primary cancers and lack of appropriate tumor micro-environment. Having acknowledged the aforementioned cons cell lines are still very useful research tools in preclinical *in vitro* studies before proceeding to animal/ human trials [208].

3.2 Proliferation and cytotoxicity assays

Uncontrolled proliferation is one of the most important characteristics of cancer cells. Successful cancer treatment depends on cytotoxicity of the treatment agents towards malignant cells. The aim of developing effective cancer treatment is therefore to kill malignant cells while keeping the normal cells unaffected. There are different methods to assess cytotoxicity of pharmacological agents *in vitro*. Some of the commonly used assays are: WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium], MTT assay, XTT sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6- nitro) benzene-sulfonic acid hydrate assay, trypan blue cell exclusion method, 5-bromo-2´-deoxyuridine (BrdU) incorporation assay, propidium iodide (PI) staining of the nuclei, alamar blue and lactade dehydrogenase (LDH) leakage assay.

The XTT assay, which is a colorimetric assay for non-radioactive quantification of cell proliferation and viability, was used in all of the papers. XTT is a tetrazolium salt that becomes formazan dye by the metabolic activity of mitochondrial dehydrogenases of live

cells. Formed formazan dye is water soluble and orange in color, which can be measured by spectrophotometers. The more viable cells present in the samples the higher will be the absorbance and vice-versa. XTT is a widely used method for its sensitivity and convenience of use. It might be important to thoroughly re-consider using this method if the therapeutic agent is expected to alter the metabolic state of the cells to avoid false positive or false negative results.

The trypan blue exclusion method was used in paper I and III. This method uses the principle that cell membrane integrity is compromised during cell death allowing penetration of dyes into the cytoplasm. Live cells are impermeable by trypan blue, and therefore glowing, unstained cytoplasms are easily visible under light microscope. This method is widely used because of its rapidity, cost-effectiveness and accuracy. It is a very stable method to count live cells, yet might not be the best choice studying cytotoxicity. Cells that are still alive but have compromised growth and cell function will not be distinguished by this method. On the contrary, cells having transient membrane permeability due to mechanical or other stress will be stained with trypan blue. During large-scale and time-course experiments, it is almost impossible to use this method as trypan blue itself is cytotoxic when used in long-term incubations.

Replication states of cells following treatment with anticancer drugs can be analyzed by staining the nuclei of cells with fluorescence dyes. Light scattering characteristics of up taken propidium iodide (PI) are capitalized in this method. According to the protocol, cell membrane lipids are dissolved using a nonionic detergent, the cell cytoskeleton and nuclear proteins are eliminated with trypsin, cellular RNAs are digested with an enzyme, and the nuclear chromatins are stabilized with spermine. PI stoichiometrically binds to the clean, isolated nuclei, which are then analyzed by flow cytometry. PI-stained nuclei emit fluorescent light primarily at wavelengths between 580 and 650 nm. The resulting histogram consists of three populations, two Gaussian curves for the G1- and G2 populations and another for S phase population. Quiescent and G1 cells have one copy of DNA and therefore have 1X fluorescence intensity, whereas cells in the G2/ M phase of the cell cycle have double DNA copies and therefore have 2X intensity. On the contrary, the cells in S phase are in the DNA synthesis phase and therefore have fluorescence intensity between the 1X and 2X populations. This is a very robust way of determining cell cycle progression and cytotoxicity but at the same time it is tedious and time consuming.

In paper II, the Annexin-V-FLUOS staining kit (Roche Diagnostics GmbH, Germany) was used to measure cell death. One of the changes occurring in the cell surface of early apoptosis is externalization of phosphatidylserine (PS) to the cell surface. PS translocation occurs by the enzyme flippase during necrosis as well but during necrosis, cell membranes become leaky and ruptured where as in early apoptosis cell membranes are intact. According to this method, fluorescein isothiocyanate (FITC) labeled Annexin-V binds to phosphatidylserine in the outer leaflet of apoptotic cell membranes, whereas PI binds to the nuclei of the necrotic cells, thereby differentiating apoptotic and necrotic or late apoptotic cells. Annexin-V, which

is a Ca^{+} dependent phospholipid binding protein has high affinity towards PS. Therefore combining annexin-V with PI will result in cell populations that are FITC-/PI- (healthy cells), FITC+/PI- (early apoptotic cells) or FITC+/PI+ (necrotic cells). These discrete cell populations are then detected by analyzing the samples in a flow-cytometer [209, 210].

Microscopy is a powerful technique to visualize morphological features of cells or tissues. In paper I and II we have used fluorescent and confocal microscopy to determine features of apoptotic cell death. During apoptosis biochemical and morphological changes occur in the cells. Cell shrinkage, surface blebbing, and chromatin condensation leading to nuclear fragmentation are some of the morphological characteristics during apoptosis. In our study we have used DAPI, which exclusively stains nuclei that is visible under fluorescent or confocal microscope by using appropriate filter. Treated cells were processed and fixed on the cover slips and visualized for changes in the nuclei.

3.3 Messenger RNA and protein assays

Real time quantitative PCR is widely used in medical research to study gene expression in many different settings including in different disease or treatment conditions. Accuracy and specificity of this method is largely dependent on design of primer-probe and use of appropriate house keeping genes. In order to choose the most stable reference gene in our study, 8 different reference genes (e.g. 18S, Actb, B2m, Gapdh, Gusb, HPRT1, Pgk1 and Tfrc) were screened. HPRT1 showed very stable expression levels therefore, we chose to continue using it [211].

In paper I we have used cell death ELISA (Roche Diagnostics, Basel, Switzerland) to detect histone and intra-nucleosomal DNA fragmentation. It is a quantitative sandwich ELISA that capitalizes specificity of two different monoclonal antibodies. This allows specific determination of mono-nucleosomes and oligo-nucleosomes in the cytoplasmic fraction of the cell lysates. The micro-plate wells were coated with the anti-histone-biotin antibody; nonspecific binding sites are saturated by treatment with blocking solution, and binds to histones H1, H2A, H2B, H3 and H4. The anti-DNA-peroxidase antibody binds with double and single stranded DNA in the cytoplasm. After removal of unbound peroxidase conjugate, the amount of peroxidase retained in the immuno-complex is photo-metrically determined with ABTS as the substrate.

In all of the papers we have used western blotting to study the expression of different proteins. Western blotting is the transfer of separated proteins from the gel to the surface of a thin support membrane matrix. The proteins are bound and immobilized on the membrane. Bound proteins are further detected by using HRP- or infra-red (IR) dye-conjugated secondary antibodies. In paper I we have used HRP-conjugated secondary antibodies whereas in paper II and III we have used IR dye conjugated antibodies along with the more sensitive LICOR imaging system. This method is very time consuming and many parameters need to be optimized. One of the main hazards is certainly availability of specific and good antibodies. To overcome this limitation it is important to validate panel of antibodies by using

positive control or recombinant protein, appropriate negative control and blocking peptide. It is also recommended to use a stable loading control protein to relate the expression of the protein of interest. In our study we have validated antibodies from different companies in order to select the suitable one.

4 RESULTS AND DISCUSSIONS

4.1 PAPER I: Inhibition of sphingosine kinase 1 enhances cytotoxicity, ceramide levels and ROS formation in liver cancer cells treated with selenite

Whereas low doses of selenium have been shown to prevent cancer [212], treatment with high doses of selenite has previously been shown selective cytotoxicity towards malignant [213] and drug-resistant cells [214]. Accumulation of ceramide subspecies and cell death in erythrocytes has also been shown after selenite treatment [215]. In our study we have shown that after treating Huh7 cells with an EC50 dose of selenite, long chain ceramide subspecies (C14-, C16-, C18- and C18: 1- ceramides) increased approximately by two-fold. This result implies ceramide formation as a contributor to selenite mediated cytotoxicity in Huh7 cells. Increased levels of C16-, C18- and C20-ceramides following cytotoxic treatment of cells of different origin have been reported earlier [216, 217].

We were also interested to know whether the observed accumulation of ceramide after selenite treatment was the result of *de novo* synthesis or the breakdown of sphingomyelin. We have observed that blocking *de novo* ceramide synthesis by myriocin, and acid sphingomyelinase by desipramine did not alter the decrease in Huh7 cells viability induced by selenite treatment. On the contrary, blocking neutral sphingomyelinase by 3-O-methyl sphingosine significantly counteracted the cytotoxicity caused by selenite treatment. In line with this observation we have shown increased activity of nSMase following selenite treatment. Cellular stress, anticancer drugs, and oxidants have earlier been shown to activate nSMase [23]. Our results imply that part of the selenite-mediated cytotoxicity towards Huh7 cells was mediated by ceramide-formation from sphingomyelin via nSMase.

We further investigated how selenite treatment affects downstream breakdown of ceramide in order to be able to increase treatment efficacy by inhibiting SK1 and GCS. We were mainly focused on SK1 and GCS due to their known contribution to promote cancer and drug resistance. Selenite treatment previously has been shown to induce reactive oxygen species (ROS) [218]. Pretreatment with an inhibitor of SK1 (SKI-II) more effectively enhanced the decreased viability following selenite treatment compared to treatment with an inhibitor of GCS. Combined treatment with selenite and SKI-II decreased the viability of the hepatocellular carcinoma cell line Huh7 without affecting the close to normal hepatocellular cell line MIHA. These results suggest that inhibition of SK1 sensitizes Huh7 cells towards selenite treatment.

Investigation of the underlying mechanisms of cytotoxicity observed after combined treatment with selenite and SKI-II revealed decreased number of cells in the G2 phase in the

cell cycle, nuclear fragmentation and internucleosomal cleavage. From the observations we have concluded that combination of selenite and SKI-II induces alteration of the cell cycle progression and apoptosis in Huh7 cells. Measurement of ceramide subspecies has shown an increase in long chain ceramides after cotreatment with SKI-II and selenite compared to the vehicle treated controls. Increase in the ceramide subspecies following inhibition of SK1 might be explained by earlier studies that N-acetylation of sphingosine by CerS generated long to very long chain ceramides [219]. Suggesting that when SK1 was inhibited S1P production was blocked and sphingosine was recycled to ceramide. We suggest that the accumulation of long chain ceramides and increased cytotoxicity after co-treatment with SKI-II and selenite in the HCC cell line is the result of ceramide generation from sphingomyelin breakdown and recycling of sphingosine. Moreover, treatment with a combination of SKI-II with selenite significantly increased ROS formation compared to treatment with SKI-II or selenite alone.

4.2 PAPER II: FTY720 (Fingolimod) sensitizes hepatocellular carcinoma cells to sorafenib-mediated cytotoxicity

In this study we wanted to sensitize HCC cells to sorafenib treatment, which is a FDA approved small molecule inhibitor to treat unresectable HCC [220] by combining with FTY720, which is also approved by FDA to treat multiple sclerosis and has cytotoxicity towards HCC cells [221]. In order to combine sorafenib with the least cytotoxic dose of FTY720, a dose titration was performed with FTY720 alone towards hepatocellular carcinoma cell lines Huh7 and HepG2 cells separately. In all the experiments an increasing doses of sorafenib were combined with the least cytotoxic dose of FTY720.

Following treatment with increasing doses of sorafenib for up to three days the viability of Huh7 and HepG2 cells was decreased. The decreased viability was further enhanced after combining the sorafenib treatment with FTY720. The effects of combined treatment increased over time in Huh7 cells that were also more sensitive to the treatment compared to HepG2 cells.

Cell cycle distribution analysis of Huh7 cells revealed that combined treatment with sorafenib and FTY720 caused a significant accumulation of cells in the G1 phase, resulting in the concurrent decrease of cells in the S and G2 phases of the cell cycle. At higher doses of sorafenib cotreatment with FTY720 has shown significant decrease of cells in G2 phase in a time dependent manner. A substantial increase in sub G1 fraction referring to cell debris has been observed with increasing doses of sorafenib treatment compared to the vehicle control, which has slightly increased after combination with FTY720.

Cell death analysis of Huh7 cells by annexin V and propidium iodide staining by flow cytometry following treatment with sorafenib and FTY720 has shown a significant increase in total cell death compared to sorafenib treatment alone. Since it can be difficult to distinguish late apoptosis from secondary necrosis, total cell death was calculated as the sum of the populations of cells stained with Annexin V only, with Annexin V and PI or with PI.

Investigation of PARP-cleavage in Huh7 cells revealed apoptosis following treatment with sorafenib alone, which was further augmented when combined with FTY720. PARP-cleavage is a well-known marker for caspase-dependent apoptosis [222]. Additionally, combined treatment with lower doses of sorafenib and FTY720 resulted in slight release of apoptosis inducing factor (AIF) and Cyt c from the mitochondria into the cytosol, which was slightly augmented after combination treatment. These findings suggest that combined treatment of Huh7 cells with sorafenib and FTY720 induces mitochondria-mediated apoptosis mostly in a caspase-independent manner but at higher doses of sorafenib, in a caspase-dependent manner.

Finally, we have investigated if autophagy also played a role in cell death mechanisms observed after sorafenib and FTY720 combination treatment. A previous study has shown that treatment with FTY720 enhanced anticancer efficacy of milatuzumab in mantle cell lymphoma by blockage of autophagy [223]. On the other hand, sorafenib treatment was associated with induction of autophagy, which might be a way of escaping cytotoxicity [224]. We observed that treatment with increasing doses of sorafenib alone slightly increased the levels LC3 II, which were further augmented following cotreatment with sorafenib and FTY720. Additionally, treatment with increasing doses of sorafenib alone decreased the levels of p62 protein, which was reverted after cotreatment with sorafenib and FTY720. These results are in line with the aforementioned studies [223, 224] implying that sorafenib treatment alone induces autophagy in Huh7 cells whereas FTY720 treatment blocks it. The resulting effects along with a summary of our study are depicted in Fig 7.

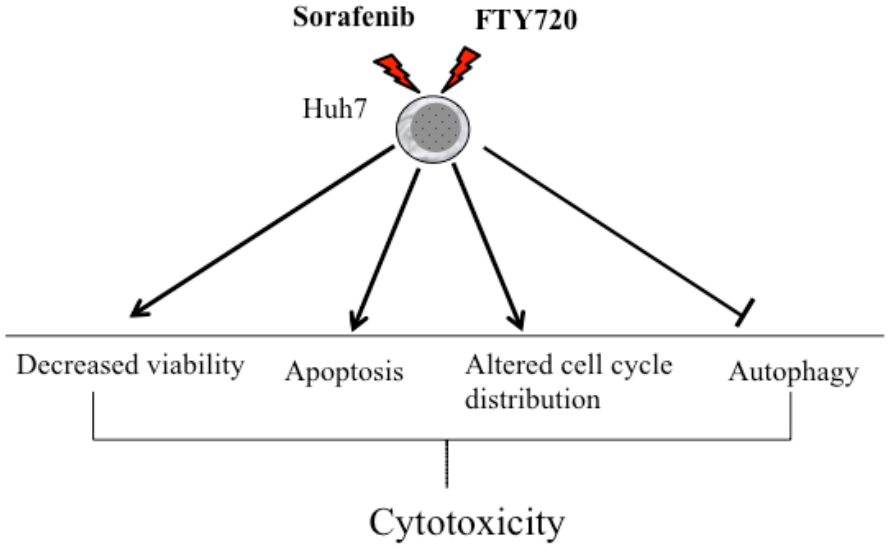

Figure 7 Summary of cotreatment effects of sorafenib and FTY720 on Huh7

4.3 PAPER III: Effects of treatment with Bacillus Calmette-Guérin and an inhibitor of sphingosine kinase 1 in urothelial bladder-cancer cells (Manuscript)

In this study, we have investigated if BCG treatment has any effects on the levels of SK1 RNA or protein as well as how cotreatment with SKI-II and BCG affects cell viability and sphingolipid metabolism in bladder cancer cells. In order to mimic *in vivo* immune response *in vitro* we have stimulated RAW264.7 macrophages with BCG and collected supernatants (SupBCG) to treat murine bladder cancer cells (MBT2) with the clear supernatants.

Previous studies have shown antitumor and cytotoxic effects of SKI-II both *in vitro* and *in vivo* [68, 97]. We have investigated if treatment with SKI-II in combination with BCG/ SupBCG will exert viability reduction in bladder cancer cells. In the human bladder cancer cell line T24, significant cytotoxicity was observed only when higher doses of SKI-II was combined with direct BCG treatment. In the murine bladder cancer cell line MBT2, a dose dependent decrease in viability was observed after treatment with increasing doses of SupBCG alone. The decrease was further enhanced after combined treatment with SKI-II compared to treatment with SupBCG or SKI-II alone.

Furthermore, we wanted to know if casepase-mediated apoptosis was involved in the cytotoxic effects after SKI-II and SupBCG treatment in MBT2 cells. Treatment with SKI-II or SupBCG alone induced substantial PARP-cleavage compared to treatment with supernatant from unstimulated macrophages (SupCtr). Nevertheless, no combined effect was observed and it should also be noted that the treatment agents did not counteract their respective effects on the levels of cleaved PARP either. This observation led to the conclusion that there are additional mechanisms of cell death than caspase-dependent apoptosis behind the observed decrease in viability following combined treatment with SupBCG and SKI-II.

Our results have shown a significant but transient increase in Sphk1 mRNA expression after SupBCG treatment. On the contrary, SK1 protein levels remained unchanged over time following treatment with SupBCG. Earlier studies have shown connection between cytotoxicity mediated by cytotoxic treatments and increased levels of different chain lengths of ceramide subspecies [216, 217]. Thus, we have investigated if SKI-II and/ or SupBCG treatment influenced the levels of ceramide subspecies in MBT2 cells. Our results have shown that treatment with SupBCG alone did not have a significant influence on the levels of analyzed ceramide subspecies. The level of dihydro C16-ceramide was increased after combined treatment with SKI-II and SupBCG, whereas the level of C16-ceramide was decreased compared to SupBCG treatment alone. Dihydroceramides are the precursors of ceramide in the *de novo* pathway, which undergo N-acylation by the enzyme Des to produce ceramide. Additionally, (dihydro) C16-ceramide are the products of both CerS5 and CerS6 [15]. Previous studies have shown accumulation of dihydroceramide following treatment with different substances and as a result of oxidative stress leading to cell cycle arrest and autophagy [225]. Accumulation of dihydroceramide level following SKI-II might also be

explained by the recent finding that SKI-II not only inhibits SK1 but also Des and led to growth arrest [101] .

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The perspective of the thesis was to study the treatment effects of sphingolipid metabolizing enzyme inhibitors: on HCC cell lines following cotreatment with selenite or sorafenib, and on bladder cancer cell lines after cotreatment with BCG. Treatment effects were studied by investigating the levels of ceramide subspecies, and by studying pathways governing cytotoxicity.

In the first study we have shown that selenite treatment of Huh7 cells increased the levels of long chain ceramide subspecies with subsequent increase in nSMase activity. This might refer to the activation of sphingomyelin breakdown pathway of ceramide generation followed by concomitant decrease in cell viability. Furthermore, cotreatment with SKI-II and selenite resulted in increased levels of ceramide subspecies, induction of reactive oxygen species formation and apoptosis, and inhibition of cell cycle progression and cell viability. These results imply that combined treatment of HCC cells with selenite and inhibition of SK1 might be an alternative option to treat HCC. Therefore, it will be required to do further investigation in animal models (e.g. humanized mouse model of liver cancer) to test the benefits and toxicities of the combination treatment. More specific and novel inhibitors of SK1with better bioavailability should be considered. Study of the CerS enzyme following combined treatment could provide valuable insights, as ceramide can be recycled from sphingosine upon inhibition of its phosphorylation or from sphingomyelin catabolism.

In the second study we have tested the combination of sorafenib and FTY720 in the treatment of HCC cells. Both of the drugs used in this study are FDA approved: sorafenib for treating advanced stage, non-resectable HCC, and FTY720 for treating relapsing multiple sclerosis. Additionally, FTY720 is a modulator of sphingolipid metabolism. In this study we showed that a marginally cytotoxic dose of FTY720 sensitized HCC cell lines towards sorafenib treatment. Combined treatment with sorafenib and FTY720 of Huh7 cells resulted in a decreased cell viability, cell cycle arrest, apoptosis and blockage of autophagy leading to cell death. This study provides solid background for continuing research on signaling pathways such as changes in the PI3K and MAPK pathways since both of the substances influence these pathways individually. More research should be done to analyze the treatment effects on different sphingolipid metabolites, and animal models (e.g. humanized mouse model of liver cancer) should be included in the future.

In the last study we have investigated the combination of a sphingosine kinase inhibitor SKI-II and supernatant from BCG stimulated RAW264.7 cells on bladder cancer cell line. Immunotherapy by BCG is approved by the FDA to treat, and prevent recurrence of high-risk superficial bladder cancer. In our study we could not detect any changes in the protein level of SK1 following SupBCG treatment. Our data provide no influence of SupBCG treatment alone on the levels of sphingolipid metabolites however cotreatment with SKI-II and SupBCG significantly increased the level of dihydro C16-ceramide. Treatment with SupBCG

or SKI-II individually induced PARP cleavage. Cotreatment with SupBCG and SKI-II has also shown increased cytotoxicity towards bladder cancer cell line. This study implies that targeting SKs adds to the cytotoxicity of BCG in bladder cancer cells. Further studies investigating autophagy and involvement of PI3K/ AKT pathway after the combined treatment are required.

6 ACKNOWLEDGEMENTS

The journey of my PhD education was full of challenges, achievements, and mixed emotions. These years of experiences gave me insights into the research world, helped me to think in a very different way and enriched my life. At the end of this path I would like to express my gratitude to all who has contributed through out this time.

I would like to thank **Jenny Flygare,** my main supervisor for seeing the potential in me to do research, for believing in me, and for all of your encouragement and support. You allowed me to grow and develop not only my scientific knowledge but also personal development. You were more than my academic supervisor sometimes you were like my friend and other time like my guardian.

My co-supervisor, **Petra de Verdier** thanks for sharing your knowledge, all the puns and encouraging words with me. I wish I could have more of your company. You never missed to give feedbacks on my works, which definitely helped me to grow and flourish.

Per Stål, my co-supervisor thanks for sharing your clinical expertise and appreciating my work. I would like to thank **Magnus Johansson**, my co-supervisor for a very important role that you played in my doctoral education even though you were not involved in my research.

Thanks to our former and present extended group members Vicky Chatzakos, Charlotta Ryk for helping me out with statistics, and Tomas Thiel for the BCG-regarding discussions.

I am very thankful to all my colleagues in the Division of Clinical Chemistry, we are small but a happy family and I am a proud member. You all are wonderful people and enriched my life in many different ways.

A big thank to Paolo Parini, former head of the division, who had a great effort to make the place suitable for every one. I highly appreciate our very efficient former and current administrators Jenny Bernström and Naradja Wissmar.

Thanks to other present and former colleagues at the division: Ingemar Björkhem, Gösta Eggersten, Ulf Diczfalusy, Stefan Alexson, Knut Steffensen, Anders Helander and Anna-Klara Rundlöf, Camilla Pramfalk, Johan Saldeen, Magnus Hansson, Robin, Anita Löfgren-Sandblom and Ulla K Andersson. Thanks and gratitude to the super women of the division Lilian & Maria, you were like the guardian angels in the lab.

Thanks & love to all of my colleagues who have been and still sitting in the PhD room: Madeleine, Zeina, Osman, Mirko, Ahmed, Dalma, Mihaela, Treska, Kristina, Xiaoli for all the discussions, supports, fika and laughs that we have shared. Thanks for tolerating me for all these years :D I would also like to show my gratitude towards all the super duper post-docs of our division Lise-Lotte, Vera, Matteo, Maura, Tomas, Ali Reza. Thanks for your care, all the lunchtime talks, Swedish lessons, and all the good times that we had together. It would have been difficult to finish the degree without having you around.

Thanks to my colleagues and friends from the division of Pathology: Göran Andersson for letting me be there at the beginning of my PhD, Agata, Christina, Sougat, Anja, Filip, Martin, Shuvojit, Raghu, Magali, Rauol, Ashish, Joman for all the good times and chats. Special thanks and love to Tunde and Ghazal for your friendship, love and support.

My Bangladeshi friends & family in Sweden, thanks for all your care, support and all the fun we had and will continue to have together in the future. Thanks Moon apu, Nibedita apu, Shanto vai, Sayka, Sagar vai, Binita & Minhaz.

Sudip Saha, my beloved life partner, my motivation and constant support thank you for always being there for me even though we are thousand miles apart most of the time. I can move mountains when you are by my side. Thanks for listening to allll the borrrring complains and FRUSTRATIONs about everything particularly during the final year of my PhD. You'll not hear the same stories again ...I promise :D

Finally, thanks and love to my parents, I became who I am today because of you. I cannot express my gratitude enough by writing it here. I am so proud to be your daughter. Thanks to all my siblings for almost raising me and for all the supports and love that you gave me.

I would also like to acknowledge Karolinska Institutet Research foundation, the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet and Swedish Society of Medicine and Cancerföreningen for supporting this study.

7 REFERENCES

[1] H.E. Carter, D.S. Galanos, Y. Fujino, Chemistry of the sphingolipides, Canadian journal of biochemistry and physiology, 34 (1956) 320-333.

[2] M. Maceyka, S. Spiegel, Sphingolipid metabolites in inflammatory disease, Nature, 510 (2014) 58-67.

[3] Y. Zhou, M.S. Salker, B. Walker, P. Munzer, O. Borst, M. Gawaz, E. Gulbins, Y. Singh, F. Lang, Acid Sphingomyelinase (ASM) is a Negative Regulator of Regulatory T Cell (Treg) Development, Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology, 39 (2016) 985-995.

[4] I.V. Tiper, J.E. East, P.B. Subrahmanyam, T.J. Webb, Sphingosine 1-phosphate signaling impacts lymphocyte migration, inflammation and infection, Pathogens and disease, 74 (2016).

[5] L.K. Ryland, T.E. Fox, X. Liu, T.P. Loughran, M. Kester, Dysregulation of sphingolipid metabolism in cancer, Cancer biology & therapy, 11 (2011) 138-149.

[6] C.R. Gault, L.M. Obeid, Y.A. Hannun, An overview of sphingolipid metabolism: from synthesis to breakdown, Adv Exp Med Biol, 688 (2010) 1-23.

[7] J. Bielawski, Z.M. Szulc, Y.A. Hannun, A. Bielawska, Simultaneous quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry, Methods, 39 (2006) 82-91.

[8] S. Sonnino, A. Prinetti, Gangliosides as regulators of cell membrane organization and functions, Adv Exp Med Biol, 688 (2010) 165-184.

[9] National Center for Biotechnology Information, PubChem Compound Database, https://pubchem.ncbi.nlm.nih.gov/compound/5283565 2017-2-10.

[10] Y.A. Hannun, L.M. Obeid, Many ceramides, J Biol Chem, 286 (2011) 27855-27862.

[11] S. Ponnusamy, M. Meyers-Needham, C.E. Senkal, S.A. Saddoughi, D. Sentelle, S.P. Selvam, A. Salas, B. Ogretmen, Sphingolipids and cancer: ceramide and sphingosine-1-phosphate in the regulation of cell death and drug resistance, Future oncology, 6 (2010) 1603-1624.

[12] D.K. Perry, Serine palmitoyltransferase: role in apoptotic de novo ceramide synthesis and other stress responses, Biochim Biophys Acta, 1585 (2002) 146-152.

[13] M.S. Wegner, S. Schiffmann, M.J. Parnham, G. Geisslinger, S. Grosch, The enigma of ceramide synthase regulation in mammalian cells, Progress in lipid research, 63 (2016) 93-119.

[14] D. Hartmann, J. Lucks, S. Fuchs, S. Schiffmann, Y. Schreiber, N. Ferreiros, J. Merkens, R. Marschalek, G. Geisslinger, S. Grosch, Long chain ceramides and very long chain ceramides have opposite effects on human breast and colon cancer cell growth, The international journal of biochemistry & cell biology, 44 (2012) 620-628.

[15] S. Grösch, S. Schiffmann, G. Geisslinger, Chain length-specific properties of ceramides, Progress in lipid research, 51 (2012) 50-62.

[16] M. Casasampere, Y.F. Ordonez, A. Pou, J. Casas, Inhibitors of dihydroceramide desaturase 1: Therapeutic agents and pharmacological tools to decipher the role of dihydroceramides in cell biology, Chemistry and physics of lipids, 197 (2016) 33-44.

[17] M.W. Holliday, Jr., S.B. Cox, M.H. Kang, B.J. Maurer, C22:0- and C24:0-dihydroceramides confer mixed cytotoxicity in T-cell acute lymphoblastic leukemia cell lines, PLoS One, 8 (2013) e74768.

[18] G. Illuzzi, C. Bernacchioni, M. Aureli, S. Prioni, G. Frera, C. Donati, M. Valsecchi, V. Chigorno, P. Bruni, S. Sonnino, A. Prinetti, Sphingosine kinase mediates resistance to the synthetic retinoid N-(4-hydroxyphenyl)retinamide in human ovarian cancer cells, J Biol Chem, 285 (2010) 18594-18602.

[19] J. Newton, S. Lima, M. Maceyka, S. Spiegel, Revisiting the sphingolipid rheostat: Evolving concepts in cancer therapy, Experimental Cell Research, 333 (2015) 195-200.

[20] G.M. Jenkins, L.A. Cowart, P. Signorelli, B.J. Pettus, C.E. Chalfant, Y.A. Hannun, Acute activation of de novo sphingolipid biosynthesis upon heat shock causes an accumulation of ceramide and subsequent dephosphorylation of SR proteins, J Biol Chem, 277 (2002) 42572-42578.

[21] R. Bose, M. Verheij, A. Haimovitz-Friedman, K. Scotto, Z. Fuks, R. Kolesnick, Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals, Cell, 82 (1995) 405-414.

[22] K. Gustafsson, B. Sander, J. Bielawski, Y.A. Hannun, J. Flygare, Potentiation of cannabinoid-induced cytotoxicity in mantle cell lymphoma through modulation of ceramide metabolism, Molecular cancer research : MCR, 7 (2009) 1086-1098.

[23] N. Marchesini, Y.A. Hannun, Acid and neutral sphingomyelinases: roles and mechanisms of regulation, Biochemistry and cell biology = Biochimie et biologie cellulaire, 82 (2004) 27-44.

[24] I. Sultan, C.E. Senkal, S. Ponnusamy, J. Bielawski, Z. Szulc, A. Bielawska, Y.A. Hannun, B. Ogretmen, Regulation of the sphingosine-recycling pathway for ceramide generation by oxidative stress, and its role in controlling c-Myc/Max function, The Biochemical journal, 393 (2006) 513-521.

[25] K. Kitatani, J. Idkowiak-Baldys, Y.A. Hannun, The sphingolipid salvage pathway in ceramide metabolism and signaling, Cellular signalling, 20 (2008) 1010-1018.

[26] M.J. Hernandez-Corbacho, D. Canals, M.M. Adada, M. Liu, C.E. Senkal, J.K. Yi, C. Mao, C. Luberto, Y.A. Hannun, L.M. Obeid, Tumor Necrosis Factor-alpha (TNFalpha)-induced Ceramide Generation via Ceramide Synthases Regulates Loss of Focal Adhesion Kinase (FAK) and Programmed Cell Death, J Biol Chem, 290 (2015) 25356-25373.

[27] B.J. Pettus, C.E. Chalfant, Y.A. Hannun, Ceramide in apoptosis: an overview and current perspectives, Biochim Biophys Acta, 1585 (2002) 114-125.

[28] S.A. Saddoughi, P. Song, B. Ogretmen, Roles of bioactive sphingolipids in cancer biology and therapeutics, Sub-cellular biochemistry, 49 (2008) 413-440.

[29] T. Kohama, A. Olivera, L. Edsall, M.M. Nagiec, R. Dickson, S. Spiegel, Molecular cloning and functional characterization of murine sphingosine kinase, J Biol Chem, 273 (1998) 23722-23728.

[30] H. Liu, M. Sugiura, V.E. Nava, L.C. Edsall, K. Kono, S. Poulton, S. Milstien, T. Kohama, S. Spiegel, Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform, J Biol Chem, 275 (2000) 19513-19520.

[31] K. Mizugishi, T. Yamashita, A. Olivera, G.F. Miller, S. Spiegel, R.L. Proia, Essential role for sphingosine kinases in neural and vascular development, Mol Cell Biol, 25 (2005) 11113-11121.

[32] M. Maceyka, S. Milstien, S. Spiegel, Sphingosine-1-phosphate: the Swiss army knife of sphingolipid signaling, J Lipid Res, 50 Suppl (2009) S272-276.

[33] M. Maceyka, H. Sankala, N.C. Hait, H. Le Stunff, H. Liu, R. Toman, C. Collier, M. Zhang, L.S. Satin, A.H. Merrill, Jr., S. Milstien, S. Spiegel, SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism, J Biol Chem, 280 (2005) 37118-37129.

[34] Y. Morita, G.I. Perez, F. Paris, S.R. Miranda, D. Ehleiter, A. Haimovitz-Friedman, Z. Fuks, Z. Xie, J.C. Reed, E.H. Schuchman, R.N. Kolesnick, J.L. Tilly, Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy, Nat Med, 6 (2000) 1109-1114.

[35] N.C. Hait, J. Allegood, M. Maceyka, G.M. Strub, K.B. Harikumar, S.K. Singh, C. Luo, R. Marmorstein, T. Kordula, S. Milstien, S. Spiegel, Regulation of Histone Acetylation in the Nucleus by Sphingosine-1-Phosphate, Science, 325 (2009) 1254-1257.

[36] S.P. Selvam, B. Ogretmen, Sphingosine kinase/sphingosine 1-phosphate signaling in cancer therapeutics and drug resistance, Handbook of experimental pharmacology, (2013) 3-27.

[37] N. Urtz, A. Olivera, E. Bofill-Cardona, R. Csonga, A. Billich, D. Mechtcheriakova, F. Bornancin, M. Woisetschlager, J. Rivera, T. Baumruker, Early activation of sphingosine kinase in mast cells and recruitment to FcepsilonRI are mediated by its interaction with Lyn kinase, Mol Cell Biol, 24 (2004) 8765-8777.

[38] A. Olivera, N. Urtz, K. Mizugishi, Y. Yamashita, A.M. Gilfillan, Y. Furumoto, H. Gu, R.L. Proia, T. Baumruker, J. Rivera, IgE-dependent activation of sphingosine kinases 1 and 2 and secretion of sphingosine 1-phosphate requires Fyn kinase and contributes to mast cell responses, J Biol Chem, 281 (2006) 2515-2525.

[39] T. Fujita, T. Okada, S. Hayashi, S. Jahangeer, N. Miwa, S. Nakamura, Delta-catenin/NPRAP (neural plakophilin-related armadillo repeat protein) interacts with and activates sphingosine kinase 1, The Biochemical journal, 382 (2004) 717-723.

[40] T.M. Leclercq, P.A. Moretti, M.A. Vadas, S.M. Pitson, Eukaryotic elongation factor 1A interacts with sphingosine kinase and directly enhances its catalytic activity, J Biol Chem, 283 (2008) 9606-9614.

[41] R.K. Barr, H.E. Lynn, P.A. Moretti, Y. Khew-Goodall, S.M. Pitson, Deactivation of sphingosine kinase 1 by protein phosphatase 2A, J Biol Chem, 283 (2008) 34994-35002.

[42] H. Hayashi, H. Nakagami, Y. Takami, H. Koriyama, M. Mori, K. Tamai, J. Sun, K. Nagao, R. Morishita, Y. Kaneda, FHL-2 Suppresses VEGF-Induced Phosphatidylinositol 3-Kinase/Akt Activation via Interaction With Sphingosine Kinase-1, Arteriosclerosis, thrombosis, and vascular biology, 29 (2009) 909.

[43] Y. Fukuda, Y. Aoyama, A. Wada, Y. Igarashi, Identification of PECAM-1 association with sphingosine kinase 1 and its regulation by agonist-induced phosphorylation, Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1636 (2004) 12-21.

[44] M. Maceyka, V.E. Nava, S. Milstien, S. Spiegel, Aminoacylase 1 is a sphingosine kinase 1-interacting protein, FEBS Letters, 568 (2004) 30-34.

[45] H. Chan, S.M. Pitson, Post-translational regulation of sphingosine kinases, Biochim Biophys Acta, 1831 (2013) 147-156.

[46] M.R. Pitman, D.H. Pham, S.M. Pitson, Isoform-selective assays for sphingosine kinase activity, Methods Mol Biol, 874 (2012) 21-31.

[47] W. Zhu, B.L. Gliddon, K.E. Jarman, P.A. Moretti, T. Tin, L.V. Parise, J.M. Woodcock, J.A. Powell, A. Ruszkiewicz, M.R. Pitman, S.M. Pitson, CIB1 contributes to oncogenic signalling by Ras via modulating the subcellular localisation of sphingosine kinase 1, Oncogene, (2016).

[48] S.M. Pitson, P.A. Moretti, J.R. Zebol, H.E. Lynn, P. Xia, M.A. Vadas, B.W. Wattenberg, Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation, Embo j, 22 (2003) 5491-5500.

[49] D. Lidington, B.F. Peter, A. Meissner, J.T. Kroetsch, S.M. Pitson, U. Pohl, S.S. Bolz, The phosphorylation motif at serine 225 governs the localization and function of sphingosine kinase 1 in resistance arteries, Arteriosclerosis, thrombosis, and vascular biology, 29 (2009) 1916-1922.

[50] S.M. Pitson, Regulation of sphingosine kinase and sphingolipid signaling, Trends Biochem Sci, 36 (2011) 97-107.

[51] S.M. Pitson, P. Xia, T.M. Leclercq, P.A. Moretti, J.R. Zebol, H.E. Lynn, B.W. Wattenberg, M.A. Vadas, Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling, J Exp Med, 201 (2005) 49-54.

[52] S.M. Pitson, J. D'Andrea R, L. Vandeleur, P.A. Moretti, P. Xia, J.R. Gamble, M.A. Vadas, B.W. Wattenberg, Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes, The Biochemical journal, 350 Pt 2 (2000) 429-441.

[53] H. Yu, Y. Shao, L. Gao, L. Zhang, K. Guo, C. Wu, X. Hu, H. Duan, Acetylation of sphingosine kinase 1 regulates cell growth and cell-cycle progression, Biochem Biophys Res Commun, 417 (2012) 1242-1247.

[54] L. Zhang, X. Liu, Z. Zuo, C. Hao, Y. Ma, Sphingosine kinase 2 promotes colorectal cancer cell proliferation and invasion by enhancing MYC expression, Tumor Biology, (2016) 1-6.

[55] N.C. Hait, A. Bellamy, S. Milstien, T. Kordula, S. Spiegel, Sphingosine kinase type 2 activation by ERK-mediated phosphorylation, J Biol Chem, 282 (2007) 12058-12065.

[56] T.A. Taha, T.D. Mullen, L.M. Obeid, A house divided: Ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death, Biochimica et Biophysica Acta (BBA) - Biomembranes, 1758 (2006) 2027-2036.

[57] O. Cuvillier, G. Pirianov, B. Kleuser, P.G. Vanek, O.A. Coso, J.S. Gutkind, S. Spiegel, Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate, Nature, 381 (1996) 800-803.

[58] M. Dany, B. Ogretmen, Ceramide induced mitophagy and tumor suppression, Biochim Biophys Acta, 1853 (2015) 2834-2845.

[59] M. Taniguchi, K. Kitatani, T. Kondo, M. Hashimoto-Nishimura, S. Asano, A. Hayashi, S. Mitsutake, Y. Igarashi, H. Umehara, H. Takeya, J. Kigawa, T. Okazaki, Regulation of autophagy and its associated cell death by "sphingolipid rheostat": reciprocal role of ceramide

and sphingosine 1-phosphate in the mammalian target of rapamycin pathway, J Biol Chem, 287 (2012) 39898-39910.

[60] G.T. Kunkel, M. Maceyka, S. Milstien, S. Spiegel, Targeting the sphingosine-1-phosphate axis in cancer, inflammation and beyond, Nature reviews. Drug discovery, 12 (2013) 688-702.

[61] D. Shida, J. Kitayama, H. Yamaguchi, H. Yamashita, K. Mori, T. Watanabe, Y. Yatomi, H. Nagawa, Sphingosine 1-phosphate transactivates c-Met as well as epidermal growth factor receptor (EGFR) in human gastric cancer cells, FEBS Lett, 577 (2004) 333-338.

[62] T. Tanimoto, Z.G. Jin, B.C. Berk, Transactivation of vascular endothelial growth factor (VEGF) receptor Flk-1/KDR is involved in sphingosine 1-phosphate-stimulated phosphorylation of Akt and endothelial nitric-oxide synthase (eNOS), J Biol Chem, 277 (2002) 42997-43001.

[63] G. Marfe, G. Mirone, A. Shukla, C. Di Stefano, Sphingosine kinases signalling in carcinogenesis, Mini reviews in medicinal chemistry, 15 (2015) 300-314.

[64] I.O. Zakharova, T.V. Sokolova, Y.A. Vlasova, V.V. Furaev, M.P. Rychkova, N.F. Avrova, GM1 ganglioside activates ERK1/2 and Akt downstream of Trk tyrosine kinase and protects PC12 cells against hydrogen peroxide toxicity, Neurochemical research, 39 (2014) 2262-2275.

[65] Y. Osawa, Y. Banno, M. Nagaki, D.A. Brenner, T. Naiki, Y. Nozawa, S. Nakashima, H. Moriwaki, TNF-alpha-induced sphingosine 1-phosphate inhibits apoptosis through a phosphatidylinositol 3-kinase/Akt pathway in human hepatocytes, J Immunol, 167 (2001) 173-180.

[66] P. Xia, J.R. Gamble, L. Wang, S.M. Pitson, P.A. Moretti, B.W. Wattenberg, R.J. D'Andrea, M.A. Vadas, An oncogenic role of sphingosine kinase, Curr Biol, 10 (2000) 1527-1530.

[67] J.R. Van Brocklyn, C.A. Jackson, D.K. Pearl, M.S. Kotur, P.J. Snyder, T.W. Prior, Sphingosine kinase-1 expression correlates with poor survival of patients with glioblastoma multiforme: roles of sphingosine kinase isoforms in growth of glioblastoma cell lines, J Neuropathol Exp Neurol, 64 (2005) 695-705.

[68] K.J. French, R.S. Schrecengost, B.D. Lee, Y. Zhuang, S.N. Smith, J.L. Eberly, J.K. Yun, C.D. Smith, Discovery and evaluation of inhibitors of human sphingosine kinase, Cancer Res, 63 (2003) 5962-5969.

[69] M. Bao, Z. Chen, Y. Xu, Y. Zhao, R. Zha, S. Huang, L. Liu, T. Chen, J. Li, H. Tu, X. He, Sphingosine kinase 1 promotes tumour cell migration and invasion via the S1P/EDG1 axis in hepatocellular carcinoma, Liver international : official journal of the International Association for the Study of the Liver, 32 (2012) 331-338.

[70] B. Uranbileg, H. Ikeda, M. Kurano, K. Enooku, M. Sato, D. Saigusa, J. Aoki, T. Ishizawa, K. Hasegawa, N. Kokudo, Y. Yatomi, Increased mRNA Levels of Sphingosine Kinases and S1P Lyase and Reduced Levels of S1P Were Observed in Hepatocellular Carcinoma in Association with Poorer Differentiation and Earlier Recurrence, PLoS One, 11 (2016) e0149462.

[71] M.G. Bayerl, R.D. Bruggeman, E.J. Conroy, J.A. Hengst, T.S. King, M. Jimenez, D.F. Claxton, J.K. Yun, Sphingosine kinase 1 protein and mRNA are overexpressed in non-Hodgkin lymphomas and are attractive targets for novel pharmacological interventions, Leuk Lymphoma, 49 (2008) 948-954.

[72] T.A. Taha, K. Kitatani, M. El-Alwani, J. Bielawski, Y.A. Hannun, L.M. Obeid, Loss of sphingosine kinase-1 activates the intrinsic pathway of programmed cell death: modulation of sphingolipid levels and the induction of apoptosis, Faseb j, 20 (2006) 482-484.

[73] J.W. Ho, K. Man, C.K. Sun, T.K. Lee, R.T. Poon, S.T. Fan, Effects of a novel immunomodulating agent, FTY720, on tumor growth and angiogenesis in hepatocellular carcinoma, Molecular cancer therapeutics, 4 (2005) 1430-1438.

[74] C.R. Gault, L.M. Obeid, Still benched on its way to the bedside: sphingosine kinase 1 as an emerging target in cancer chemotherapy, Critical reviews in biochemistry and molecular biology, 46 (2011) 342-351.

[75] C. Watson, J.S. Long, C. Orange, C.L. Tannahill, E. Mallon, L.M. McGlynn, S. Pyne, N.J. Pyne, J. Edwards, High expression of sphingosine 1-phosphate receptors, S1P1 and S1P3, sphingosine kinase 1, and extracellular signal-regulated kinase-1/2 is associated with development of tamoxifen resistance in estrogen receptor-positive breast cancer patients, Am J Pathol, 177 (2010) 2205-2215.

[76] S. Pyne, J. Edwards, J. Ohotski, N. Pyne, Sphingosine 1-phosphate receptors and sphingosine kinase 1: novel biomarkers for clinical prognosis in breast, prostate, and hematological cancers, Frontiers in Oncology, 2 (2012).

[77] J.K. Venkata, N. An, R. Stuart, L.J. Costa, H. Cai, W. Coker, J.H. Song, K. Gibbs, T. Matson, E. Garrett-Mayer, Z. Wan, B. Ogretmen, C. Smith, Y. Kang, Inhibition of sphingosine kinase 2 downregulates the expression of c-Myc and Mcl-1 and induces apoptosis in multiple myeloma, Blood, 124 (2014) 1915-1925.

[78] M. Nagahashi, J. Tsuchida, K. Moro, M. Hasegawa, K. Tatsuda, I.A. Woelfel, K. Takabe, T. Wakai, High levels of sphingolipids in human breast cancer, J Surg Res, 204 (2016) 435-444.

[79] E. Degagne, A. Pandurangan, P. Bandhuvula, A. Kumar, A. Eltanawy, M. Zhang, Y. Yoshinaga, M. Nefedov, P.J. de Jong, L.G. Fong, S.G. Young, R. Bittman, Y. Ahmedi, J.D. Saba, Sphingosine-1-phosphate lyase downregulation promotes colon carcinogenesis through STAT3-activated microRNAs, J Clin Invest, 124 (2014) 5368-5384.

[80] Y.Y. Liu, G.A. Patwardhan, P. Xie, X. Gu, A.E. Giuliano, M.C. Cabot, Glucosylceramide synthase, a factor in modulating drug resistance, is overexpressed in metastatic breast carcinoma, Int J Oncol, 39 (2011) 425-431.

[81] Y.Y. Liu, T.Y. Han, A.E. Giuliano, M.C. Cabot, Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers adriamycin resistance in human breast cancer cells, J Biol Chem, 274 (1999) 1140-1146.

[82] Y. Lavie, H. Cao, S.L. Bursten, A.E. Giuliano, M.C. Cabot, Accumulation of glucosylceramides in multidrug-resistant cancer cells, J Biol Chem, 271 (1996) 19530-19536.

[83] M. Stefanovic, A. Tutusaus, G.A. Martinez-Nieto, C. Barcena, E. de Gregorio, C. Moutinho, E. Barbero-Camps, A. Villanueva, A. Colell, M. Mari, C. Garcia-Ruiz, J.C. Fernandez-Checa, A. Morales, Targeting glucosylceramide synthase upregulation reverts sorafenib resistance in experimental hepatocellular carcinoma, Oncotarget, 7 (2016) 8253-8267.

[84] J.L. Roh, J.Y. Park, E.H. Kim, H.J. Jang, Targeting acid ceramidase sensitises head and neck cancer to cisplatin, Eur J Cancer, 52 (2016) 163-172.

[85] I.G. Rivera, M. Ordonez, N. Presa, P. Gangoiti, A. Gomez-Larrauri, M. Trueba, T. Fox, M. Kester, A. Gomez-Munoz, Ceramide 1-phosphate regulates cell migration and invasion of human pancreatic cancer cells, Biochem Pharmacol, 102 (2016) 107-119.

[86] K. Kitatani, K. Sheldon, V. Anelli, R.W. Jenkins, Y. Sun, G.A. Grabowski, L.M. Obeid, Y.A. Hannun, Acid beta-glucosidase 1 counteracts p38delta-dependent induction of interleukin-6: possible role for ceramide as an anti-inflammatory lipid, J Biol Chem, 284 (2009) 12979-12988.

[87] A.J. Snider, K.A. Orr Gandy, L.M. Obeid, Sphingosine kinase: Role in regulation of bioactive sphingolipid mediators in inflammation, Biochimie, 92 (2010) 707-715.

[88] L.A. Hoeferlin, D.S. Wijesinghe, C.E. Chalfant, The role of ceramide-1-phosphate in biological functions, Handbook of experimental pharmacology, (2013) 153-166.

[89] R.C. Ekyalongo, H. Nakayama, K. Kina, N. Kaga, K. Iwabuchi, Organization and functions of glycolipid-enriched microdomains in phagocytes, Biochim Biophys Acta, 1851 (2015) 90-97.

[90] A. Gomez-Muñoz, N. Presa, A. Gomez-Larrauri, I.-G. Rivera, M. Trueba, M. Ordoñez, Control of inflammatory responses by ceramide, sphingosine 1-phosphate and ceramide 1-phosphate, Progress in lipid research, 61 (2016) 51-62.

[91] C.-I. Lin, C.-N. Chen, P.-W. Lin, H. Lee, Sphingosine 1-phosphate regulates inflammation-related genes in human endothelial cells through S1P(1) and S1P(3), Biochemical and Biophysical Research Communications, 355 (2007) 895-901.

[92] K. Hamada, H. Nakamura, T. Oda, T. Hirano, N. Shimizu, H. Utiyama, Involvement of Mac-1-mediated adherence and sphingosine 1-phosphate in survival of phorbol ester-treated U937 cells, Biochem Biophys Res Commun, 244 (1998) 745-750.

[93] L. Wang, R. Cummings, P. Usatyuk, A. Morris, K. Irani, V. Natarajan, Involvement of phospholipases D1 and D2 in sphingosine 1-phosphate-induced ERK (extracellular-signal-regulated kinase) activation and interleukin-8 secretion in human bronchial epithelial cells, The Biochemical journal, 367 (2002) 751-760.

[94] P. Keul, S. Lucke, K. von Wnuck Lipinski, C. Bode, M. Graler, G. Heusch, B. Levkau, Sphingosine-1-phosphate receptor 3 promotes recruitment of monocyte/macrophages in inflammation and atherosclerosis, Circulation research, 108 (2011) 314-323.

[95] T. Geng, A. Sutter, M.D. Harland, B.A. Law, J.S. Ross, D. Lewin, A. Palanisamy, S.B. Russo, K.D. Chavin, L.A. Cowart, SphK1 mediates hepatic inflammation in a mouse model of NASH induced by high saturated fat feeding and initiates proinflammatory signaling in hepatocytes, J Lipid Res, 56 (2015) 2359-2371.

[96] A.J. Snider, T. Kawamori, S.G. Bradshaw, K.A. Orr, G.S. Gilkeson, Y.A. Hannun, L.M. Obeid, A role for sphingosine kinase 1 in dextran sulfate sodium-induced colitis, FASEB J, 23 (2009) 143-152.

[97] K.J. French, J.J. Upson, S.N. Keller, Y. Zhuang, J.K. Yun, C.D. Smith, Antitumor activity of sphingosine kinase inhibitors, J Pharmacol Exp Ther, 318 (2006) 596-603.

[98] L. Yang, W. Weng, Z.X. Sun, X.J. Fu, J. Ma, W.F. Zhuang, SphK1 inhibitor II (SKI-II) inhibits acute myelogenous leukemia cell growth in vitro and in vivo, Biochem Biophys Res Commun, 460 (2015) 903-908.

[99] L. Aurelio, C.V. Scullino, M.R. Pitman, A. Sexton, V. Oliver, L. Davies, R.J. Rebello, L. Furic, D.J. Creek, S.M. Pitson, B.L. Flynn, From Sphingosine Kinase to Dihydroceramide

Desaturase: A Structure-Activity Relationship (SAR) Study of the Enzyme Inhibitory and Anticancer Activity of 4-((4-(4-Chlorophenyl)thiazol-2-yl)amino)phenol (SKI-II), J Med Chem, 59 (2016) 965-984.

[100] F. Cingolani, M. Casasampere, P. Sanllehi, J. Casas, J. Bujons, G. Fabrias, Inhibition of dihydroceramide desaturase activity by the sphingosine kinase inhibitor SKI II, J Lipid Res, 55 (2014) 1711-1720.

[101] M. McNaughton, M. Pitman, S.M. Pitson, N.J. Pyne, S. Pyne, Proteasomal degradation of sphingosine kinase 1 and inhibition of dihydroceramide desaturase by the sphingosine kinase inhibitors, SKi or ABC294640, induces growth arrest in androgen-independent LNCaP-AI prostate cancer cells, Oncotarget, 7 (2016) 16663-16675.

[102] J. Noack, J. Choi, K. Richter, A. Kopp-Schneider, A. Regnier-Vigouroux, A sphingosine kinase inhibitor combined with temozolomide induces glioblastoma cell death through accumulation of dihydrosphingosine and dihydroceramide, endoplasmic reticulum stress and autophagy, Cell death & disease, 5 (2014) e1425.

[103] K.J. French, Y. Zhuang, L.W. Maines, P. Gao, W. Wang, V. Beljanski, J.J. Upson, C.L. Green, S.N. Keller, C.D. Smith, Pharmacology and antitumor activity of ABC294640, a selective inhibitor of sphingosine kinase-2, J Pharmacol Exp Ther, 333 (2010) 129-139.

[104] V. Beljanski, C.S. Lewis, C.D. Smith, Antitumor activity of sphingosine kinase 2 inhibitor ABC294640 and sorafenib in hepatocellular carcinoma xenografts, Cancer biology & therapy, 11 (2011) 524-534.

[105] C. White, H. Alshaker, C. Cooper, M. Winkler, D. Pchejetski, The emerging role of FTY720 (Fingolimod) in cancer treatment, Oncotarget, 7 (2016) 23106-23127.

[106] M.R. Pitman, J.M. Woodcock, A.F. Lopez, S.M. Pitson, Molecular targets of FTY720 (fingolimod), Current molecular medicine, 12 (2012) 1207-1219.

[107] F. Tonelli, K.G. Lim, C. Loveridge, J. Long, S.M. Pitson, G. Tigyi, R. Bittman, S. Pyne, N.J. Pyne, FTY720 and (S)-FTY720 vinylphosphonate inhibit sphingosine kinase 1 and promote its proteasomal degradation in human pulmonary artery smooth muscle, breast cancer and androgen-independent prostate cancer cells, Cellular signalling, 22 (2010) 1536-1542.

[108] S. Pyne, R. Bittman, N.J. Pyne, Sphingosine kinase inhibitors and cancer: seeking the golden sword of Hercules, Cancer Res, 71 (2011) 6576-6582.

[109] S. Lahiri, H. Park, E.L. Laviad, X. Lu, R. Bittman, A.H. Futerman, Ceramide synthesis is modulated by the sphingosine analog FTY720 via a mixture of uncompetitive and noncompetitive inhibition in an Acyl-CoA chain length-dependent manner, J Biol Chem, 284 (2009) 16090-16098.

[110] E.V. Berdyshev, I. Gorshkova, A. Skobeleva, R. Bittman, X. Lu, S.M. Dudek, T. Mirzapoiazova, J.G. Garcia, V. Natarajan, FTY720 inhibits ceramide synthases and up-regulates dihydrosphingosine 1-phosphate formation in human lung endothelial cells, J Biol Chem, 284 (2009) 5467-5477.

[111] H. Chen, J.T. Tran, A. Eckerd, T.P. Huynh, M.H. Elliott, R.S. Brush, N.A. Mandal, Inhibition of de novo ceramide biosynthesis by FTY720 protects rat retina from light-induced degeneration, J Lipid Res, 54 (2013) 1616-1629.

[112] Y. Xing, Z.H. Wang, D.H. Ma, Y. Han, FTY720 enhances chemosensitivity of colon cancer cells to doxorubicin and etoposide via the modulation of P-glycoprotein and multidrug resistance protein 1, J Dig Dis, 15 (2014) 246-259.

[113] G. Marvaso, A. Barone, N. Amodio, L. Raimondi, V. Agosti, E. Altomare, V. Scotti, A. Lombardi, R. Bianco, C. Bianco, M. Caraglia, P. Tassone, P. Tagliaferri, Sphingosine analog fingolimod (FTY720) increases radiation sensitivity of human breast cancer cells in vitro, Cancer biology & therapy, 15 (2014) 797-805.

[114] S.K. Pal, H.A. Drabkin, J.A. Reeves, J.D. Hainsworth, S.E. Hazel, D.A. Paggiarino, J. Wojciak, G. Woodnutt, R.S. Bhatt, A phase 2 study of the sphingosine-1-phosphate antibody sonepcizumab in patients with metastatic renal cell carcinoma, Cancer, 123 (2017) 576-582.

[115] L. Zhang, X. Wang, A.J. Bullock, M. Callea, H. Shah, J. Song, K. Moreno, B. Visentin, D. Deutschman, D.C. Alsop, M.B. Atkins, J.W. Mier, S. Signoretti, M. Bhasin, R.A. Sabbadini, R.S. Bhatt, Anti-S1P Antibody as a Novel Therapeutic Strategy for VEGFR TKI-Resistant Renal Cancer, Clin Cancer Res, 21 (2015) 1925-1934.

[116] M.R. Stratton, P.J. Campbell, P.A. Futreal, The cancer genome, Nature, 458 (2009) 719-724.

[117] Q. Song, S.D. Merajver, J.Z. Li, Cancer classification in the genomic era: five contemporary problems, Hum Genomics, 9 (2015) 27.

[118] P. Gascard, T.D. Tlsty, Carcinoma-associated fibroblasts: orchestrating the composition of malignancy, Genes Dev, 30 (2016) 1002-1019.

[119] N.A. Bhowmick, E.G. Neilson, H.L. Moses, Stromal fibroblasts in cancer initiation and progression, Nature, 432 (2004) 332-337.

[120] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell, 144 (2011) 646-674.

[121] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell, 100 (2000) 57-70.

[122] D. Hanahan, Rethinking the war on cancer, Lancet, 383 (2014) 558-563.

[123] R. Dutta, R.I. Mahato, Recent Advances in Hepatocellular Carcinoma Therapy, Pharmacology & therapeutics, (2017).

[124] S. Singh, P.P. Singh, L.R. Roberts, W. Sanchez, Chemopreventive strategies in hepatocellular carcinoma, Nature reviews. Gastroenterology & hepatology, 11 (2014) 45-54.

[125] K.J. Lafaro, A.N. Demirjian, T.M. Pawlik, Epidemiology of Hepatocellular Carcinoma, Surgical Oncology Clinics of North America, 24 (2015) 1-17.

[126] A.B. Ryerson, C.R. Eheman, S.F. Altekruse, J.W. Ward, A. Jemal, R.L. Sherman, S.J. Henley, D. Holtzman, A. Lake, A.-M. Noone, R.N. Anderson, J. Ma, K.N. Ly, K.A. Cronin, L. Penberthy, B.A. Kohler, Annual Report to the Nation on the Status of Cancer, 1975-2012, featuring the increasing incidence of liver cancer, Cancer, (2016) n/a-n/a.

[127] EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma, Journal of hepatology, 56 (2012) 908-943.

[128] P.A. Farazi, R.A. DePinho, Hepatocellular carcinoma pathogenesis: from genes to environment, Nat Rev Cancer, 6 (2006) 674-687.

[129] J.U. Marquardt, J.B. Andersen, S.S. Thorgeirsson, Functional and genetic deconstruction of the cellular origin in liver cancer, Nat Rev Cancer, 15 (2015) 653-667.

[130] J.M. Llovet, J. Zucman-Rossi, E. Pikarsky, B. Sangro, M. Schwartz, M. Sherman, G. Gores, Hepatocellular carcinoma, Nat Rev Dis Primers, 2 (2016) 16018.

[131] J.C. Nault, J. Calderaro, L. Di Tommaso, C. Balabaud, E.S. Zafrani, P. Bioulac-Sage, M. Roncalli, J. Zucman-Rossi, Telomerase reverse transcriptase promoter mutation is an early somatic genetic alteration in the transformation of premalignant nodules in hepatocellular carcinoma on cirrhosis, Hepatology, 60 (2014) 1983-1992.

[132] J. Huang, Q. Deng, Q. Wang, K.Y. Li, J.H. Dai, N. Li, Z.D. Zhu, B. Zhou, X.Y. Liu, R.F. Liu, Q.L. Fei, H. Chen, B. Cai, B. Zhou, H.S. Xiao, L.X. Qin, Z.G. Han, Exome sequencing of hepatitis B virus-associated hepatocellular carcinoma, Nat Genet, 44 (2012) 1117-1121.

[133] Y. Duan, L. Tian, Q. Gao, L. Liang, W. Zhang, Y. Yang, Y. Zheng, E. Pan, S. Li, N. Tang, Chromatin remodeling gene ARID2 targets cyclin D1 and cyclin E1 to suppress hepatoma cell progression, Oncotarget, (2016).

[134] Z. Kan, H. Zheng, X. Liu, S. Li, T.D. Barber, Z. Gong, H. Gao, K. Hao, M.D. Willard, J. Xu, R. Hauptschein, P.A. Rejto, J. Fernandez, G. Wang, Q. Zhang, B. Wang, R. Chen, J. Wang, N.P. Lee, W. Zhou, Z. Lin, Z. Peng, K. Yi, S. Chen, L. Li, X. Fan, J. Yang, R. Ye, J. Ju, K. Wang, H. Estrella, S. Deng, P. Wei, M. Qiu, I.H. Wulur, J. Liu, M.E. Ehsani, C. Zhang, A. Loboda, W.K. Sung, A. Aggarwal, R.T. Poon, S.T. Fan, J. Wang, J. Hardwick, C. Reinhard, H. Dai, Y. Li, J.M. Luk, M. Mao, Whole-genome sequencing identifies recurrent mutations in hepatocellular carcinoma, Genome Res, 23 (2013) 1422-1433.

[135] J.S. Lee, The mutational landscape of hepatocellular carcinoma, Clinical and molecular hepatology, 21 (2015) 220-229.

[136] S.P. Cleary, W.R. Jeck, X. Zhao, K. Chen, S.R. Selitsky, G.L. Savich, T.X. Tan, M.C. Wu, G. Getz, M.S. Lawrence, J.S. Parker, J. Li, S. Powers, H. Kim, S. Fischer, M. Guindi, A. Ghanekar, D.Y. Chiang, Identification of driver genes in hepatocellular carcinoma by exome sequencing, Hepatology, 58 (2013) 1693-1702.

[137] S.L. Ding, Z.W. Yang, J. Wang, X.L. Zhang, X.M. Chen, F.M. Lu, Integrative analysis of aberrant Wnt signaling in hepatitis B virus-related hepatocellular carcinoma, World J Gastroenterol, 21 (2015) 6317-6328.

[138] J.M. Llovet, A. Villanueva, A. Lachenmayer, R.S. Finn, Advances in targeted therapies for hepatocellular carcinoma in the genomic era, Nat Rev Clin Oncol, 12 (2015) 436.

[139] C. Guichard, G. Amaddeo, S. Imbeaud, Y. Ladeiro, L. Pelletier, I.B. Maad, J. Calderaro, P. Bioulac-Sage, M. Letexier, F. Degos, B. Clement, C. Balabaud, E. Chevet, A. Laurent, G. Couchy, E. Letouze, F. Calvo, J. Zucman-Rossi, Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma, Nat Genet, 44 (2012) 694-698.

[140] K. Schulze, S. Imbeaud, E. Letouze, L.B. Alexandrov, J. Calderaro, S. Rebouissou, G. Couchy, C. Meiller, J. Shinde, F. Soysouvanh, A.L. Calatayud, R. Pinyol, L. Pelletier, C. Balabaud, A. Laurent, J.F. Blanc, V. Mazzaferro, F. Calvo, A. Villanueva, J.C. Nault, P. Bioulac-Sage, M.R. Stratton, J.M. Llovet, J. Zucman-Rossi, Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets, Nat Genet, 47 (2015) 505-511.

[141] L. Bao, P.K. Chandra, K. Moroz, X. Zhang, S.N. Thung, T. Wu, S. Dash, Impaired autophagy response in human hepatocellular carcinoma, Experimental and molecular pathology, 96 (2014) 149-154.

[142] R. Mathew, C.M. Karp, B. Beaudoin, N. Vuong, G. Chen, H.Y. Chen, K. Bray, A. Reddy, G. Bhanot, C. Gelinas, R.S. Dipaola, V. Karantza-Wadsworth, E. White, Autophagy suppresses tumorigenesis through elimination of p62, Cell, 137 (2009) 1062-1075.

[143] A. Umemura, F. He, K. Taniguchi, H. Nakagawa, S. Yamachika, J. Font-Burgada, Z. Zhong, S. Subramaniam, S. Raghunandan, A. Duran, J.F. Linares, M. Reina-Campos, S. Umemura, M.A. Valasek, E. Seki, K. Yamaguchi, K. Koike, Y. Itoh, M.T. Diaz-Meco, J. Moscat, M. Karin, p62, Upregulated during Preneoplasia, Induces Hepatocellular Carcinogenesis by Maintaining Survival of Stressed HCC-Initiating Cells, Cancer cell, 29 (2016) 935-948.

[144] K. Taniguchi, S. Yamachika, F. He, M. Karin, p62/SQSTM1-Dr. Jekyll and Mr. Hyde that prevents oxidative stress but promotes liver cancer, FEBS Lett, 590 (2016) 2375-2397.

[145] T. Saito, Y. Ichimura, K. Taguchi, T. Suzuki, T. Mizushima, K. Takagi, Y. Hirose, M. Nagahashi, T. Iso, T. Fukutomi, M. Ohishi, K. Endo, T. Uemura, Y. Nishito, S. Okuda, M. Obata, T. Kouno, R. Imamura, Y. Tada, R. Obata, D. Yasuda, K. Takahashi, T. Fujimura, J. Pi, M.-S. Lee, T. Ueno, T. Ohe, T. Mashino, T. Wakai, H. Kojima, T. Okabe, T. Nagano, H. Motohashi, S. Waguri, T. Soga, M. Yamamoto, K. Tanaka, M. Komatsu, p62/Sqstm1 promotes malignancy of HCV-positive hepatocellular carcinoma through Nrf2-dependent metabolic reprogramming, Nat Commun, 7 (2016).

[146] M. Komatsu, H. Kurokawa, S. Waguri, K. Taguchi, A. Kobayashi, Y. Ichimura, Y.S. Sou, I. Ueno, A. Sakamoto, K.I. Tong, M. Kim, Y. Nishito, S. Iemura, T. Natsume, T. Ueno, E. Kominami, H. Motohashi, K. Tanaka, M. Yamamoto, The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1, Nat Cell Biol, 12 (2010) 213-223.

[147] L. Sanz, M.T. Diaz-Meco, H. Nakano, J. Moscat, The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway, EMBO J, 19 (2000) 1576-1586.

[148] G. Grammatikos, N. Schoell, N. Ferreiros, D. Bon, E. Herrmann, H. Farnik, V. Koberle, A. Piiper, S. Zeuzem, B. Kronenberger, O. Waidmann, J. Pfeilschifter, Serum sphingolipidomic analyses reveal an upregulation of C16-ceramide and sphingosine-1-phosphate in hepatocellular carcinoma, Oncotarget, 7 (2016) 18095-18105.

[149] R. Savic, X. He, I. Fiel, E.H. Schuchman, Recombinant human acid sphingomyelinase as an adjuvant to sorafenib treatment of experimental liver cancer, PLoS One, 8 (2013) e65620.

[150] S. Wilhelm, C. Carter, M. Lynch, T. Lowinger, J. Dumas, R.A. Smith, B. Schwartz, R. Simantov, S. Kelley, Discovery and development of sorafenib: a multikinase inhibitor for treating cancer, Nature reviews. Drug discovery, 5 (2006) 835-844.

[151] L. Montella, G. Palmieri, R. Addeo, S. Del Prete, Hepatocellular carcinoma: Will novel targeted drugs really impact the next future?, World J Gastroenterol, 22 (2016) 6114-6126.

[152] M. Moriguchi, A. Umemura, Y. Itoh, Current status and future prospects of chemotherapy for advanced hepatocellular carcinoma, Clinical journal of gastroenterology, 9 (2016) 184-190.

[153] J. Friemel, M. Rechsteiner, L. Frick, F. Bohm, K. Struckmann, M. Egger, H. Moch, M. Heikenwalder, A. Weber, Intratumor heterogeneity in hepatocellular carcinoma, Clin Cancer Res, 21 (2015) 1951-1961.

[154] M.A. Powell, M.W. Sill, P.J. Goodfellow, D.M. Benbrook, H.A. Lankes, K.K. Leslie, Y. Jeske, R.S. Mannel, M.A. Spillman, P.S. Lee, J.S. Hoffman, D.S. McMeekin, P.M. Pollock, A phase II trial of brivanib in recurrent or persistent endometrial cancer: an NRG Oncology/Gynecologic Oncology Group Study, Gynecol Oncol, 135 (2014) 38-43.

[155] J.M. Llovet, A.M. Di Bisceglie, J. Bruix, B.S. Kramer, R. Lencioni, A.X. Zhu, M. Sherman, M. Schwartz, M. Lotze, J. Talwalkar, G.J. Gores, H.C.C.D.C.T. Panel of Experts in, Design and endpoints of clinical trials in hepatocellular carcinoma, J Natl Cancer Inst, 100 (2008) 698-711.

[156] M. Nagata, S. Muto, S. Horie, Molecular Biomarkers in Bladder Cancer: Novel Potential Indicators of Prognosis and Treatment Outcomes, Disease markers, 2016 (2016) 8205836.

[157] S. Antoni, J. Ferlay, I. Soerjomataram, A. Znaor, A. Jemal, F. Bray, Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends, Eur Urol, (2016).

[158] G.H. Olle Bergman, Elizabeth Johansson, Cancer i siffror 2013, Socialstyrelsen, Stockholm, Sweden, 2013.

[159] M.G. Cumberbatch, M. Rota, J.W. Catto, C. La Vecchia, The Role of Tobacco Smoke in Bladder and Kidney Carcinogenesis: A Comparison of Exposures and Meta-analysis of Incidence and Mortality Risks, Eur Urol, 70 (2016) 458-466.

[160] P. Vineis, D. Marinelli, H. Autrup, J. Brockmoller, I. Cascorbi, A.K. Daly, K. Golka, H. Okkels, A. Risch, N. Rothman, E. Sim, E. Taioli, Current smoking, occupation, N-acetyltransferase-2 and bladder cancer: a pooled analysis of genotype-based studies, Cancer Epidemiol Biomarkers Prev, 10 (2001) 1249-1252.

[161] H.N. Tawfik, Carcinoma of the urinary bladder associated with schistosomiasis in Egypt: the possible causal relationship, Princess Takamatsu Symp, 18 (1987) 197-209.

[162] S. Eissa, R. Ali-Labib, A. Khalifa, Deletion of p16 and p15 genes In schistosomiasis-associated bladder cancer (SABC), Clin Chim Acta, 300 (2000) 159-169.

[163] M.A. Knowles, C.D. Hurst, Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity, Nat Rev Cancer, 15 (2015) 25-41.

[164] E. Pasin, D.Y. Josephson, A.P. Mitra, R.J. Cote, J.P. Stein, Superficial bladder cancer: an update on etiology, molecular development, classification, and natural history, Rev Urol, 10 (2008) 31-43.

[165] M. Babjuk, A. Böhle, M. Burger, O. Capoun, D. Cohen, E.M. Compérat, V. Hernández, E. Kaasinen, J. Palou, M. Rouprêt, B.W.G. van Rhijn, S.F. Shariat, V. Soukup, R.J. Sylvester, R. Zigeuner, EAU Guidelines on Non–Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2016, European Urology.

[166] J. Alfred Witjes, T. Lebret, E.M. Compérat, N.C. Cowan, M. De Santis, H.M. Bruins, V. Hernández, E.L. Espinós, J. Dunn, M. Rouanne, Y. Neuzillet, E. Veskimäe, A.G. van der Heijden, G. Gakis, M.J. Ribal, Updated 2016 EAU Guidelines on Muscle-invasive and Metastatic Bladder Cancer, European Urology.

[167] G. Redelman-Sidi, M.S. Glickman, B.H. Bochner, The mechanism of action of BCG therapy for bladder cancer[mdash]a current perspective, Nat Rev Urol, 11 (2014) 153-162.

[168] R. Crispen, History of BCG and its substrains, Prog Clin Biol Res, 310 (1989) 35-50.

[169] F. Luelmo, BCG vaccination, Am Rev Respir Dis, 125 (1982) 70-72.

[170] R. Pearl, On the Pathological Relations Between Cancer and Tuberculosis, Proceedings of the Society for Experimental Biology and Medicine, 26 (1928) 73-75.

[171] G. Mathe, J.L. Amiel, L. Schwarzenberg, M. Schneider, A. Cattan, J.R. Schlumberger, M. Hayat, F. De Vassal, Active immunotherapy for acute lymphoblastic leukaemia, Lancet, 1 (1969) 697-699.

[172] J.E. Coe, J.D. Feldman, Extracutaneous delayed hypersensitivity, particularly in the guinea-pig bladder, Immunology, 10 (1966) 127-136.

[173] A. Morales, D. Eidinger, A.W. Bruce, Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors, J Urol, 116 (1976) 180-183.

[174] D.L. Lamm, D.E. Thor, S.C. Harris, J.A. Reyna, V.D. Stogdill, H.M. Radwin, Bacillus Calmette-Guerin immunotherapy of superficial bladder cancer, J Urol, 124 (1980) 38-40.

[175] B. Ehdaie, R. Sylvester, H.W. Herr, Maintenance bacillus Calmette-Guerin treatment of non-muscle-invasive bladder cancer: a critical evaluation of the evidence, Eur Urol, 64 (2013) 579-585.

[176] L.J. Old, D.A. Clarke, B. Benacerraf, Effect of Bacillus Calmette-Guerin infection on transplanted tumours in the mouse, Nature, 184(Suppl 5) (1959) 291-292.

[177] A.M. Kamat, J. Briggman, D.L. Urbauer, R. Svatek, G.M. Nogueras Gonzalez, R. Anderson, H.B. Grossman, F. Prat, C.P. Dinney, Cytokine Panel for Response to Intravesical Therapy (CyPRIT): Nomogram of Changes in Urinary Cytokine Levels Predicts Patient Response to Bacillus Calmette-Guerin, Eur Urol, 69 (2016) 197-200.

[178] T.L. Ratliff, J.O. Palmer, J.A. McGarr, E.J. Brown, Intravesical Bacillus Calmette-Guerin therapy for murine bladder tumors: initiation of the response by fibronectin-mediated attachment of Bacillus Calmette-Guerin, Cancer Res, 47 (1987) 1762-1766.

[179] Y. Luo, M.J. Knudson, Mycobacterium bovis Bacillus Calmette-Guérin-Induced Macrophage Cytotoxicity against Bladder Cancer Cells, Clinical and Developmental Immunology, 2010 (2010) 357591.

[180] V.T. Packiam, S.C. Johnson, G.D. Steinberg, Non-muscle-invasive bladder cancer: Intravesical treatments beyond Bacille Calmette-Guerin, Cancer, 123 (2017) 390-400.

[181] E.C. De Boer, W.H. De Jong, P.A. Steerenberg, A.P. Van der Meijden, L.A. Aarden, F.M. Debruyne, E.J. Ruitenberg, Leukocytes and cytokines in the urine of superficial bladder cancer patients after intravesical immunotherapy with bacillus Calmette-Guerin, In Vivo, 5 (1991) 671-677.

[182] E.C. de Boer, W.H. de Jong, A.P. van der Meijden, P.A. Steerenberg, F. Witjes, P.D. Vegt, F.M. Debruyne, E.J. Ruitenberg, Leukocytes in the urine after intravesical BCG treatment for superficial bladder cancer. A flow cytofluorometric analysis, Urol Res, 19 (1991) 45-50.

[183] E.C. De Boer, W.H. De Jong, A.P. Van Der Meijden, P.A. Steerenberg, J.A. Witjes, P.D. Vegt, F.M. Debruyne, E.J. Ruitenberg, Presence of activated lymphocytes in the urine of patients with superficial bladder cancer after intravesical immunotherapy with bacillus Calmette-Guerin, Cancer Immunol Immunother, 33 (1991) 411-416.

[184] K. Pryor, J. Goddard, D. Goldstein, P. Stricker, P. Russell, D. Golovsky, R. Penny, Bacillus Calmette-Guerin (BCG) enhances monocyte- and lymphocyte-mediated bladder tumour cell killing, Br J Cancer, 71 (1995) 801-807.

[185] J. Klostergaard, M.E. Leroux, S.M. Ezell, F.C. Kull, Jr., Tumoricidal effector mechanisms of murine Bacillus Calmette-Guerin-activated macrophages: mediation of

cytolysis, mitochondrial respiration inhibition, and release of intracellular iron by distinct mechanisms, Cancer Res, 47 (1987) 2014-2019.

[186] J. Klostergaard, P.A. Stoltje, F.C. Kull, Jr., Tumoricidal effector mechanisms of murine BCG-activated macrophages: role of TNF in conjugation-dependent and conjugation-independent pathways, J Leukoc Biol, 48 (1990) 220-228.

[187] J. Klostergaard, M.E. Leroux, M.C. Hung, Cellular models of macrophage tumoricidal effector mechanisms in vitro. Characterization of cytolytic responses to tumor necrosis factor and nitric oxide pathways in vitro, J Immunol, 147 (1991) 2802-2808.

[188] S. Atkinson, E. Valadas, S.M. Smith, P.T. Lukey, H.M. Dockrell, Monocyte-derived macrophage cytokine responses induced by M. bovis BCG, Tuber Lung Dis, 80 (2000) 197-207.

[189] J. Wang, J. Wakeham, R. Harkness, Z. Xing, Macrophages are a significant source of type 1 cytokines during mycobacterial infection, J Clin Invest, 103 (1999) 1023-1029.

[190] Y. Luo, H. Yamada, D.P. Evanoff, X. Chen, Role of Th1-stimulating cytokines in bacillus Calmette-Guerin (BCG)-induced macrophage cytotoxicity against mouse bladder cancer MBT-2 cells, Clin Exp Immunol, 146 (2006) 181-188.

[191] D.L. Lamm, B.A. Blumenstein, E.D. Crawford, J.E. Montie, P. Scardino, H.B. Grossman, T.H. Stanisic, J.A. Smith, Jr., J. Sullivan, M.F. Sarosdy, et al., A randomized trial of intravesical doxorubicin and immunotherapy with bacille Calmette-Guerin for transitional-cell carcinoma of the bladder, The New England journal of medicine, 325 (1991) 1205-1209.

[192] P.U. Malmstrom, H. Wijkstrom, C. Lundholm, K. Wester, C. Busch, B.J. Norlen, 5-year followup of a randomized prospective study comparing mitomycin C and bacillus Calmette-Guerin in patients with superficial bladder carcinoma. Swedish-Norwegian Bladder Cancer Study Group, J Urol, 161 (1999) 1124-1127.

[193] R.L. Steinberg, L.J. Thomas, M.A. O'Donnell, Bacillus Calmette-Guerin (BCG) Treatment Failures in Non-Muscle Invasive Bladder Cancer: What Truly Constitutes Unresponsive Disease, Bladder Cancer, 1 (2015) 105-116.

[194] A.P. van der Meijden, R.J. Sylvester, W. Oosterlinck, W. Hoeltl, A.V. Bono, Maintenance Bacillus Calmette-Guerin for Ta T1 bladder tumors is not associated with increased toxicity: results from a European Organisation for Research and Treatment of Cancer Genito-Urinary Group Phase III Trial, Eur Urol, 44 (2003) 429-434.

[195] E.J. Askeland, M.R. Newton, M.A. O'Donnell, Y. Luo, Bladder Cancer Immunotherapy: BCG and Beyond, Adv Urol, 2012 (2012) 181987.

[196] I. Hosen, P.S. Rachakonda, B. Heidenreich, P.J. de Verdier, C. Ryk, G. Steineck, K. Hemminki, R. Kumar, Mutations in TERT promoter and FGFR3 and telomere length in bladder cancer, Int J Cancer, 137 (2015) 1621-1629.

[197] Y. Allory, W. Beukers, A. Sagrera, M. Flandez, M. Marques, M. Marquez, K.A. van der Keur, L. Dyrskjot, I. Lurkin, M. Vermeij, A. Carrato, J. Lloreta, J.A. Lorente, E. Carrillo-de Santa Pau, R.G. Masius, M. Kogevinas, E.W. Steyerberg, A.A. van Tilborg, C. Abas, T.F. Orntoft, T.C. Zuiverloon, N. Malats, E.C. Zwarthoff, F.X. Real, Telomerase reverse transcriptase promoter mutations in bladder cancer: high frequency across stages, detection in urine, and lack of association with outcome, Eur Urol, 65 (2014) 360-366.

[198] E. Christensen, K. Birkenkamp-Demtroder, I. Nordentoft, S. Hoyer, K. van der Keur, K. van Kessel, E. Zwarthoff, M. Agerbaek, T.F. Orntoft, J.B. Jensen, L. Dyrskjot, Liquid

Biopsy Analysis of FGFR3 and PIK3CA Hotspot Mutations for Disease Surveillance in Bladder Cancer, Eur Urol, (2017).

[199] D. Smolensky, K. Rathore, M. Cekanova, Molecular targets in urothelial cancer: detection, treatment, and animal models of bladder cancer, Drug Des Devel Ther, 10 (2016) 3305-3322.

[200] Q. Dong, L. Fu, Y. Zhao, S. Tan, E. Wang, Derlin-1 overexpression confers poor prognosis in muscle invasive bladder cancer and contributes to chemoresistance and invasion through PI3K/AKT and ERK/MMP signaling, Oncotarget, (2017).

[201] A.M. Blanca Pedregosa, A. Sanchez-Gonzalez, J. Carrasco Valiente, J.M. Ruiz Garcia, E. Gomez Gomez, A. Lopez Beltran, M.J. Requena Tapia, Expression of proteins FGFR3, PI3K, AKT, p21Waf1/Cip1 and cyclins D1 and D3 in patients with T1 bladder tumours: clinical implications and prognostic significance, Actas urologicas espanolas, (2016).

[202] X.D. Meng, Z.S. Zhou, J.H. Qiu, W.H. Shen, Q. Wu, J. Xiao, Increased SPHK1 expression is associated with poor prognosis in bladder cancer, Tumor Biology, 35 (2014) 2075-2080.

[203] S. Kawamura, C. Ohyama, R. Watanabe, M. Satoh, S. Saito, S. Hoshi, S. Gasa, S. Orikasa, Glycolipid composition in bladder tumor: a crucial role of GM3 ganglioside in tumor invasion, Int J Cancer, 94 (2001) 343-347.

[204] R. Watanabe, C. Ohyama, H. Aoki, T. Takahashi, M. Satoh, S. Saito, S. Hoshi, A. Ishii, M. Saito, Y. Arai, Ganglioside G(M3) overexpression induces apoptosis and reduces malignant potential in murine bladder cancer, Cancer Res, 62 (2002) 3850-3854.

[205] C. Ohyama, S. Kawamura, K. Suzuki, K. Numahata, S. Tokuyama, A. Ito, M. Satoh, S. Saito, K. Yoshikawa, S. Hoshi, S. Orikasa, G(M3) inhibits murine MET-2 tumor invasion and growth, Int J Oncol, 8 (1996) 809-813.

[206] B. Bressac, K.M. Galvin, T.J. Liang, K.J. Isselbacher, J.R. Wands, M. Ozturk, Abnormal structure and expression of p53 gene in human hepatocellular carcinoma, Proc Natl Acad Sci U S A, 87 (1990) 1973-1977.

[207] B. Chen, M. Sirota, H. Fan-Minogue, D. Hadley, A.J. Butte, Relating hepatocellular carcinoma tumor samples and cell lines using gene expression data in translational research, BMC Medical Genomics, 8 (2015) 1-10.

[208] J.L. Wilding, W.F. Bodmer, Cancer cell lines for drug discovery and development, Cancer research, 74 (2014).

[209] I. Vermes, C. Haanen, H. Steffens-Nakken, C. Reutelingsperger, A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, J Immunol Methods, 184 (1995) 39-51.

[210] W. Gorczyca, Cytometric analyses to distinguish death processes, Endocr Relat Cancer, 6 (1999) 17-19.

[211] T. Thiel, C. Ryk, V. Chatzakos, K. Hallen Grufman, N. Bavand-Chobot, J. Flygare, N.P. Wiklund, P.J. de Verdier, Secondary stimulation from Bacillus Calmette-Guerin induced macrophages induce nitric oxide independent cell-death in bladder cancer cells, Cancer Lett, 348 (2014) 119-125.

[212] L.C. Clark, G.F. Combs, Jr., B.W. Turnbull, E.H. Slate, D.K. Chalker, J. Chow, L.S. Davis, R.A. Glover, G.F. Graham, E.G. Gross, A. Krongrad, J.L. Lesher, Jr., H.K. Park, B.B. Sanders, Jr., C.L. Smith, J.R. Taylor, Effects of selenium supplementation for cancer

prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group, JAMA, 276 (1996) 1957-1963.

[213] B. Husbeck, L. Nonn, D.M. Peehl, S.J. Knox, Tumor-selective killing by selenite in patient-matched pairs of normal and malignant prostate cells, Prostate, 66 (2006) 218-225.

[214] S. Cao, F.A. Durrani, Y.M. Rustum, Selective Modulation of the Therapeutic Efficacy of Anticancer Drugs by Selenium Containing Compounds against Human Tumor Xenografts, Clinical Cancer Research, 10 (2004) 2561.

[215] M. Sopjani, M. Foller, E. Gulbins, F. Lang, Suicidal death of erythrocytes due to selenium-compounds, Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology, 22 (2008) 387-394.

[216] L.J. Siskind, T.D. Mullen, K. Romero Rosales, C.J. Clarke, M.J. Hernandez-Corbacho, A.L. Edinger, L.M. Obeid, The BCL-2 protein BAK is required for long-chain ceramide generation during apoptosis, J Biol Chem, 285 (2010) 11818-11826.

[217] C.E. Senkal, S. Ponnusamy, M.J. Rossi, J. Bialewski, D. Sinha, J.C. Jiang, S.M. Jazwinski, Y.A. Hannun, B. Ogretmen, Role of human longevity assurance gene 1 and C18-ceramide in chemotherapy-induced cell death in human head and neck squamous cell carcinomas, Molecular cancer therapeutics, 6 (2007) 712-722.

[218] W. Zhong, T.D. Oberley, Redox-mediated effects of selenium on apoptosis and cell cycle in the LNCaP human prostate cancer cell line, Cancer Res, 61 (2001) 7071-7078.

[219] S. Schiffmann, K. Birod, J. Mannich, M. Eberle, M.S. Wegner, R. Wanger, D. Hartmann, N. Ferreiros, G. Geisslinger, S. Grosch, Ceramide metabolism in mouse tissue, The international journal of biochemistry & cell biology, 45 (2013) 1886-1894.

[220] R. Kim, M.T. Byrne, A. Tan, F. Aucejo, What is the indication for sorafenib in hepatocellular carcinoma? A clinical challenge, Oncology (Williston Park, N.Y.), 25 (2011) 283-291, 295.

[221] T.K. Lee, K. Man, J.W. Ho, C.K. Sun, K.T. Ng, X.H. Wang, Y.C. Wong, I.O. Ng, R. Xu, S.T. Fan, FTY720 induces apoptosis of human hepatoma cell lines through PI3-K-mediated Akt dephosphorylation, Carcinogenesis, 25 (2004) 2397-2405.

[222] P.J. Duriez, G.M. Shah, Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death, Biochemistry and cell biology = Biochimie et biologie cellulaire, 75 (1997) 337-349.

[223] L. Alinari, R.A. Baiocchi, M. Praetorius-Ibba, FTY720-induced blockage of autophagy enhances anticancer efficacy of milatuzumab in mantle cell lymphoma: is FTY720 the next autophagy-blocking agent in lymphoma treatment?, Autophagy, 8 (2012) 416-417.

[224] G. Mazzoccoli, L. Miele, J. Oben, A. Grieco, M. Vinciguerra, Biology, Epidemiology, Clinical Aspects of Hepatocellular Carcinoma and the Role of Sorafenib, Current drug targets, 17 (2016) 783-799.

[225] V. Gagliostro, J. Casas, A. Caretti, J.L. Abad, L. Tagliavacca, R. Ghidoni, G. Fabrias, P. Signorelli, Dihydroceramide delays cell cycle G1/S transition via activation of ER stress and induction of autophagy, The international journal of biochemistry & cell biology, 44 (2012) 2135-2143.