UNMARKED MANUSCRIPT

Sphingolipids and glycerophospholipids – the "ying and yang" of lipotoxicity in metabolic diseases.

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Abstract

Sphingolipids in general and ceramides in particular, contribute to pathophysiological mechanisms by modifying signalling and metabolic pathways. Here, we present the available evidence for a bidirectional homeostatic crosstalk between sphingolipids and glycerophospholipids, whose dysregulation contribute to lipotoxicity induced metabolic stress. The initial evidence for this crosstalk originates from simulated models designed to investigate the biophysical properties of sphingolipids in plasma membrane representations. In this review, we reinterpret some of the original findings and conceptualise them as a sort of *"ying/yang"* interaction model of opposed/complementary forces, which is consistent with the current knowledge of lipid homeostasis and pathophysiology. We also propose that the dysregulation of the balance between sphingolipids and glycerophospholipids results in a lipotoxic insult relevant in the pathophysiology of common metabolic diseases, typically characterised by their increased ceramide/sphingosine pools.

Abbreviations

[AA] Arachidonic acid, **[AKT]** RAC-alpha serine/threonine-protein kinase, **[CCTα]** Choline-phosphate cytidylyltransferase A, **[CERK]** Ceramide Kinase, **[C1P]** Ceramide-1-phosphate, **[CerS]** Ceramide Synthase, **[CERT]** ceramide transport protein, **[HDL]** High-density lipoproteins, **[PAF]** Platelet activating factor, **[PC]** phosphatidylcholine, **[PE]** Phosphatidylethanolamine, **[PH]** Pleckstrin homology domain, **[PI]** Phosphatidylinositol, **[PI3K]** Phosphatidylinositol 3-kinase, **[PLA2]** Phospholipase A2, **[PLC]**, Phospholipase C, **[PLD]** Phospholipase D, **[PS]** Phosphatidylserine, **[S1P]** sphingosine-1-phosphate, **[SM]** Sphingomyelin, **[SMase]** Sphingomyelinase **[SMS]** Sphingomyelin synthase **[Sphk1]** Sphingosine kinase 1, **[SPTLC]** Serine palmitoyltransferase, **[SRE]** Sterol regulatory element, **[SREBP1]** Sterol regulatory element-binding protein 1, **[TG]** Triglyceride.

INTRODUCTION

In the context of common metabolic diseases such as obesity, diabetes or non-alcoholic fatty liver disease (NAFLD), the concept of *"lipotoxicity"* refers to the inappropriate ectopic accumulation of lipids in non-adipose organs causing metabolic stress and dysfunction. Lipotoxicity in liver, skeletal muscle, heart, pancreas or brain has been identified as an important pathogenic contributor to their metabolic dysfunction. Lipotoxicity can operate at multiple levels spanning from cellular to organ levels and involves a repertoire of characteristic biochemical mediators. The severity of the lipotoxic insult is modulated by the specific cellular genetic vulnerability to the toxicity induced by lipids. When at physiological concentrations, most of these lipid species exert important physiological functions that contribute to structural, signalling or cellular homeostasis. Hence, these lipids *per se* only become toxic when: **a)** they accumulate in excessive quantities as a result of exacerbated biosynthesis and/or impaired turnover; **b)** they exhibit distorted qualitative properties (e.g. both biologically or chemically induced) and/or **c)** when their spatio-temporal location in the cell is atypical (*being in the wrong place at the wrong time*).

When in excess, sphingolipids behave as lipotoxic lipid species. Amongst them, ceramides and sphingosines are considered the "*usual pathogenic suspects"*. The biochemical processes of biosynthesis and remodelling of ceramides/sphingolipids are undoubtedly complex involving at least three well-characterised pathways described in detail elsewhere [1, 2]. These biosynthetic pathways are highly compartmentalised within the cell, which leads to the formation of discrete organelle lipid pools accumulating specific ceramide and other sphingolipids species. Their topographic localisation within the cell determines their supplied/targeted structures and also affects their signalling responses to extracellular and/or intracellular stimuli. Thus, it is the biochemical repertoire of sphingolipid molecules (e.g. sphingomyelin, ceramide, C1P, sphingosine, S1P as well as other complex sphingolipids), but also their topographic localisation and relative concentrations that determine their metabolic effects [3-8].

As typically observed in insulin resistant diabetes and also in some cancers, excessive accumulation of sphingolipids causes toxicity. In addition to their absolute abundance and localisation, the *relative proportions* of their specific biochemical species is also pathologically relevant. This concept has recently been coined as the ***"sphingolipid rheostat"*** [9], which refers to the relevance of the coordinated regulation and balance of specific sphingolipids (ceramides, sphingosine and S1P) for the control of cellular responses. This concept was originally studied in the field of cell fate and cancer, but recent investigation has demonstrated its validity for metabolic pathologies such as cardiovascular diseases [10, 11] and diabetes [12]. Therefore, this paradigm needs to be revisited to include new regulators of sphingolipid metabolism as well as the paracrine action of some of those sphingolipids [13].

The dysregulation of *the rheostat* either by increasing or decreasing specific types of sphingolipid species may exacerbate the pathology, may be used as biomarker of phenotypes or stages within the natural history of the disease, and could be helpful to identify nosological entities. Thus, sphingolipid profiles could be used as diagnostic, prognostic and therapeutic responsiveness biomarkers of metabolic stress and disease.

The current interest on the pathophysiological role of sphingolipids has been sparked by enabling technologies such as cellular and *in vivo* knockout models and improved lipidomic platforms. Technologies such as genetic engineering/editing and mass spectrometry have helped to elucidate the function of sphingolipid and glycerophospholipid regulated pathways, recognise their direct contribution to toxic cellular effects, and have identified new allostatic pathways designed to restore or maintain cellular homeostasis. The two most recognised sphingolipid-mediated toxic effects at cellular level are mitochondrial and endoplasmic reticulum stress and apoptosis [14, 15]. Another important but underestimated target of sphingolipid/ceramide mediated toxicity is ***the perturbation of glycerophospholipids homeostasis and their associated signalling events,*** the main focus of this review.

Using systems biology, Koberlin [16] and Vonkova [17] have provided new evidence for the close bidirectional interaction between sphingolipids and glycerophospholipids. Koberlin et al. have shown an intimate interaction between sphingolipids and glycerophospholipid metabolism conforming

a circular network of co-regulated lipids [16]. Their elegant approach has revealed a fundamental inter-lipid regulatory network that controls membrane lipid composition. Interestingly, these membrane lipid perturbations predicted the inflammatory responses in patients derived cells; enabling the functional assignment of lipids to specific Toll-like-receptor (TLR) signalling. Moreover, Vonkova and colleagues have shown the existence of cooperative (and also a few deterring) interactions between phosphatidylinositides (acting as driver ligands), and sphingolipids (in an auxiliary role), that mediate the recruitment of PH containing proteins to the membranes in both yeast and higher eukaryotic models. Moreover, in many cases, these cooperative events are highly specific and restricted to specific pairs of signalling lipids [17]. Thus, accumulating evidence indicates that both, sphingolipid and glycerophospholipid metabolism is essential for cellular membrane dynamics. Both types of lipids mutually regulate their biosynthesis and signalling paths within eukaryotic cellular models.

Our perception after reviewing the subject is that both, sphingolipid and glycerophospolipid metabolism are highly connected, integrated and driven in opposed directions, representing a harmonic *"Ying and Yang"* model of regulation contributing to membrane lipid homeostasis and lipid signalling events. Below, we provide the current experimental evidence supporting the existence of this regulatory crosstalk.

1. Evidence that sphingolipid metabolism is essential for cellular membrane dynamics and signalling.

Sphingomyelins and ceramides are present in the lipid rafts and caveolae domains of the plasma membrane. The relative concentration of sphingolipids, phospholipids and cholesterol [18-25] determines its biophysical properties varying from gel to liquid-ordered and to liquid-disordered structures [26, 27]. Other sphingolipid species, such as sphingosine or ceramide-1-phosphate, are also present in membranes at lower concentrations. Despite their low concentration, these lipids play essential roles as determinants of membrane dynamics and signalling pathways. For instance, sphingosine permeabilises phospholipid bilayers and increases vesicle leakage [28]; S1P modifies the gel-fluid transition of glycerophospholipids and stabilises the lipid bilayer structure [29]. Moreover C1P

is required for the formation of a negative membrane curvature [30] and it is not segregated into lateral lipid domains in phospholipid bilayers [31].

The use of artificial liposomes and *in vitro* cellular models have documented how temperature [32] and/or other mechanical stresses affect the interaction between ceramides and the other constituents of the plasma membrane [33]. There is evidence from these *in vitro* models that both, quantitative (relative percentage) and qualitative changes (length and unsaturation of fatty acid moieties) [34, 35] in sphingolipids (mostly ceramides and sphingomyelins) [36-43] perturb the biophysical properties of the plasma membrane (e.g. fluidity, lateral pressure and diffusion). These are non-subtle membrane perturbations and are likely to compromise metabolite transport and diffusion, receptor signalling, sorting and trafficking, formation of pores (e.g. in the outer membrane of the mitochondria [44]) and activation of apoptotic stimuli.

At physiological levels, SMs stabilise the interactions with sterols in bilayer membranes [45, 46] and regulate the trans bilayer movement and distribution of diacylglycerols (DAGs) [47] and galactosylceramides [48]. In contrast, ceramides -when in excess- associate with SM/PC and destabilise/ displace cholesterol within the membrane (reviewed in [26, 49, 50]). Ceramides facilitate the transbilayer lipid movement in membranes, which occurs irrespectively of whether ceramides are externally sourced or obtained by metabolising SM via SMases [51]. Additionally, there is evidence that ceramides spontaneously translocate (with limited capacity) from the outer to the inner leaflet of the plasma membrane [52, 53], which is relevant for signalling and trafficking. The position of ceramides in the inner or outer side of the membrane is important given their role promoting plasma membrane repair in response to injury [54] and control of exocytosis or endocytosis [55], processes that require the involvement of a functional plasma membrane.

Genetically modified murine models have helped to identify the primary effects on energy homeostasis of sphingolipid-related gene ablation. These experiments have also revealed robust compensatory mechanisms aiming to maintain cellular and tissue homeostasis, some of them of therapeutic potential. For instance, the SMS2KO mouse exhibited a healthy phenotype characterised by impaired fatty acid uptake and lipid droplet formation in response to high fat diet and protection against insulin resistance

[39]. Alterations in membrane lipid microdomains associated with CD36/FATP1 transporters and CAV1 [40] ultimately lead to decreased lipid storage. Conversely, overexpression of sphingomyelin synthases, SMS1 and SMS2, caused a pro-atherogenesis phenotype characterised by aggregation of lipoprotein particles [56]. Also interesting is the effect of sphingomyelin ablation on cellular signalling. Particularly, SMS deficiency or overexpression are expected to change membrane microdomains (lipid rafts, caveolae) enriched in SM, affect their biophysical properties and compromise transport, secretion, and signalling mechanisms such as apoptosis and cell death [57]. Furthermore, SMS1 and SMS2 deficiency/overexpression also affect the levels of sphingosine and S1P [58, 59] that could potentially act as second messengers. In the light of recent investigations it has been suggested that S1P may contribute to the development of insulin resistance in mice [60-63]. Thus, it is tempting to speculate that changes in S1P in those models may contribute to their overall phenotype.

Whereas the *in vivo* evidence for the crosstalk between sphingolipids and glycerophospholipids is still limited, the *in vitro* models have shown that sphingolipids and glycerophospholipids interact at a functional level. Moreover, this crosstalk goes beyond what could have initially been anticipated given the relatively marginal structural disruption observed from gain or loss of function genetic experiments. These *in vitro* studies show the direct regulation of glycerophospholipids metabolism by sphingolipids by interfering with major phospholipases, such as PLA2, PLC and PLD, releasing lysophospholipids and fatty acids that could act as signalling metabolites.

2. Evidence of the crosstalk between sphingolipid and glycerophospholipid metabolism.

Beyond the linear lipid biosynthesis pathways, accumulating evidence shows that these pathways crosstalk and intertwine with others metabolic networks. These interactions are evolutionary preserved in plants, fungi, protozoa, yeast as well as in invertebrates and vertebrates (reviewed in [64]).

One important crosstalk between sphingolipid and glycerophospholipid metabolism is the metabolic pathway modulating the conversion of sphingolipids to glycerophospholipids regulated by the S1P lyase recently reviewed by Kihara [65]. Briefly, degradation of S1P and sphinganine (dihydrosphingosine) by S1P lyase yields fatty acid aldehydes (hexadecenal and hexadecanal respectively) and

phosphoethanolamine. When these fatty aldehydes are oxidized and converted into palmitoyl-CoA by the concerted action of several enzymes, it enters the biosynthesis pathway of glycerophospholipids. Recent evidence also suggests that degradation of sphingosine and sphinganine may contribute to the biosynthesis of etherlipids (ether analogues of phospholipids) when the activity of the fatty aldehyde dehydrogenase (e.g. ALDH3A2) is inactive [66]. This suggests that interlipid conversion may be a common allostatic response in pathologies characterised by sphingolipid alterations. Interestingly, this lipid interconversion may lead to lipotoxicity as the hexadecenal is able to form aldehyde-derived DNA adducts [67]. The relevance of this in pathophysiological scenarios has not been investigated yet.

In the same line of evidence, Kondo et colleague have shown that phytosphingosine can also be metabolised to odd-numbered fatty acids and incorporated into glycerophospholipids, both in yeast and mammalian cells, or alternatively undergo mitochondria oxidation [68]. However, further investigation will be required to fully understand the biological significance of this pathway in overall lipid homeostasis.

2.1 Evidence for a cross talk between sphingolipids and phosphatidylethanolamine

Phosphoethanolamine, the other degradation product of S1P and sphinganine, can enter the CDP-ethanolamine pathway to generate phosphatidylethanolamine (PE) [69-71]. Moreover, PE can be further converted to other glycerophospholipids such as PC and PS, via PEMT and PDSS2 enzymes respectively.

Proof of this close relationship between sphingolipids and phosphatidylethanolamine is the evidence that genetic manipulation of sphingolipids biosynthesis impairs PE homeostasis by limiting substrate availability. For instance, the simultaneous ablation of Sphk1 and Sphk2 [72] (the enzymes responsible of the phosphorylation of sphingosine to S1P) and the S1P lyase *in vivo* [72, 73] decreased PE levels and accumulated sphingosines, ceramides and sphingomyelins -as a result of the direct reutilization of the sphingosine backbone- [73] These data indicates that the biosynthesis of glycerophospholipids from sphingolipids intermediates is essential for the degradation of sphingolipids [74] and that perturbations in the S1P pool affects glycerophospholipid reservoirs. Interestingly, this interconversion is of particular

significance for the survival of the protozoan parasite *leishmania* being the major route for ethanolamine synthesis [75].

Additional evidence showing that sphingolipid metabolism affects glycerophospholipid pools comes from studies showing that ceramides also inhibit the CDP-ethanolamine 1,2 diradyl-sn-glycerol ethanolamine phosphotransferase (EPT) and reduce the levels of PE [69, 76]. Moreover, several *in vitro* and *in vivo* models of impaired biosynthesis of ceramides have shown both quantitative [16] and qualitative changes in the pools of PEs [43, 77] suggesting the presence of compensatory mechanisms. Whether this opposed direct action of ceramides vs. S1P regarding PE metabolism is part of a regulated homeostatic rheostat is at the present moment unknown.

Intriguingly, similar to PC (see section below), PE contributes to the biosynthesis of ethanolamine containing analogues of SM [78]. Thus, it is also conceivable that PE levels may limit the biosynthesis of specific sphingolipid species, in the same way that PCs do.

2.2 Sphingolipid mediated inhibition of phosphatidylcholine biosynthesis.

Short chain ceramides but not their structural analogues dihydroceramides [79], prevent the biosynthesis of PCs by inhibiting the enzyme CCTα [76, 80-82]. Inhibition of CCTα requires activation of cPLA2 and subsequent release of lysophosphatidylcholine (LPC) [83]. CTTα activity and mRNA gene expression is also repressed by sphingosine [84]. Interestingly, the inhibitory effect of sphingosine on CCTα is reversed in the presence of other glycerophospholipids such as PS, PI and PG [85].

The inhibitory effect of ceramides on PC biosynthesis is tightly controlled and apparently independent of the source of ceramides. Thus, *in vitro* experiments in lung epithelia overexpressing ceramide synthase 5 (CerS5) and/or sphingomyelinase (SMase) have shown an additive effect mediating the inhibition of the biosynthesis of phosphatidylcholine. Conversely, PC biosynthesis is stimulated when Cers5 is downregulated by SiRNA [86]. These data suggest that the PC biosynthetic pathway "senses" ceramide pools rather than only responding to a supraphysiological stress.

Similarly, SM analogues also inhibit the translocation and activation of CTT [87, 88]. Thus, the impairment in PC biosynthesis resulting from increased level of ceramides may be relevant to disease pathogenesis and has been proposed as one of many mechanisms for how aSMase may contribute to steatohepatitis [89]. Conversely, glycosylceramides have been shown to activate CTT as observed in *in vitro* models of Gaucher disease [90].

In line with these findings, global lipidomic analysis has lent further support to the existence of a solid crosstalk between sphingolipids and PC. For instance, the knock-down of *sptlc2* in RAW264 macrophages increases the levels of PC [16]. Similarly, the total knock-out of *sptlc123* in Huh7 hepatocytes results in increased phospholipids with an enrichment in PC species [91]. Paradoxically, the *sptlc2* heterozygote [39] and the heart specific *sptlc2* KO [92] mice exhibited a decrease in the PC levels as well as qualitative changes in the FA of the PC fraction. These data suggest that the changes observed in the PC pools in these models are likely secondary homeostatic responses directed to preserve cellular or tissue viability.

Moreover, sphingolipids also regulate the pool of PCs through indirect mechanisms. An illustrative example of this is the influence of SM levels on the hydrolysis of PC pools from VLDL, LDL and HDL lipoproteins by hepatic lipase (HL) (see also section 5.2). Thus, SM depletion stimulates the hydrolysis of PC (mainly PUFA-PC) as well as TG, indicating that SM acts as an inhibitor of HL [93]. Another effect relates to the activation of the ABCB4 receptor expressed in the canalicular membrane of hepatocytes whose function is to export PC into the bile. In this regard, Zhao and colleagues have shown that the cellular content of SM is critical for ABCB4 to facilitate the efflux of PC, opposite to the effects of other members of the ABC family such as ABCB1 [91].

PCs also reciprocally regulate sphingolipid metabolism. Examples of this regulation include: a) the activity of PCs acting as phosphocholine donors for SM biosynthesis [94-97]; b) the fact that choline deficient media/diets [98] or that pharmacological inhibition of choline kinase [99], both impair the synthesis of SM while concomitantly increasing ceramide levels. Conversely, the action of SMase on SM releases phosphocholine that can be incorporated into the cytidine biosynthetic pathway for the

generation of phosphatidylcholine [100]. Altogether these data strengthen the concept of a strong co-regulation between PC and SM biosynthetic and metabolic pathways.

2.3 Evidence for a crosstalk between sphingolipids and phosphatidylserine.

Serine is a common component of both PS and ceramides, suggesting that both types of lipids may be metabolically connected. This biochemical link has been confirmed by *in vitro* where labelled serine originated from PS was identified in ceramides [101]. Moreover, sphingosine, S1P and lyso-sphingomyelin increase the biosynthesis of PS and block the conversion of PS to PE by decarboxylation [102, 103] mediated by a PKC independent mechanism [104, 105]. In support of this regulatory loop, the ablation of sptlc2 gene in RAW264 macrophage caused the expected impairment in the *de novo* biosynthesis of ceramides but also decreased the levels of PS. Similarly, the elevation of SM mass by different mechanisms leads to the depletion of the PS pool from the plasma membrane [106] leading to mislocalisation of k-ras [37], this was prevented by inhibition of SM synthesis suggesting the existence of a concerted mechanism to regulate the levels of both lipids in the plasma membrane.

In addition to SM, other sphingolipids also affect the location of PS in the plasma membrane as well as some of the biochemical processes regulated by PS. Thus, the ceramides generated by activation of SMSase facilitate the synthesis

of PS and also contribute to the translocation of PS to the outer membrane, a requirement for platelet secretion and thrombus formation [107]. Other sphingolipids such as sphingosines, due to their positive charge, prevent the binding of calcium to PS enriched vesicles [108-110] and/or negatively modulate substrate phosphorylation by PKC by directly competing with PS [111, 112]. This evidence indicates that ceramides facilitate PS biosynthesis and optimise cellular location, whereas other sphingolipids elicit a negative effect on PS homeostasis.

It is worth noting that a recent investigation in the forebrain of CerS6KO mice [113] has shown the paradoxical increase in PS levels. This may be interpreted as against ceramides having a positive effect on the PS pool. Nevertheless, in this model there is a specific depletion of C16:0 ceramides rather than a global depletion, and as the molecular mechanism to support this finding is unknown, further research is required.

In terms of our ying/yang concept, the yang of this regulation is that phosphatidylserine reciprocally modulates sphingolipid metabolism. For instance, PS increases the activity and translocation of sphingosine kinase to membranes [114, 115] and also activates enzymes involved in the degradation of complex sphingolipids [116, 117] . Whether PS interferes with the activity of SMase is less clear as some studies have shown that PS inhibits PKC mediated activation of neutral SMase [118], whereas others indicate that PS stimulates neutral [119] and alkaline [120] SMSase activities.

PS also reverses some of the negative effects of sphingolipids on enzymatic activities. PS (as well as PI but not PC) reverses sphingosine mediated inhibition of transmembrane protein tyrosylprotein sulfotransferase (TPST), the enzyme that catalyzes the sulfation of tyrosines. Despite the molecular mechanism not being well characterised, the authors concluded that the positive charges of sphingosine and a long alkyl chain are fundamental for impairing the affinity of the protein-substrate and that PS is able to reverse that inhibition in a competitive manner [121].

Similarly, PS also reduces the inhibitory effect of sphingosine on the autophosphorylation of the insulin receptor tyrosine kinase [122]. In this regard, it has been suggested that the high affinity for PS may protect the receptor from interacting with the amine group of sphingosine. Globally, these

investigations suggest that acidic phospholipids, such as PS, may prevent the interaction of sphingosine with the targeted proteins. Given that sphingosine inhibits other kinases such as SRC kinase [123], it is conceivable that this competitive interaction between sphingosine and PS may be common in other similar kinases. Further research will be needed to evaluate the biological significance of this competitive interaction.

2. 4 Bidirectional Crosstalk between Sphingolipid metabolism and Platelet Activating Factor.

Platelet Activating Factor (PAF) is an ether-glycerosphospholipid important for immune cell activation. The first insight for an interaction between PAF and sphingolipid biosynthetic pathways came from the discovery of a CoA independent transacetylase that mediates the transfer of an acetyl group from PAF in the synthesis of N acetylsphingosine [124-126]. More recently, we have learned that S1P promotes PAF synthesis [127, 128] and modulation of cell chemotaxis and inflammatory responses in immune cells. Conversely, PAF increases SMase activity and facilitates the generation of ceramide microdomains, which are important for inflammation, apoptosis, activation of eNOS in EC cells [129], development of pulmonary oedema [130], and the activation of the enzyme scramblase that facilitates the exposure of PS in the outer membrane [131].

3. Sphingolipids modulation of phospholipid biosynthesis via SREBP activation.

How sphingolipids regulate the biosynthesis of phospholipids at a molecular level is a key question. SREBPS are important mediators of the biosynthesis of fatty acids, cholesterol and TGs. SREBPS also regulates the levels of phosphatidylcholines by regulating the expression of pcyt1a (the gene that encodes CTTα) at transcriptional and posttranscriptional mechanisms level [132, 133]. SREBPS may also indirectly increase pcyt1a expression and activity secondarily to the concomitant biosynthesis of fatty acids. Genome-wide analysis of SREBP1 binding in mouse liver has shown the presence of putative SRE motifs in the phosphatidylinositol synthase gene [134], suggesting that the regulation by srebp1 might not be restricted to PC biosynthesis. This concept is further reinforced by novel research in flies [135] and metazoans [136].

Sphingolipids control SREBP1 cleavage and activation by different mechanisms: a) the breakdown of sphingomyelins to ceramides in the plasma membrane displaces cholesterol to intracellular compartments where cholesterol represses the cleavage and activation of srebp1 and 2 [137]. Additional evidence indicates that high levels of ceramides resulting from SM hydrolysis inhibit SRE mediated gene transcription independently of changes in intracellular cholesterol trafficking through a mechanism not yet defined [138] and b) S1P stimulates SREBP1 cleavage and nuclear translocation via a S1P receptor [139].

Sphingolipids also regulate SREBP1 expression. For instance, ceramides have been reported to increase the levels of the precursor SREBP and decrease the levels of mature SREBP [140]. Moreover, accumulation of SM (either from external origin or as a result of the inhibition of SMase) represses srebp1 expression via a caveolin and Ras-ERK-MAPK-CREB signalling pathway in adipocytes [141]. Accordingly, dietary administration of sphingomyelin decreases hepatic lipid levels due to inactivation of the LXR-SREBP1 pathway [142]. The potential pathophysiological relevance of these findings is highlighted by a recent study showing that increased levels of SMs are associated with decreased levels of srebp1 mRNA in the adipose tissue of obese women [141].

Altogether, this indicates that sphingolipids may regulate lipid homeostasis (including PC biosynthesis), by controlling both the expression and activation of SREBP1. This may be of particular relevance in tissues such as liver and adipose tissue where SREBP1 is an important regulator of lipid synthesis, and where its dysregulation contributes to the development of NAFLD and obesity associated adipose tissue dysfunction.

4. Sphingolipids interfere with phospholipid mediated signalling.

4.1 Sphingolipids modulate the biosynthesis and activity of phophatidylinositols.

Compared to the robust effect of sphingolipids on other phospholipids, the evidence that sphingolipids may interfere with the *de novo* biosynthesis of phosphatidylinositol phospholipids in high eukaryotic cells is still preliminary. However, it is known that the small subunit of the serine palmitoyltransferase (ssSPT) [143] is also a lysophosphatidylinositol acyltransferase 1-interacting

protein [144]. Thus, this protein could be a pivotal signal co-regulating both the synthesis of ceramides and the biosynthesis and remodelling of PI species. Of relevance, we have recently observed that in adipocytes engineered with a knock down of the DEGS1 enzyme, the enzyme controlling the final step of the *de novo* biosynthesis of ceramides by promoting the desaturation of dihydroceramides to ceramides [79], results in decreased levels of glycerophospholipids, specially etherlipids and phosphatidylinositols. Whether the downregulation of these lipids is the result of the decreased ceramide pool, increased dihydroceramides, or alternatively occurs secondarily to homeostatic changes, is currently under investigation (Rodriguez-Cuenca, *unpublished*).

4.1.1 Sphingolipids modulate the synthesis and signalling mediated by Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2).

In terms of its concentration, **phosphatidylinositol-4,5-bisphosphate** is a relatively minor phospholipid in cell membranes. However, it is functionally important as a substrate for signalling proteins. Sphingosine - at low doses - activates the enzyme phosphatidylinositol-4 kinase that converts PI into PI4P. However, this reaction is repressed when sphingosine is present at high concentrations as a result of the biosynthesis of sphingosine phosphate [145, 146]. Additionally, gangliosides also activate PIP5K and increase PI(4,5)P_2, which contributes to the inflammatory response via NFkB activity [147]. Thus, these data strongly support that sphingolipids both regulate the biosynthesis of PI derivatives and interfere with their signalling events.

Quantitative changes in the sphingolipid pools of the plasma membrane are pathophysiologically relevant as they perturb the microenvironment and compartmentalization of PI(4,5)P2, and compromise the recruitment of specific signalling proteins to the membrane. Some illustrative examples of this link between sphingolipids and impaired signalling are: **a)** increased levels of ceramides resulting from the ablation of Ceramide Kinase (CERK) directly impair PI(4,5)P_2 processing by PLC and prevent the recruitment of photoreceptors in drosophila cells [148]; **b)** depletion of SM in otherwise typically rich domains, disperse PI(4,5)P_2 and inhibit the recruitment of GTPases leading to abnormal cytokinesis in Hela cells [149].

Moreover, sphingolipids directly compete but also occasionally synergise with the effect exerted by $PI(4,5)P_2$ on different enzymes and proteins. For instance, whereas $PI(4,5)P_2$ facilitates the phosphorylation of ERM proteins in the plasma membrane (which plays a crucial role in the organization of membrane domains through interaction with transmembrane proteins and the cytoskeleton), ceramides exert the opposite effect and repress ERM phosphorylation by activating phosphatases [150]. Another example of the opposing effects between sphingolipids and $PI(4,5)P_2$, comes from studies on phospholipases (see section 5). Here, ceramides inhibit PLD activity by competing for its catalytic core with PIP_2 (activator) [151]. Similarly, SMs also compete -against PIP_2- and repress PLCδ1 [152]. However, in its phosphorylated form, C1P, does not compete but actually synergises with PIP_2 activation of PLA2G6 by modulating their interaction with PC in the membrane, and/or by increasing the catalytic efficiency respectively [153]. In this regard, an interesting finding in yeasts, which are theoretically transferable to higher eukaryotic cells, is that $PI(4,5)P_2$ (as well as other phosphoinositides such as $PI(3,5)P_2$, PI(5)P, PI(3)P) cooperates with and in a few cases interferes with a variety of sphingolipids such as dihydroceramides, dihydrosphingosines in targeting PH domains to membranes [17].

4.1.2 Sphingolipids modulate the signalling mediated by Phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$).

Phosphatidylinositol 3,4,5-trisphosphate is the product of phosphorylation of phosphatidylinositol (4,5)-bisphosphate by PI3K and localises to the plasma membrane. One of the key signalling events regulated by PI3K is the activation of AKT. The activation of PI3K converts $PI(4,5)P_2$ into $PI(3,4,5)P_3$ and facilitates the binding of $PI(3,4,5)P_3$ to the pleckstrin homology domain of protein kinase B/AKT. This induces both a conformational change and translocation as well as further activation by Ser473 phosphorylation via PDK1. Like AKT, PDK1 also possesses a $PI(3,4,5)P_3$/$PI(3,4)P_2$ binding Pleckstrin homology domain (PH) domain, necessary for PDK1-mediated phosphorylation of Thr308 and full AKT activation [154-159].

From the pathophysiological point of view, one of the most studied lipotoxic effects of ceramides in the context of insulin resistance, is the spatio-temporal dependent impairment of

PI3K/AKT [160, 161]. The mechanisms through which ceramides interfere with PI3K signalling include the PPA2 mediated dephosphorylation of AKT Ser473 [160, 162-165] and the inhibition of the recruitment of CAV1 to PI(3)K-associated receptor complexes within lipid raft microdomains [166]. Of note, ceramides disable the binding of $PI(3,4,5)P_3$ to the plecktrin homology domain of PKB/AKT, an effect mediated by activated PKCz and the subsequent phosphorylation of PKB/AKT [160, 162-164, 167]. Through this mechanism, ceramides impair the activation and translocation of insulin induced AKT kinase [168] and contribute to insulin resistance. Interestingly, the long chain ceramides directly interact with the catalytic domain of the PI3KC2b subunit, which disturbs its compartmentalization and suppresses its activation [169]. Additionally, the negative effect of ceramides on $PI(3,4,5)P_3$ related signalling also involves other domains that impair the translocation of certain $PI(3,4,5)P_3$-binding proteins such as GRP1 (general receptor for phosphoinositides-1), even in the presence of $PI(3,4)P_2$ or $PI(3,4,5)P_3$ [170].

S1P activates the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway via S1P receptors (coupled with Gi) and modulates a variety of cellular processes including cell proliferation, migration and stress response [171-173]. This contrasts with the observation that S1P reduces the ability of insulin to induce AKT phosphorylation via S1P2 receptor in muscle cells [63, 174] and highlights the differences amongst the three classes of PI3K members (class I that produces $PI(3,4,5)P_3$, and class II and III that produces PI(3P) and the cell specific peculiarities of similar signalling pathways.

In summary, the above data suggest that sphingolipids interfere with PI derivatives by affecting their *biosynthesis* and *metabolism*, and by *competing* with their signalling processes. This supports the concept that lipotoxic effects mediated by sphingolipids involve physicochemical changes in membranes as well as impairments in both signalling and metabolic reactions and contributes to common disorders such as insulin resistance and diabetes. The impact of sphingolipids on other phosphatidylinositol related signalling events in the context of disease has not yet been elucidated.

4.2 Phosphoinositides regulate sphingolipid metabolism.

There are also reciprocal effects between phosphatidylinositol metabolites and sphingolipid metabolism. This is particularly relevant in yeast, where PI provides phosphoinositol for the generation of inositolphosphorylceramides [175]. In addition to this direct metabolic link found in yeast, there is also a regulatory mechanism mediated by Pkh1, Torc2 and Ypk1 [176-178] that coordinates the biosynthesis of PI/phosphoinositides and sphingolipids. This co-regulation is essential to adjust the membrane lipid composition and maintain its integrity particularly in response to environmental stress.

In higher eukaryotic cells for instance, $PI(3,5)P_2$ is a potent and specific inhibitor of acid sphingomyelinase (aSMase) and therefore blocks the generation of pro-apoptotic ceramides [179-181]. However, phosphatidylinositol ether lipid analogues exert the opposite effect inducing apoptosis via activation of SMase and concomitantly increase in ceramide levels [182]. Another mechanism mediating this crosstalk is illustrated by $PI(4,5)P_2$, which has been shown to interact with the PH domain of ceramide kinase (CERK) and regulate its location to the Golgi and plasma membrane targeting. The localisation of CERK is important as it determines the levels of C1P in the plasma membrane [183]. Additionally, $PI(4,5)P_2$ and PI4P have been shown to modulate the activity of Sphingosine kinases 1 and 2 [184, 185].

Another example of multifaceted crosstalk between sphingolipids and PI derivatives is that PI4P - generated by a PI4 kinase IIIbeta- regulates the flow of ceramides between the ER and Golgi, and favours the synthesis of sphingomyelin by binding to the N-terminal pleckstrin homology domain of a ceramide transport protein (CERT) [186, 187]. This process is regulated by cholesterol as it affects the activity of PI4KIIa- by altering its membrane microenvironment- and by facilitating the recruitment of CERT to the Golgi where it promotes SM biosynthesis [188]. Additionally, the availability of PI4P synthesised by PI4 kinase IIIbeta and PI4KIIa also contributes to the delivery of beta-glucocerebrosidase (an enzyme relevant for the degradation of glucosylceramide) to the lysosome [189].

Overall, these examples show that both sphingolipids and PI derivatives, exert bidirectional regulatory roles in signalling. Intriguingly, the existence of cross-regulation between these lipid families suggests the existence of a tight homeostatic mechanism designed to regulate the relative abundance of

each other lipid species. This raises the possibility that pathologies characterised by defects in sphingolipid metabolism may benefit from strategies targeting phosphatidylinositol metabolites and *viceversa*.

5. Sphingolipids modulate phospholipid composition and signalling via regulating phospholipase activity.

In addition to the direct effect of sphingolipids on the biosynthesis of glycerophospholipids and their derivatives, there is also evidence that *modulation of signalling pathways* by sphingolipid is directly dependent on the metabolisation of phospholipids by major phospholipases as outlined below.

5.1 Sphingolipids regulate Phospholipase C (PLC) activity.

Phospholipase C includes a group of lipases that catalyse the hydrolysis of the linkage between glycerol and phosphate in glycerophospholipids, releasing DAG and choline-P from PC, and inositol 1,4,5 triphosphate from PIP_2. SM as well as glycosphingolipids have been reported to directly inhibit PLCδ1 and PLCγ through a mechanism dependent on the chain length and unsaturation index of the fatty acid moieties [190-193]. Conversely, in liposome based studies, sphingosine has been described to activate PI-PLC by direct physical interaction with PLC- in a calcium dependent manner [194, 195]. Alternatively, sphingosine as well as S1P were reported to activate PLC, enhancing PI turnover and calcium mobilization via G protein interaction [196-198], which supports an extracellular action of S1P. The discovery of S1P receptors (for a detailed review see [199, 200]) as mediators of such effects was described later on [201]. In this regard, the coupling of several S1P receptors (S1P1-4) to particular G proteins (Gi and Gq) has been demonstrated to activate PLC in a variety of cellular models [202, 203]. In the same line of evidence, C1P also activates PI-PLC and increase the levels of S1P [204], unfortunately the precise molecular mechanism is currently unknown.

An example of sphingolipids modifying PLC activity occurs in response to HDL associated sphingolipids. HDL in addition to glycerophospholipids, steroids, triacylglycerides and cholesteryl esters carries several sphingolipids including sphingomyelin, ceramides and lysosphingolipids [205] and their dysregulation has been associated to vascular dysfunction and Metabolic Syndrome [206].

Among them, S1P is the best characterised in relation to their impact in vascular biology [207]. Other biologically active lysophospholipids in HDL are *lysosulfatide* and *sphingosylphosphorylcholine*. Interestingly, these two lysophingolipids trigger specifically PI-PLC activation and stimulate cell proliferation in contrast with the Apo AI activation of PC-PLC associated with induced cholesterol efflux [208]. Here, authors suggested that these lysophingolipids could account for some anti-inflammatory effects mediated by HDL. These data support the concept that qualitative changes in the HDL sphingolipidome modify the physico-chemical properties of lipoproteins, impact signalling cascades mediated by PLC (as well as other lipases and phospholipases) and ameliorate or exacerbate the atherogenic risk. In this regard, despite the evidence in rodent models that overexpression /repression of SMS increase/decrease the atherogenic potential respectively [56, 58] the contribution of secondary changes in the glycerophospholipids and their metabolites was not evaluated.

Another revealing example of how sphingolipid metabolism interacts with glycerophospholipids has been shown for the intestinal alkaline SMase. Besides acting on SM, the alkaline SMase also directly hydrolyses PAF working as putative phospholipase C, removing the phosphocholine head group and generating 1-O-alkyl-2-acetyl-sn-glycerol. Interestingly, the hydrolysis of PAF and SM can be counterregulated by the presence of SM and PAF respectively [209]. If this apparent promiscuity is part of a regulatory mechanism that controls the levels of specific glycerophospholipids by different members of the sphingolipid family and enzymatic repertoire will need to be addressed in the future.

5.2 Sphingolipids regulate Phospholipase D (PLD) activity.

Phospholipase D catalyses the cleavage of the phosphodiester bond of structural phospholipids and releases phosphatidic acid (PA), a well-established second messenger**.** Sphingosine and sphingosine-1-phosphate, both activate PLD and directly compete with the inhibitory effect exerted by PE and PC [210-215], through both PKC dependent and independent mechanisms [216]. One of the mechanisms described for S1P as an activator of PLD is via activation of the SP1 receptor, as suggested by its role in control of IL8 secretion in human bronchial epithelial cells [217].

Sphingolipids also exert inhibitory effects on PLD. Ceramides for example inhibit phospholipase D, through a change in membrane biophysical properties which impair PKC translocation to the membrane, a required step for PLD activation [218-223]. Moreover, ceramides also repress the transcription of PLD mRNA in several cellular models [224-226]. This inhibitory effect of ceramides on PLD activity/expression is pathophysiologically relevant as indicated by its effects promoting: **a)** insulin resistance in hepatocytes [227], **b)** senescence [228] and **c)** apoptosis in granulosa cells [229]. However, the inhibitory effect of ceramides on PLD has been contested by some studies showing that it is the conversion from SM to ceramide in lipid rafts which activates PLD [230]. Therefore, it cannot be excluded that the activation of PLD is driven by the secondary conversion of ceramides to other bioactive sphingolipids.

Conversely, the PA released upon PLD activation regulates sphingolipid homeostasis. For instance, PA inhibits C1P stimulated macrophage migration [231], facilitates the translocation of Sphingosine kinase1 to membrane compartments [232], and modulates the activation of protein phosphatase 1 by ceramides [233]. Recently, Demirkan et al. identified a strong association between a SNP in PLD2 (rs12051548) and the ratio of sphingomyelins [SM(d18:1/23:0)] / [SM(d18:1/16:1)] in a GWAS study [234]. Nevertheless, the possible molecular mechanism responsible for such interaction is currently unknown. Globally considered, these data strongly support the existence of a bidirectional cross talk between sphingolipids and derivatives of glycerophospholipids that determine the biochemical repertoire and concentrations of second messengers.

5.3 Sphingolipids regulate phospholipase A2 activity.

PLA2 enzymes play an important role in lipid mediated inflammatory processes, signalling and phospholipid remodelling. Specifically, PLA2 phospholipases hydrolyse the bond at the second carbon group of glycerol, releasing the fatty acid moiety located in the sn2 position (e.g. arachidonic acid) and the corresponding lysophospholipid [235]. Several families of enzymes exist according to their dependence on calcium, location, substrate preference, specific roles in signalling, immune response and phospholipid remodelling.

5.3.1 Sphingolipids regulate cytosolic and calcium-independent phospholipase A2

Ceramide, ceramide-1-phosphate, sphingosines and other complex sphingolipids regulate the activity of cytosolic PLA2 (cPLA2) and the release of arachidonic acid (AA) in many cell types. For instance, C1P, sphingosine and lactosylceramide directly bind to the calcium binding (C2) domain of cPLA2a (PLA2G4A), facilitating its translocation from cytosol to Golgi (into PC rich areas) and activating the release of AA from substrate phospholipids [236-244]. This phenomenon is essential for biological processes such mast cell degranulation [245], stimulation of cell adhesion pathways between monocytes and endothelial cells [246], and vasodilation [247]. Most importantly, they contribute to the activation of prostaglandin biosynthesis in response to calcium and inflammatory agonists such as TNFα, ILβ1 [248, 249]. Another much less studied cPLA2, the plasmalogen selective PLA2, seems to be activated by ceramides in the brain [260, 261]. Interestingly, recent investigations in the liver of alcohol-dependent patients have proposed the activation of cPLA2 as a potential mechanism linking the increased levels of ceramides as consequence of the high activity of aSMase and the increase in hepatic LPC observed in that subjects [250]. Interestingly, increased levels of both LPC [251, 252] and ceramides [253] have been also shown in NAFLD patients. Whether the increase in LPC is due to a ceramide mediated activation of PLA2 or part of secondary homeostatic response/or lipotoxic insult will required further research.

In addition to the direct activation of cPLA2 by ceramides and derivatives, cPLA2 is also activated through indirect mechanisms via PKC activation [254, 255]. Moreover, cPLA2 is activated by C1P via JNK [256]. This latter mechanism has been considered as a cellular strategy for recycling structural phospholipids into energy generating substrates in CHO cells [257]. S1P also activates PLA2G6, releasing AA and LPC, which subsequently activate TRP5 cationic channels in HEK293 cells [258]. Conversely, other members of the sphingolipid family exert deterring effects on phospholipase A2 activity. For example, SM decreases the activity of cPLA2 by disturbing its binding to glycerophospholipids, which impairs the release of AA [259, 260]. Gangliosides are another example of inhibitors of PLA2, doing so by altering the biophysical properties of the membrane [261, 262].

Despite the fact that most of the research focused in PLA2 and ceramides has aimed to investigate the signalling/inflammatory events mediated by the release of fatty acids (e.g arachidonic acid) and lysophospholipids, it is conceivable that others aspects of PLA2 biology such as their role in the remodelling of phospholipids in partnership with LPLATs (lands' cycle) will be also affected. This is an area which our lab is currently exploring in relation to obesity and associated comorbidities.

5.3.2 Sphingolipids also regulate Secretory PLA2

Similarly to their effect on cPLA2, ceramides modify the fatty acid specificity exhibited by different secretory phospholipases by inducing structural defects in membrane bilayers [263, 264]. Additionally, ceramides mediate the TNFα induced upregulation of sPLA2 (and cox2) in mesangial cells through NFκB activation and increase production of PGE2, strongly suggesting that this pathway is important in the pathogenesis of renal injury [265].

What could be considered the "yang" effect on this occasion would be SM mediated inhibition of the activity of secretory phospholipase A2. This has been shown for sPLA2-V and results in the reduction of the release of AA [241, 266]. SM may also modulate the binding of PLA2 to membranes, a process that is highly dependent on membrane cholesterol levels [267-269].

The opposed effect of ceramides and sphingomyelins on the activity of sPLA2 is of biological relevance as it affects the clearance and metabolisation of lipoproteins. When HDL and LDL are enriched with exogenous long chain ceramides and/or ceramides obtained from the degradation of the SM pool, this stimulates the activity of sPLA2V [270-272], which facilitates the release of fatty acids. This effect is important as it forms cholesteryl esters in macrophages [273] and promotes aggregation and fusion of LDL [274]. As before, SMs exerts the opposite regulatory effect by inhibiting several secretory PLA2s even in the presence of oxidised phospholipids, which are well known activators of PLA2s [270-272].

Globally considered, these data indicate that the balance between the ceramide and SM pools accumulated within lipoproteins are important determinants of the release of pro-inflammatory lipids

as well as regulators of oxLDL-induced cholesterol esterification and therefore should be considered modulators of the atherogenic properties of lipoproteins.

5.3.3 Phospholipase A2 reciprocally modulates sphingolipid metabolism.

There is evidence that certain PLA2s exert a regulatory role on sphingolipid metabolism. For instance **a)** The activation of iPLA2b (PLA2G6) during ER stress seems to activate neutral sphingomyelinase and promotes the generation of ceramides, causing mitochondrial dysfunction and activating of mitochondrial apoptotic pathways [275, 276], **b)** another example is pancreatic phospholipase A2 (sPLA2IB) which stimulates the expression of neutral sphingomyelinase and acid ceramidase via a sPLA2 receptor [277]; in this regard, these phospholipases regulate the production of lipid mediators by regulating the expression of key enzymes in phospholipid and sphingolipid metabolism. All together, these studies support the existence of a closely regulated bidirectional feed-back system between sphingolipids and PLA2s.

Concluding remarks

Here we have brought together the biochemistry of sphingolipids and glycerophospholipids and identified current evidence supporting the existence of a bidirectional crosstalk between them. Its importance stems from the fact that their dysregulation can influence the progression of metabolic diseases. Despite the literature supporting the association between dysregulated levels of sphingolipids and profound changes in glycerophospholipid species at multiple levels, there is little knowledge about how these perturbations occur and how they may contribute to the metabolic lipotoxic burden exerted by sphingolipids at a cellular, tissue or organismal level, and more importantly how these changes specifically contribute to disease models.

Future studies

The development of new and more precise high-throughput analytical tools in the field of lipid biochemistry in combination with other disciplines such as biophysics, will expand our understanding of the consequences of small qualitative pathophysiological changes in the lipid composition of specific

organelles. These “new technological windows” should provide a more detailed picture of the existence and relevance of the bidirectional crosstalk between sphingolipids and glycerophospholipids.

The development of appropriate bioinformatics tools for data analysis is also essential to integrate their heterogeneity into workable testable models. The recent identification by Bjorkolm and colleagues of several sphingolipid binding motifs in mammalian membrane proteins using a probability algorithm (MOPRO) [278] will facilitate the identification of new targets of sphingolipid action. Interestingly, among the 672 novel candidates identified there are genes relevant for lipid homeostasis such as *scap* (a regulator of srebp1), and *mboat1* and *mboat2* (remodelling of glycerophospholipids). In the same line of evidences, the recent identification of 234 new candidate ceramide binding protein fragments by cDNA display and deep sequencing [279] reveals the presence of important genes for both sphingolipid and glycerophospholipids metabolism such as *asah1* (acid ceramidase) and *mboat7* (lysophosphatidylinositol acyltransferase) and *ptgds* (prostaglandin d2 synthase). We envisage an active phase of experimental validation of those candidate genes and others that will fuel our understanding of the complex crosstalk between sphingolipids and glycerophospholipids biology.

We believe that the use of genetically modified models to selectively increase or decrease pools of specific subsets of sphingolipids in a particular time/spatial frame (conditional tools) and in a precise organ (tissue specific) will help to dissect the relevance of sphingolipid related lipotoxicity on global lipid homeostasis and metabolism. These experimental models will assist in the understanding of the abnormal lipid profiles observed in multiple metabolic diseases such as obesity and insulin resistance, where both sphingolipids and glycerophospholipids are disturbed, and where, currently, it is challenging to specifically define a causative role for the changes in lipotoxic species (e.g. increase ceramides in insulin resistance models).

Globally considered, we are optimistic that with the power of new enabling technologies elucidating the regulatory networks controlling sphingolipid and glycerophospholipids homeostasis, specific targets will become feasible to exploit for therapeutic purposes.

NB. *During the review of this manuscript Matsuzaki et colleagues showed that cardiolipin bind to Ceramide kinase and regulate its activity and C1P levels in vitro [280], the pathophysiological consequences of such interaction need to be elucidated.*

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Figure 1. Structure of the major sphingolipids [A] and glycerophospholipids [B].

Figure 2. Schematic representation of some of the mechanisms described for sphingolipid interaction with glycerophospholipid metabolism. [A] Modulation of plasma membrane dynamics. [A] S1P lyase pathway [C] Phosphatidylinositides biosynthesis and modulation of enzyme/receptor actions by sphingolipids. [D] Interaction with PLC, PLA2 and PLD activity. Cer reads for ceramides, SM reads for sphingomyelin, and S reads for sphingosine and S1P reads for sphingosine-1-phosphate.

Figure 3. SREBP transcriptional and post-translational regulation: SREBP1 activity/inhibition is regulated depending on the environmental demands of the cells. Increase levels of ceramides has been shown to alter transcriptional levels of srebf1 as well as impair the maturation of SREBP1 in ER-Golgi (see text for additional details).

Figure 4. Summary of key references reporting direct effects of sphingolipids on glycerophospholipid biosynthesis and *vice versa*: positive effect (green) and negative effect (red).

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MARKED MANUSCRIPT

Sphingolipids and glycerophospholipids – the "ying and yang" of lipotoxicity in metabolic diseases.

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KEYWORDS: Sphingolipids, glycerophospholipids, lipotoxicity**.**

Abstract

Sphingolipids in general and ceramides in particular, contribute to pathophysiological mechanisms by modifying signalling and metabolic pathways. Here, we present the available evidence for a bidirectional homeostatic crosstalk between sphingolipids and glycerophospholipids, whose dysregulation contributes to lipotoxicity induced metabolic stress. The initial evidence for this crosstalk originates from simulated models designed to investigate the biophysical properties of sphingolipids in plasma membrane representations. In this review, we reinterpret some of the original findings and conceptualise them as a sort of *"ying/yang"* interaction model of opposed/complementary forces, which is consistent with the current knowledge of lipid homeostasis and pathophysiology. We also propose that the dysregulation of the balance between sphingolipids and glycerophospholipids results in a lipotoxic insult relevant in the pathophysiology of common metabolic diseases, typically characterised by their increased ceramide/sphingosine pools.

Abbreviations

[AA] Arachidonic acid, **[AKT]** RAC-alpha serine/threonine-protein kinase, **[CCTα]** Choline-phosphate cytidylyltransferase A, **[CERK]** Ceramide Kinase, **[C1P]** Ceramide-1-phosphate, **[CerS]** Ceramide Synthase, **[CERT]** ceramide transport protein, **[HDL]** High-density lipoproteins, **[PAF]** Platelet activating factor, **[PC]** phosphatidylcholine, **[PE]** Phosphatidylethanolamine, **[PH]** Pleckstrin homology domain, **[PI]** Phosphatidylinositol, **[PI3K]** Phosphatidylinositol 3-kinase, **[PLA2]** Phospholipase A2, **[PLC]**, Phospholipase C, **[PLD]** Phospholipase D, **[PS]** Phosphatidylserine, **[S1P]** sphingosine-1-phosphate, **[SM]** Sphingomyelin, **[SMase]** Sphingomyelinase **[SMS]** Sphingomyelin synthase **[Sphk1]** Sphingosine kinase 1, **[SPTLC]** Serine palmitoyltransferase, **[SRE]** Sterol regulatory element, **[SREBP1]** Sterol regulatory element-binding protein 1, **[TG]** Triglyceride.

INTRODUCTION

In the context of common metabolic diseases such as obesity, diabetes or non-alcoholic fatty liver disease (NAFLD), the concept of *"lipotoxicity"* refers to the inappropriate ectopic accumulation of lipids in non-adipose organs causing metabolic stress and dysfunction. Lipotoxicity in liver, skeletal muscle, heart, pancreas or brain has been identified as an important pathogenic contributor to their metabolic dysfunction. Lipotoxicity can operate at multiple levels spanning from cellular to organ levels and involves a repertoire of characteristic biochemical mediators. The severity of the lipotoxic insult is modulated by the specific cellular genetic vulnerability to the toxicity induced by lipids. When at physiological concentrations, most of these lipid species exert important physiological functions that contribute to structural, signalling or cellular homeostasis. Hence, these lipids *per se* only become toxic when: **a)** they accumulate in excessive quantities as a result of exacerbated biosynthesis and/or impaired turnover; **b)** they exhibit distorted qualitative properties (e.g. both biologically or chemically induced) and/or **c)** when their spatio-temporal location in the cell is atypical (*being in the wrong place at the wrong time*).

When in excess, sphingolipids behave as lipotoxic lipid species. Amongst them, ceramides and sphingosines are considered the "*usual pathogenic suspects"*. The biochemical processes of biosynthesis and remodelling of ceramides/sphingolipids are undoubtedly complex involving at least three well-characterised pathways described in detail elsewhere [1, 2]. These biosynthetic pathways are highly compartmentalised within the cell, which leads to the formation of discrete organelle lipid pools accumulating specific ceramide and other sphingolipids species. Their topographic localisation within the cell determines their supplied/targeted structures and also affects their signalling responses to extracellular and/or intracellular stimuli. Thus, it is the biochemical repertoire of sphingolipid molecules (e.g. sphingomyelin, ceramide, C1P, sphingosine, S1P as well as other complex sphingolipids), but also their topographic localisation and relative concentrations that determine their metabolic effects [3-8].

As typically observed in insulin resistant diabetes and also in some cancers, excessive accumulation of sphingolipids causes toxicity. In addition to their absolute abundance and localisation, the *relative proportions* of their specific biochemical species is also pathologically relevant. This concept has recently been coined as the ***"sphingolipid rheostat"*** [9], which refers to the relevance of the coordinated regulation and balance of specific sphingolipids (ceramides, sphingosine and S1P) for the control of cellular responses. This concept was originally studied in the field of cell fate and cancer, but recent investigation has demonstrated its validity for metabolic pathologies such as cardiovascular diseases [10, 11] and diabetes [12]. Therefore, this paradigm needs to be revisited to include new regulators of sphingolipid metabolism as well as the paracrine action of some of those sphingolipids [13].

The dysregulation of *the rheostat* either by increasing or decreasing specific types of sphingolipid species may exacerbate the pathology, may be used as biomarker of phenotypes or stages within the natural history of the disease, and could be helpful to identify nosological entities. Thus, sphingolipid profiles could be used as diagnostic, prognostic and therapeutic responsiveness biomarkers of metabolic stress and disease.

The current interest on the pathophysiological role of sphingolipids has been sparked by enabling technologies such as cellular and *in vivo* knockout models and improved lipidomic platforms. Technologies such as genetic engineering/editing and mass spectrometry have helped to elucidate the function of sphingolipid and glycerophospholipid regulated pathways, recognise their direct contribution to toxic cellular effects, and have identified new allostatic pathways designed to restore or maintain cellular homeostasis. The two most recognised sphingolipid-mediated toxic effects at cellular level are mitochondrial and endoplasmic reticulum stress and apoptosis [14, 15]. Another important but underestimated target of sphingolipid/ceramide mediated toxicity is ***the perturbation of glycerophospholipids homeostasis and their associated signalling events,*** the main focus of this review.

Using systems biology, Koberlin [16] and Vonkova [17] have provided new evidence for the close bidirectional interaction between sphingolipids and glycerophospholipids. Koberlin et al. have shown an intimate interaction between sphingolipids and glycerophospholipid metabolism conforming

a circular network of co-regulated lipids [16]. Their elegant approach has revealed a fundamental inter-lipid regulatory network that controls membrane lipid composition. Interestingly, these membrane lipid perturbations predicted the inflammatory responses in patients derived cells; enabling the functional assignment of lipids to specific Toll-like-receptor (TLR) signalling. Moreover, Vonkova and colleagues have shown the existence of cooperative (and also a few deterring) interactions between phosphatidylinositides (acting as driver ligands), and sphingolipids (in an auxiliary role), that mediate the recruitment of PH containing proteins to the membranes in both yeast and higher eukaryotic models. Moreover, in many cases, these cooperative events are highly specific and restricted to specific pairs of signalling lipids [17]. Thus, accumulating evidence indicates that both, sphingolipid and glycerophospholipid metabolism is essential for cellular membrane dynamics. Both types of lipids mutually regulate their biosynthesis and signalling paths within eukaryotic cellular models.

Our perception after reviewing the subject is that both, sphingolipid and glycerophospolipid metabolism are highly connected, integrated and driven in opposed directions, representing a harmonic *"Ying and Yang"* model of regulation contributing to membrane lipid homeostasis and lipid signalling events. Below, we provide the current experimental evidence supporting the existence of this regulatory crosstalk.

3. Evidence that sphingolipid metabolism is essential for cellular membrane dynamics and signalling.

Sphingomyelins and ceramides are present in the lipid rafts and caveolae domains of the plasma membrane. The relative concentration of sphingolipids, phospholipids and cholesterol [18-25] determines its biophysical properties varying from gel to liquid-ordered and to liquid-disordered structures [26, 27]. Other sphingolipid species, such as sphingosine or ceramide-1-phosphate, are also present in membranes at lower concentrations. Despite their low concentration, these lipids play essential roles as determinants of membrane dynamics and signalling pathways. For instance, sphingosine permeabilises phospholipid bilayers and increases vesicle leakage [28]; S1P modifies the gel-fluid transition of glycerophospholipids and stabilises the lipid bilayer structure [29]. Moreover C1P

is required for the formation of a negative membrane curvature [30] and it is not segregated into lateral lipid domains in phospholipid bilayers [31].

The use of artificial liposomes and *in vitro* cellular models have documented how temperature [32] and/or other mechanical stresses affect the interaction between ceramides and the other constituents of the plasma membrane [33]. There is evidence from these *in vitro* models that both, quantitative (relative percentage) and qualitative changes (length and unsaturation of fatty acid moieties) [34, 35] in sphingolipids (mostly ceramides and sphingomyelins) [36-43] perturb the biophysical properties of the plasma membrane (e.g. fluidity, lateral pressure and diffusion). These are non-subtle membrane perturbations and are likely to compromise metabolite transport and diffusion, receptor signalling, sorting and trafficking, formation of pores (e.g. in the outer membrane of the mitochondria [44]) and activation of apoptotic stimuli.

At physiological levels, SMs stabilise the interactions with sterols in bilayer membranes [45, 46] and regulate the trans bilayer movement and distribution of diacylglycerols (DAGs) [47] and galactosylceramides [48]. In contrast, ceramides -when in excess- associate with SM/PC and destabilise/ displace cholesterol within the membrane (reviewed in [26, 49, 50]). Ceramides facilitate the transbilayer lipid movement in membranes, which occurs irrespectively of whether ceramides are externally sourced or obtained by metabolising SM via SMases [51]. Additionally, there is evidence that ceramides spontaneously translocate (with limited capacity) from the outer to the inner leaflet of the plasma membrane [52, 53], which is relevant for signalling and trafficking. The position of ceramides in the inner or outer side of the membrane is important given their role promoting plasma membrane repair in response to injury [54] and control of exocytosis or endocytosis [55], processes that require the involvement of a functional plasma membrane.

Genetically modified murine models have helped to identify the primary effects on energy homeostasis of sphingolipid-related gene ablation. These experiments have also revealed robust compensatory mechanisms aiming to maintain cellular and tissue homeostasis, some of them of therapeutic potential. For instance, the SMS2KO mouse exhibited a healthy phenotype characterised by impaired fatty acid uptake and lipid droplet formation in response to high fat diet and protection against insulin resistance

[39]. Alterations in membrane lipid microdomains associated with CD36/FATP1 transporters and CAV1 [40] ultimately lead to decreased lipid storage. Conversely, overexpression of sphingomyelin synthases, SMS1 and SMS2, caused a pro-atherogenesis phenotype characterised by aggregation of lipoprotein particles [56]. Also interesting is the effect of sphingomyelin ablation on cellular signalling. Particularly, SMS deficiency or overexpression are expected to change membrane microdomains (lipid rafts, caveolae) enriched in SM, affect their biophysical properties and compromise transport, secretion, and signalling mechanisms such as apoptosis and cell death [57]. Furthermore, SMS1 and SMS2 deficiency/overexpression also affect the levels of sphingosine and S1P [58, 59] that could potentially act as second messengers. In the light of recent investigations it has been suggested that S1P may contribute to the development of insulin resistance in mice [60-63]. Thus, it is tempting to speculate that changes in S1P in those models may contribute to their overall phenotype.

Whereas the *in vivo* evidence for the crosstalk between sphingolipids and glycerophospholipids is still limited, the *in vitro* models have shown that sphingolipids and glycerophospholipids interact at a functional level. Moreover, this crosstalk goes beyond what could have initially been anticipated given the relatively marginal structural disruption observed from gain or loss of function genetic experiments. These *in vitro* studies show the direct regulation of glycerophospholipids metabolism by sphingolipids by interfering with major phospholipases, such as PLA2, PLC and PLD, releasing lysophospholipids and fatty acids that could act as signalling metabolites.

4. Evidence of the crosstalk between sphingolipid and glycerophospholipid metabolism.

Beyond the linear lipid biosynthesis pathways, accumulating evidence shows that these pathways crosstalk and intertwine with others metabolic networks. These interactions are evolutionary preserved in plants, fungi, protozoa, yeast as well as in invertebrates and vertebrates (reviewed in [64]).

One important crosstalk between sphingolipid and glycerophospholipid metabolism is the metabolic pathway modulating the conversion of sphingolipids to glycerophospholipids regulated by the S1P lyase recently reviewed by Kihara [65]. Briefly, degradation of S1P and sphinganine (dihydrosphingosine) by S1P lyase yields fatty acid aldehydes (hexadecenal and hexadecanal respectively) and

phosphoethanolamine. When these fatty aldehydes are oxidized and converted into palmitoyl-CoA by the concerted action of several enzymes, it enters the biosynthesis pathway of glycerophospholipids. Recent evidence also suggests that degradation of sphingosine and sphinganine may contribute to the biosynthesis of etherlipids (ether analogues of phospholipids) when the activity of the fatty aldehyde dehydrogenase (e.g. ALDH3A2) is inactive [66]. This suggests that interlipid conversion may be a common allostatic response in pathologies characterised by sphingolipid alterations. Interestingly, this lipid interconversion may lead to lipotoxicity as the hexadecenal is able to form aldehyde-derived DNA adducts [67]. The relevance of this in pathophysiological scenarios has not been investigated yet.

In the same line of evidence, Kondo et colleague have shown that phytosphingosine can also be metabolised to odd-numbered fatty acids and incorporated into glycerophospholipids, both in yeast and mammalian cells, or alternatively undergo mitochondria oxidation [68]. However, further investigation will be required to fully understand the biological significance of this pathway in overall lipid homeostasis.

2.1 Evidence for a cross talk between sphingolipids and phosphatidylethanolamine

Phosphoethanolamine, the other degradation product of S1P and sphinganine, can enter the CDP-ethanolamine pathway to generate phosphatidylethanolamine (PE) [69-71]. Moreover, PE can be further converted to other glycerophospholipids such as PC and PS, via PEMT and PDSS2 enzymes respectively.

Proof of this close relationship between sphingolipids and phosphatidylethanolamine is the evidence that genetic manipulation of sphingolipids biosynthesis impairs PE homeostasis by limiting substrate availability. For instance, the simultaneous ablation of Sphk1 and Sphk2 [72] (the enzymes responsible of the phosphorylation of sphingosine to S1P) and the S1P lyase *in vivo* [72, 73] decreased PE levels and accumulated sphingosines, ceramides and sphingomyelins -as a result of the direct reutilization of the sphingosine backbone- [73] These data indicates that the biosynthesis of glycerophospholipids from sphingolipids intermediates is essential for the degradation of sphingolipids [74] and that perturbations in the S1P pool affects glycerophospholipid reservoirs. Interestingly, this interconversion is of particular

significance for the survival of the protozoan parasite *leishmania* being the major route for ethanolamine synthesis [75].

Additional evidence showing that sphingolipid metabolism affects glycerophospholipid pools comes from studies showing that ceramides also inhibit the CDP-ethanolamine 1,2 diradyl-sn-glycerol ethanolamine phosphotransferase (EPT) and reduce the levels of PE [69, 76]. Moreover, several *in vitro* and *in vivo* models of impaired biosynthesis of ceramides have shown both quantitative [16] and qualitative changes in the pools of PEs [43, 77] suggesting the presence of compensatory mechanisms. Whether this opposed direct action of ceramides vs. S1P regarding PE metabolism is part of a regulated homeostatic rheostat is at the present moment unknown.

Intriguingly, similar to PC (see section below), PE contributes to the biosynthesis of ethanolamine containing analogues of SM [78]. Thus, it is also conceivable that PE levels may limit the biosynthesis of specific sphingolipid species, in the same way that PCs do.

2.2 Sphingolipid mediated inhibition of phosphatidylcholine biosynthesis.

Short chain ceramides but not their structural analogues dihydroceramides [79], prevent the biosynthesis of PCs by inhibiting the enzyme CCTα [76, 80-82]. Inhibition of CCTα requires activation of cPLA2 and subsequent release of lysophosphatidylcholine (LPC) [83]. CTTα activity and mRNA gene expression is also repressed by sphingosine [84]. Interestingly, the inhibitory effect of sphingosine on CCTα is reversed in the presence of other glycerophospholipids such as PS, PI and PG [85].

The inhibitory effect of ceramides on PC biosynthesis is tightly controlled and apparently independent of the source of ceramides. Thus, *in vitro* experiments in lung epithelia overexpressing ceramide synthase 5 (CerS5) and/or sphingomyelinase (SMase) have shown an additive effect mediating the inhibition of the biosynthesis of phosphatidylcholine. Conversely, PC biosynthesis is stimulated when Cers5 is downregulated by SiRNA [86]. These data suggest that the PC biosynthetic pathway "senses" ceramide pools rather than only responding to a supraphysiological stress.

Similarly, SM analogues also inhibit the translocation and activation of CTT [87, 88]. Thus, the impairment in PC biosynthesis resulting from increased level of ceramides may be relevant to disease pathogenesis and has been proposed as one of many mechanisms for how aSMase may contribute to steatohepatitis [89]. Conversely, glucosylceramides have been shown to activate CTT as observed in in vitro models of Gaucher disease [90].

In line with these findings, global lipidomic analysis has lent further support to the existence of a solid crosstalk between sphingolipids and PC. For instance, the knock-down of *sptlc2* in RAW264 macrophages increases the levels of PC [16]. Similarly, the total knock-out of *sptlc123* in Huh7 hepatocytes results in increased phospholipids with an enrichment in PC species [91]. Paradoxically, the *sptlc2* heterozygote [39] and the heart specific *sptlc2* KO [92] mice exhibited a decrease in the PC levels as well as qualitative changes in the FA of the PC fraction. These data suggest that the changes observed in the PC pools in these models are likely secondary homeostatic responses directed to preserve cellular or tissue viability.

Moreover, sphingolipids also regulate the pool of PCs through indirect mechanisms. An illustrative example of this is the influence of SM levels on the hydrolysis of PC pools from VLDL, LDL and HDL lipoproteins by hepatic lipase (HL) (see also section 5.2). Thus, SM depletion stimulates the hydrolysis of PC (mainly PUFA-PC) as well as TG, indicating that SM acts as an inhibitor of HL [93]. Another effect relates to the activation of the ABCB4 receptor expressed in the canalicular membrane of hepatocytes whose function is to export PC into the bile. In this regard, Zhao and colleagues have shown that the cellular content of SM is critical for ABCB4 to facilitate the efflux of PC, opposite to the effects of other members of the ABC family such as ABCB1 [91].

PCs also reciprocally regulate sphingolipid metabolism. Examples of this regulation include: a) the activity of PCs acting as phosphocholine donors for SM biosynthesis [94-97]; b) the fact that choline deficient media/diets [98] or that pharmacological inhibition of choline kinase [99], both impair the synthesis of SM while concomitantly increasing ceramide levels. Conversely, the action of SMase on SM releases phosphocholine that can be incorporated into the cytidine biosynthetic pathway for the

generation of phosphatidylcholine [100]. Altogether these data strengthen the concept of a strong co-regulation between PC and SM biosynthetic and metabolic pathways.

2.3 Evidence for a crosstalk between sphingolipids and phosphatidylserine.

Serine is a common component of both PS and ceramides, suggesting that both types of lipids may be metabolically connected. This biochemical link has been confirmed by *in vitro* where labelled serine originated from PS was identified in ceramides [101]. Moreover, sphingosine, S1P and lyso-sphingomyelin increase the biosynthesis of PS and block the conversion of PS to PE by decarboxylation [102, 103] mediated by a PKC independent mechanism [104, 105]. In support of this regulatory loop, the ablation of sptlc2 gene in RAW264 macrophage caused the expected impairment in the *de novo* biosynthesis of ceramides but also decreased the levels of PS. Similarly, the elevation of SM mass by different mechanisms leads to the depletion of the PS pool from the plasma membrane [106] leading to mislocalisation of k-ras [33], this was prevented by inhibition of SM synthesis suggesting the existence of a concerted mechanism to regulate the levels of both lipids in the plasma membrane.

In addition to SM, other sphingolipids also affect the location of PS in the plasma membrane as well as some of the biochemical processes regulated by PS. Thus, the ceramides generated by activation of SMSase facilitate the synthesis of PS and also contribute to the translocation of PS to the outer membrane, a requirement for platelet secretion and thrombus formation [107]. Other sphingolipids such as sphingosines, due to their positive charge, prevent the binding of calcium to PS enriched vesicles [108-110] and/or negatively modulate substrate phosphorylation by PKC by directly competing with PS [111, 112]. This evidence indicates that ceramides facilitate PS biosynthesis and optimise cellular location, whereas other sphingolipids elicit a negative effect on PS homeostasis.

It is worth noting that a recent investigation in the forebrain of CerS6KO mice [113] has shown the paradoxical increase in PS levels. This may be interpreted as against ceramides having a positive effect on the PS pool. Nevertheless, in this model there is a specific depletion of C16:0 ceramides rather than a global depletion, and as the molecular mechanism to support this finding is unknown, further research is required.

In terms of our ying/yang concept, the yang of this erregulation is that phosphatidylserine reciprocally modulates sphingolipid metabolism. For instance, PS increases the activity and translocation of sphingosine kinase to membranes [114, 115] and also activates enzymes involved in the degradation of complex sphingolipids [116, 117] . Whether PS interferes with the activity of SMase is less clear as some studies have shown that PS inhibits PKC mediated activation of neutral SMase [118], whereas others indicate that PS stimulates neutral [119] and alkaline [120] SMSase activities.

PS also reverses some of the negative effects of sphingolipids on enzymatic activities. PS (as well as PI but not PC) reverses sphingosine mediated inhibition of transmembrane protein tyrosylprotein sulfotransferase (TPST), the enzyme that catalyzes the sulfation of tyrosines. Despite the molecular mechanism not being well characterised, the authors concluded that the positive charges of sphingosine and a long alkyl chain are fundamental for impairing the affinity of the protein-substrate and that PS is able to reverse that inhibition in a competitive manner [121].

Similarly, PS also reduces the inhibitory effect of sphingosine on the autophosphorylation of the insulin receptor tyrosine kinase [122]. In this regard, it has been suggested that the high affinity for PS may protect the receptor from interacting with the amine group of sphingosine. Globally, these investigations suggest that acidic phospholipids, such as PS, may prevent the interaction of sphingosine with the targeted proteins. Given that sphingosine inhibits other kinases such as SRC kinase [123], it is conceivable that this competitive interaction between sphingosine and PS may be common in other similar kinases. Further research will be needed to evaluate the biological significance of this competitive interaction.

2. 4 Bidirectional Crosstalk between Sphingolipid metabolism and Platelet Activating Factor.

Platelet Activating Factor (PAF) is an ether-glycerosphospholipid important for immune cell activation. The first insight for an interaction between PAF and sphingolipid biosynthetic pathways came from the discovery of a CoA independent transacetylase that mediates the transfer of an acetyl group from PAF in the synthesis of N acetylsphingosine [124-126]. More recently, we have learned that S1P promotes PAF synthesis [127, 128] and modulation of cell chemotaxis and inflammatory responses

in immune cells. Conversely, PAF increases SMase activity and facilitates the generation of ceramide microdomains, which are important for inflammation, apoptosis, activation of eNOS in EC cells [129], development of pulmonary oedema [130], and the activation of the enzyme scramblase that facilitates the exposure of PS in the outer membrane [131].

3. Sphingolipids modulation of phospholipid biosynthesis via SREBP activation.

How sphingolipids regulate the biosynthesis of phospholipids at a molecular level is a key question. SREBPS are important mediators of the biosynthesis of fatty acids, cholesterol and TGs. SREBPS also regulates the levels of phosphatidylcholines by regulating the expression of pcyt1a (the gene that encodes CTTα) at transcriptional and posttranscriptional mechanisms level [132, 133]. SREBPS may also indirectly increase pcyt1a expression and activity secondarily to the concomitant biosynthesis of fatty acids. Genome-wide analysis of SREBP1 binding in mouse liver has shown the presence of putative SRE motifs in the phosphatidylinositol synthase gene [134], suggesting that the regulation by srebp1 might not be restricted to PC biosynthesis. This concept is further reinforced by novel research in flies [135] and metazoans [136].

Sphingolipids control SREBP1 cleavage and activation by different mechanisms: a) the breakdown of sphingomyelins to ceramides in the plasma membrane displaces cholesterol to intracellular compartments where cholesterol represses the cleavage and activation of srebp1 and 2 [137]. Additional evidence indicates that high levels of ceramides resulting from SM hydrolysis inhibit SRE mediated gene transcription independently of changes in intracellular cholesterol trafficking through a mechanism not yet defined [138] and b) S1P stimulates SREBP1 cleavage and nuclear translocation via a S1P receptor [139].

Sphingolipids also regulate SREBP1 expression. For instance, ceramides have been reported to increase the levels of the precursor SREBP and decrease the levels of mature SREBP [140]. Moreover, accumulation of SM (either from external origin or as a result of the inhibition of SMase) represses srebp1 expression via a caveolin and Ras-ERK-MAPK-CREB signalling pathway in adipocytes [141]. Accordingly, dietary administration of sphingomyelin decreases hepatic lipid levels due to inactivation

of the LXR-SREBP1 pathway [142]. The potential pathophysiological relevance of these findings is highlighted by a recent study showing that increased levels of SMs are associated with decreased levels of srebp1 mRNA in the adipose tissue of obese women [141].

Altogether, this indicates that sphingolipids may regulate lipid homeostasis (including PC biosynthesis), by controlling both the expression and activation of SREBP1. This may be of particular relevance in tissues such as liver and adipose tissue where SREBP1 is an important regulator of lipid synthesis, and where its dysregulation contributes to the development of NAFLD and obesity associated adipose tissue dysfunction.

4. Sphingolipids interfere with phospholipid mediated signalling.

4.1 Sphingolipids modulate the biosynthesis and activity of phophatidylinositols.

Compared to the robust effect of sphingolipids on other phospholipids, the evidence that sphingolipids may interfere with the *de novo* biosynthesis of phosphatidylinositol phospholipids in high eukaryotic cells is still preliminary. However, it is known that the small subunit of the serine palmitoyltransferase (ssSPT) [143] is also a lysophosphatidylinositol acyltransferase 1-interacting protein [144]. Thus, this protein could be a pivotal signal co-regulating both the synthesis of ceramides and the biosynthesis and remodelling of PI species. Of relevance, we have recently observed that in adipocytes engineered with a knock down of the DEGS1 enzyme, the enzyme controlling the final step of the *de novo* biosynthesis of ceramides by promoting the desaturation of dihydroceramides to ceramides [79], results in decreased levels of glycerophospholipids, specially etherlipids and phosphatidylinositols. Whether the downregulation of these lipids is the result of the decreased ceramide pool, increased dihydroceramides, or alternatively occurs secondarily to homeostatic changes, is currently under investigation (Rodriguez-Cuenca, *unpublished*).

4.1.1 Sphingolipids modulate the synthesis and signalling mediated by Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$).

In terms of its concentration, **phosphatidylinositol-4,5-bisphosphate** is a relatively minor phospholipid in cell membranes. However, it is functionally important as a substrate for signalling

proteins. Sphingosine - at low doses - activates the enzyme phosphatidylinositol-4 kinase that converts PI into PI4P. However, this reaction is repressed when sphingosine is present at high concentrations as a result of the biosynthesis of sphingosine phosphate [145, 146]. Additionally, gangliosides also activate PIP5K and increase $PI(4,5)P_2$, which contributes to the inflammatory response via NKFB activity [147]. Thus, these data strongly support that sphingolipids both regulate the biosynthesis of PI derivatives and interfere with their signalling events.

Quantitative changes in the sphingolipid pools of the plasma membrane are pathophysiologically relevant as they perturb the microenvironment and compartmentalization of PI(4,5)P2, and compromise the recruitment of specific signalling proteins to the membrane. Some illustrative examples of this link between sphingolipids and impaired signalling are: **a)** increased levels of ceramides resulting from the ablation of Ceramide Kinase (CERK) directly impair $PI(4,5)P_2$ processing by PLC and prevent the recruitment of photoreceptors in drosophila cells [148]; **b)** depletion of SM in otherwise typically rich domains, disperse $PI(4,5)P_2$ and inhibit the recruitment of GTPases leading to abnormal cytokinesis in Hela cells [149].

Moreover, sphingolipids directly compete but also occasionally synergise with the effect exerted by $PI(4,5)P_2$ on different enzymes and proteins. For instance, whereas $PI(4,5)P_2$ facilitates the phosphorylation of ERM proteins in the plasma membrane (which plays a crucial role in the organization of membrane domains through interaction with transmembrane proteins and the cytoskeleton), ceramides exert the opposite effect and repress ERM phosphorylation by activating phosphatases [150]. Another example of the opposing effects between sphingolipids and $PI(4,5)P_2$, comes from studies on phospholipases. Here, ceramides inhibit PLD activity by competing for its catalytic core with PIP_2 (activator) [151]. Similarly, SMs also compete -against PIP_2- and repress PLCδ1 [152]. However, in its phosphorylated form, ceramide-1-phosphate, does not compete but actually synergises with PIP_2 activation of PLA2G6 by modulating their interaction with PC in the membrane, and/or by increasing the catalytic efficiency respectively [153]. In this regard, an interesting finding in yeasts, which are theoretically transferable to higher eukaryotic cells, is that $PI(4,5)P_2$ (as well as other phosphoinositides such as $PI(3,5)P_2$, PI(5)P, PI(3)P) cooperates with and in a few cases interferes with

a variety of sphingolipids such as dihydroceramides, dihydrosphingosines in targeting PH domains to membranes [17].

4.1.2 Sphingolipids modulate the signalling mediated by Phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P_3).

Phosphatidylinositol 3,4,5-trisphosphate is the product of phosphorylation of phosphatidylinositol (4,5)-bisphosphate by PI3K and localises to the plasma membrane. One of the key signalling events regulated by PI3K is the activation of AKT. The activation of PI3K converts PI(4,5)P_2 into PI(3,4,5)P_3 and facilitates the binding of PI(3,4,5)P_3 to the pleckstrin homology domain of protein kinase B/AKT. This induces both a conformational change and translocation as well as further activation by Ser473 phosphorylation via PDK1. Like AKT, PDK1 also possesses a PI(3,4,5)P_3/PI(3,4)P_2 binding Pleckstrin homology domain (PH) domain, necessary for PDK1-mediated phosphorylation of Thr308 and full AKT activation [154-159].

From the pathophysiological point of view, one of the most studied lipotoxic effects of ceramides in the context of insulin resistance, is the spatio-temporal dependent impairment of PI3K/AKT [160, 161]. The mechanisms through which ceramides interfere with PI3K signalling include the PPA2 mediated dephosphorylation of AKT Ser 473 [160, 162-165] and the inhibition of the recruitment of CAV1 to PI(3)K-associated receptor complexes within lipid raft microdomains [166]. Of note, ceramides disable the binding of PI(3,4,5)P_3 to the plecktrin homology domain of PKB/AKT, an effect mediated by activated PKCz and the subsequent phosphorylation of PKB/AKT [160, 162-164, 167]. Through this mechanism, ceramides impair the activation and translocation of insulin induced AKT kinase [168] and contribute to insulin resistance. Interestingly, the long chain ceramides directly interact with the catalytic domain of the PI3KC2b subunit, which disturbs its compartmentalization and suppresses its activation [169]. Additionally, the negative effect of ceramides on PI(3,4,5)P_3 related signalling also involves other domains that impair the translocation of certain PI(3,4,5)P_3-binding proteins such as GRP1 (general receptor for phosphoinositides-1), even in the presence of PI(3,4)P_2 or PI(3,4,5)P_3 [170].

S1P activates the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway via S1P receptors (coupled with Gi) and modulates a variety of cellular processes including cell proliferation, migration and stress response [171-173]. This contrasts with the observation that S1P reduces the ability of insulin to induce AKT phosphorylation via S1P2 receptor in muscle cells [63, 174] and highlights the differences amongst the three classes of PI3K members (class I that produces $PI(3,4,5)P_3$, and class II and III that produces PI(3P) and the cell specific peculiarities of similar signalling pathways.

In summary, the above data suggest that sphingolipids interfere with PI derivatives by affecting their *biosynthesis* and *metabolism*, and by *competing* with their signalling processes. This supports the concept that lipotoxic effects mediated by sphingolipids involve physicochemical changes in membranes as well as impairments in both signalling and metabolic reactions and contributes to common disorders such as insulin resistance and diabetes. The impact of sphingolipids on other phosphatidylinositol related signalling events in the context of disease has not yet been elucidated.

4.2 Phosphoinositides regulate sphingolipid metabolism.

There are also reciprocal effects between phosphatidylinositol metabolites and sphingolipid metabolism. This is particularly relevant in yeast, where PI provides phosphoinositol for the generation of inositolphosphorylceramides [175]. In addition to this direct metabolic link found in yeast, there is also a regulatory mechanism mediated by Pkh1, Torc2 and Ypk1 [176-178] that coordinates the biosynthesis of PI/phosphoinositides and sphingolipids. This co-regulation is essential to adjust the membrane lipid composition and maintain its integrity particularly in response to environmental stress.

In higher eukaryotic cells for instance, $PI(3,5)P_2$ is a potent and specific inhibitor of acid sphingomyelinase (aSMase) and therefore blocks the generation of pro-apoptotic ceramides [179-181]. However, phosphatidylinositol ether lipid analogues exert the opposite effect inducing apoptosis via activation of SMase and concomitantly increase in ceramide levels [182]. Another mechanism mediating this crosstalk is illustrated by $PI(4,5)P_2$, which has been shown to interact with the PH domain of ceramide kinase (CERK) and regulate its location to the Golgi and plasma membrane targeting. The localisation of CERK is important as it determines the levels of C1P in the plasma membrane [183].

Additionally, $PI(4,5)P_2$ and PI4P have been shown to modulate the activity of Sphingosine kinases 1 and 2 [184, 185].

Another example of multifaceted crosstalk between sphingolipids and PI derivatives is that PI4P - generated by a PI4 kinase IIIbeta- regulates the flow of ceramides between the ER and Golgi, and favours the synthesis of sphingomyelin by binding to the N-terminal pleckstrin homology domain of a ceramide transport protein (CERT) [186, 187]. This process is regulated by cholesterol as it affects the activity of PI4KIIa- by altering its membrane microenvironment- and by facilitating the recruitment of CERT to the Golgi where it promotes SM biosynthesis [188]. Additionally, the availability of PI4P synthesised by PI4 kinase IIIbeta and PI4KIIa also contributes to the delivery of beta-glucocerebrosidase (an enzyme relevant for the degradation of glucosylceramide) to the lysosome [189].

Overall, these examples show that both sphingolipids and PI derivatives, exert bidirectional regulatory roles in signalling. Intriguingly, the existence of cross-regulation between these lipid families suggests the existence of a tight homeostatic mechanism designed to regulate the relative abundance of each other lipid species. This raises the possibility that pathologies characterised by defects in sphingolipid metabolism may benefit from strategies targeting phosphatidylinositol metabolites and *viceversa*.

5. Sphingolipids modulate phospholipid composition and signalling via regulating phospholipase activity.

In addition to the direct effect of sphingolipids on the biosynthesis of glycerophospholipids and their derivatives, there is also evidence that *modulation of signalling pathways* by sphingolipid is directly dependent on the metabolisation of phospholipids by major phospholipases as outlined below.

5.1 Sphingolipids regulate Phospholipase C (PLC) activity.

Phospholipase C includes a group of lipases that catalyse the hydrolysis of the linkage between glycerol and phosphate in glycerophospholipids, releasing DAG and choline-P from PC, and inositol 1,4,5 triphosphate from PIP_2. SM as well as glycosphingolipids have been reported to directly inhibit

PLCδ1 and PLCγ through a mechanism dependent on the chain length and unsaturation index of the fatty acid moieties [190-193]. Conversely, in liposome based studies, sphingosine has been described to activate PI-PLC by direct physical interaction with PLC- in a calcium dependent manner [194, 195]. Alternatively, sphingosine as well as S1P were reported to activate PLC, enhancing PI turnover and calcium mobilization via G protein interaction [196-198], which supports an extracellular action of S1P. The discovery of S1P receptors (for a detailed review see [199, 200]) as mediators of such effects was described later on [201]. In this regard, the coupling of several S1P receptors (S1P1-4) to particular G proteins (Gi and Gq) has been demonstrated to activate PLC in a variety of cellular models [202, 203]. In the same line of evidence, C1P also activates PI-PLC and increase the levels of S1P [204], unfortunately the precise molecular mechanism is currently unknown.

An example of sphingolipids modifying PLC activity occurs in response to HDL associated sphingolipids. HDL in addition to glycerophospholipids, steroids, triacylglycerides and cholesteryl esters carries several sphingolipids including sphingomyelin, ceramides and lysosphingolipids [205] and their dysregulation has been associated to vascular dysfunction and Metabolic Syndrome [206]. Among them, S1P is the best characterised in relation to vascular biology [207]. Other biologically active lysophospholipids in HDL are *lysosulfatide* and *sphingosylphosphorylcholine*. Interestingly, these two lysophingolipids trigger specifically PI-PLC activation and stimulate cell proliferation in contrast with the Apo AI activation of PC-PLC associated with induced cholesterol efflux [208]. Here authors suggested that these lysophingolipids could account for some anti-inflammatory effects mediated by HDL. These data support the concept that qualitative changes in the HDL sphingolipidome modify the physico-chemical properties of lipoproteins, impact signalling cascades mediated by PLC (as well as other lipases and phospholipases) and ameliorate or exacerbate the atherogenic risk. In this regard, despite the evidence in rodent models that overexpression /repression of SMS increase/decrease the atherogenic potential respectively [56, 58] the contribution of secondary changes in the glycerophospholipids and their metabolites was not evaluated.

Another revealing example of how sphingolipid metabolism interacts with glycerophospholipids has been shown for the intestinal alkaline SMase. Besides acting on SM, the

alkaline SMase also directly hydrolyses PAF working as putative phospholipase C, removing the phosphocholine head group and generating 1-O-alkyl-2-acetyl-sn-glycerol. Interestingly, the hydrolysis of PAF and SM can be counterregulated by the presence of SM and PAF respectively [209]. If this apparent promiscuity is part of a regulatory mechanism that controls the levels of specific glycerophospholipids by different members of the sphingolipid family and enzymatic repertoire will need to be addressed in the future.

5.2 Sphingolipids regulate Phospholipase D (PLD) activity.

Phospholipase D catalyses the cleavage of the phosphodiester bond of structural phospholipids and releases phosphatidic acid (PA), a well-established second messenger**.** Sphingosine and sphingosine-1-phosphate, both activate PLD and directly compete with the inhibitory effect exerted by PE and PC [210-215], through both PKC dependent and independent mechanisms [216]. One of the mechanisms described for S1P as an activator of PLD is via activation of the SP1 receptor, as suggested by its role in control of IL8 secretion in human bronchial epithelial cells [217].

Sphingolipids also exert inhibitory effects on PLD. Ceramides for example inhibit phospholipase D, through a change in membrane biophysical properties which impair PKC translocation to the membrane, a required step for PLD activation [218-223]. Moreover, ceramides also repress the transcription of PLD mRNA in several cellular models [224-226]. This inhibitory effect of ceramides on PLD activity/expression is pathophysiologically relevant as indicated by its effects promoting: **a)** insulin resistance in hepatocytes [227], **b)** senescence [228] and **c)** apoptosis in granulosa cells [229]. However, the inhibitory effect of ceramides on PLD has been contested by some studies showing that it is the conversion from SM to ceramide in lipid rafts which activates PLD [230]. Therefore, it cannot be excluded that the activation of PLD is driven by the secondary conversion of ceramides to other bioactive sphingolipids.

Conversely, the PA released upon PLD activation regulates sphingolipid homeostasis. For instance, PA inhibits C1P stimulated macrophage migration [231], facilitates the translocation of Sphingosine kinase1 to membrane compartments [232], and modulates the activation of protein

phosphatase 1 by ceramides [233]. Recently, Demirkan et al. identified a strong association between a SNP in PLD2 (rs12051548) and the ratio of sphingomyelins [SM(d18:1/23:0)] / [SM(d18:1/16:1)] in a GWAS study [234]. Nevertheless, the possible molecular mechanism responsible for such interaction is currently unknown. Globally considered, these data strongly support the existence of a bidirectional cross talk between sphingolipids and derivatives of glycerophospholipids that determine the biochemical repertoire and concentrations of second messengers.

5.3 Sphingolipids regulate phospholipase A2 activity.

PLA2 enzymes play an important role in lipid mediated inflammatory processes, signalling and phospholipid remodelling. Specifically, PLA2 phospholipases hydrolyse the bond at the second carbon group of glycerol, releasing the fatty acid moiety located in the sn2 position (e.g. arachidonic acid) and the corresponding lysophospholipid [235]. Several families of enzymes exist according to their dependence on calcium, location, substrate preference, specific roles in signalling, immune response and phospholipid remodelling.

5.3.1 Sphingolipids regulate cytosolic and calcium-independent phospholipase A2

Ceramide, ceramide-1-phosphate, sphingosines and other complex sphingolipids regulate the activity of cytosolic PLA2 (cPLA2) and the release of arachidonic acid (AA) in many cell types. For instance, C1P, sphingosine and lactosylceramide directly bind to the calcium binding (C2) domain of cPLA2a (PLA2G4A), facilitating its translocation from cytosol to Golgi (into PC rich areas) and activating the release of AA from substrate phospholipids [236-244]. This phenomenon is essential for biological processes such mast cell degranulation [245], stimulation of cell adhesion pathways between monocytes and endothelial cells [246], and vasodilation [247]. Most importantly, they contribute to the activation of prostaglandin biosynthesis in response to calcium and inflammatory agonists such as TNFα, ILβ1 [248, 249]. Another much less studied cPLA2, the plasmalogen selective PLA2, seems to be activated by ceramides in the brain [260, 261]. Interestingly, recent investigations in the liver of alcohol-dependent patients have proposed the activation of cPLA2 as a potential mechanism linking the increased levels of ceramides in that subjects as consequence of the high activity of aSMase and the

increase in hepatic LPC [250]. Interestingly, increased levels of both LPC [251, 252] and ceramides [253] have been also shown in NAFLD patients. Whether this increase in LPC is secondary to a ceramide mediated activation of PLA2 or part of secondary homeostatic response/or lipotoxic insult will required further research.

In addition to the direct activation of cPLA2 by ceramides and derivatives, cPLA2 is also activated through indirect mechanisms via PKC activation [254, 255]. Moreover, cPLA2 is activated by C1P via JNK mediated activation/phosphorylation of Ser505 [256]. This latter mechanism has been considered as a cellular strategy for recycling structural phospholipids into energy generating substrates in CHO cells [257]. S1P also activates PLA2G6, releasing AA and LPC, which subsequently activate TRP5 cationic channels in HEK293 cells [258]. Conversely, other members of the sphingolipid family exert deterring effects on phospholipase A2 activity. For example, SM decreases the activity of cPLA2 by disturbing its binding to glycerophospholipids, which impairs the release of AA [259, 260]. Gangliosides are another example of inhibitors of PLA2, doing so by altering the biophysical properties of the membrane [261, 262].

Despite the fact that most of the research focused in PLA2 and ceramides has aimed to investigate the signalling/inflammatory events mediated by the release of fatty acids (e.g arachidonic acid) and lysophospholipids, it is conceivable that others aspects of PLA2 biology such as their role in the remodelling of phospholipids in partnership with LPLATs (lands' cycle) will be also affected. This is an area which our lab is currently exploring in relation to obesity and associated comorbidities.

5.3.2 Sphingolipids also regulate Secretory PLA2

Similarly to their effect on cPLA2, ceramides modify the fatty acid specificity exhibited by different secretory phospholipases by inducing structural defects in membrane bilayers [263, 264]. Additionally, ceramides mediate the TNFα induced upregulation of sPLA2 (and cox2) in mesangial cells through NFκB activation and increase production of PGE2, strongly suggesting that this pathway is important in inflammation and the pathogenesis of renal injury [265].

What could be considered the "yang" effect on this occasion would be SM mediated inhibition of the activity of secretory phospholipase A2. This has been shown for sPLA2-V and results in the reduction of the release of AA [241, 266]. SM may also modulate the binding of PLA2 to membranes, a process that is highly dependent on membrane cholesterol levels [267-269].

The opposed effect of ceramides and sphingomyelins on the activity of sPLA2 is of biological relevance as it affects the clearance and metabolisation of lipoproteins. When HDL and LDL are enriched with exogenous long chain ceramides and/or ceramides obtained from the degradation of the SM pool, this stimulates the activity of sPLA2V [270-272], which facilitates the release of fatty acids. This effect is important as it forms cholesteryl esters in macrophages [273] and promotes aggregation and fusion of LDL [274]. As before, SMs exerts the opposite regulatory effect by inhibiting several secretory PLA2s even in the presence of oxidised phospholipids, which are well known activators of PLA2s [270-272].

Globally considered, these data indicate that the balance between the ceramide and SM pools accumulated within lipoproteins are important determinants of the release of pro-inflammatory lipids as well as regulators of oxLDL-induced cholesterol esterification and therefore should be considered modulators of the atherogenic properties of lipoproteins.

5.3.3 Phospholipase A2 reciprocally modulates sphingolipid metabolism.

There is evidence that certain PLA2s exert a regulatory role on sphingolipid metabolism. For instance **a)** The activation of iPLA2b (PLA2G6) during ER stress seems to activate neutral sphingomyelinase and promotes the generation of ceramides, causing mitochondrial dysfunction and activating of mitochondrial apoptotic pathways [275, 276], **b)** another example is pancreatic phospholipase A2 (sPLA2IB) which stimulates the expression of neutral sphingomyelinase and acid ceramidase via sPLA2 receptor [277], In this regard, these phospholipases regulate the production of lipid mediators by regulating the expression of key enzymes in phospholipid and sphingolipid metabolism through activation of their membrane receptors. All together, these studies support the existence of a closely regulated bidirectional feed-back system between sphingolipids and PLA2s.

Concluding remarks

Here we have brought together the biochemistry of sphingolipids and glycerophospholipids and identified current evidence supporting the existence of a bidirectional crosstalk between them. Its importance stems from the fact that their dysregulation can influence the progression of metabolic diseases. Despite the literature supporting the association between dysregulated levels of sphingolipids and profound changes in glycerophospholipid species at multiple levels, there is little knowledge about how these perturbations occur and how they may contribute to the metabolic lipotoxic burden exerted by sphingolipids at a cellular, tissue or organismal level, and more importantly how these changes specifically contribute to disease models.

Future studies

The development of new and more precise high-throughput analytical tools in the field of lipid biology and biochemistry, the availability of sophisticated bioinformatics integrative tools, in combination with other disciplines such as biophysics, will expand our understanding of the consequences of small qualitative pathophysiological changes in the lipid composition of specific organelles (plasma membrane, ER, mitochondria). These "new technological windows" should provide a more detailed picture of the existence and relevance of the bidirectional crosstalk between sphingolipids and glycerophospholipids, and also unravel both the impact of qualitative changes in particular subsets of lipids, as well as their spatio-temporal compartimentalization.

The development of appropriate bioinformatics tools for data analysis is also essential to integrate their heterogeneity into workable testable models. The recent identification of several sphingolipid binding motifs in mammalian membrane proteins using a tailored motif probability algorithm (MOPRO) developed by Bjorkolm and colleagues [278] will facilitate the identification of new targets of sphingolipid action. Interestingly, among the 672 novel candidates identified, there are receptors for IP3 and LPA involved in lipid related signalling as well as for *plc*, *scap* (a regulator of srebp1), and *mboat1* and *mboat2*, relevant for the remodelling of glycerophospholipids. Moreover, as new *in silico* evidence emerge, as the recent identification by cDNA display and deep sequencing of 234 new

candidate ceramide binding protein fragments among them *asah1* (acid ceramidase) and *mboat7* (lysophosphatidylinositol acyltransferase) and *ptgds* (prostaglandin d2 synthase) [279], we envisage an active phase of experimental validation to understand the complex crosstalk between sphingolipids and glycerophospholipids biology at multiple levels.

We believe that the use of genetically modified models to selectively increase or decrease pools of specific subsets of sphingolipids in a particular time/spatial frame (conditional tools) and in a precise organ (tissue specific) will help to dissect the relevance of sphingolipid related lipotoxicity on global lipid homeostasis and metabolism. These experimental models will assist in the understanding of the abnormal lipid profiles observed in multiple metabolic diseases such as obesity and insulin resistance, where both sphingolipids and glycerophospholipids are disturbed, and where, currently, it is challenging to specifically define a causative role for the changes in lipotoxic species (e.g. increase ceramides in insulin resistance models).

Globally considered, we are optimistic that with the power of new enabling technologies elucidating the regulatory networks controlling sphingolipid and glycerophospholipids homeostasis, and how these lipid families crosstalk, specific targets will become feasible to exploit for therapeutic purposes.

NB. *During the review of this manuscript Matsuzaki et colleagues showed that cardiolipin bind to Ceramide kinase and regulate its activity and C1P levels in vitro [280], the pathophysiological consequences of such interaction need to be elucidated.*

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Figure 1. Structure of the major sphingolipids [A] and glycerophospholipids [B].

Figure 2. Schematic representation of some of the mechanisms described for sphingolipid interaction with glycerophospholipid metabolism. [A] Modulation of plasma membrane dynamics. [B] Phosphatidylinositides biosynthesis and modulation of enzyme/receptor actions by sphingolipids. [C] Interaction with PLC, PLA2 and PLD activity. C reads for ceramides, SM reads for sphingomyelin, and S reads for sphingosine and S1P reads for sphingosine-1-phosphate

Figure 3. SREBP transcriptional and post-translational regulation: SREBP1 activity/inhibition is regulated depending on the environmental demands of the cells. SREBP regulates synthesis of the of the major long chain fatty acids that can be incorporated into triglycerides and phospholipids. Under conditions of high sterol and PUFA levels SREBP1 remains endoplasmic reticulum membrane as part of a tripartite complex with SCAP Insig1 protein preventing its maturation in the Golgi and its activation.

Figure 4. Summary of references reporting direct effects of sphingolipids on glycerophospholipid levels and *vice versa*: positive effect (green) and negative effect (red).

Figure 5. Diagram representing the potential mechanism by which dysregulation of the sphingolipids/glycerophospholipid crosstalk leads to modification of cell membrane property, altered cell functions and subsequent impairment in metabolic organs.

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Figure 1

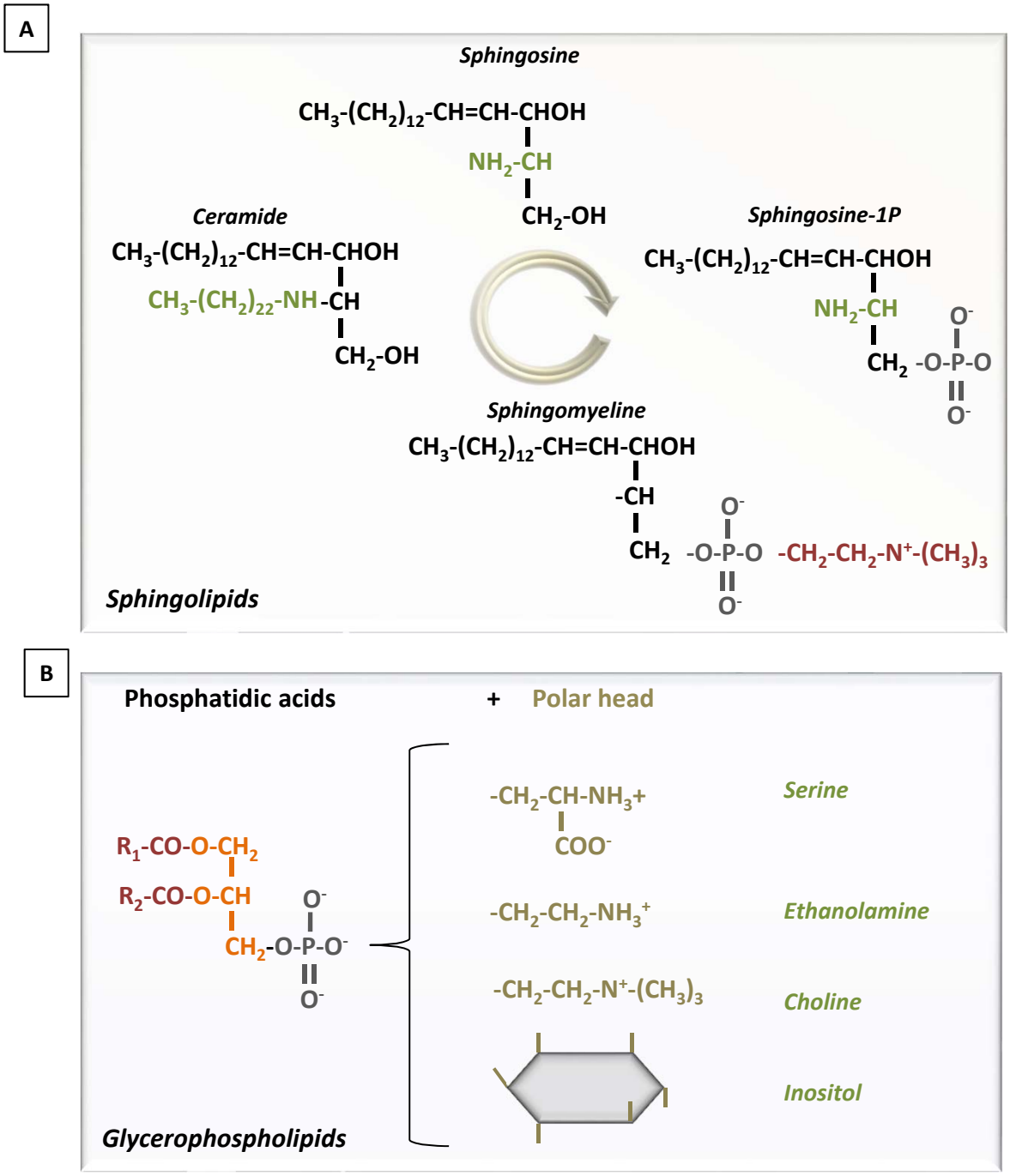

A
Sphingosine
CH3-(CH2)12-CH=CH-CHOH
NH2-CH
CH2-OH
Ceramide
CH3-(CH2)12-CH=CH-CHOH
CH3-(CH2)22-NH-CH
CH2-OH
Sphingosine-1P
CH3-(CH2)12-CH=CH-CHOH
NH2-CH
CH2 -O-P-O
Sphingomyeline
CH3-(CH2)12-CH=CH-CHOH
-CH
CH2 -O-P-O -CH2-CH2-N+-(CH3)3
Sphingolipids
B
Phosphatidic acids + Polar head
R1-CO-O-CH2
R2-CO-O-CH
CH2-O-P-O-
-CH2-CH-NH3+
COO-
Serine
-CH2-CH2-NH3+
Ethanolamine
-CH2-CH2-N+-(CH3)3
Choline
Inositol
Glycerophospholipids

Figure 2

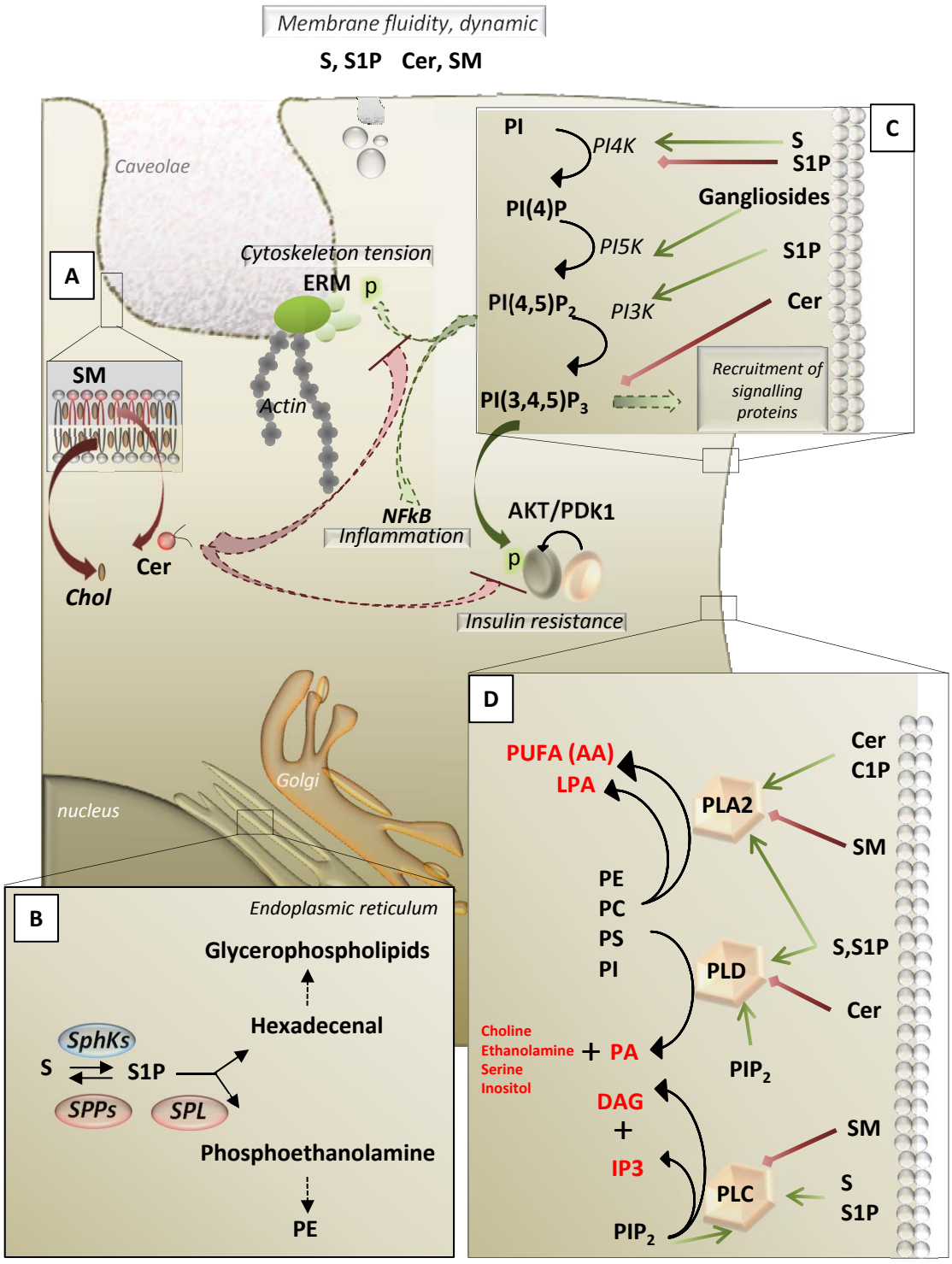

Membrane fluidity, dynamic
S, S1P Cer, SM
Caveolae
A
SM
Cytoskeleton tension
ERM
p
Actin
NFkB
Inflammation
Cer
Chol
AKT/PDK1
p
Insulin resistance
C
PI
PI4K
S
S1P
PI(4)P
Gangliosides
PI5K
S1P
PI(4,5)P2
PI3K
Cer
PI(3,4,5)P3
Recruitment of signalling proteins
nucleus
Golgi
B
Endoplasmic reticulum
Glycerophospholipids
Hexadecenal
SphKs
S
S1P
SPPs
SPL
Phosphoethanolamine
PE
D
PUFA (AA)
LPA
PE
PC
PS
PI
Choline
Ethanolamine
Serine
Inositol
+ PA
DAG
+
IP3
PIP2
PLA2
Cer
C1P
SM
PLD
S,S1P
Cer
PIP2
PLC
SM
S
S1P

Figure 3

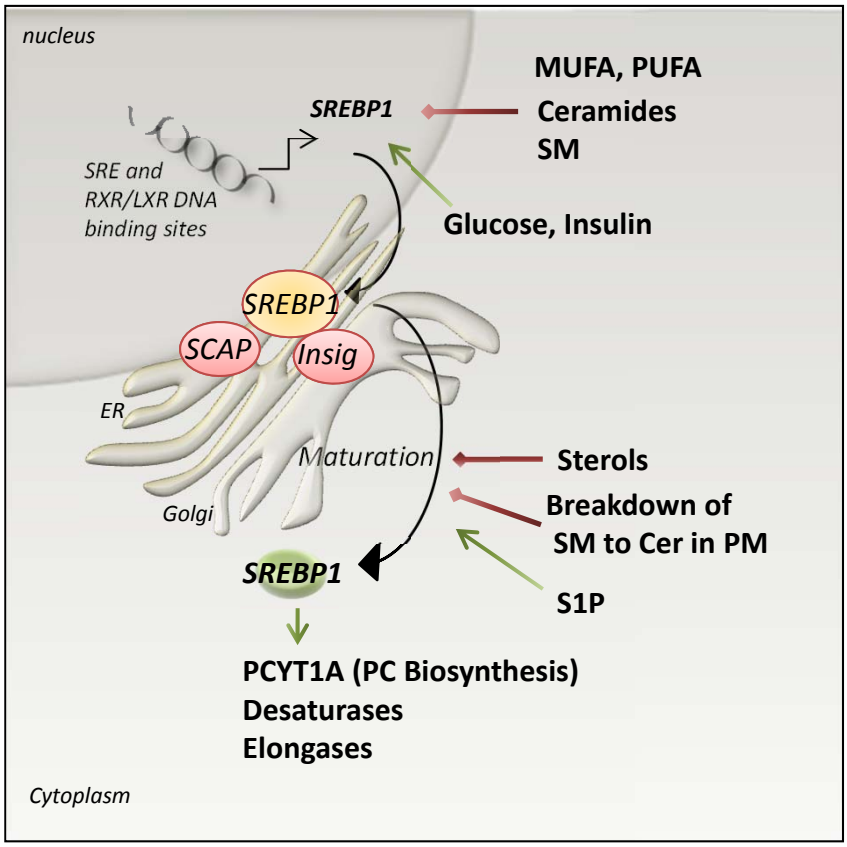
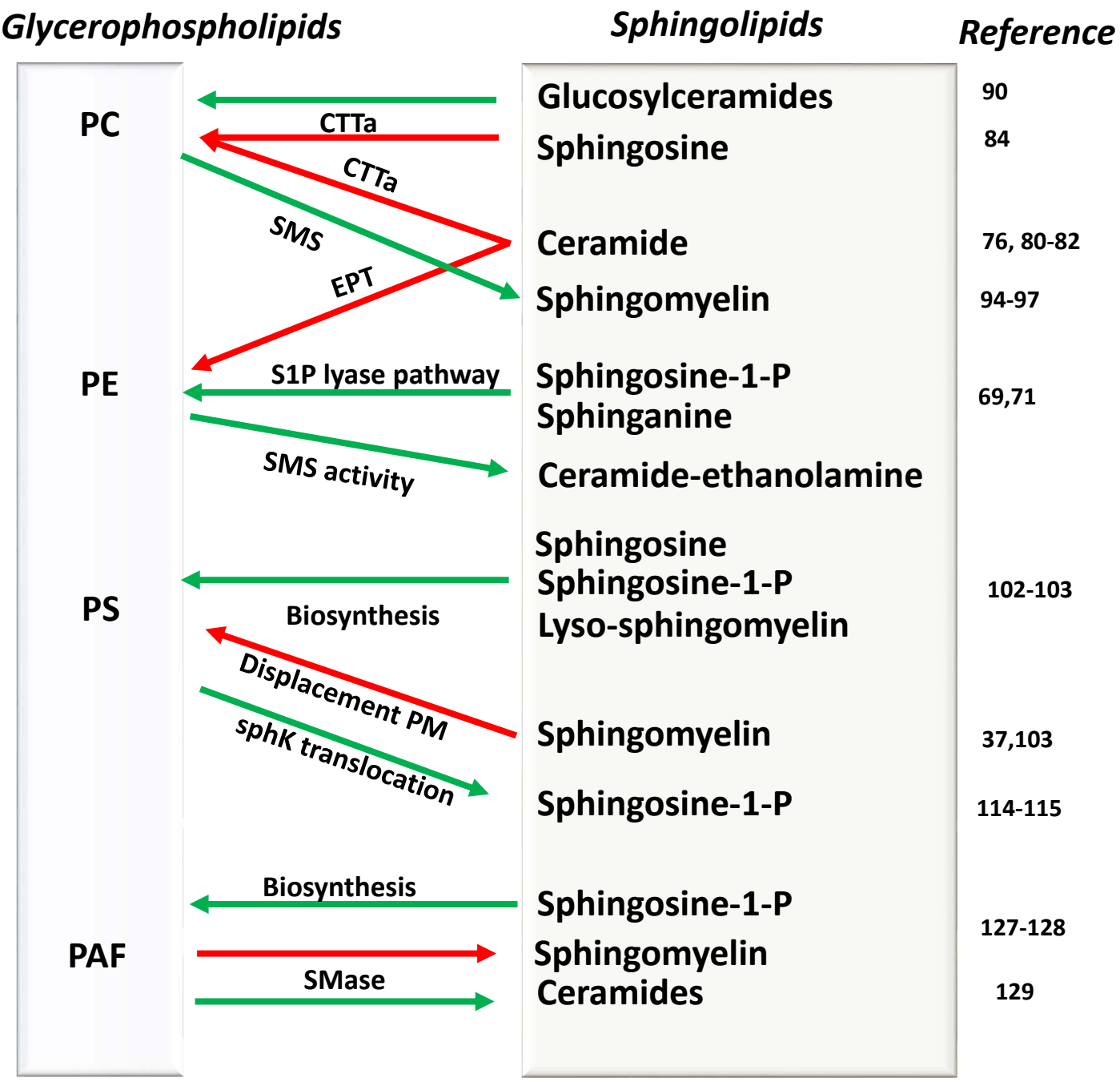

nucleus
MUFA, PUFA
SREBP1
Ceramides
SM
SRE and
RXR/LXR DNA
binding sites
Glucose, Insulin
SREBP1
SCAP
Insig
ER
Maturation
Sterols
Breakdown of
SM to Cer in PM
Golgi
SREBP1
S1P
PCYT1A (PC Biosynthesis)
Desaturases
Elongases
Cytoplasm

Figure 4


Glycerophospholipids
Sphingolipids
Reference
PC
PE
PS
PAF
CTTa
CTTa
SMS
EPT
S1P lyase pathway
SMS activity
Biosynthesis
Displacement PM
sphK translocation
Biosynthesis
SMase
Glucosylceramides
Sphingosine
Ceramide
Sphingomyelin
Sphingosine-1-P
Sphinganine
Ceramide-ethanolamine
Sphingosine
Sphingosine-1-P
Lyso-sphingomyelin
Sphingomyelin
Sphingosine-1-P
Sphingosine-1-P
Sphingomyelin
Ceramides
90
84
76, 80-82
94-97
69,71
102-103
37,103
114-115
127-128
129