# Lipids and lipid transporters

Chapter 8

# General discussion and summary

In my PhD project, I investigated the role of lipids and lipid transporters in several pathophysiological pathways with the aim to narrow the knowledge gap of their involvement in membrane dynamics, neuroinflammation and Alzheimer's disease.

In my work, I have been able to show that CERTs, the ceramide transfer proteins, not only participate in the biogenesis and lipid composition of EV but are also present in the cellular membrane, specifically in the lipid rafts (see Chapter 3). Furthermore, I discovered that CERTs are binding partners of several proteins involved in diverse cellular cascades mainly related to oxidative stress and immune response (see Chapter 4). My experiments, shown in Chapter 4, indicate that CERTL binds to the transcription factor STAT6 forming a complex in the cytoplasm. The co-expression of STAT6 and CERTL decreases gene expression, protein levels of CERTL and reducing also the SL composition in the cells, confirming the potential role of CERTs in involvement the immune response via different pathways.

Finally, my experiments described in chapter 7 helped to identify SL-related genes differentially expressed in AD patients which also display multiple types of epigenetic alterations affecting different cytosine states, corroborating the relevance of SL alterations in AD. Taken together, these findings provide insights into links between membrane dynamics, neuroinflammation and Alzheimer's disease and may serve as the basis for further research, and possible future intervention to slow and/or repair components of the pathology of AD and other neurodegenerative disorders. Below, I discuss some of my key findings in a larger framework and I provide a viewpoint.

**CERTS involved in cell membrane dynamic.**

The sphingolipid ceramide is important for membrane structure and extracellular vesicles (EVs) in cells [1]. Even if there is evidence on the regulation of the EVs formation by ceramide [2], it is still not clear how the lipid and proteins interact in the membrane to generate EVs and how the lipid profile of the vesicles is determined.

In **Chapter 2**, we demonstrated that CERTs are involved in the formation of EVs and controls the ceramide levels associated to EVs. Firstly, it was demonstrated that CERTs can be released extracellularly via multi vesicular endosomes (MVE) and that CERT forms a complex with Tsg101, protein which is part of the endosomal sorting complex required for transport (ESCRT) [3] and triggers generation of EV. Ceramide is necessary for the formation of the complex between Tsg101 and the START domain of CERTs. Thus, the reduction of Ceramide biosynthesis reduced the CERTs-

Tsg101 complex. In line with this, we demonstrated that the over-expression of CERTs in neuronal cells increases EV secretion while inhibition of CERTs with the drug HPA-12 (ceramide analogue) reduced EV formation and the concentration of ceramide and sphingomyelin in EV. Importantly, our results provide evidence on the fundamental role of CERTs in the formation and lipid composition of the EV. These findings open a new area for therapeutic strategies aimed to reduce biogenesis and ceramide enrichment of EV in disease conditions of the brain.

After demonstrating in **chapter 2** that CERTs are part of EV, and can be released extracellularly, in **chapter 3** we showed that CERTs are also localized in the lipid rafts. Lipid rafts are proteins-lipid domains present in the external leaflet of the plasma membrane composed by several class of lipids and diverse proteins [4]. The exact composition is still unclear, nevertheless, many studies demonstrated that these microdomains are sites of important physiologic regulations of many pathways [5] . To monitor lipid rafts composition is one of the biggest challenges nowadays and it if of extreme importance for better understanding particular molecular events [6]. Therefore, we developed a method to monitor lipid rafts dynamic using CERTs targeting antibodies. Antibodies are more accessible and reliable tools to target these multi factorial domains compared to the methods regularly used to record their composition and changes.

**CERTs and SLs play a role in inflammatory pathways and neurodegenerative processes**

The localization of CERTs in the lipid rafts strengthen the existing findings that these proteins are involved in several biological functions. Nevertheless, it is still not clear which is the role in triggering or modulating pathophysiological cascades, mainly during inflammation and neurodegeneration. In our previous studies we have demonstrated that CERTs are able to bind and activate C1q [7], central protein of the complement system cascade and the amyloid precursor protein (APP), fundamental protein for familial AD pathology [8]. Therefore, in order to elucidate other possible binding partners of CERTs in **chapter 4** we have performed a Y2H system analysis with the long isoform of CERTs and a human brain cDNA library. We have demonstrated that CERTL binds several proteins involved in oxidative stress and inflammatory cascades and neurodegenerative processes. In particular, we have further investigated the interaction of CERTL with the transcription factor STAT6. We have demonstrated that STAT6 and CERTL co-localize in the cytoplasm of the cells. However, we were unable to confirm the interaction with co-immunoprecipitation. CERTs are known to self-aggregate, in certain condition, compromising the interpretation of the immunoprecipitation results [9]. Nevertheless, we have showed that the expression of CERTL modulates the gene expression and the

protein levels of STAT6 and vice versa. Future studies could fruitfully explore these results, further trying to understand if CERTL can bind to DNA activating or repressing gene expression. Moreover, we showed that the presence of STAT6 and CERTL in cells modulate the SLs composition towards a reduction of short-chain ceramides and SM. Probably, the decrease in ceramide and SM is a consequence of an increase of S1P, known to have an anti-apoptotic and protective effect [10]. Future studies would need to explore the possible increase of S1P levels in the presence of CERTL and STAT6 in order to have a comprehensive picture of the pathways. Nevertheless, in line with previous findings the modulation of SLs upon the presence of CERTs and STAT6 might be of great importance in several pathophysiological conditions as for example AD or lipid storage disease [11] [12]. Overall, these promising results, together with the important function of CERTL STAT6 and SLs in immune reactions and neurodegenerative processes, would open up new research on new treatment avenues for brain diseases.

Based on the results of the **chapter 4**, we acquire a better understanding on the underling role of SLs in inflammatory processes with a focus on AD. Therefore, in **chapter 5** we have summarized the current available knowledge of SL species and their implications in neuroinflammation and neurodegeneration, in particular during AD. The review presents an integrated overview of the background concerning their properties and functions contributing to modulation of neuroinflammatory processes and neurodegenerative diseases. Moreover, we gave a comprehensive overview on compounds that are currently used to target SLs and their metabolites as promising molecules to treat neurodegenerative disorders and neuroinflammatory events.

As aforementioned, SL metabolism is strongly involved in the onset and progression of neurodegenerative processes. Therefore, in **chapter 6**, we have examined the current state of knowledge regarding the implication of SLs and CERT in neurodegeneration and which are the current promising molecules available in the clinic and in clinical research targeting the SLs and aiming to revert the pathology of AD. In AD, in fact, the excessive ceramide levels contribute to the pathology of the disease while ceramide metabolites, in particular S1P, seem to be protective [13]. Therefore, lately, several studies revealed that targeting SLs metabolism, specifically reducing ceramide levels and increasing S1P concentration, can be a valid therapeutic approach in AD [14]. This review allowed us to critically examine the available potential drugs targeting SLs and make new working hypothesis on how to treat AD modulating the SL pathway.

Since the involvement of SLs in the pathophysiology of the disease is becoming largely evident [15] [16] [17] in **chapter 7** we provided more insight into the genes and mechanisms behind the dysregulation of specific SL pathways in AD. We were able to identify SL-related genes differentially expressed in AD patients which also display multiple types of epigenetic alterations affecting different cytosine states, corroborating the relevance of SL alterations in AD. In this chapter we revealed a significant enrichment of SL-related gene expression alterations in the middle temporal gyrus (MTG) of AD patients in comparison to age- and sex-matched controls. Given the results from previous studies showing significant alterations of SL-related genes in AD patients, we aimed to provide a more comprehensive and detailed characterization of these alterations at the gene regulatory network level.

The data presented here may serve as a starting point to help filling the current knowledge gaps concerning the role of SLs in AD. Follow-up studies using extensive molecular profiling analyses across multiple brain regions in combination with perturbation experiments using in-silico and in-vivo AD models are needed to obtain a more comprehensive characterization and mechanistic understanding of the role of SLs in AD. Proteomic and lipidomic analysis could be performed in order to have a complete picture of dysregulation in the MTG of AD patients.

**Conclusions and future prospective**

In conclusion our findings suggest that SLs and SL transporters have a key role in modulating different biological pathways ranging from physiological cascades to pathological events.

First, we demonstrated that CERTs can influence EV formation and control their lipid composition and that this modulation is dependent on the ceramide production. Our findings open a new area for therapeutic strategies aimed to reduce biogenesis and sphingolipid enrichment of EV in disease condition of the brain.

Moreover, we have shown that CERTs are localized in the lipid rafts, and CERT targeting antibodies could potentially be used to monitor membrane dynamics and lipid rafts composition.

Secondly, we have then showed that CERTs are binding partner of several proteins, present in the brain and already known to participate in neuroinflammatory events and neurodegenerative processes. Moreover, we have shown that CERTs co-localize with STAT6, a transcription factor involved in adaptive immunity by transducing signals from extracellular cytokine in an immune response [18]. The overexpression of both proteins in the cell leads to a modulation of the CERTL

and STAT6 gene expression and a downregulation of ceramide and sphingomyelin levels. Our preliminary findings, shows that CERTs can modulate the microglia activation status, lowering their pro-inflammatory phenotype. The interaction and downstream effects of the STAT6 and CERTL complex, might act as a "switch-on" for several genes that potentially activates an anti-inflammatory and an anti-apoptotic signal. Future research should further develop and confirm these initial findings by analysing the interaction STAT6-CERT in different cell types. For example, myeloid cells or brain resident macrophages could be used as cell models in order to assess a putative role of CERTs and SLs in inflammation. Additionally, it would be important to develop new animal models.

Furthermore, we showed that SL-related genes are differentially expressed in AD patients displaying multiple types of epigenetic alterations and affecting different cytosine states, corroborating the relevance of SL alterations in AD. Our integrative analyses have revealed novel candidate AD-associated genes strengthening the hypothesis that the regulation of SLs and their metabolism is a key event in the exacerbation of the disease. CAV1, for example, is one of the genes that seems having a key role in reversing the disease. CAV1 has been linked with alterations in AD and inflammatory pathways. Our results open up new research lines investigating the connection between the CAV1 dysfunction in AD and inflammatory pathways. Cell or animal models, knock-out/in studies could be used to address possible role of CAV1 in AD. As lipid binding protein, CAV1 could be potentially used as a pharmacological target to treat AD. Importantly, future studies should aim to replicate these results in larger cohorts and to investigate the possible dysregulation of SLs related

All in all, my work provides some insight on the involvement of lipids and lipid transporters in pathophysiological mechanisms in the brain.

**References**


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