Identification of novel lyso-PS lipase in mammalian tissues

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by

Theja Sajeevan
20151057

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road, Pashan, Pune 411008, INDIA.

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Supervisor: Dr. Siddhesh S. Kamat

Certificate

This is to certify that this dissertation entitled **Identification of novel lyso-PS lipases in mammalian tissues** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Theja Sajeevan (20151057)** at **Indian Institute of Science Education and Research, Pune (IISER Pune)**, under the supervision of **Dr. Siddhesh S. Kamat, Associate Professor, Department of Biology**, during the academic year **2019-2020**.

Dr. Siddhesh S. Kamat
Associate Professor
Department of Biology
IISER Pune

Committee:

Guide: Dr. Siddhesh S. Kamat

TAC Member: Dr. Thomas Pucadyil

This thesis is dedicated to the five years at IISER.

Declaration

I hereby declare that the matter embodied in the report entitled **Identification of novel lyso-PS lipases in mammalian tissues** are the results of the work carried out by me at the **Department of Biology**, **Indian Institute of Science Education and Research, Pune (IISER Pune)**, under the supervision of **Dr. Siddhesh S. Kamat** and the same has not been submitted elsewhere for any other degree.

Theja Sajeevan
20151057

Date: 28th March, 2020

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Abstract

Serine hydrolases are one of the largest family of enzymes known to play a major role in many of the patho-physiological processes. ABHD12, a major brain lyso-PS lipase, belongs to the same family of proteins, are seen to prefer very long chain (VLC) lipids as their substrates in in vitro and endogenous experiments. At the same time the (S)-configuration of these lipids do not show any activity with this enzyme. From the identification of ABHD12, which is associated with a disease PHARC, and Bio-GPS data it was hypothesized that there may exist more lyso-PS lipases in other metabolic tissues. With this lead in hand we tested several metabolic tissues in the human body. From my studies, I could show that there are indeed lyso-PS lipases present in the membrane fraction of these tissue lysates which needs to be identified (annotated if necessary) and studied. As serine hydrolases are a major drug target in many diseases, identification and characterisation of new lipases will help in finding if there are diseases associated with these proteins.

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Chapter 1: Introduction

Lipids are a major class of biomolecules that are mainly seen to play a role in structural components, energy storage and signalling in the cells. A subtype of lipids are phospholipids, lipids with a phosphate head group attached to the glycerol backbone and acylation with free fatty acids. The deacylated derivative of phospholipids, lysophospholipids (or lipid moieties with only one hydrophobic fatty acid tail) are produced by phospholipases, enzyme that cleaves of the fatty acid chain from the glycerol backbone. Phospholipases known to do these are either phospholipase A1 (PLA1) or phospholipase A2 (PLA2) depending on position of hydrolysis. Lysophospholipids have been detected in plasma membrane and majority of them acts as a signalling molecule (1).

Phosphatidylserine (PS) is an important component of cell membrane, usually present in the inner leaflet of the membrane and are recognised to give the membrane curvature and act as anchor point for series of signalling molecules. PS also acts as a precursor for lysophosphatidylserine (lyso-PS) that is deacylated form of PS and is known to act as a signalling molecule. PS specific PLA1 secreted from platelets are known to be the major PS-lipase responsible for lyso-PS production in platelets. There are known to be 22 distinct PLA1 proteins. (2)

Lyso-PS is of growing interest due to its profound impact on cellular functions. It also acts as a lipid mediator and gives distinct divergent signals in spite of having a similar structure. Lyso-PS plays role in both in-vitro and in-vivo cellular responses. It mediates inflammation by inducing mast cell degranulation and histamine release at consequent. (22) (23) Other functions associated to lyso-PS are enhancement of nerve growth factor (NGF) induced dendritic sprouting, phagocytosis of apoptotic cells via neutrophils and initiation of acute inflammation. Few studies show involvement of lyso-PS in neurite outgrowth, migration of glioma cells and calcium mobilization. (24)

It has been shown previously that acyl transferases, MBOAT 1 and 5 used lyso-PS as a substrate. Decrease in this action of acyl transferases and lyso-PS lipases lead to the accumulation of lyso-PS in cells. (2) Accumulation of any molecule above a certain

limit is toxic to the cell and thus needs to be broken down. Blankman and her colleagues had shown accumulation of lyso-PS in PHARC (a neurodegenerative disorder) mouse models leading to neuro-inflammation and neurobehavioral defects. (3) PHARC or also known as Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract is a rare autosomal recessive genetic disorder which affects both central and peripheral nervous system. (4)

This disease was first reported in a Norwegian family, as a result of which 19 patients from 4 different families underwent clinical manifestation and homozygosity mapping was done to identify the particular gene responsible for this disease. (4) The result of this was a gene named ABHD12 (α/β hydrolase domain–containing protein 12) present on chromosome 20 (Chromosome 20p11 in humans). (5) 5 different mutations of ABHD12 were found out of which 4 resulted in abolishment or reduced activity of the gene. (4) ABHD12 is a member of the metabolic serine hydrolase family of enzymes. ABHD12 was known to exhibit MAG (Mono Acyl Glycerol) lipase activity in vitro. After rigorous untargeted and targeted metabolomics study on ABHD12 +/+, +/- and -/- mouse brains, it was seen that lyso-PS species especially one with longer chain lengths were highly elevated in the -/- ABHD12 mouse brains than the +/+. Thus from this it was concluded that ABHD12 hydrolyses lyso-PS and act as lyso-PS lipase in mammalian brain. (3)

Identification ABHD12 as an important lyso-PS lipase and accumulation of higher chain length lyso-PS species generated in mammalian brain tissues generated interest in thorough biochemical characterization of this enzyme. The substrate profiling of this enzyme is under investigated and in this project I tried do profile substrates for this enzyme based on the chain length and unsaturation of the hydrophobic fatty acid chains.

Lipases are a large category of enzymes which carry out the hydrolysis of lipids. Serine hydrolases form a major part of these lipases, which in itself is a diverse class of enzymes. These are further divided into 2 classes called 1. Serine proteases and 2. Metabolic serine Hydrolases. Serine proteases are usually present in their inactive pro-enzyme form which is activated by cleavage inside the human body by the action

of another enzyme. Metabolic serine hydrolases have a serine nucleophile at the active site of the enzyme forming the catalytic triad. The mechanism is carried forward by the formation of an acyl intermediate followed by saponification of the product by water molecule. The serine residue is regenerated for the next cycle. (6)

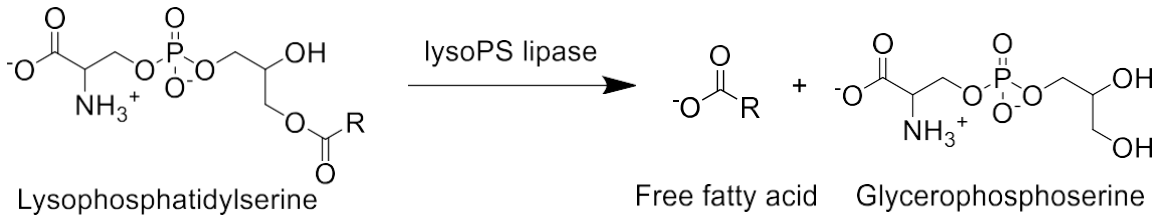


Fig: 1.1 Biochemical mechanism of lipases: Lipase reaction carried out by lipases with lyso-PS as the substrate (R= fatty acyl chain)

Few lipases belonging to serine hydrolase family have the alpha beta hydrolase fold with beta sheets flanked on either sides by alpha helices. (7) Serine hydrolases play an important role in many physiological processes and thus is targeted by several drugs to treat obesity, neurodegenerative diseases, etc. But the fundamental understanding, biological activities or inhibitor studies is lacking in more than hundreds of these serine hydrolases.

Biochemical characterisation of serine hydrolases are mainly done using activity based protein profiling. This method targets the active site of the enzyme through a covalent interaction. Fluorophosphonate (FP) are used as the reactive group due to its high electrophilic property which allows it to bind to the serine nucleophilic center covalently. FP reacts with the active enzyme and not with the inactive precursor or inhibitor bound forms. Rhodamine or biotin can be used as the tag for fluorescence study or pull down assays respectively. (8)(9) Since, Bio-GPS shows comparative mRNA levels to brain ABHD12 mRNA levels but negligible expression of ABHD12 enzymes in these mammalian metabolic tissues (e.g. lungs, liver, kidney, heart and muscle) made us hypothesize that there are likely other tissue specific lyso-PS lipases in the aforementioned tissues, as lyso-PS is known to exist in these tissues and remains unchanged in ABHD12 knockout mice.

As ABHD12 is the major lyso-PS lipase in brain, the ABHD12$^{-/-}$ mice model would be used as a control to annotate a different tissue specific lyso-PS lipase. Activity based protein profiling, inhibitor activity assay, pull down assays, transfection and overexpression experiments would help in identification and characterisation of the enzyme. Discovering a novel lyso-PS lipase will lead to annotating a novel function to an incompletely characterised or identifying a new serine hydrolase and thus decoding a new pathway for metabolism of this lipid mediator.

Chapter 2: Materials and methods

2.1 ABHD12 expression in HEK293T cells – Cell culture and transfection

Full length WT ABHD12 cDNA was cloned in pCMV-Sport6 vector between NotI and SalI restriction sites with ampicillin antibiotic resistance. Catalytically inactive S246A ABHD12, (serine residue at 246th position is changed to alanine) was generated using whole plasmid PCR and DpnI digestion based site directed mutagenesis. The HEK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1X penicillin streptomycin at 37 °C with 5% (v/v) CO_2. The cells were allowed to grow to reach a confluency of 20-30% (in 15 cm plates) and then transiently transfected with the above plasmids in the presence of polyethyleneimine (1:3; plasmid: polyethyleneimine). Plasmids were cultured in serum free DMEM and incubated for 45 minutes and transferred to growing HEK cell plates. 15 µg of DNA plasmid was used for each 15 cm plate.

Mock control cells were transfected with empty vector using same protocol. The cells were allowed to grow till 100% confluency was attained. For harvesting the cells, the plate was washed in sterile DPBS (Dulbecco's phosphate buffer saline) and scrapped. The collected cells were centrifuged at 4 °C and the pellet was stored in -80 °C. The transfected cells were thawed on ice and re-suspended in 500 µL of PBS (phosphate buffer saline) solution. The cells were lysed by probe sonication for 50 seconds with 5 seconds on and 5 seconds off pulse at an amplitude of 60%. The lysed cells were ultra-centrifuged at 4 °C for 45 minutes at a speed of 1,00,000 g. The supernatant was discarded and the pellet re-suspended in 500 µL of PBS and sonicated till the pellet gets dissolved (for 5 seconds, 1 on 1 off). Expression and activity check for the cells were done using western blotting and activity based protein profiling (ABPP) respectively. Protein estimation using BCA kit was done and each sample was diluted to 1mg/ml concentration for a volume of 100 µL.

2.2 Protein estimation

Protein concentration estimation was done using BCA protein assay kit by comparing with standards. 200 µL of the BCA reagent is incubated with 10 µL of protein after appropriate dilution in a 96 well plate at 37 °C for 30 minutes and absorbance was measured using a varioscan at 562 nm. After estimation, final samples were prepared at 1 mg/mL concentration (with PBS buffer).

2.3 Expression and activity check

For expression check, samples were run on a resolved 10% SDS PAGE gel and transferred to a PVDF membrane in 1X transfer buffer overnight at 4 °C at constant 60 Amp. After transfer the membrane was blocked for around 2 hours in 5% (w/v) milk in PBST (0.1% (w/v) Tween) (phosphate buffered saline with tween 20) and then probed overnight with primary antibody, anti-ABHD12 in 1:1000 dilution. The membrane was washed for 3 times in PBST and incubated in anti-rabbit secondary antibody at a dilution of 1:10,000 for 1 hour at room temperature (RT). The membrane is washed again in PBST three times and blot was developed using HRP sensitive chemiluminescent substrate and imaged in G-Box. The blot was stripped and reprobed for GAPDH as the loading control.

For activity check, gel based activity profiling was done. Membrane proteomes (for mock and WT ABHD12 cells) were incubated with 2 µM of FP-Rhodamine (FP-fluorophore) and incubated at 37°C at 650 rpm for 45 minutes and then quenched with 4X loading dye (40 µL of 4X loading dye for 100 µL of sample) and heated at 95°C for 15 minutes and loaded on to a 10% SDS PAGE gel. The activity gel is imaged using a Syngene G-Box.

To see the trend of ABHD12 in different tissues, membrane proteomes for heart, kidney, lungs and spleen from WT and KO mice were prepared in 1 mg/ml concentration from the stock after protein estimation using BCA reagent. The samples were run on 10% SDS-PAGE gel along with brain membrane proteome of WT and KO mice as controls. Western blotting was done for the lysates using ABHD12 primary antibody and anti-rabbit secondary antibody and the blot was additionally probed for GAPDH to get loading control.

2.4 Preparation of mouse tissue lysates

Mice were anesthetized with isoflurane and sacrificed by cervical dislocation and tissues such as brain, lungs, liver, heart, kidney, muscles were harvested and stored at -80°C. Stored tissues were thawed on ice before processing and hair particles were removed by washing thoroughly in cold PBS. The tissues were chopped using scissors and carefully collected into an eppendoff. The tissue is suspended in 500 µL of sterile DPBS with 1 scoop of glass beads (for brain) or zirconium beads (for lungs or stainless steel beads for muscles) and homogenised using a tissue homogeniser at a speed setting of 8 for 3 minutes twice at 4°C. The tissue sample is then sonicated for 30 seconds (2 on and 2 off) and centrifuged at 200 g for 5 minutes at 4°C to pellet unlysed cells. The supernatant is collected and ultra-centrifuged at 4 °C for 45 minutes at a speed of 1,00,000 g. The supernatant is separated and both supernatant (soluble) and pellet (membrane) fractions are stored in -80 °C.

2.5 Lipid substrate assay

Lipid substrate assay were done with 100 µM lipid and 20 µg protein for every 100 µL of reaction in glass vials. 20 µg lysate, with a concentration of 1 mg/ml were incubated in 100 µM of Lyso-PS lipid at 37°C for 30 minutes with shaking (inside incubator). The reaction was quenched by adding 250 µL of internal standard (2:1 v/v chloroform: methanol containing 0.5 nmol of C 15:0 free fatty acids) and centrifuged at 1000g for 10 minutes. 2 phases were separated and organic layer (bottom layer) was collected. The extract was dried in N_2 stream and LC-MS analysis done for the samples.

1. For kinetics assay 20 µg proteome from mock and ABHD12 overexpressed HEK293T cell membrane lysates was incubated with the desired lipid (R and S configuration of synthesized Me-lyso-PS of chain length C10:0 to C24:0). This assay was performed with 8 different concentrations of substrate (lipid) ranging from 0-400 µM.

2. For endogenous mouse brain tissue experiments, WT and KO mouse membrane lysates at 1 mg/ml concentration is incubated with different lipids (R

and S configuration of synthesized Me-lyso-PS of chain length C10:0 to C24:0). Here denatured mouse brain membrane lysate was used as control for the experiment. For denaturing, processed brain membrane lysate (in PBS) is heated to a temperature of 95 °C for 10 minutes and then immediately cooled on ice for 10 mins. This is repeated three times.

3. To check the presence of serine hydrolases in different tissues by activity check using FP-Rhodamine (FP-Rh) for tissue lysates. ABHD12 WT and KO tissues membrane lysates for all the different tissues were prepared with 1 mg/ml concentration and was incubated with 2 µM of FP-Rh at 37°C for 30 minutes. After the FP-Rh incubation lipid substrate assay was performed with 50 µM of lyso-PS C17:1 lipid. The four different conditions here are WT membrane and soluble lysate with and without FP-Rh and similarly with ABHD12 KO tissue membrane lysates.

2.6 Proteomics and MS sample preparation

For analyzing tissue membrane samples in MS, lysates at a concentration of 2 mg/ml for 1 ml of sample (for brain) and 1 mg/ml for 1 ml sample (for lungs and liver) were incubated with 200 µM of FP-Biotin at 37°C for 45 minutes. After this 2:0.5:1 v/v ratio of ice cold methanol, chloroform and PBS respectively is added, vortexed and centrifuged at 4000 rpm for 15 minutes. The protein disc obtained from this is washed thrice with 1:1 v/v methanol: chloroform and the solvent is aspirated. This protein disc is then re-suspended in 500 µL of 6 M urea in PBS, 20 µL of 10% SDS and 50 µL of premixed TCEP (in DPBS) and 600 mM K_2CO_3 (in DPBS) (with final concentrations 200 mM of TCEP and 600 mM K_2CO_3) and sonicated in a water bath until the disc dissolves and forms a clear solution. To this solution, 70 µL of IAA (55mM) is added and incubated for 30 minutes at room temperature in the dark. Finally, 120 µL of 10% SDS solution and 4.5 mL DPBS is added to the sample. 100 µL of avidin beads for each sample (washed thrice with 0.2% SDS solution) is re-suspended in 1 mL of DPBS and added to the FP-Biotin enriched sample and incubated for 1.5 hours at room temperature while rotating.

After beads incubation, the sample was centrifuged in a swing bucket centrifuge at 2000 g for 15 mins and solvent was aspirated and the beads were washed three times each with 10 ml of 0.2 % SDS solution followed by 10 ml DPBS and 10 mL milli Q. The beads are then transferred to low binding tubes in 100 mM TEAB solution. To this trypsin resuspended in 200 ul of 2 M urea in 100 mM TEAB is added and incubated overnight at 37°C. Samples were acidified the next day with 0.1% TFA and centrifuged to collect the supernatant. This supernatant was desalted using C18 column and final sample collected and solvents were evaporated using a centrivap machine. The final samples were run on LC-MS and analysis for the samples were done.

2.7 Analysis

1. MS data analysis for substrate assays

   The spectral data for the experiments are obtained. The rate of Lyso-PS hydrolysis in the experiments were measured. The rate of hydrolysis for each species was calculated by measuring the area under the curve normalizing it w.r.t the internal standard to quantify the free fatty acids released. These rate values were subtracted from the mock rate values for the final corrected enzymatic rates. These final values were plotted to fit the classic Michaelis–Menten enzyme kinetics equation with ± SD for each concentration point.

2. MS analysis for SHs identification

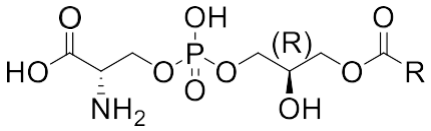
   MS-MS ion search is done on the MS data obtained. Protein-pilot software is used for the analysis of MS data obtained. Because mouse tissue samples are being analysed, the parameter chosen is Mouse ID and database (already prepared) for mouse is selected. Iodoacetamide (IAA) is chosen in cys-alkylation option (as we have used iodoacetamide as the alkylating agent in the sample preparation) and trypsin digestion is selected. The runs are done in 99% false discovery rate (FDR). After the run the results are exported in an excel file format. The results we require for further analysis is in the peptide summary section.

   The program matches the run data to the already available Mouse ID data and gives details of all the identified peptides in the sample. First step for further

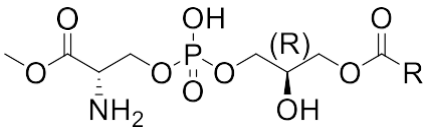
analysis id to delete the duplicates. We get all the identified proteins in excel file and then to get the list of all the SHs, we do a ‘VLOOKUP’ excel function (function to look up and retrieve data from specific columns) with the accession number and name of the SHs list for mouse. Thus we get the list of all the SHs in our sample.

Chapter 3: Biochemical characterization of ABHD12

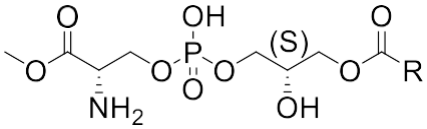
ABHD12 had been identified as 2-AG (2-Arachidonoylglycerol) and MAG (Monoacylglycerol) lipase in the brain. Blankman et. al. in their studies showed that ABHD12 has more preference for lyso-PS than MAG. Thus with untargeted and targeted mass spectroscopic studies and animal models they concluded that ABHD12 is a major brain lyso-PS lipase. (3) But lack of proper biochemical characterization for the same further led to rigorous biochemical studies in our lab using MAG as the main substrate molecule as it had already been reported as a MAG lipase. A library of 1-MAG molecules with different chain lengths and unsaturation were synthesized and subjected to biochemical assays. Joshi et. al., shows substrate profiling of ABHD12 for MAG and comparing MAG to lyso-PS hydrolysis and their preference of long chain or very long chain lipids in recombinant and endogenous ABHD12. Cellular localization of ABHD12 was shown to be at the endoplasmic reticulum (ER) in mammalian cells. (5)



Lysophosphatidylserine ((R)-lysoPS)



Methyl-Lysophosphatidylserine ((R)-Me-lysoPS)



Methyl-Lysophosphatidylserine ((S)-Me-lysoPS)

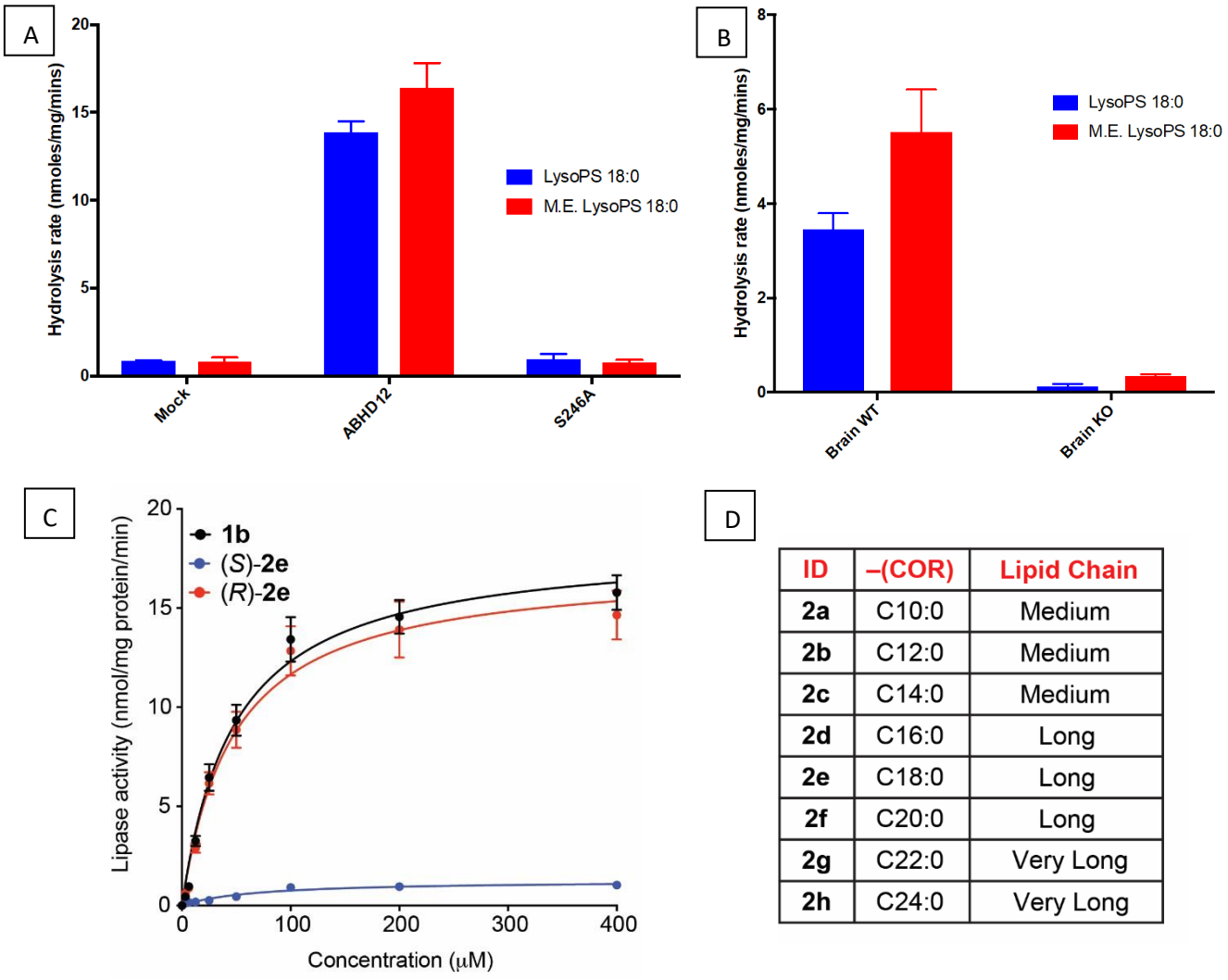
Fig:3.1 Chemical Structures of Me-lyso-PS and R- and S- configuration (synthesized library R=C10 to C24)

A comparative study using 1-MAG, 2-MAG and commercially available lyso-PS was performed and this showed much increased preference of ABHD12 towards lyso-PS than MAG, which lead us forward to use lyso-PS as substrates. Synthesis of a library of lyso-PS substrates with different chain lengths were done in the lab and substrate and kinetics assays were performed for the same. Synthesis of lyso-PS is difficult and challenging task. From the chemical structure of lyso-PS (**Fig:3.1**) we can see that the carboxylate of the amino acid can cross react and thus the synthetic scheme was modified by using methyl ester analog of the substrate. Thus methyl ester lyso-PS (Me-lyso-PS) of varying chain length and unsaturation (to be done) were synthesized in the lab (by Minhaj and Amol). This modification did not hinder the biochemical assays as the methyl ester linkage got hydrolyzed inside the cell leading to the formation of lyso-PS.

Results:

3.1 Comparison of commercial lyso-PS and Me-lyso-PS synthesized.

After synthesis of Me-lyso-PS, it was necessary to check the activity of the lipid substrate if it matched to the commercially available analog. Thus, we went ahead by performing substrate assay on recombinant ABHD12 cells and ABHD12 WT and KO mouse brain lysates and analyzing the rate of free fatty acid (FFA) release using mass spectrometry. Based on previous experiments, 20 µg of cell and tissue membrane lysate was used for all the further experiments. 100 µM each of Me-lyso-PS C18:0 and commercial lyso-PS 18:0 (Avanti polar) was incubated with 20 µg of membrane lysates of mock (empty plasmid HEK cells), ABHD12 (overexpressed HEK293T cells), S246A (catalytic mutant HEK293T cells), ABHD12 WT and KO mouse brain for 30 minutes. An internal standard of 0.5 nmoles of C15:0 FFA was added to each reaction mixture as a standard to normalize the final rate of hydrolysis. Triplicates of each condition were done for precision.



ID	–(COR)	Lipid Chain
2a	C10:0	Medium
2b	C12:0	Medium
2c	C14:0	Medium
2d	C16:0	Long
2e	C18:0	Long
2f	C20:0	Long
2g	C22:0	Very Long
2h	C24:0	Very Long

Fig: 3.2 Comparing synthesized Me-lyso-PS and commercial lyso-PS A. Hydrolysis rates compared for mock, WT and S246A catalytic mutant. B. Hydrolysis rates compared for ABHD12 WT and KO brain membrane lysates. C. Kinetics assay comparing the lipase activity for commercial lyso-PS, R-Me-lyso-PS and S-Me-lyso-PS in mock and ABHD12 recombinant cells. D. **Table 3.1** library of synthesized R- and S-Me-lyso-PS

The rate of FFA release in each reaction were normalized to the internal standard and the rate of hydrolysis was plotted for each condition. Comparing both the synthesized Me-lyso-PS and commercially available lyso-PS it was seen that there is no significant difference in the hydrolysis rate of the two compounds (**Fig: 3.2** A. and B.) in both recombinantly expressed cells and ABHD12 WT and KO tissue samples and thus we concluded that our synthetic Me-lyso-PS analog had the same activity and thus can

be used in future experiments for the biochemical characterization. The WT showed activity whereas the catalytic mutant and the KO did not show activity (due to the absence of ABHD12 in them). After this a library of Me-lyso-PS compounds were synthesized with varying chain lengths and unsaturation.

Naturally occurring lyso-PS bear R-conformation at glycerol backbone and hence we synthesized both R- and S- conformation of Me-lyso-PS as substrate for ABHD12 activity. Both R- and S- form of Me-Lyso-PS were synthesized for various chain lengths, C10:0 to C24:0 (**Fig: 3.2** D). As a confirmation to the activity of commercial and synthesized lyso-PS compounds, I performed kinetics assay on substrates- C 18:0 chain length of commercial lyso-PS (1b), R-Me-lyso-PS (R-2e) and S-Me-lyso-PS (S-2e) with mock and ABHD12 recombinant cells. R- and S- configuration were also assayed to check if both the forms are active or not. From the results we can see that commercial lyso-PS and R-Me-lyso-PS show similar hydrolysis rate again confirming the previous results. The other prominent result we obtained from this is that the S- form of Me-lyso-PS is inactive (**Fig: 3.2** C). The S-Me-lyso-PS showed almost negligible rate of free fatty acid hydrolysis in the sample showing that S- form of lyso-PS cannot be hydrolyzed by ABHD12 and only R- form is the active form.

3.2 Kinetics Assay for varying chain lengths of Me-lyso-PS

Kinetics help us study the rate and understand the catalytic mechanism of enzymes. These assays help in quantifying the Vmax and Km of a particular enzyme. Vmax of an enzyme is the point at which there is the exact amount of substrate molecules to fill the enzyme active sites. This measurement helps in reflecting how fast the particular enzyme catalyzes a reaction. Similarly, Km is that substrate concentration when half of the enzyme active sites are occupied. Higher the Km, more substrate required to saturate the enzyme.

Lyso-PS species	Fatty acid chain length	Lyso-PS species	Fatty acid chain length
(*R*)-2a	10:0	(*S*)-2a	10:0
(*R*)-2b	12:0	(*S*)-2b	12:0
(*R*)-2c	14:0	(*S*)-2c	14:0
(*R*)-2d	16:0	(*S*)-2d	16:0
(*R*)-2e	18:0	(*S*)-2e	18:0
(*R*)-2f	20:0	(*S*)-2f	20:0
(*R*)-2g	22:0	(*S*)-2g	22:0
(*R*)-2h	24:0	(*S*)-2h	24:0

Table 3.2: Lyso-PS species terminology

Here the assays were carried out at 8 different concentrations of the synthesized configuration of Me-Lyso-PS (0-400um). Substrates used were R- and S- forms of Me-Lyso-PS C10:0, C12:0, C14:0, C16:0, C18:0, C20:0, C22:0 and C24:0. Each concentration of substrate was incubated with 20 µg of mock and ABHD12 expressed HEK293T cells and an internal standard of 0.5 nmol of C15:0 FFA was used.

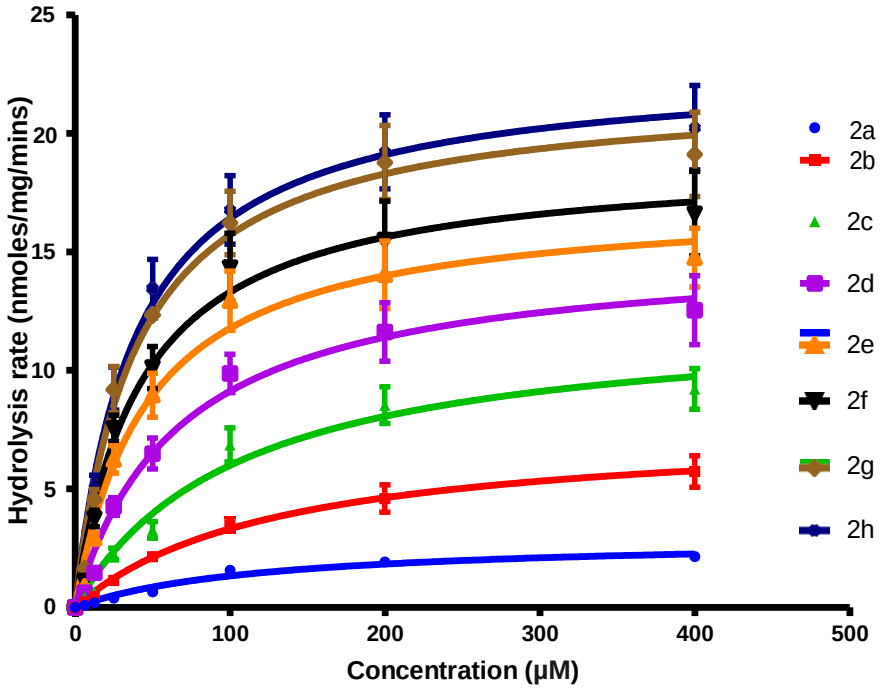


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Fig: 3.3 Enzyme kinetic assays for membrane lysates with varying chain lengths of (R)-Me-lyso-PS

Enzymatic rate for each reaction mixture with particular substrate concentration was corrected by substracting the rate of mock from WT rate and plotting the data to fit the Michaelis–Menten kinetics equation. (**Fig: 3.3**)

Table 3.2 gives all the kinetics constant data for Vmax and Km for all the substrates. The trend for Vmax and Vmax/Km for R-Me-lyso-PS goes as C24:0 ≈ C22:0 > C20:0 > C18:0 > C16:0 > C14:0 >C12:0 > C10:0. Similarly C10:0 > C12:0 > C14:0 > C16:0 > C18:0 > C20:0 > C22:0 ≈ C24:0 for Km values. Based on these kinetics data we can conclude that WT ABHD12 has a strong preference for very long chain (VLC) lipids. From the kinetics constant we can see that there is no significance difference in the values of Vmax and Vmax/Km for the different chain lengths suggesting that the S form of Me-lyso-PS is inactive and ABHD12 does not have any activity towards these.

Lyso-PS species	V_{max} (nmol/mg protein/min)	$K_{M\ (\mu M)}$	V_{max}/K_M (nmol/mg protein/min/M) (x 10^5)
(*R*)-2a	2.9 ± 0.2	126 ± 18	0.2 ± 0.04
(*R*)-2b	7.6 ± 0.3	131 ± 14	0.6 ± 0.06
(*R*)-2c	12.2 ± 0.6	106 ± 14	1.1 ± 0.1
(*R*)-2d	15.2 ± 0.6	67 ± 7	2.3 ± 0.3
(*R*)-2e	17.2 ± 0.6	47 ± 5	3.7 ± 0.5
(*R*)-2f	19.0 ± 0.6	43 ± 4	4.4 ± 0.5
(*R*)-2g	22.0 ± 0.6	40 ± 4	5.5 ± 0.6
(*R*)-2h	22.9 ± 0.6	40 ± 4	5.7 ± 0.6
(*S*)-2a	0.2 ± 0.02	275 ± 32	0.007 ± 0.001
(*S*)-2b	0.4 ± 0.05	255 ± 35	0.016 ± 0.002
(*S*)-2c	0.6 ± 0.1	215 ± 28	0.028 ± 0.003
(*S*)-2d	0.8 ± 0.1	187 ± 24	0.043 ± 0.006
(*S*)-2e	1.2 ± 0.1	178 ± 25	0.067 ± 0.008
(*S*)-2f	1.3 ± 0.2	165 ± 23	0.078 ± 0.009
(*S*)-2g	1.2 ± 0.2	167 ± 26	0.072 ± 0.009
(*S*)-2h	1.2 ± 0.3	176 ± 31	0.068 ± 0.008

Table 3.3: Kinetics constants for varying chain lengths of (R)- and (S)-Me-lyso-PS

3.3 Endogeneous lipid hydrolysis assays

To provide a stronger evidence for the kinetics assay results, we performed endogeneous assays with mouse brain. From previous studies we know that brain express the highest amount of ABHD12, thus WT and KO mouse brain lysates were used to do the experiments. KO brain lysates were used as a control for this specific activity as they have shown to accumulate lyso-PS in the brain due to absence of ABHD12. As ABHD12 showed specific lipase like activity against lyso-PS in in-vitro studies, we hypothesized to see a similar treand in this activity too. Here 20 µg of ABHD12 WT and KO mice brain membrane lysates were incubated with 100 µM of Me-lyso-PS (library) of varying chain lengths. All synthesized substrates Me-lyso-PS C10:0 to C24:0 were used for the experiment. After 30 minutes of incubation, internal standard was added, lipids extracted and samples prepared was run in LC-MS.

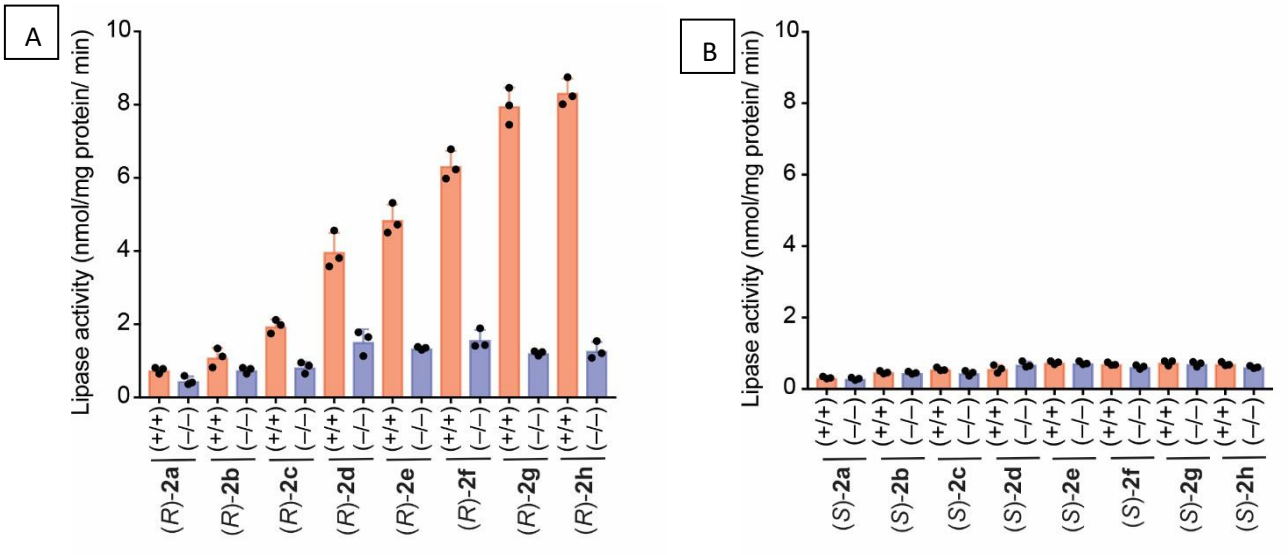

Fig: 3.4: Endogenous Lipid hydrolysis assays for A. (R)-Me-lyso-PS B. (S)-Me-lyso-PS against brain membrane of wild type (+/+) and ABHD12 knockout (–/–) mice.

From the plot we can evidently see that when comparing the WT and KO for each lipid, WT have more hydrolysing activity than KO confirming ABHD12 lipase activity. If we compare WT plots of the different chain lengths, we can also see that with the increase in the chain length the rate of hydrolysis is also increasing showing that endogenously

ABHD12 have preference for longer chain fatty acids than short chain lengths. This helps us in confirming the kinetics assay and establishing that ABHD12 has a preference for very long chain or VLC lipids.

Future perspectives:

Next step to this project is to do substrate profiling of Me-lyso-PS with unsaturation in the hydrophobic fatty acid chains and analyse the ABHD12 preference for the same.

Chapter 4: Identification of lyso-PS Lipases

It is already known that lyso-PS is a potent signaling molecule and its accumulation in brain leads to PHARC syndrome. Thorough research and studies on this disease lead to the finding of ABHD12 as a lyso-PS lipase. (3) ABHD12 belonged to mSH family of enzymes and also had a nucleophilic serine residue at the active site. All our metabolic tissues (like lungs, liver, kidney, heart and muscle) have abundant lyso-PS in them. We also know that there are other PS lipases present in the body, upon whose action lyso-PS are formed. Bio GPS studies also shows that, ABHD16A, a PS lipase enzyme is present in metabolic tissues which results in formation of lyso-PS. But ABHD12, the known lyso-PS lipase is absent in these tissues and thus allowing us to hypothesize that there are other lyso-PS lipases that exist in these tissues which help in the hydrolysis of lyso-PS and avoid their accumulation in these tissues. We planned to target serine hydrolase family to identify any lyso-PS lipase enzyme other than ABHD12. This was due to the established tools and resource available in the lab to investigate serine hydrolases.

Serine hydrolase (SH) is a diverse family of enzymes which are known to perform a diverse range of functions. These are an important class of enzymes as they participate in several physiological and there are several drugs for diseases which target these SHs. (3) The main and unique characteristic of an enzyme in the SH family is the highly conserved serine residue as the nucleophile at their active site.
(10) The enzymes of this family usually have a unique fold known as the α/β hydrolase fold. This fold usually has a Nucleophile-His-Base catalytic triad at the active site. Structurally this fold has 8 beta sheets flanged on each sides with alpha helices and the whole structure is bent to form a half barrel shape. The enzymes have diverged to obtain new functions from a common ancestor but core structure is similar to preserve the catalytic residues. (7)

SHs are widely characterized into two subfamilies, namely serine proteases and metabolic serine hydrolases (mSH). Serine proteases (or trypsin/ Chymotrypsin class of serine proteases) are approximately around 125 in number in humans. They are usually present in an inactive precursor form in the body and needs to be externally acted upon by other enzymes to form the active form of enzyme. The mSH subfamily includes several types of enzymes like lipases, esterases, amidases, etc. The enzymes of this subfamily is associated with several (patho)-physiological process and even drugs are designed to target these moieties. (3) Even after having such diverse functions, we lack biological knowledge about SHs, one of the main reason being the unavailability of inhibitors to probe and study the same. More than 80% of the mSH do not have recognized inhibitors for further biochemical studies.

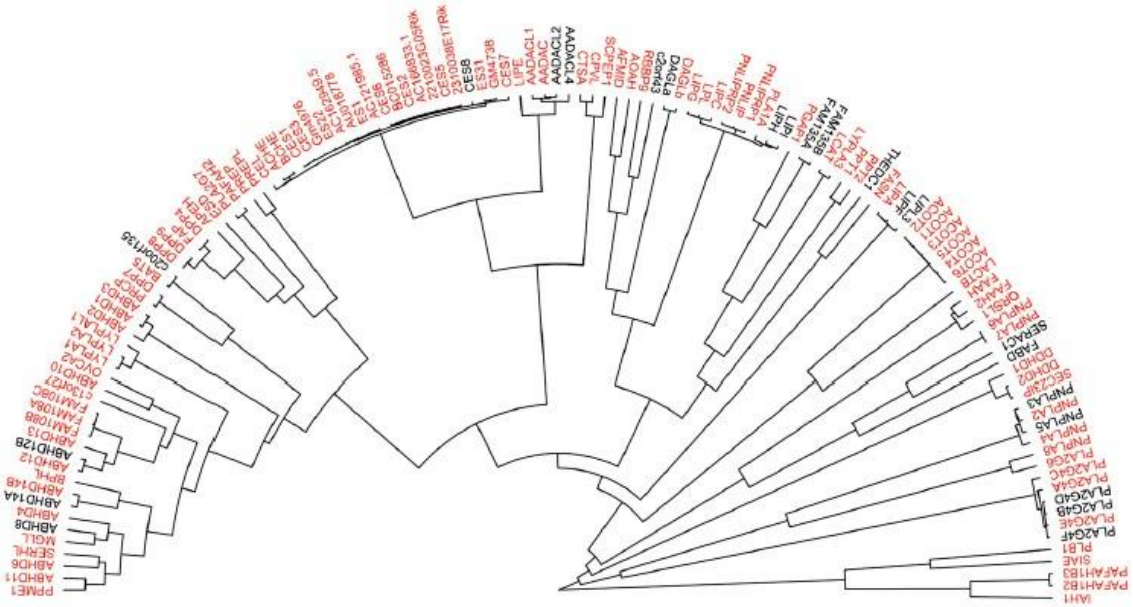

Fig: 4.1 Serine hydrolases identified in mouse tissues

As the isolation and identification of SH were difficult a new method of single active site directed probe was introduced. Fluorophosphonate or FP probes were designed which selectively bound to the active site serine residue and thus SHs could be isolated. (8) FP probes bound to both serine proteases and mSH. As FP probes resulted in successful marking of SHs, the next step taken was to isolate these

enzymes and for the same biotinylated FP probes were synthesized with a biotin reporter tag. These could be used to isolate SHs and pull down them using avidin beads.

4.1 Activity Based Protein Profiling (ABPP)

As Serine Hydrolases (SHs) play a major role in many physiological functions, characterization of these is an important task to be performed. An innovative way to study these enzymes was by doing collective characterization by pulling down the entire family of enzymes rather than trying to isolate a single SH. Characterization of a protein/ enzyme is necessary 1. for assigning a specific function for that particular protein/ enzyme. 2. to identify whether this could be a potential pharmaceutical target for any disease. An effective way to obtain this abovementioned is to perturb the activity of these enzymes with the help of inhibitors and understand the expression dynamics and identify the functions associated to these.

Activity Based Protein Profiling is an established method of using an active site directed probe to pull down all the enzymes of the specific subset of family of enzymes and analyse them. This helps in providing a quantitative readout of enzymes in the family. It is already known that mSHs have a serine residue at the active site of the enzyme and usually forms a catalytic dyad (Ser-Lys or Ser-Asp) or traid (Ser-His-Asp or Ser-Ser-Lys) for carrying out enzymatic reactions. A nucleophilic centre is formed at this catalytic dyad or triad, so appropriate inhibitor for this enzyme needs to be electrophilic in nature which would covalently bind to the active site. FP being highly electrophilic in nature could form a strong covalent interaction with the active site serine residue and thus could be used as a successful active site directed affinity label.

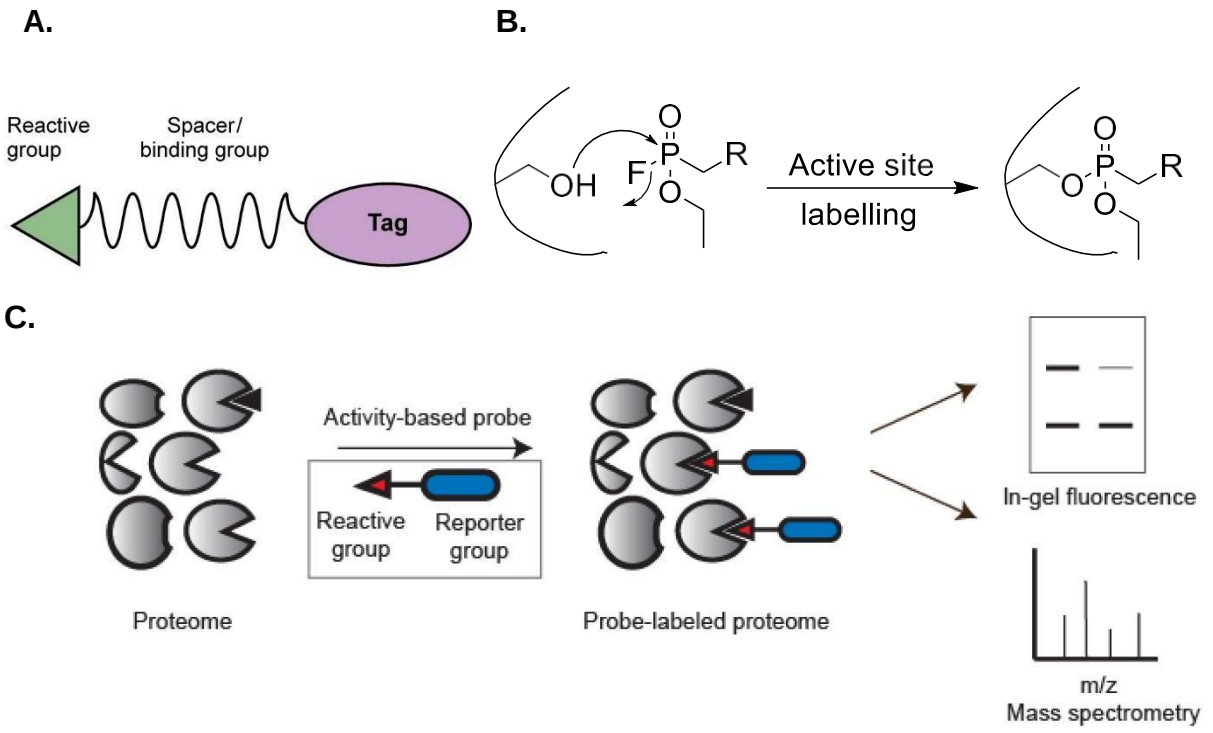

Fig: 4.2 Activity based protein profiling A. ABPP probe B. FP group reacting to the active site serine residue (R=linker and tag) C. ABPP experiments with in-gel fluorescence and Mass spectroscopy

An essential property that the FP probe follows is that it gets covalently attached only to the catalytically active site. Experiments were performed with serine proteases, which are usually in their inactive state, to accommodate the probe in their active site and results showed that there was significantly very low probe labelling confirming that active site is a necessary condition for FP probe binding. Other major reasons why FP could be a favourable probe is that it can detect sub-nanomolar concentrations of the enzyme. As the same probe reacts with numerous SHs, only a single probe can be used for the entire family of proteins. This probe has the ability to differently record the functional state of the enzyme and the expression levels.

A FP probe (**Fig: 4.2** A) is comprised of 3 different parts 1. an electrophilic group, the fluorophosphanate group (in other cases any photoreactive group). 2. A linker group. 3. Reporter tag, for example rhodamine, biotin, alkyne, etc. for recognition. This technique of ABPP with site directed probes are mainly used for characterizing unannotated enzymes, doing comparative studies to identify targets for specific enzymes and also for competitive studies of inhibitor discovery and designing. Usually

ABPP is done for gel based analysis where either in gel fluorescence or western blotting can be used to identify the enzyme. (**Fig: 4.2** C) Then this technique was combined with other techniques like LC-MS to improve the resolution of these probes and carry out both qualitative and quantitative studies.

4.2 Targeted lipid measurement in tissues

Blankman and her colleagues performed targeted lipid profiling on mouse tissues to see the difference in the lyso-PS and PS lipid measurement in ABHD12 WT and KO tissues. They had concentrated on brain tissue as they were trying to show how ABHD12 affected lyso-PS amounts in PHARC disease, which showed accumulation of VLC-lyso-PS in cerebellum of the KO animal brains, which lead them to attribute ABHD12 as a major brain lyso-PS lipase. Along with the brain tissues they had performed this targeted lipidomics in other metabolic tissues as well, data is given in supplementary data, to which they have mentioned that they could see no significant difference in the levels of lyso-PS and PS in other tissues. As a start to our hypothesis, I first used this data to analyze and confirm their conclusion regarding the other metabolic tissues they had done MS studies on.

4.3 FP analysis of tissues

After a lead that there is a presence of other proteins in these tissues for lyso-PS hydrolysis, our next aim was to see if that enzyme belongs to serine hydrolase family. We tested this using serine hydrolases inhibitor FP - a known inhibitor, as it covalently binds to the active site serine residue. Here rhodamine (Rh) was used as the reporter tag.

1. Effect of ABHD12 on tissues

To confirm that ABHD12 did not have any effect on other tissues when knocked out, all metabolic tissues (heart, kidney, liver, lungs and muscles) from ABHD12 WT and KO were incubated with FP-Rh followed by incubation with lipid substrate, lyso-PS C17:1 (commercial) and internal standard of 0.5 nmoles of C15:0 FFA was added. The FFA were obtained in the organic phase and dried using N_2 stream and MS analysis

was done. Brain tissue lysates was used as a control in the experiment as it has already been studied.

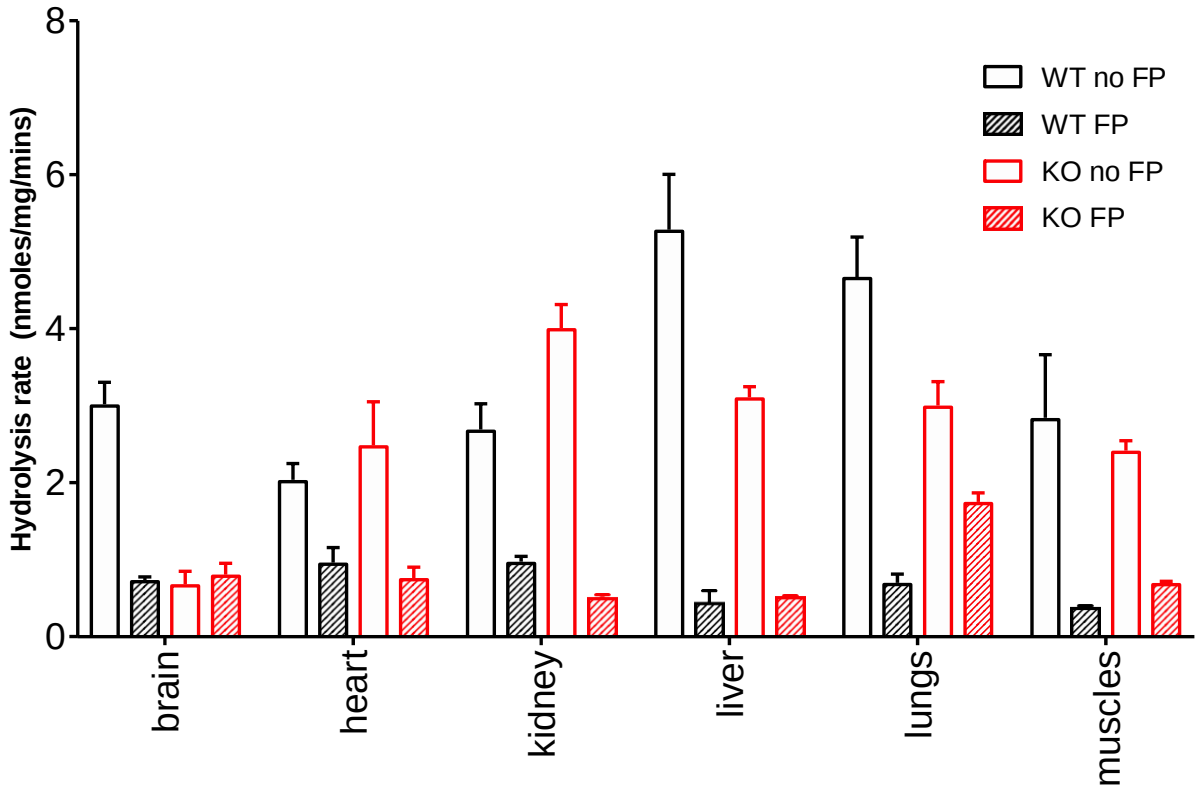

Fig: 4.3 FP analysis assays with ABHD12 WT and KO tissue lysates in the presence and absence of FP-Rh probe with brain as control

In the plot (**Fig: 4.3**), the rate of hydrolysis into FFA is plotted in the y-axis w.r.t each tissue (in x-axis) with and without FP-Rh incubation. From the plot if we observe the WT and KO without FP-Rh, there is a decrease in the levels of FFA in the brain while other tissues do not show any significant difference. This helps us confirm ABHD12 KO does not have any effect on other metabolic tissue. Now if we observe the with and without FP plots for each tissue we can see difference confirming that there is presence of some serine hydrolase which is getting inhibited when incubated with the FP probe. From here we could confirm that there is some other SH other than ABHD12 present in these tissue that compensate for the hydrolysis of the lyso-PS formed in these.

2. Proteome fractionation of tissues

Once the presence of SH was confirmed, our next aim was to see if this SHs is present in the soluble or membrane fraction of these tissues. Thus in the next experiment we took tissue lysates of WT ABHD12 mouse and separated the soluble and membrane fractions and incubated these with FP-Rh and carried out the same scheme and looked for the FFA produced in each sample.

From the plot, (**Fig: 4.4**) when we compare the membrane and soluble fraction without FP incubation, the rate of hydrolysis is much more in membrane than soluble. Similarly, we observe the FP incubated and not incubated membrane fractions, there is a significant decrease in the hydrolysis rate in the FP incubated fraction whereas almost no difference in the soluble fraction. From all these observations we concluded that there can be novel SH/SHs (other than ABHD12) present in the membrane fraction of these tissues.

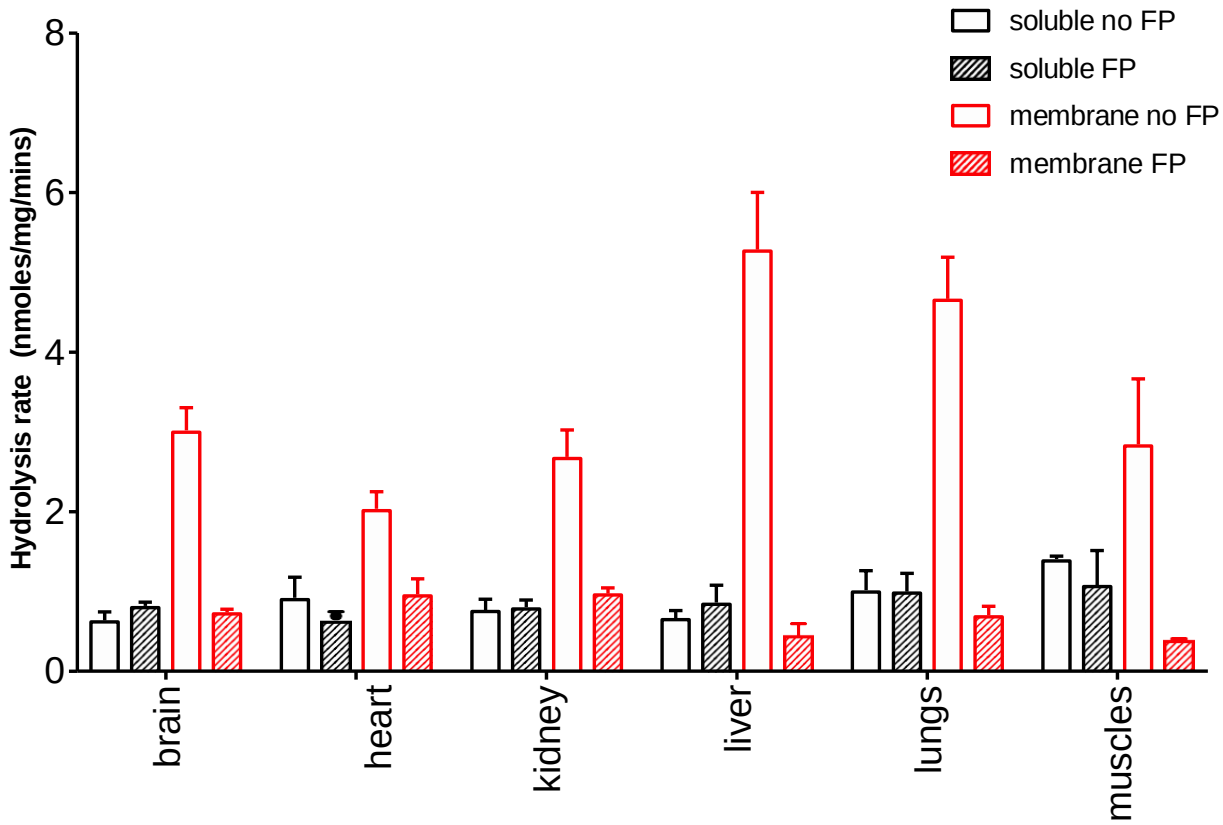

Fig: 4.4 FP assays with membrane and soluble fractions of ABHD12 WT tissue lysates in the presence and absence of FP-Rh probe with brain as control

4.4. Selection of tissues

As the presence of lyso-PS lipase had been confirmed from the experiments, the next step is to select the tissue for the further studies and trying to identify any novel lyso-PS lipase present in them. For shortlisting the tissues, we took into consideration the soluble vs membrane substrate assay results. After proper examination of the membrane fraction plots we can see that there is a very significant difference in the rate of hydrolysis in the with and without FP samples of liver and lungs tissues. Thus from this we confirmed to go ahead with liver and lung tissues for the critical examination and identification of the lipase.

We had also gone through Bio-GPS data. Bio-GPS is an online portal with all the genetic and genomic details of any gene (gives information about the mRNA levels). Bio-GPS can be used to search any particular gene of interest, view any gene annotation report or even built your own gene report. We searched Bio-GPS data for ABHD12 and saw that ABHD12 mRNA is present in both liver and lungs tissues. Comparing the mRNA level to brain where ABHD12 is present in abundance and also a major brain Lyso-PS lipase, the levels in liver and brain were very low, approximately 5 to 8-fold low. From this we can say that even after presence of mRNA, ABHD12 does not show the lipase activity and thus we can infer that there are other novel lyso-PS lipases abundant to these tissues.

4.5 Optimization of FP probe

The next step towards the project was to somehow pull down all the SHs present and identify the enzyme. For this we wanted a probe which can inhibit SHs and at the same time be pulled down for further analysis. We used a FP probe as the active site of the enzyme family has a serine residue. Here as we wanted to pull down all the possible SH, FP probe was designed with a biotin reporter tag which can then be pulled down easily using avidin beads. The FP-Biotin probe was synthesized in our lab by Amol (8). As this probe was synthesized in lab, optimization of the probe was necessary step to be done at the first place. We decided to do this by using this probe on mouse brain tissue lysates. As thorough studies have been carried out on brain, if the probe

pulled down all the essential SHs present in the brain tissue lysate we could declare the probe to be functional and optimize the concentration it should be used.

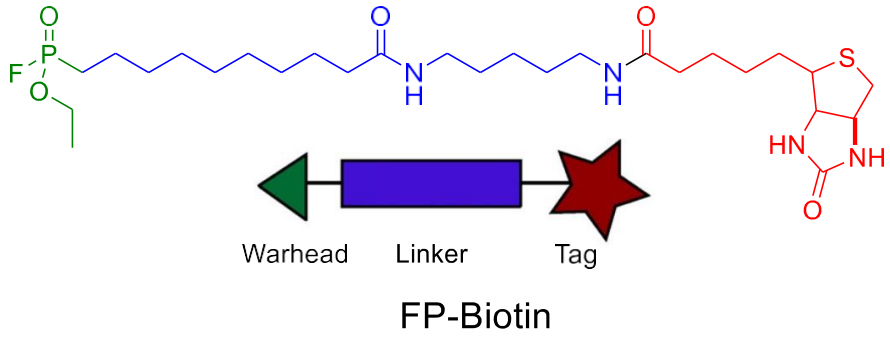

Fig: 4.5: FP-Biotin probe

We used 2 step process in this optimization, first being gel based and western blotting and the second step by pull down assay and MS analysis. By western blotting we tried to optimize the concentration of probe required to optimally label the proteome into the samples. This was done by labelling the lysate with FP-Biotin and probing for biotin with anti-biotin antibody on immune blots. Another approach we took for this was a chase experiment. Here we incubated the samples with FP-Biotin probe and chased the incubated samples with FP-Rh and visualized it on a fluorescence SDS-PAGE gel. Following, gel based optimizations for our in house FP-biotin probe, we used liquid chromatography coupled mass spectrometry (LC-MS) to list out all the SHs that can potentially be pulled down using this probe. The FP-Biotin incubated samples were prepared and run on a QTOF MS couple with nano LC system. All spectra were collected in discovery mode analyzed spectral counts were analyzed for the SHs present in them. If the probe could pull down the important SHs such as ABHD12, ABHD16A, MAG, etc. which are known to be present in the brain tissue in abundance can be said to be functional.

Brain tissue lysates for membrane and soluble fractions were used for all these experiments. We wanted to optimize on the concentration of the probe, the concentration of the lysate and the incubation time to be used for each sample

preparation. In every cycle of experiment samples were divided into 2 parts after incubation, one part was used for western blotting to confirm the biotin incubation and the other part was used for MS sample preparation.

For optimizing the concentration of probe, we decided to use 2 mg/ml concentration of brain membrane and soluble fractions and tried 3 different concentrations of 200, 250 and 500 µM of FP-Biotin probe. Western blotting and MS samples were prepared and run and from the results we concluded to use 250 µM of FP-Biotin probe per 2 mg/ml of sample. Next we tried to see if lower concentration of lysates resulted in same trend. Thus 2 mg/ml, 1mg/ml and 0.5 mg/ml lysate samples were tried and we observed good results with 1 mg/ml of tissue lysates. Thus finally we had optimized to use the probe to be used at a concentration of 250 µM for 1 mg/ml of freshly prepared tissue lysate with probe incubation time of 45 minutes.

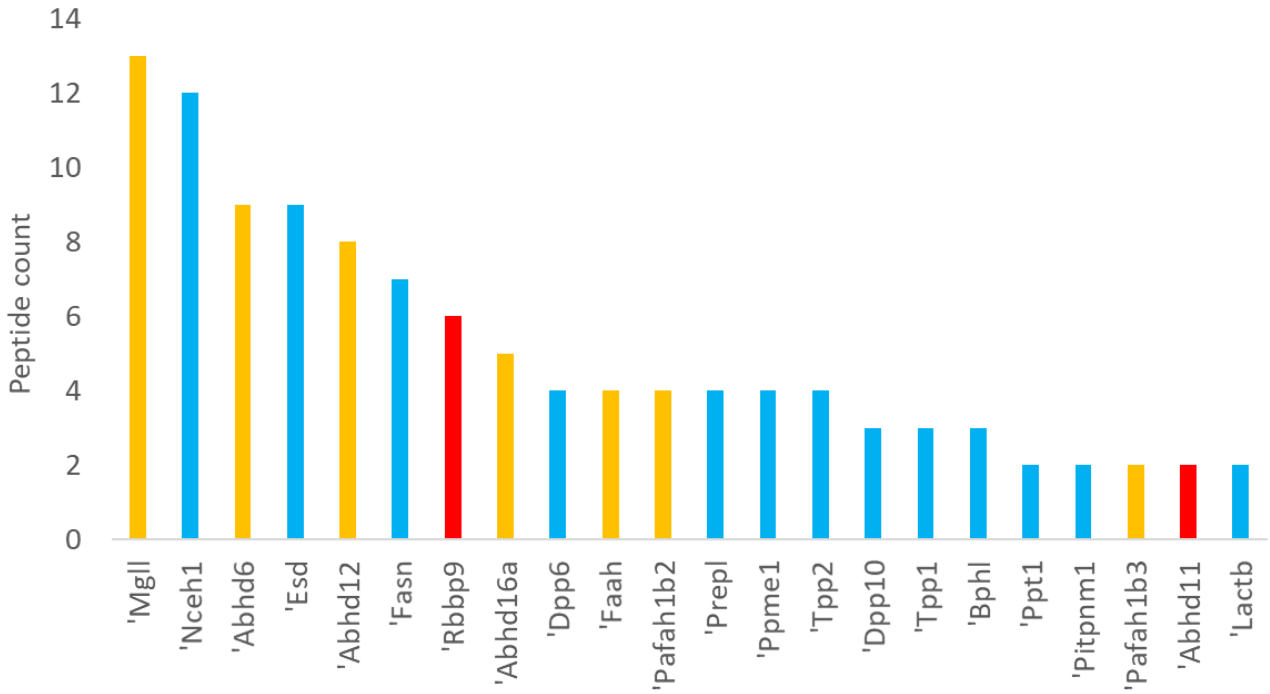

Fig 4.6: FP-Biotin pull down for brain lysates. Color code: 1. Blue-SHs 2. Yellow-Already identified lipases 3. Red- Unannotated SHs (y axis = peptide counts)

Fig 4.6 lists out the common SHs pulled down in replicates of brain lysates and we can see that the ABHD12, ABHD16A, MAG, FAAH, the important and abundant

SHs have been pulled down by the probe and picked up in the analysis by using the above mentioned conditions for sample preparation. Thus from all these we confirmed to go ahead with the synthesized probe for identification of a novel lyso-PS lipase in the selected tissues - liver and lungs.

4.6 Critical examination of tissues

Serine hydrolases being a large family of proteins, several inhibitor studies have been actively carried out. Bachovchin and colleagues (3) had also carried out inhibitor studies on SHs using carbamate inhibitors to develop a pharmacological probe to inhibit these SHs. In this study of his, he and his colleagues have provided with a library of SHs on the basis of expression in each tissue of the body, for example, brain, heart, kidney, liver, lungs, pancreas in their supplementary data. We have taken this data for our studies and did an extensive literature digging and categorized all the serine hydrolases they have provided into different subfamilies and tried to identify and classify all the lipases in this list.

The list had all the SHs identified from both membrane and soluble fraction of the tissues with the corresponding spectral count obtained in the readings. As from our results we had already identified that the serine hydrolase we are looking for is present in the membrane fraction, we decided to not consider the spectral counts obtained in the soluble fractions. From here first I arranged the proteins in the order from highest to lowest spectral counts obtained. We did not consider any protein which had spectral count in the soluble fraction but absent in the membrane fraction. I then plotted a graph for all these proteins. Following this I did a thorough literature check for each proteins and identified all the lipases from the already present SHs list. Once I had the lipases list I further tried to identify and classify these as phospholipases and neutral lipases and find out all the information that was present about the particular lipase, like the substrate preference, possible functions performed, abundance of the protein, etc. Thus I could generate a list all the unannotated serine hydrolases in these tissues. From this list after rigorous brainstorming we tried to select a few proteins and do further literature reading for the same.

To complement our list generated from literature we tried to pull down the SHs in these tissues using our in house FP-Biotin probe. We started with liver and lung lysates to pull down SHs and generate a list as obtained by chemical proteomics using LC-MS. The data obtained was run through the already available mouse protein list for identification in protein-pilot (Sciex) program. The final results obtained were compared to the lipases list we had prepared from the already present data and all the SHs pulled down were spotted and color coded to make our own list of all the SHs we had pulled down. All the replicate studies we had performed were also compared with each other.

A. Tissue type: Liver

Liver being one of the major metabolic tissues in the body which play an active role in metabolism. There have been previous studies showing SHs playing a major role in the secretion and transport of triglycerides rich chylomicron. From our preliminary results it was observed that maximum difference in the level of SHs measured by FP-Rh assays arised from the membrane and soluble fractions of liver tissue lysates.

A.1 Literature

From Bachovchin and colleagues list of SHs for liver, there were a total of 67 SHs reported (the least spectral count for the membrane fraction was 2.33). Out of these there were 63 known and 4 unknown SHs. The 63 known and annotated SHs also contained SHs that are functionally unannotated. **Fig: 4.7** shows all the SHs plotted together. From this list after thorough literature reading I identified 20 lipases, 11 neutral lipases and 8 phospholipases (also includes lysophospholipases). From this list we narrowed down to 4 lipases which I plan to look more in detail into the literature and get all the possible information about the same.

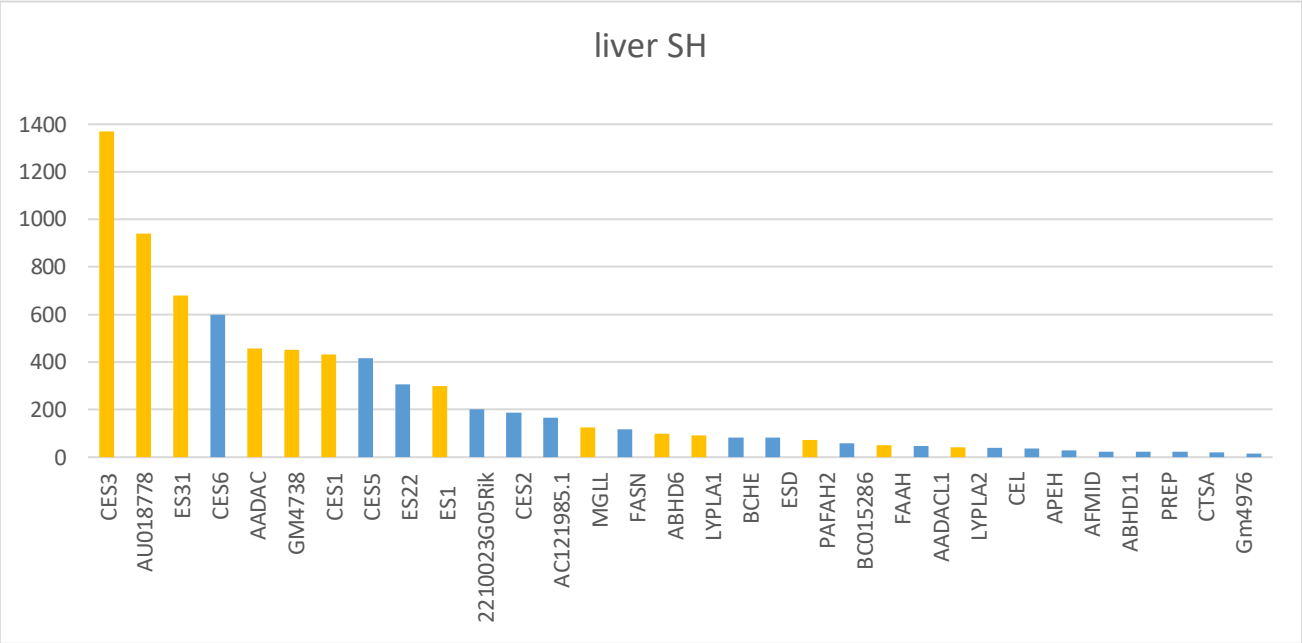

Fig: 4.7: Graph of the SHs present in liver tissue. Color code: 1. Blue-SHs 2. Yellow-annotated lipases (y axis = peptide counts)

Lipases	Phospholipases (lyso)
CES3	ABHD6
CES1F	LYPLA1
ES31	PAFAH2
AADAC	LYPLA2
GM4738	LIPE
CES1	PNPLA7
ES1	ABHD3
MGLL	LYPLA3
FAAH	
Gm4976	
LIPC	

Table: 4.1: Lipase list categorized as phospholipase and neutral lipase in liver

A.2 FP-Biotin pull down

For FP-Biotin pull down MS sample preparation, I had used 1 mg/ml liver membrane lysate in 1 ml volume incubated with 250 µM of FP-Biotin probe. After the incubation the sample preparation protocol was followed - obtaining the protein disc, treatment

with TCEP/K2CO3, IAA (Iodoacetamide), incubation on avidin beads and finally obtain peptides by trypsin digestion. The trypsin digested sample is desalted and MS (triple TOF) runs for the sample are performed.

99% FDR					95% FDR			
total	683	total	688		total	683	total	688
serine hydrolase	33	serine hydrolase	32		serine hydrolase	38	serine hydrolase	35
lipases	15	lipases	12		lipases	15	lipases	13
NP_075872	'Aadac	NP_075872	'Aadac		NP_075872	'Aadac	NP_075872	'Aadac
NP_941074	'Ces3a	NP_659179	'Ces1f		NP_941074	'Ces3a	NP_659179	'Ces1f
NP_444430	'Ces1d	NP_444430	'Ces1d		NP_444430	'Ces1d	NP_444430	'Ces1d
NP_659179	'Ces1f	NP_941074	'Ces3a		NP_659179	'Ces1f	NP_941074	'Ces3a
NP_653094	'Ces3b	NP_653094	'Ces3b		NP_653094	'Ces3b	NP_653094	'Ces3b
NP_598721	'Ces2a	NP_031966	'Ephx2		NP_598721	'Ces2a	NP_031966	'Ephx2
NP_031966	'Ephx2	NP_598721	'Ces2a		NP_031966	'Ephx2	NP_598721	'Ces2a
NP_766347	'Ces2e	NP_058599	'Esd		NP_766347	'Ces2e	NP_058599	'Esd
NP_058599	'Esd	NP_766347	'Ces2e		NP_058599	'Esd	NP_766347	'Ces2e
NP_067431	'Ces1g	NP_663578	'Ces2c		NP_067431	'Ces1g	NP_663578	'Ces2c
NP_663578	'Ces2c	NP_034275	'Ephx1		NP_444473	'Prss1	NP_034275	'Ephx1
NP_031980	'Ces1c	NP_031980	'Ces1c		NP_663578	'Ces2c	NP_031980	'Ces1c
XP_006530714	'Ces1e	NP_034303	'Faah		NP_031980	'Ces1c	NP_034303	'Faah
NP_932116	'Ces2g	NP_067431	'Ces1g		XP_006530714	'Ces1e	NP_067431	'Ces1g
NP_034303	'Faah	NP_032014	'Fasn		NP_932116	'Ces2g	NP_032014	'Fasn
NP_080788	'Bphl	NP_444473	'Prss1		NP_034303	'Faah	NP_444473	'Prss1
NP_034275	'Ephx1	XP_006515238	'Iah1		NP_080788	'Bphl	XP_006530714	'Ces1e
NP_035974	'Mgll	NP_080788	'Bphl		NP_034275	'Ephx1	XP_006515238	'Iah1
XP_006518136	'Abhd6	XP_011237576	'Dpp4		NP_035974	'Mgll	NP_080788	'Bphl
NP_032892	'Lypla1	NP_032892	'Lypla1		XP_006518136	'Abhd6	XP_011237576	'Dpp4
NP_032932	'Ctsa	NP_035286	'Prep		NP_032892	'Lypla1	NP_032892	'Lypla1
NP_032014	'Fasn	XP_006518136	'Abhd6		NP_032932	'Ctsa	NP_035286	'Prep
XP_011237576	'Dpp4	NP_031545	'Baat		NP_032014	'Fasn	XP_006518136	'Abhd6
NP_080623	'Iah1	NP_109642	'Lactb		XP_011237576	'Dpp4	NP_031545	'Baat
NP_598891	'Abhd3	NP_036072	'Lypla2		NP_080623	'Iah1	NP_109642	'Lactb
NP_666338	'Apeh	XP_006504555	'Abhd11		XP_006504555	'Abhd11	NP_036072	'Lypla2
XP_006537628	'Baat	XP_006511786	'Apeh		NP_598891	'Abhd3	XP_006504555	'Abhd11
XP_006538941	'Lypla2	XP_011239970	'Tpp1		NP_666338	'Apeh	XP_006511786	'Apeh
NP_666218	'Lyplal1	NP_598891	'Abhd3		XP_006537628	'Baat	XP_017175376	'Pafah2
NP_056569	'Rbbp9	NP_598949	'Acot2		XP_006538941	'Lypla2	XP_011239970	'Tpp1
XP_011239970	'Tpp1	NP_032932	'Ctsa		NP_666218	'Lyplal1	NP_598891	'Abhd3
NP_109642	'Lactb	NP_083058	'Lonp1		NP_056569	'Rbbp9	NP_598949	'Acot2
NP_032903	'Plg				XP_011239970	'Tpp1	NP_032932	'Ctsa
					NP_075822	'2210010C	NP_083058	'Lonp1
					NP_109642	'Lactb	NP_056569	'Rbbp9
					NP_032903	'Plg		
					XP_006524701	'Ppt2		
					NP_033444	'Tpp2		

Table: 4.2: MS results for liver- comparing replicates for 99% and 95% FDR

The tissue lysates were always freshly prepared and 3 replicates were done for each experiment. After the runs the MS data was analyzed in protein-pilot. Thus we get the list of all the SHs in our sample. We then arranged them in descending order of the number of peptides obtained to see the abundance of particular SHs.

The **Table 4.2** shows the MS result of liver membrane lysates. The results obtained for 99% and 95% FDR have been compared in the table. The proteins which are common in all the replicates have been color coded in green and all the lipases identified have been color coded in orange. The proteins which were picked up in 95% FDR and not in 99% FDR are color coded in violet. Around 680 total proteins were identified in almost each replicates and an approximate of 30 SHs were identified from this list out of which 12 to 15 SHs were identified in these replicates and these lipases were compared to the lipases list we had prepared.

From the MS data and the lipases data we had prepared from literature were analyzed and we decided to select the following lipases for further intensive studying – Ces1f (Carboxylesterase 1F), Ces1a (Carboxylic ester hydrolase 1A), Pnpla7 (Patatin-like phospholipase domain-containing protein 7) and Pla2g15 (Group XV phospholipase A2).

B. Tissue type: Lungs

Once we had data on liver our next tissue to work on was lungs. SHs have been previously associated to cause lung cancers. Increased levels of Lyso-PS were found in the plasma of the cancer patients.

B.1 literature

From the list of SHs obtained from untargeted proteomics experiment from Bachovchin and colleagues for lungs, 63 SHs were already identified. As I had done for liver, I had

arranged the SHs abundance in the tissue in the descending order of spectral counts in the membrane fraction (**Fig:4.8**) and looked into each of the SHs and categorized them into lipases. A total of 28 lipases were identified from this list out of which 7 were hydrolases which were suspected to have lipase activity due to the sequence similarity and 1 probable lipase. I then categorized the rest of the lipases as neutral and phospholipases, 11 phospholipases and 9 neutral lipases.

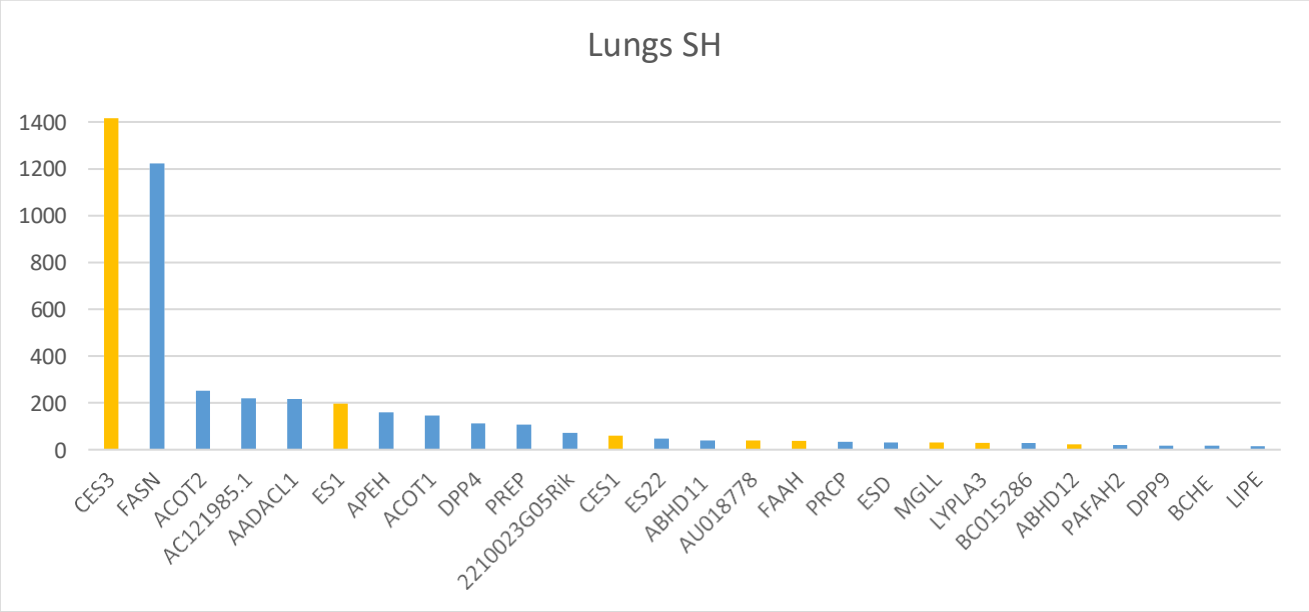

Fig: 4.8: Graph of the SHs present in lungs tissue. Color code: 1. Blue-SHs 2. Yellow-Already identified lipases (y axis = peptide counts)

Lipases	Phospholipases (Lyso)
CES3	ABHD12
ES1	PNPLA6
CES1	LYPLA1
CES1f	PNPLA8
MGLL	PNPLA7
LIPE	PLA2G7
CES1a	LCAT
LPL	BAT5
ABHD6	PLA1A
	LIPC
	LYPLAL1

Table 4.3: Lipase list for lungs tissue categorized as phospholipase and neutral

B.2 FP-Biotin pull down

For FP pull down MS assays, samples were prepared with 1 mg/ml in 1 ml volume of lungs membrane lysates and incubated in 250 µM of FP-Biotin probe and MS sample preparation (according to the protocol) was done by obtaining the protein disc and denaturing, reducing and alkylating the samples followed by avidin enrichment and trypsin digestion to obtain the peptides. To remove salts present in the sample, desalting of the samples was done using C18 disc and samples were run on triple TOF MS and results were obtained.

total	1007
seine hydrolase	23
lipases	7
NP_032014	'Fasn
NP_034204	'Dpp4
NP_034275	'Ephx1
NP_444430	'Ces1d
NP_031980	'Ces1c
NP_032903	'Plg
NP_035974	'Mgll
NP_035286	'Prep
NP_056569	'Rbbp9
NP_848887	'Nceh1
NP_598949	'Acot2
XP_017175463	'Faah
NP_058599	'Esd
XP_017177158	'Gm5409
NP_032535	'Lpl
NP_080455	'Abhd5
XP_006511786	'Apeh
XP_006531454	'Ces2g
XP_006495535	'Lypla1
NP_034036	'Tpp1
NP_032932	'Ctsa
NP_034500	'Gzma
XP_006515240	'Iah1
	'Prss1
	'Pafah2

Table: 4.4 MS results for lungs- comparing replicates for 99% and 95% FDR

The table below (**Table:4.4**) shows the results obtained after performing protein-pilot analysis for 99% and 95% FDR. As we had observed less amount of MS-MS peptide count and less number of SHs identified in other replicates of the lungs membrane lysates samples, we suspected that there was a problem in the sample preparation and decided to perform these experiments again to confirm with the other replicated result that is obtained. From the table we can see that a total of 1007 peptides were picked up out of which 22 SHs were identified out of which 7 were lipases, identified by comparing to the already prepared list of lipases.

After obtaining a better MS replicates result, we plan to compare these to the already present literature and narrow down on the lipases we want to do a focused study.

4.7 Future perspective

In future we plan to try and fetch out potential hits for lyso-PS lipase for both liver and lungs tissues (by FP-Biotin probe pull downs). Once this is done we could recombinantly express these enzymes in HEK cells and biochemically characterize using synthesized Me-lyso-PS and commercially available lyso-PS as substrates. Additionally, knock down and knock out studies can be performed on cell lines belonging to tissues of interest to look for any cellular defects exhibited due to loss of function of these enzymes. It can be extended to mouse knock out studies to understand their broader roles in physiology. It would also help us decipher the role of lyso-PS signaling in lungs and liver basally or under patho-physiological conditions.

Chapter 5: Conclusions

Being associated with the disease PHARC, ABHD12, a major brain lyso-PS lipase needed thorough biochemical characterization. For rigorous assaying of substrates, we synthesized a library of methyl analog of lyso-PS, Me-lyso-PS with both R- and S-configuration in our lab and performed kinetics and substrate assays. The synthesized Me-lyso-PS showed similar hydrolyzing capacity as the commercially available lyso-PS. Our studies also compared the R- and S- configuration and showed that R-configuration, being the naturally occurring form showed hydrolyzing capacity while S-is inactive. Chain length of lipids is an important criterion which also plays a major role in deciding the functions of particular lipids. Thus to see if difference in chain length had any effect on substrate preference of ABHD12, we assayed the library of lipids synthesized. Performing in-vitro kinetics study and endogenous assays we constantly observed that there was a higher hydrolyzing rate for very long chain (VLC) lipids than others. Thus from our study we could establish that ABHD12 prefers very long chain (VLC) lipids as their substrates. Our next aim is to establish that if ABHD12 has any effect due to presence of unsaturation in the lyso-PS lipid chains. If unsaturation in lyso-PS lipid chains affect their preference for ABHD12 as substrate and in signaling pathways remain to be investigated due to lack of unavailability of unsaturated lyso-PS species.

Lyso-PS being an important signaling molecule needs to be regulated. Accumulation of these have shown to cause diseases (like PHARC). Bio-GPS studies showed presence of ABHD12 mRNA in the metabolic tissues, but lacks expression. So it can be anticipated that there are other lyso-PS lipases in these metabolic tissues which keep a check on lyso-PS accumulations. Lipases belong to the serine hydrolase family thus FP/ fluorophosphanate derived probes are used to characterize these. We synthesized these probes in lab and optimized the conditions for the same. Several experiments with metabolic tissues isolated from ABHD12 WT and KO mouse let us confirm that there are lipases (SHs) present in the membrane fraction of these metabolic tissues.

We selected liver and lungs because these tissues are metabolically active and have abundant lyso-PS independent of ABHD12 activity. Additionally, these tissues showed the maximum difference in my inhibitor studies. Thus these tissues were ideal for fishing lyso-PS lipases alternative to ABHD12. Thorough literature search was carried out to organize the already identified lipases in these tissues. Annotated and unannotated lipases and hydrolase known to show sequence similarity with lipases were enlisted. Untargeted proteomics were performed with FP-Biotin probe on these tissue lysates to pull down and identify all the serine hydrolases present in these tissues and perform a comparative study to the already identified lipases. We are now at this stage for liver samples and more FP-Biotin pull down experiments need to be performed for lungs. From this we have narrowed down on few lipases and we plan to look deeper in literature as well as carry out more experiments to try and identify a novel lyso-PS lipase/s in these tissues.

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Image References:

1. **Fig 4.1** - Blankman, J. L., Long, J. Z., Trauger, S. A., Siuzdak, G., & Cravatt, B. F. (2013). ABHD12 controls brain lysophosphatidylserine pathways that are deregulated in a murine model of the neurodegenerative disease PHARC. *Proceedings of the National Academy of Sciences*, *110*(4), 1500-1505.

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