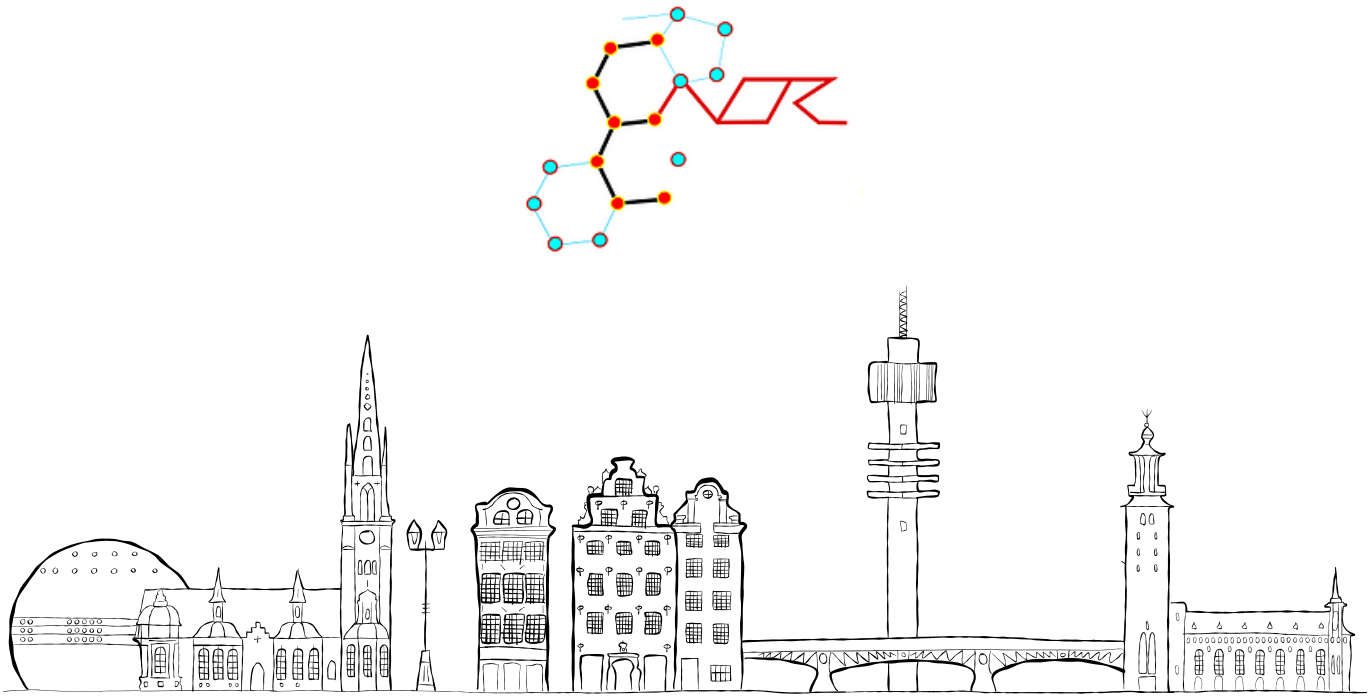
15th Meeting of the European Network for Oxysterol Research

Stockholm, Sweden
September 14th - 15th, 2026



Oxysterols: signaling molecules, therapeutic targets, and biomarkers in health and disease

Avanti Research

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Hosted by Silvia Maioli laboratory,
Division of Neurogeriatrics, Center for Alzheimer Research
Department of Neurobiology, Care Sciences and Society
Karolinska Institutet

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ABSTRACTS

Oral presentations

DAY 1:

Monday, September 14th

A PUBLICLY ACCESSIBLE STEROL AND STEROID ATLAS OF MOUSE BRAIN

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The brain is the most sterol rich organ in the body of mammals. Here we present a publicly accessible *Sterol and Steroid Atlas of Mouse Brain* containing mass spectrometry imaging (MSI) data for sagittal sections of male and female C57BL6J mouse brains at an age of 56 days. Data is presented for twenty sections (plates) cut across a hemisphere of mouse brain at 200 µm intervals and mapped to the Allan Mouse Brain Atlas (AMBA). High resolution MALDI-MSI data is presented for cholesterol (50 µm) and for neurosteroids (100 µm), and low-resolution liquid-extraction for surface analysis (LESA)-MSI data for cholesterol precursors and oxysterols (800 µm). Data was normalised to isotope labelled standards sprayed on before a derivatisation step and prior to matrix application. The atlas shows high spatial resolution quantitative imaging data, plate by plate, for 13 neurosteroids, including progesterone, corticosterone and androstenedione; cholesterol, and at low resolution the cholesterol precursors desmosterol and the oxysterols 24S-hydroxycholesterol and 24S,25-epoxycholesterol. Quantitative data can be perused compound by AMBA region, compound by plate number and compound by sex. Some notable observations are that cholesterol is most abundant in fibre tracts and hind brain, while its major metabolite is most abundant in the regions of striatum and palladium. We aim to extend the Atlas to young (14 day) and old (24 month) mouse brain and to mouse models of neurodegenerative disease.

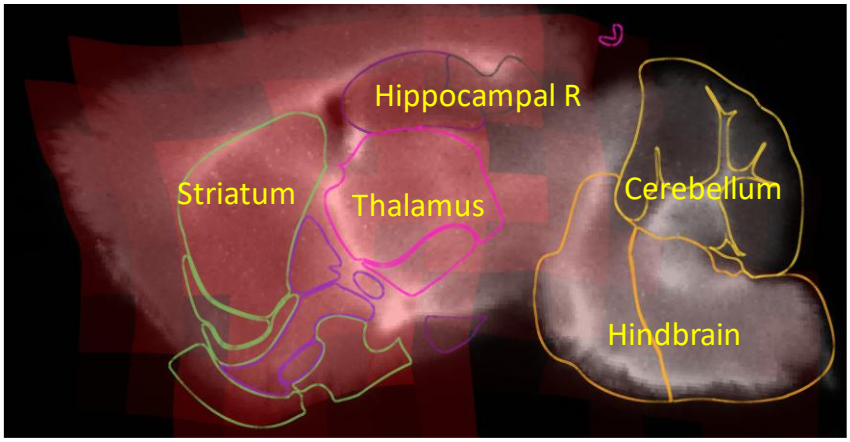

Figure. Image of a sagittal section of mouse brain showing a high-resolution image of cholesterol (in white) overlayed with low-resolution 24S-HC data in red. While cholesterol is most abundant in fibre tracks and hindbrain 24S-HC is most abundant in striatum and thalamus.

MICROBIAL TRANSFORMATION OF BILE ACIDS IN THE GUT: QUANTIFICATION OF OXO- AND ISO-BILE ACIDS BY LC–MS/MS

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Bile acids that reach the colon are extensively metabolized by the gut microbiota. Deconjugation is catalyzed by bile salt hydrolases (BSHs), whereas 7α-dehydroxylation proceeds via a multi-enzyme pathway. Further transformations — including oxidation and epimerization — are catalyzed by microbial hydroxysteroid dehydrogenases (HSDHs), which mediate the oxidation and reduction of hydroxyl groups on bile acids. Both primary and secondary bile acids can undergo such modifications at the C-3, C-7, and C-12 positions, in which hydroxyl groups in the α-orientation are oxidized to oxo-intermediates and subsequently reduced to the β-orientation — a net epimerization that proceeds through individually reversible redox steps.

For example, in the urso-bile acid pathway, CDCA is converted into the oxo-intermediate 7-oxochenodeoxycholic acid (7-oxoCDCA) and subsequently into ursodeoxycholic acid (UDCA) through the sequential action of 7α-HSDH and 7β-HSDH. The secondary bile acids lithocholic acid (LCA) and deoxycholic acid (DCA) are generated via the multi-step 7α-dehydroxylation of CDCA and CA, respectively. In the iso-bile acid pathway, DCA is transformed into 3-oxoDCA by 3α-HSDH and subsequently into isoDCA by 3β-HSDH. In addition, DCA can be converted into 12-oxoDCA by 12α-HSDH and further into epiDCA by 12β-HSDH. Notably, microbial bile acid 3α- and 3β-HSDHs generally show a preference for dihydroxy bile acids, such as DCA- and CDCA-derived bile acids, over trihydroxy bile acids derived from CA.

Although the physiological role of microbial HSDHs is not yet fully understood, increasing evidence suggests that oxo- and iso-bile acids contribute to host signaling pathways. In particular, metabolites of the iso-bile acid pathway have been shown to interact with several host bile acid receptors. For example, 3-oxoLCA and LCA can each activate the bile acid receptors FXR, VDR, and PXR, whereas 12-oxoDCA, 7-oxoCDCA, and UDCA exhibit little or no activation of FXR or VDR [1–3]. These findings suggest that microbial bile acid transformations may have important consequences for host physiology and bile acid-mediated signaling.

In this study, we present an LC–MS/MS method for the accurate quantification of bile acids, including oxo- and iso-forms, in fecal samples, and demonstrate its application in a human cohort study. Iso-bile acids can constitute up to approximately 50% of the total fecal bile acid pool. Among the oxo-bile acids, 12-oxoDCA is one of the most abundant species detected in human feces, reaching concentrations of up to approximately half the level of DCA. These findings highlight the substantial contribution of microbial bile acid transformations to the composition, diversity, and signaling potential of the intestinal bile acid pool.

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OXYSTEROL PROFILES IN THE LUNG REFLECT HOST–PATHOGEN CO-METABOLISM AND IDENTIFY ACTIVE TUBERCULOSIS

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Tuberculosis (TB) is caused by the bacterial pathogen *Mycobacterium tuberculosis* (Mtb) and remains a leading cause of infectious mortality worldwide. The pathogenesis of Mtb is tightly linked to cholesterol metabolism in the lung, where the pathogen promotes the formation of lipid-overloaded macrophages that serve as a replicative niche for the bacilli. Mtb and a select group of actinobacteria encode machinery to oxidize the cholesterol side chain and break down the sterol rings into intermediates that fuel bacterial metabolism.[1,2] We previously identified an unusual oxidized cholesterol metabolite, cholestenone, that accumulates in sputum during active tuberculosis. The Mtb enzyme 3β-Hsd converts cholesterol to cholestenone and can modify a variety of oxysterol substrates *in vitro*, prompting us to investigate whether Mtb modifies oxysterols in people.[3,4] Oxysterols integrate lipid metabolism, host defense, and inflammatory responses, yet their role in TB remains poorly defined. To understand how TB impacts the oxysterol repertoire in the lungs, we profiled oxysterols in sputum from 78 HIV-negative adults from Peru and Vietnam with active TB or with compatible symptoms without Mtb infection using liquid chromatography-tandem mass spectrometry. This revealed a unique sputum oxysterol signature, in which 27-hydroxycholesterol, a major lung-derived liver X receptor agonist, and cholestenoic acid are converted to 27-hydroxy-4-cholesten-3-one and 3-oxo-4-cholestenoic acid and accumulate during active TB. Additional intermediates in the acidic pathway of bile acid synthesis including 3β,7α-dihydroxycholest-5-enoic acid and 7α-hydroxy-3-oxo-4-cholestenoic acid were enriched in TB sputum relative to symptomatic controls. In an independent cohort, sputum 27-hydroxy-4-cholesten-3-one and 3-oxo-4-cholestenoic acid decreased significantly with effective TB therapy. Further, we found that the Mtb enzyme 3β-Hsd is required to convert 27HC to 27-hydroxy-4-cholesten-3-one and cholestenoic acid to 3-oxo-4-cholestenoic acid in macrophages *ex vivo,* and that 27-hydroxy-4-cholesten-3-one lacks the ability to activate liver X receptor signaling. These findings point to CYP27A1–3β-Hsd co-metabolites as biomarkers of TB disease and provide mechanistic insight into a virulence strategy of Mtb that impairs liver X receptor signaling to fuel bacterial growth, impair cholesterol efflux, and reshape local immunity.

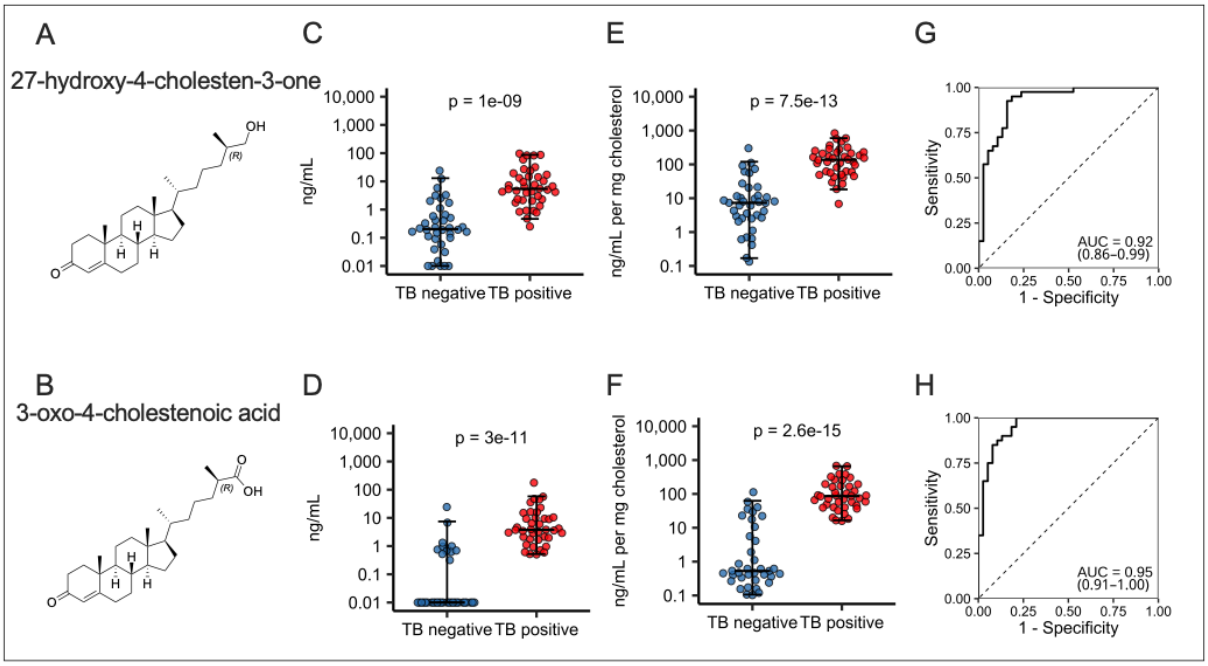

Figure: 27-hydroxy-4-cholesten-3-one and 3-oxo-4-cholestenoic acid accumulate in sputum during active TB and predict TB status. Sputum samples from 78 subjects with active TB or symptomatic controls were processed and measured using liquid chromatography-tandem mass spectrometry. (A-B) Structures, (C-D) raw sputum concentrations, (E-F) cholesterol-normalized sputum concentrations, and (G-H) ROC curves showing ability of 27-hydroxy-4-cholesten-3-one and 3-oxo-4-cholestenoic acid to predict TB status. Statistical significance was assessed using Wilcoxon rank-sum tests and plotted as median with 95% confidence intervals (C-F). ROC curves are shown with area under the curve and 95% confidence intervals estimated by the DeLong method (G-H).

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MINIATURIZED LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) PLATFORM FOR STUDYING OXYSTEROLS IN PFAS-EXPOSED LIVER ORGANOIDS

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Metabolic dysfunction-associated steatotic liver disease (MASLD) is characterized by excessive lipid accumulation in the liver and can progress to steatohepatitis and cirrhosis. The disease is driven by altered cholesterol metabolism, and exposure to environmental pollutants such as per- and polyfluoroalkyl substances (PFAS) has been associated with an increased risk of MASLD[1]. Oxysterols have been proposed as potential biomarkers, and profiling these metabolites could provide more insight into the link between PFAS exposure and MASLD. New Approach Methodologies (NAMs), such as organoids, are increasingly used to investigate human health effects in more translationally relevant models compared to animal systems. This requires tailoring analytical workflows to these complex, heterogeneous, and minute samples, e.g., through miniaturization of sample preparation and chromatographic methods.

Our previous study, using LC-MS, demonstrated elevated oxysterol levels in steatotic liver organoid models compared to healthy controls[2]. Building on this, we optimized and miniaturized the sample preparation for Girard T derivatization of oxysterols, enabling the quantification of 26-hydroxycholesterol in a single liver organoid[3]. To further improve sensitivity and expand oxysterol coverage, we are developing a capillary LC-MS method using a 0.3 mm inner diameter column with online sample clean-up. Experimental design strategies are applied to optimize sample loading and MS parameters. The downscaled method enables the separation of nine oxysterols, with preliminary results showing promising linearity and repeatability, and limits of detection in the sub-picogram range. Importantly, our combined oxysterol workflow, including sample preparation, significantly reduces reagent consumption and mobile phase usage compared to conventional LC-MS workflows.

The method will be applied to PFAS-exposed human liver organoids to explore the potential links between environmental contaminants and lipid dysregulation in liver disease.

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STATIN-INDUCED ALTERATIONS OF FREE STEROLS AND TOCOPHEROLS IN HUMAN PLASMA QUANTIFIED BY RP-UHPLC/MS/MS

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Precise monitoring of the free sterols and tocopherols profile is often interfered by the poor ionization efficiency and extensive structural isomerism of these molecules. By utilizing a targeted chemical derivatization strategy, we can reduce in-source fragmentation and essentially improve sensitivity by the reversed-phase ultra-high performance liquid chromatography coupled with tandem mass spectrometry (RP-UHPLC/MS/MS). We have recently introduced our derivatization approach (1) on untargeted sterols, tocopherols, and acylglycerols species. Since then, we have further optimized RP-UHPLC conditions and extraction efficiency for free sterols and tocopherols species only, resulting in 12 min total run time, resolving critical isomeric and isobaric species, such as cholesterol vs. lathosterol (**Figure**). Moreover, multiple reaction monitoring (MRM) transitions combined with our specific derivatization tag and retention dependencies assure high confident identification resulting in identification of 26 free sterols and 3 tocopherols in pooled human plasma. Method was validated following recommendation for bioanalytical methods, and eight internal standards were used for accurate quantitation analysis of NIST SRM 1950. In total, 17 free sterols and 2 tocopherols were quantified with high correlation of reported literature concentrations in human plasma.

The validated method revealed significant concentration differences between statin-treated individuals and controls: endogenous sterols were downregulated in the statin group, while exogenous species showed non-significant changes, confirming that statins primarily target biosynthetic precursors rather than phytosterols or other dietary sterols. The results demonstrated the methodology's capacity to resolve complex sterol profiling, providing an excellent tool for investigating drug-induced alterations in cholesterol biosynthetic pathways without the interference of esterified fractions. This approach provides a more physiologically relevant metabolic information and prevents other sensitive sterol species from degrading during sample preparation. Overall, this study underscores the necessity of isomer-resolved lipidomic analysis for understanding drug-induced alterations in plasma lipid composition.

This work was supported by the ERC Advanced grant No. 101095860 (European Research Council).

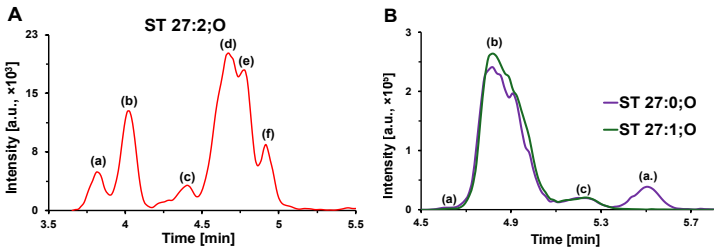

Figure. Extracted ion chromatograms of the derivatized sterol species, where A) shows separation of six derivatized isomeric molecules (a – f) with the structure level of ST 27:2;O and B) shows separation of derivatized isobaric molecules – the $[M+2]^-$ ions of ST 27:1;O (a – c) from $[M]^-$ ions of ST 27:0;O (a.).

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BEYOND INSTRUMENTAL PERFORMANCE: REASSESSING ANALYTICAL UNCERTAINTY IN STEROL OXIDATION PRODUCT DETERMINATION

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Sterol oxidation products (SOPs), including cholesterol oxidation products (COPs) and phytosterol oxidation products (POPs), are increasingly investigated as markers of lipid oxidation and potential bioactive compounds. Considerable efforts have been devoted to improving chromatographic separation and mass spectrometric sensitivity; however, the reliability and comparability of SOP datasets remain a matter of debate. The present work provides a critical reassessment of SOP determination by integrating experimental evidence generated through three complementary studies focused on reference material availability, analytical calibration strategies and sample preparation artefacts.

A first methodological breakthrough was achieved through the isolation of fifteen individual POPs derived from β-sitosterol, campesterol and stigmasterol using semi-preparative HPLC. The purified compounds showed purities ranging from 90.2 to 97.5% and enabled the validation of an LC-Orbitrap-HRMS method characterized by limits of detection between 0.012 and 0.093 ng/g, recoveries of 82–98%, and repeatability below 12%. Application of the method revealed POP concentrations up to 15.0 mg/kg in refined high-oleic sunflower oil and demonstrated that refining processes may increase POP formation by 13–29%, depending on the oil matrix.

The availability of authentic POP standards subsequently allowed a direct evaluation of the widespread practice of using COPs as surrogate reference materials. Significant differences ($p<0.05$) in detector response were observed by both LC-Orbitrap-HRMS and GC-MS. In refined high-oleic sunflower oil, the use of COP-based calibration resulted in total POP concentrations of 10.6–15.3 mg/kg by LC-MS, whereas authentic POP calibration led values between 1.8 and 2.8 mg/kg. Similar discrepancies were observed by GC-MS, where POP concentrations increased from 1.3–1.6 mg/kg to 4.2–5.1 mg/kg depending on the calibration strategy adopted.

A further source of uncertainty emerged from sample preparation. Cold saponification, commonly considered the reference procedure for SOP extraction, induced substantial degradation of 7-keto derivatives. Under the harshest conditions investigated (4 mol/L KOH, 18 h), recoveries decreased up to 12% for 7-ketocholesterol, 12% for 7-ketocampesterol, 13% for 7-ketostigmasterol and 33% for 7-ketositosterol. In contrast, 5,6β-epoxy and 7β-hydroxy derivatives exhibited significantly greater stability. Similar trends were confirmed in model lipid systems (medium chain triacylglycerols) and real food matrices (powdered milk and French fries), where losses of phytosterol 7-keto derivatives reached approximately 40–50%.

Collectively, these findings demonstrate that analytical uncertainty in SOP determination is primarily driven by compound-specific biases originating from reference material selection and sample preparation rather than instrumental performance itself. Current analytical protocols may therefore simultaneously overestimate epoxy- and hydroxy-derivatives while underestimating 7-keto compounds. The results highlight the need for harmonized methodologies based on authentic POP standards and optimized extraction procedures, providing a framework for improving the accuracy, comparability and regulatory relevance of future SOP datasets.

SIX DECADES WITH OXYSTEROLS – INGEMAR BJÖRKHEM'S SCIENTIFIC WORK

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Ingemar Björkhem's research on cholesterol metabolites began in the early 1960s at the Department of Medical Chemistry, right when he began his medical studies at the Karolinska Institute at Solna. When we look back at the history of cholesterol and bile acid metabolism today, several names from this department are inseparably linked with major milestones. The name of our honored guest, Prof. Ingemar Björkhem, stands at the very top of one unique chapter: oxysterols.

This review starts before the era of molecular biochemistry. At that time, clinical chemistry was mainly based on organic and analytical chemistry. However, thin layer chromatography, liquid lipid extraction methods, ultracentrifugation, high-performance liquid chromatography, and mass spectrometry were already well established. These analytical tools were used alongside animal models, such as bile fistula rats, to investigate bile acid metabolism using custom-synthesized, stable-isotope-labeled or radiolabeled tracer compounds.

Ingemar's main interest was directed toward a specific cholesterol metabolite, that had already fascinated other scientists in his institute before him: 7α-hydroxy-4-cholesten-3-one. We will learn how his fascination with this compound has shaped his career over the decades. His work spanned from its chemical synthesis to the mechanism of its enzymatic formation, its metabolism, and its role in bile acid biosynthesis and pathogenetic mechanisms. Furthermore, he developed highly selective and specific methods for its quantitative detection, establishing it as a reliable marker for bile acid synthesis in both healthy states and rare inborn diseases.

After specialising in Clinical Chemistry, he moved to the Department of Clinical Chemistry at the Karolinska Institute, Huddinge Hospital. There, he developed highly accurate isotope dilution methods for cholesterol, bile acids, hormones, anabolic steroids and numerous other compounds to validate existing enzymatic methods or radioimmunoassays. Additionally, he built up a highly successful molecular biology team. This work opened the door to understanding the enzymatic processes in bile acid synthesis that control the initial, critical steps of cholesterol breakdown in the liver. He well deservedly received a professorship in „Biochemical Research on Atherosclerosis" and became the head of the Department of Clinical Chemistry and Clinical Research Centre, Karolinska Institute, Huddinge Hospital. Together with his colleagues from the Department of Clinical Pharmacology, he built up the anti-doping laboratory at Huddinge Hospital.

After intensive analytical work to establish a method for the assay of major oxysterols in human plasma, the global foundation for oxysterol analysis was laid. Interest in studying ring- and side-chain oxidized cholesterol metabolites grew immensely. This research expanded far beyond gastroenterology and hepatology into medical specialties such as oncology, neurology, psychiatry, angiology, cardiology, and endocrinology. Oxysterols were no longer considered merely biological waste products of their common substrate cholesterol, but rather critical, highly potent signaling and regulatory molecules in the human body. Today, the enzyme systems producing these oxidized oxysterols serve as novel drug targets to treat neurodegenerative diseases. Furthermore, transgenic and knock-out mouse models became available to support ongoing studies in this rapidly evolving field.

Ingemar Björkhem remained loyal to Karolinska Institute well past his retirement. His career is highlighted by sixty years of generosity as a colleague and mentor, relentless curiosity, scientific excellence, and intellectual pioneering work, which among others laid the scientific groundwork for ENOR (the European Network for Oxysterol Research).

THE ROLE OF CYP46A1 IN BRAIN HEALTH AND ALZHEIMER'S DISEASE RISK IN WOMEN

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CYP46A1 is a brain-specific enzyme that plays a key role in cholesterol elimination by converting cholesterol into 24(S)-hydroxycholesterol (24SOH). In recent years, CYP46A1 and 24SOH have attracted increasing attention as potential therapeutic targets and biomarkers in neurodegenerative diseases, including Alzheimer's disease (AD).

Our group has shown that overexpressing *CYP46A1* and increasing 24SOH levels can protect the brain in a sex-specific manner, promote estrogen signaling, and counteract age- and menopause-related memory loss (1). We also found that higher 24SOH levels in cerebrospinal fluid of patients from a memory clinic cohort were associated with markers of neurodegeneration in women, such as tau and phospho-tau (1). Our data led us to hypothesize that *CYP46A1* and 24SOH may have particular relevance to AD risk in females.

We are currently investigating the effects of *CYP46A1* overexpression in male and female mouse models of AD (App knock-in) across aging. We examined behavior, brain cholesterol metabolism, neuroinflammation, and brain lipid changes through lipidomics analyses. In parallel, we analyzed the relationship between circulating oxysterols and sex hormones in approximately 1,900 postmenopausal women from the Women's Health Initiative Study.

CYP46A1 overexpression in App knock-in mouse models had both sex- and age-dependent effects. Overall, our data suggest that females showed improved cholesterol metabolism and reduced glial reactivity, whereas males showed increased neuroinflammation and greater lipid dysregulation with aging. In the human cohort, higher levels of bioavailable estrogens were associated with higher circulating oxysterols, including 24SOH and 27OH, particularly in women carrying APOE4 (2).

Together, these findings highlight important sex differences in the response to activation of brain cholesterol metabolism and support *CYP46A1* as a potential therapeutic target. They also suggest that 24SOH and related oxysterols may have value as sex-specific biomarkers for risk of neurodegeneration, particularly in women.

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CYP46A1 LINKS CHOLESTEROL METABOLISM TO AUTOPHAGY–LYSOSOME DYNAMICS IN NIEMANN–PICK TYPE C DISEASE

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The neuronal specific cholesterol 24-hydroxylase (CYP46A1) is responsible for the major pathway of cholesterol elimination from the brain, through the conversion of unesterified cholesterol to 24(S)-hydroxycholesterol. We have previously demonstrated that a gene therapy-based delivery of CYP46A1 in the $Npc1^{tm(I1061T)}$ mice model was able to improve various Niemann-Pick Type C disease (NPC) features[1].

Herein, we aimed to determine a potential role of CYP46A1 in rescuing compromised autophagy. Our results show that adeno-associated virus CYP46A1 ectopic expression in $Npc1^{tm(I1061T)}$ mice led to a decrease in p62 levels in the cerebellum of both male and female mice, and in the cortex of female mice. These changes in female mice cortex are accompanied by a decrease of Microtubule-associated protein 1A/1B-light chain 3 (LC3) lipidation and an increase in phosphorylation levels of AMP-activated protein kinase (AMPK) and Beclin-1.

In agreement, ectopic expression of CYP46A1 decreased LC3 lipidation in fibroblasts from NPC1 patients ($NPC1^{I1061T/I1061T}$) and in HeLa NPC1 knock-out cells (NPC1-KO). The maturation of autophagosomes to autolysosomes was evaluated in NPC1-KO cells transfected with the tandem-fluorescent-tagged *mCherry-GFP-LC3* reporter. We observed an increase in the number of autophagosomes in NPC1-KO cells that is unaffected by CYP46A1 expression, nevertheless the number of autolysosomes significantly increased after CYP46A1 overexpression. Moreover, immunofluorescence assays show an increase in the percentage of vesicles that stain for LC3 and Lysosome-Associated Membrane Protein 2 (LAMP2), relative to the population of LAMP2 positive vesicles, further suggesting that CYP46A1 expression favours autolysosome formation. Moreover, CYP46A1 expression leads to a decrease in perinuclear index of LAMP2-positive puncta, with a decrease in the number and size of LAMP2 positive vesicles in the perinuclear region, and an increase in the number of LAMP2 compartment in the periphery, accompanied by an increase in CYP46A1-dependent β-hexosaminidase release in NPC1-KO cells after being challenged with ionomycin and $CaCl_2$.

Overall, in addition to promoting autolysosome fusion, CYP46A1 alters lysosome spatial organization, decreasing perinuclear clustering and redistributing lysosomes toward the cell periphery. This relocalization is coupled with a reduction in lysosome size in the perinuclear region, further supporting a shift toward a more dynamic and functional lysosomal population. Importantly, CYP46A1 also enhances lysosomal exocytosis, indicating that lysosomal activation extends beyond degradation to include fusion with the plasma membrane and extracellular release of lysosomal contents. This suggests that CYP46A1 promotes a global lysosomal clearance program encompassing fusion, degradation, and exocytosis and is well positioned to be tested in combination with other therapeutic agents.

This work was supported by FEDER and by National Funds through Fundação para a Ciência e a Tecnologia (FCT) under project PTDC/MED-NEU/29455/2017, project UID/04138/2025 (https://doi.org/10.54499/UID/04138/2025), and PhD grant UI/BD/153696/2022 (IC), and Bolsa de Investigação da Sociedade Portuguesa de Doenças Metabólicas (SPDM).

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INVESTIGATING 27-HYDROXYCHOLESTEROL-INDUCED CHOLESTEROL CRYSTAL FORMATION IN RETINAL CELLS WITH NPC1 AND NPC2 DEFICIENCY

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Cholesterol crystallization is highly associated with cardiovascular diseases, lipid dysregulation, and inflammatory activation, yet the molecular mechanism driving the crystal nucleation remains unknown [1]. Oxysterols are known to act as metabolic messengers that influence lipid homeostasis, nuclear receptor signalling, oxidative stress, and immunity [2]. In recent studies, we have found that 27-hydroxycholesterol (27HC) can promote the formation of needle-like cholesterol crystals in various cell lines. Here, we present a comparison of crystal formation in wild-type human retinal pigment epithelium cells (ARPE 19 cells) and with the Niemann Pick disease-associated proteins NPC1 and NPC2 knocked out. We observe that wild-type cells can handle higher amounts of 27HC compared to the knockouts. To investigate this, we use a fluorescent version of cholesterol, cholestatrienol (CTL), and a combination of UV widefield fluorescent microscopy and reflection in combination with stimulated emission depletion microscopy. These approaches provide a framework for examining the early stages of cholesterol crystal formation in cells.

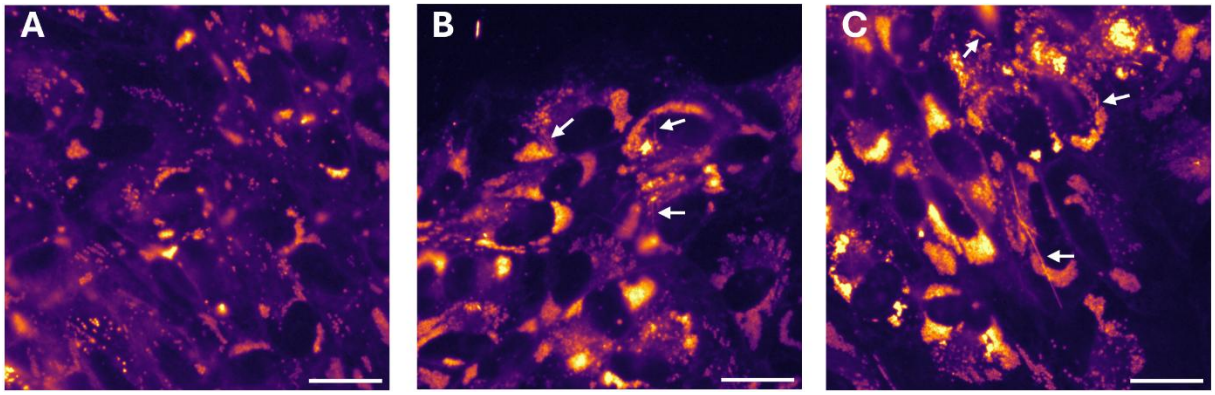

Figure: CTL distribution after 48 hr of 40 µM 27HC treatment in **A** wild-type ARPE19 cells, **B** NPC1 knockout ARPE19 cells, and **C** NPC2 knockout ARPE19 cells. The arrows in **B** and **C** exemplify CTL crystals. Scale bars are 20 µm.

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OLIVE OIL-DERIVED HYDROXYTYROSOL PROTECTS AGAINST OXYSTEROL-INDUCED NEUROINFLAMMATION IN ALZHEIMER'S DISEASE THROUGH MODULATION OF THE SIRT1/TLR4/NFKB PATHWAY

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Oxysterols play a critical role in Alzheimer's disease (AD), by promoting neuroinflammation [1]. Given the lack of effective therapeutic options for AD, increasing attention has been directed toward preventive strategies. Growing experimental evidence indicates that adherence to the Mediterranean diet is associated with a reduced risk of dementia. Notably, olive oil contains hydroxytyrosol (HXT), a potent polyphenol widely recognized as a bioactive compound capable of improving cognitive function [2] and reducing Aβ-induced toxicity [3]. However, limited information is currently available regarding the anti-inflammatory effects of HXT in the context of AD.

The present study aimed to evaluate the ability of HXT to prevent neuroinflammation induced by an oxysterol mixture, whose composition represents the oxysterol levels previously quantified in severe AD brain samples, using human neuroblastoma SK-N-BE cells. We also investigated the involvement of sirtuin 1 (SIRT1)-dependent anti-inflammatory pathways underlying the effects of HXT.

Our results showed that oxysterol exposure significantly increased the expression and production of inflammatory mediators (IL-1β, IL-6, IL-8, TNFα, IFNγ, and MCP-1). This pro-inflammatory response was markedly attenuated by HXT pre-treatment. Furthermore, oxysterols were found to trigger neuroinflammation through TLR4-mediated NFκB activation. HXT treatment increased SIRT1 mRNA and protein levels, as well as its enzymatic activity. Through SIRT1 activation, HXT inhibited oxysterol-induced TLR4 upregulation and the subsequent nuclear translocation of NFκB p65. Moreover, HXT promoted p65 deacetylation, thereby suppressing NFκB transcriptional activity. Importantly, the anti-inflammatory effects of HXT were abolished in the presence of sirtinol, a specific SIRT1 inhibitor.

Overall, these findings demonstrate that HXT mitigates oxysterol-induced neuroinflammation through modulation of the SIRT1/TLR4/NFκB pathway, supporting its potential as a nutraceutical approach for preventing neuroinflammation and subsequent neurodegeneration in AD (Figure 1).

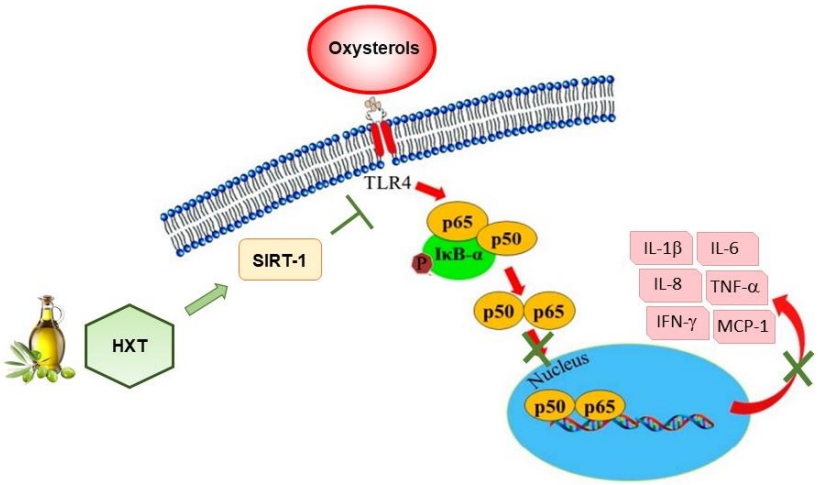

Figure 1. Proposed mechanism of the anti-inflammatory activity of HXT.

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FUCOSTEROL MODULATES CHOLESTEROL METABOLISM AND PREVENTS COGNITIVE DECLINE IN APPSWEPS1ΔE9 MICE

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Liver X Receptor (LXR) activation emerges as a promising strategy for alleviating symptoms associated with Alzheimer's Disease (AD). We have previously demonstrated ameliorating effects of *Himanthalia elongata* and *Sargassum fusiforme* extracts, enriched with natural LXR activators, on cognitive impairment and inflammation-related glia activation in an AD mouse model. These extracts increased the concentration of desmosterol, an endogenous LXR agonist with well-established anti-inflammatory properties, which may underlie the observed reduction in glia activation. Seaweed-derived phytosterol fucosterol demonstrated to increase desmosterol in cell lines. Therefore, we hypothesize that fucosterol contributed to the protective effects of the seaweed extracts on glia activation and cognitive performance in AD mice. Male APPswePS1ΔE9 (AD) and wildtype (WT) mice received either a standard chow or fucosterol-supplemented chow from 5.5 months of age and for a duration of 12 weeks. The cognitive performance was investigated using the object recognition task (ORT) and object location task (OLT). Amyloid-β and microglia (Iba1) and astrocyte (GFAP) markers were quantified using immunohistochemistry (IHC). Sterols were determined in food, serum, liver, and cerebellum using gas chromatography-mass spectrometry. Dietary supplementation with fucosterol increased desmosterol concentrations in liver but not in cerebellum. Fucosterol supplementation prevented cognitive deterioration in APPswePS1ΔE9 mice, despite demonstrating no effect on Aβ plaque load or the expression of microglia and astrocyte activation markers. Fucosterol did not affect body weight or triglyceride concentrations in liver or serum. Our data demonstrate that dietary supplementation with fucosterol prevents cognitive decline in APPswePS1ΔE9 mice. However, fucosterol does not seem to account for the previously observed ameliorative effects of *Himanthalia elongata* and *Sargassum fusiforme* extracts on glia activation. Considering fucosterol's capacity to increase the desmosterol concentrations in liver and circulation, fucosterol emerges as a promising candidate for alleviating peripheral inflammation.

OXYSTEROLS AID MICROGLIAL ACTIVATION AND CHEMOTAXIS OF IMMUNE CELLS TO PROMOTE NEURODEGENERATION IN A MOUSE MODEL OF TAUOPATHY

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Alzheimer's disease (AD), the most prevalent neurodegenerative disorder, is neuropathologically defined by amyloid plaques and tau tangles, with neuroinflammation playing a critical role in disease progression. Brain tissue from AD patients and from mouse models of amyloid and tau pathology show disease-associated microglia with elevated expression of *Ch25h*, which encodes cholesterol 25-hydroxylase responsible for producing the immune-modulatory oxysterol 25-hydroxycholesterol (25HC)[1]. We have shown that 25HC enhances IL-1β production by activated microglia[2] and alters lipid metabolism in astrocytes[3]. Notably, *Ch25h* deficiency in a mouse model of tauopathy (PS19 mice) is associated with reduced neuroinflammation, neurodegeneration, and hippocampal T-cell infiltration, suggesting a link between lipid metabolism and immune responses in AD[4]. 25HC is metabolized by Cyp7B1 to 7α,25-dihydroxycholesterol (7α25diHC)[5], a potent ligand for the chemotactic receptor *Gpr183* (EBI2), which mediates brain infiltration of T-cells in autoimmune encephalomyelitis models[6]. Here, we examine the role of microglial Ch25h with myeloid-specific Ch25h deletion and Gpr183-mediated chemotaxis of immune cells in PS19 mice. Our results implicate a pathogenic role for the 25HC–7α25diHC–Gpr183 oxysterol pathway in AD pathogenesis.

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APOE GENOTYPE-DEPENDENT ALTERATIONS IN OXYSTEROL PROFILES IN HUMAN IPSC-DERIVED ASTROCYTES

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Background: Apolipoprotein E (ApoE) genotype is one of the strongest genetic determinants of Alzheimer's disease risk, with the ApoE4 allele associated with increased susceptibility, enhanced neuroinflammation, and disruption of lipid metabolism. Astrocytes are the principal source of ApoE in the brain and play a key role in regulating cholesterol transport, lipid homeostasis, and inflammatory signalling within the central nervous system. Oxysterols, oxygenated derivatives of cholesterol, are emerging as important bioactive lipids that influence neuroinflammatory responses, oxidative stress, and neuronal function. However, the extent to which ApoE genotype alters oxysterol metabolism in human astrocytes remains poorly understood.

Aim: This study aims to characterise genotype-dependent differences in oxysterol profiles between ApoE3/3 and ApoE4/4 induced pluripotent stem cell (iPSC)-derived human astrocytes, providing insight into how ApoE4-associated lipid dysregulation may contribute to Alzheimer's disease pathology.

Methods: Human iPSC-derived astrocytes carrying ApoE3/3 or ApoE4/4 genotypes were cultured under controlled conditions and subjected to lipid extraction for targeted oxysterol analysis. Oxysterols were quantified using mass spectrometry-based approaches, enabling comparison of genotype-specific abundance patterns and pathway-level differences. Data was analysed to identify oxysterols that are significantly altered in ApoE4/4 astrocytes relative to ApoE3/3 controls and to explore their potential relationship with inflammatory and lipid metabolic pathways.

Results and discussion: ApoE4/4 astrocytes displayed a distinct oxysterol signature, reflecting altered cholesterol handling and potentially enhanced pro-inflammatory lipid signalling. APOE 4/4 astrocytes also showed impaired mitochondrial metabolism. By focusing on human iPSC-derived astrocytes, the study provides a disease-relevant platform for investigating lipid-mediated mechanisms underlying ApoE4-associated risk.

Oral presentations

DAY 2:

Tuesday, September 15th

SIGNALING ROLES OF OXYSTEROLS IN LIPID HOMEOSTASIS AND IMMUNITY

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Most of the cholesterol in the plasma membranes (PMs) of animal cells is sequestered through interactions with phospholipids and transmembrane domains of proteins. However, as cholesterol concentration rises above the PM's sequestration capacity, a new pool of cholesterol, called accessible cholesterol, emerges. The transport of accessible cholesterol between the PM and the endoplasmic reticulum (ER) is critical to maintain lipid homeostasis. This pathway has also been implicated in the suppression of 7ybacterial and viral pathogens by immunomodulatory oxysterols. Here, I will describe a mechanism of depletion of accessible cholesterol from PMs by the oxysterol 25-hydroxycholesterol (25HC), which is rapidly produced in macrophages during acute infections. We show that 25HC-mediated activation of acyl coenzyme A: cholesterol acyltransferase (ACAT) in the ER creates an imbalance in the equilibrium distribution of accessible cholesterol between the ER and PM. This imbalance triggers the rapid internalization of accessible cholesterol from the PM, and this depletion is sustained for long periods of time through 25HC-mediated suppression of SREBPs and continued activation of ACAT. In support of a physiological role for this mechanism, 25HC failed to suppress Zika virus and human coronavirus infection in ACAT-deficient cells, and Listeria monocytogenes infection in ACAT-deficient cells and mice. We propose that selective depletion of accessible PM cholesterol triggered by ACAT activation and sustained through SREBP suppression underpins the immunological activities of 25HC and a functionally related class of oxysterols.

DECIPHERING THE IMPACT OF INFLAMMATION AND OXYSTEROL-MEDIATED METABOLIC REGULATION ON BLOOD–BRAIN BARRIER (DYS)FUNCTIONS

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The blood–brain barrier (BBB) is a critical interface maintaining central nervous system (CNS) homeostasis[1]. Its dysfunction is an early feature of neuroinflammatory and neurodegenerative disorders, but the molecular mechanisms linking inflammation, lipid metabolism, and BBB integrity remain incompletely understood.

Using a human in vitro BBB model composed of brain-like endothelial cells (hBLECs) and human brain pericytes (hBP)[2,3], we investigated the impact of inflammatory signaling on BBB function and cholesterol metabolism. We demonstrated that tumor necrosis factor-α (TNF-α) induces BBB disruption through tight junction alterations and dysregulation of cholesterol homeostasis, involving activation of the SREBP-2 pathway and changes in cholesterol transport mechanisms[4,5]. Interestingly, the oxysterol 25-hydroxycholesterol partially prevented TNF-α-induced BBB leakage and modulated inflammatory cholesterol-related pathways, suggesting a partial protective role in BBB functions[5].

Targeted metabolomic analyses further revealed cell-specific metabolic adaptations following inflammatory and oxysterol stimulation[6]. TNF-α induced metabolic alterations associated with phenylalanine metabolism, while oxysterols mainly affected hBP glycolytic and lipid pathways. These findings highlight metabolic reprogramming as an important mechanism regulating BBB responses to inflammatory stress.

Overall, our studies provide new insights into the mechanisms underlying BBB dysfunction and identify oxysterol-mediated metabolic regulation as a promising therapeutic strategy for neuroinflammatory and neuro(vascular)degenerative diseases.

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24*S*-HYDROXYCHOLESTEROL ARRESTED GLIOBLASTOMA CELLS IN THE G2 PHASE IN AN LXR-DEPENDENT MANNER

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Glioblastoma is the most malignant primary brain tumor with a poor prognosis. Recent studies have shown that the expression level of cholesterol 24-hydroxylase (CYP46A1), which converts cholesterol into 24*S*-hydroxycholesterol (24S-OHC)[1], is negatively correlated with glioblastoma malignancy[2]. Although 24S-OHC has been reported to suppress glioblastoma cell growth, the underlying mechanisms remain unclear.

In this study, we investigated the effects of 24S-OHC on human glioblastoma T98G cells with low CYP46A1 expression and explored the underlying mechanisms. Treatment with 24S-OHC (1–20 µM) for 72 h decreased cell viability in a concentration-dependent manner. Cell cycle analysis using PI-EdU assay revealed a significant increase in the G2/M phase population following treatment with 5 µM 24S-OHC.

Western blot analysis demonstrated that 24S-OHC significantly decreased Cyclin B1 expression and increased the level of phosphorylated CDK1 (inactive form), indicating G2 phase arrest. Furthermore, since 24S-OHC is a ligand of liver X receptor (LXR), the involvement of LXR was examined using the LXR inverse agonist, SR9243. Co-treatment with SR9243 significantly inhibited 24S-OHC-induced reductions in cell number. Moreover, the reduction in Cyclin B1 expression and the increase in phosphorylated CDK1 expression were suppressed by co-treatment with SR9243.

Mechanistically, 24S-OHC decreased the mRNA and protein expression levels of the transcription factor FoxM1 and its target gene Cyclin B1. Additionally, CDC25B expression, a phosphatase that activates CDK1, was significantly reduced. These effects were reversed by co-treatment with SR9243.

Taken together, these findings suggest that 24S-OHC induces G2-phase arrest in glioblastoma cells through LXR activation, leading to the suppression of FoxM1 and its downstream targets, Cyclin B1 and CDC25B, and to the consequent accumulation of inactive CDK1. This study highlights the potential of the 24S-OHC–LXR pathway as a therapeutic target for glioblastoma.

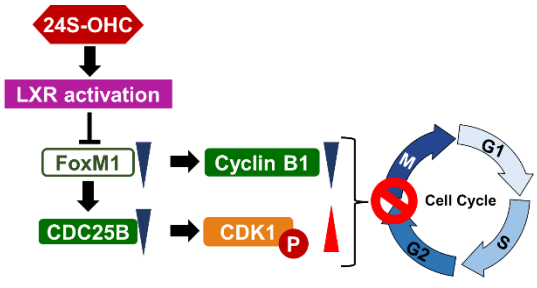

Figure: 24S-OHC suppresses glioblastoma proliferation in an LXR-dependent manner.

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LXR LINKS CHOLESTEROL METABOLISM TO FERROPTOTIC SUSCEPTIBILITY IN MACROPHAGES

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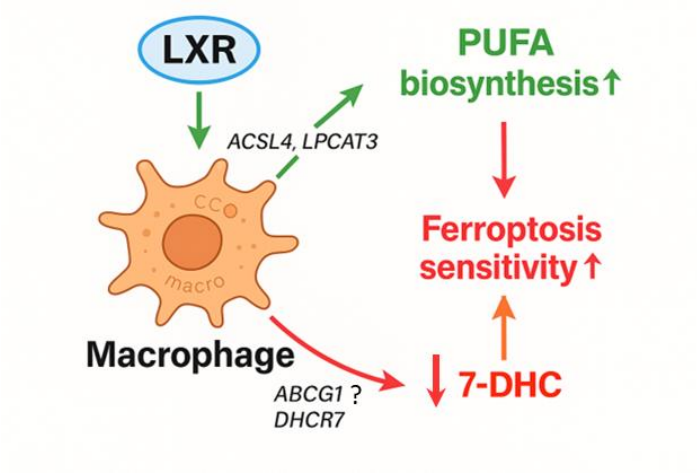
Background
Ferroptosis is a form of regulated cell death characterized by the peroxidation of membrane lipids and is implicated in numerous diseases, including cardiovascular, neurodegenerative, and cancer-related disorders. Macrophages, which can adapt their lipid composition in response to inflammatory signals, play key roles in these processes, particularly in atherosclerosis. The Liver X Receptor (LXR), a nuclear receptor activated by oxidized cholesterol derivatives, is a major regulator of lipid metabolism and may influence macrophage susceptibility to ferroptosis.

Methods
Primary human and murine macrophages were treated with the LXR agonist GW3965 and the ferroptosis inducer RSL3. Ferroptosis sensitivity was assessed alongside the silencing of LXR transcriptional targets involved in polyunsaturated fatty acid (PUFA) metabolism, including LPCAT3 and ELOVL5, as well as cholesterol efflux through ABCG1. Lipid composition and sterol levels, including 7-dehydrocholesterol (7-DHC), were analyzed by mass spectrometry. To better mimic a pathological context, macrophages were also treated with atherosclerotic plaque extracts, leading to cellular loading with oxysterols and phospholipids.

Results
LXR activation significantly increased macrophage sensitivity to RSL3-induced ferroptosis. This effect was associated with enhanced PUFA biosynthesis and reduced intracellular levels of 7-DHC. However, silencing of LPCAT3 or ELOVL5 only partially reduced ferroptosis sensitivity, suggesting the involvement of additional mechanisms independent of lipid remodeling. Furthermore, modulation of cholesterol efflux appeared to sensitize cells to ferroptosis.
In addition, LXR activation induced a redistribution of 7-DHC, characterized by decreased intracellular levels and increased extracellular levels, resulting in an elevated extracellular-to-intracellular 7-DHC ratio. Increased extracellular 7-DHC was associated with reduced ferroptosis sensitivity, suggesting a protective role for this compound.
In the context of atherosclerosis, foam macrophages undergo alterations in lipid composition that are also associated with changes in ferroptosis sensitivity.

Conclusion
These findings reveal a link between LXR activation, cholesterol metabolism, and macrophage susceptibility to ferroptosis. Beyond PUFA remodeling, modulation of cholesterol metabolism appears to be a key determinant of ferroptotic vulnerability. Ongoing studies aim to further investigate the role of these mechanisms in the development and progression of atherosclerosis.

Figure.



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LXR PATHWAY ACTIVATION IN PREGNANCY DRIVES MATERNAL ADAPTATIONS THAT INCREASE LIPID SUPPLY TO THE FETUS

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During a normal pregnancy, maternal metabolism undergoes striking adaptations to meet the nutritional demands of the developing fetus. Anabolic pathways in early pregnancy promote the net accumulation of nutrients within maternal tissues, primarily in the form of triglycerides. A mid-gestation catabolic switch then drives the breakdown of nutrient stores, ensuring the circulating availability of fatty acids and glucose for fetal uptake. These dynamically regulated shifts in maternal nutrient allocation are vital for a healthy pregnancy, as poor adaptations, commonly seen in obese pregnancy, can profoundly disrupt the development and long-term metabolic health of the child (1).

Fatty acids are crucial components at every stage of fetal development, not just as an energy source, but also to provide building blocks and key signals for organogenesis. Although adipose-derived non-esterified fatty acids can be directly transported across the placenta, the majority are transferred to the maternal liver, a highly adaptive, yet poorly characterised metabolic tissue in pregnancy. Hepatic re-esterification of fatty acids into triglycerides and export into the circulation within lipoproteins underlies the characteristic rise in plasma triglycerides in late gestation and is considered the primary source of fatty acids for the fetus (2). However, the molecular mechanisms driving this process are not well understood.

We recently profiled the liver and plasma transcriptome and lipidome of virgin and pregnant mice as an unbiased discovery approach for lipid pathways in the maternal liver that are associated with maternal fatty acid provision to the conceptus. We discovered a late pregnancy-specific, selective activation of the Liver X Receptor signalling pathway which dramatically increases maternal supply of fatty acids and cholesterol (3). Deletion of LXRα/β in female mice prevents many aspects of metabolic adaptation and ultimately compromises fetal mass and placental function. We are currently investigating how the LXR pathway is activated in pregnancy, and how it functions across tissues to co-ordinate maternal metabolism.

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ROLE OF THE HDL SCAVENGER RECEPTOR CLASS B TYPE 1 (SCARB1) IN METASTAZING PROSTATE CANCER

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Cholesterol plays a role in prostate cancer (PC) progression (1), particularly to metastasis and castration-resistant PC (CRPC), as well as being found accumulated in carcinoma and secondary sites (2). The receptor for HDL cholesterol uptake SCARB1 has been found overexpressed PC proliferative and metastatic cell phenotypes (3). This overexpression is linked to lower levels of tumor suppressors like PTEN, TP53 and SMAD4, key players in the process of prostate cancerogenesis.

In vivo experiments confirm the role of SCARB1 in cancer progression and metastatization by signicatively reducing *knocked-out* cell proliferation in orthotopic mice models. In parallel, we are developing a novel syngeneic orthotopic mouse model to study metastasis drivers like PTEN, SMAD4, Tp53 loss. Additionally, we are investigating SCARB1 orthologs in *Drosophila melanogaster*'s tumorigenesis in the accessory gland (ortholog of the human prostate, 4).

Future studies will explore lipid metabolism within tumors, further defining the precise role of SCARB1 and HDL-cholesterol transport on metastasis.

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Part of this project has been supported by the European Union's Horizon Europe research and innovation program under the Marie Sklodowska-Curie grant agreement No 101120283.

Funded by the European Union

25-HYDROXYDIHYDROLANOSTEROL AS A BIOACTIVE OXYSTEROL LINKING DISRUPTED CHOLESTEROL BIOSYNTHESIS TO HEPATIC TRANSCRIPTIONAL REMODELING

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Sterol intermediates of cholesterol biosynthesis are increasingly recognized as bioactive molecules with functions beyond their role as cholesterol precursors. Using CRISPR–Cas9-engineered HepG2 models with knockout of *CYP51A1*, *DHCR24* or *SC5D*, we previously showed that disruption of individual steps in late cholesterol biosynthesis produces distinct sterol accumulation patterns and largely non-overlapping transcriptional responses. *CYP51A1* knockout caused marked accumulation of lanosterol and 24,25-dihydrolanosterol, accompanied by changes in cell-cycle regulation, LEF1 induction, and enrichment of WNT, NF-κB and PI3K-Akt-related pathways. These findings initially suggested that lanosterol and/or 24,25-dihydrolanosterol may directly mediate the observed signalling changes.

Recent work, however, identified 25-hydroxydihydrolanosterol (25-OH-DHL) as a major oxysterol formed by CYP27A1-mediated oxidation of 24,25-dihydrolanosterol. Using targeted LC–MS analysis, we detected 25-OH-DHL in HepG2 *CYP51A1* knockout cells and in liver tissue from *Cyp51*-deficient mouse models, whereas it was absent from native HepG2 cells and wild-type liver tissue. Importantly, 25-OH-DHL was not detected in inducible *CYP51A1* silencing models, suggesting that stable sterol accumulation and/or metabolic adaptation may be required for its formation.

To assess its biological activity, liver-derived cell lines were treated with synthetic 25-OH-DHL. Treatment induced pronounced transcriptomic remodelling, including suppression of mevalonate and sterol biosynthesis genes and modulation of pathways overlapping with those observed in *CYP51A1* knockout cells. Functionally, 25-OH-DHL reduced cell proliferation in a dose- and time-dependent manner, with signs of cytotoxicity at higher concentrations.

Together, these findings identify 25-OH-DHL as a previously uncharacterized oxysterol generated under conditions of disrupted cholesterol biosynthesis. Ongoing studies aim to define the signalling pathways targeted by 25-OH-DHL, determine whether it accounts for transcriptional changes attributed to lanosterol/24,25-dihydrolanosterol accumulation, and clarify its effects on hepatic proliferation, viability and sterol homeostasis.

24-HYDROXYCHOLESTEROL SUPPRESSES STEROID 5α-REDUCTASE-CATALYZED CONVERSION IN HUMAN GLIOBLASTOMA CELL LINES: A NOVEL LINK BETWEEN BRAIN CHOLESTEROL HOMEOSTASIS AND ANDROGEN METABOLISM

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Glioblastoma is the most aggressive primary brain tumor in adults. Androgens are reported to influence the development of glioblastoma. Dihydrotestosterone (DHT) is formed from testosterone by action of the enzyme steroid 5α-reductase and is the most potent growth-inducing androgen metabolite.
24-Hydroxycholesterol, another steroid in the brain, is pivotal for brain cholesterol homeostasis and has been suggested to influence glioblastoma cells. However, a connection between 24-hydroxycholesterol and androgen metabolism related to glioblastoma has not previously been reported. The present study reports that human T98G glioblastoma cells metabolize testosterone into DHT, 3α-androstanediol and androstenedione. The 5α-reductase pathway converted testosterone to DHT and further to 3α-androstanediol. The 17β-hydroxysteroid dehydrogenase pathway metabolized testosterone to androstenedione. Results indicated that the 5α-reductase pathway is the major pathway for testosterone metabolism in this cell line. 24-Hydroxycholesterol significantly suppressed the conversion of testosterone to DHT and 3α-androstanediol, to a similar degree as the synthetic 5α-reductase inhibitor finasteride. Suppression of DHT formation resulted in increased metabolism to androstenedione. Similar effects on DHT formation were observed with the LXR agonist T0901317 as with 24-hydroxycholesterol. In addition, 24-hydroxycholesterol suppressed DHT formation in patient-derived primary GB cell lines U3009 and U3013, indicating that the observed connection between 24-hydroxycholesterol and androgen metabolism is not unique for T98G cells. Furthermore, 24-hydroxycholesterol-mediated suppression of DHT formation was also observed in human neuroblastoma SH-SY5Y cells.
To summarize, the present data provide information on androgen metabolism in glioblastoma cells and indicate a previously unknown link between cholesterol homeostasis and growth-inducing androgens in glioblastoma and potentially other cell types.

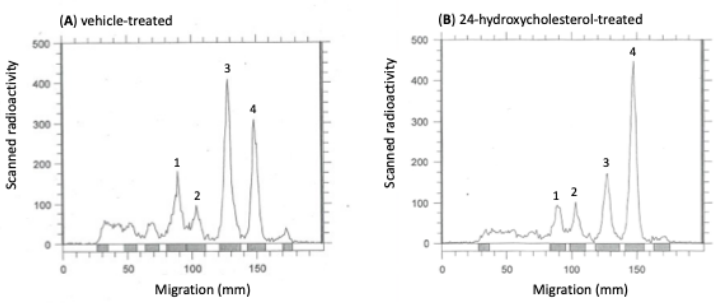

Fig. 1. Comparison of testosterone metabolite patterns in two incubations with glioblastoma T98G cells, either in the presence of **(A)** vehicle (DMSO) or **(B)** 24-hydroxycholesterol (10 μM), analyzed by TLC. Cells were incubated with 3 μg of ^{14}C-testosterone for 48 h.
1. 3α-androstanediol, 2. testosterone, 3. DHT, 4. androstenedione.
Steroid formation in incubation mixtures was confirmed by UPSFC–APCI–MS/MS.

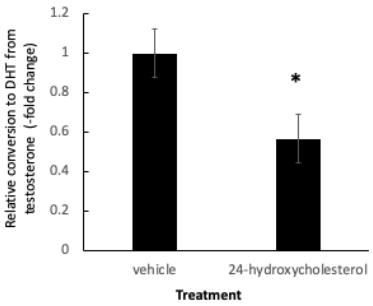

Fig. 2. Effect of 24-hydroxycholesterol (10 μM) on the conversion of testosterone to DHT in glioblastoma T98G cells incubated for 48 h with 3 μg of ^{14}C-testosterone.
*Statistically significant difference compared to vehicle-treated control, $p < 0.001$ (n=5)

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INVESTIGATING THE ROLE OF OXIDISED CHOLESTEROLS IN THE IMMUNE RESPONSE TO *MYCOBACTERIUM TUBERCULOSIS (MTB)* INFECTION

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Tuberculosis (TB), caused by the pathogen *Mycobacterium tuberculosis (Mtb),* remains the leading cause of death by a single pathogen and accounted for over 1.25 million deaths and 10.8 million new cases in 2023 (1). Our laboratory was the first to establish the role of oxysterols and the oxysterol-sensing receptor GPR183 in the immune response to TB (2, 3) and COVID-19 (4). However, the role of the oxysterol-producing enzymes in the lung remains poorly understood. Here, we investigate the effects of the oxysterol-producing enzymes cholesterol 25-hydroxylase (Ch25h), cytochrome p450 family 7 subfamily B member 1 (Cyp7B1) and cytochrome p450 family 27 subfamily A member 1 (Cyp27a1), hypothesising that they modulate the immune response to *Mtb* infection.

Firstly, 8-week old *Ch25h*$^{-/-}$ and *Cyp7b1*$^{-/-}$ mice were aerosol infected with $H_{37}R_v$ *Mtb* at either a low dose (100 colony forming units (CFUs)) or high dose (800 CFUs) and bacterial burden was determined at 1, 3 and 8 weeks post-infection. No significant differences in the lung CFUs were noted at low dose infection, while at higher infection dose sex-specific effects in *Ch25h*$^{-/-}$ females exhibiting significantly lower bacterial burden in the lungs were observed. Irrespective of sex, a change in the immune cell frequencies was observed in the *Ch25h*$^{-/-}$ mice, specifically a decrease in the frequency of B cells, while the CD8 T cells and dendritic cells (DCs) were increased. However, the cytokine profile remained unchanged. *Cyp7b1*$^{-/-}$ mice had higher B cell frequency in the lung at 1-week post-infection, however, this effect was lost at later timepoints. Additionally, *Cyp7b1*$^{-/-}$ mice exhibited a distinct cytokine profile with a peak in tumour necrosis factor (TNF) 3-weeks post-infection before declining at the 8-week timepoint. Similarly, at the 8-week time-point, interferon-γ (IFN- γ) and interleukin-10 (IL-10) concentration was also significantly reduced.

We next investigated the pathway which generates the oxysterols 27-hydroxycholesterol (27-OHC) and 7a,27-dihydroxycholesterol (7a,27-OHC). Expression of the 27-OHC generating enzyme (*Cyp27a1*) was analysed in WT samples. Cyp27a1 gene expression was significantly increased 1-week post Mtb infection. Correlation analyses showed cyp27a1 expression inversely correlated with bacterial burden in the lung, TNF and IFN-γ concentration and DC frequency, and positively correlated with eosinophil frequency. These findings will be supplemented with data from mass spectrometry quantification of oxysterol levels and spatial transcriptomics data, in relation to the host immune responses.

Altogether, our findings show that the deletion of *Ch25h* and *Cyp7b1* primarily modulates the host inflammatory immune response to Mtb infection but has no effect on bacterial load, with the exception of a female *Ch25h*$^{-/-}$ mice. The expression of *Cyp27a1* is significantly upregulated early post Mtb infection, correlating with both pathogen clearance and inflammatory profiles, highlighting the need for further research regarding other oxysterol pathways in the immune response to *Mtb* infection.

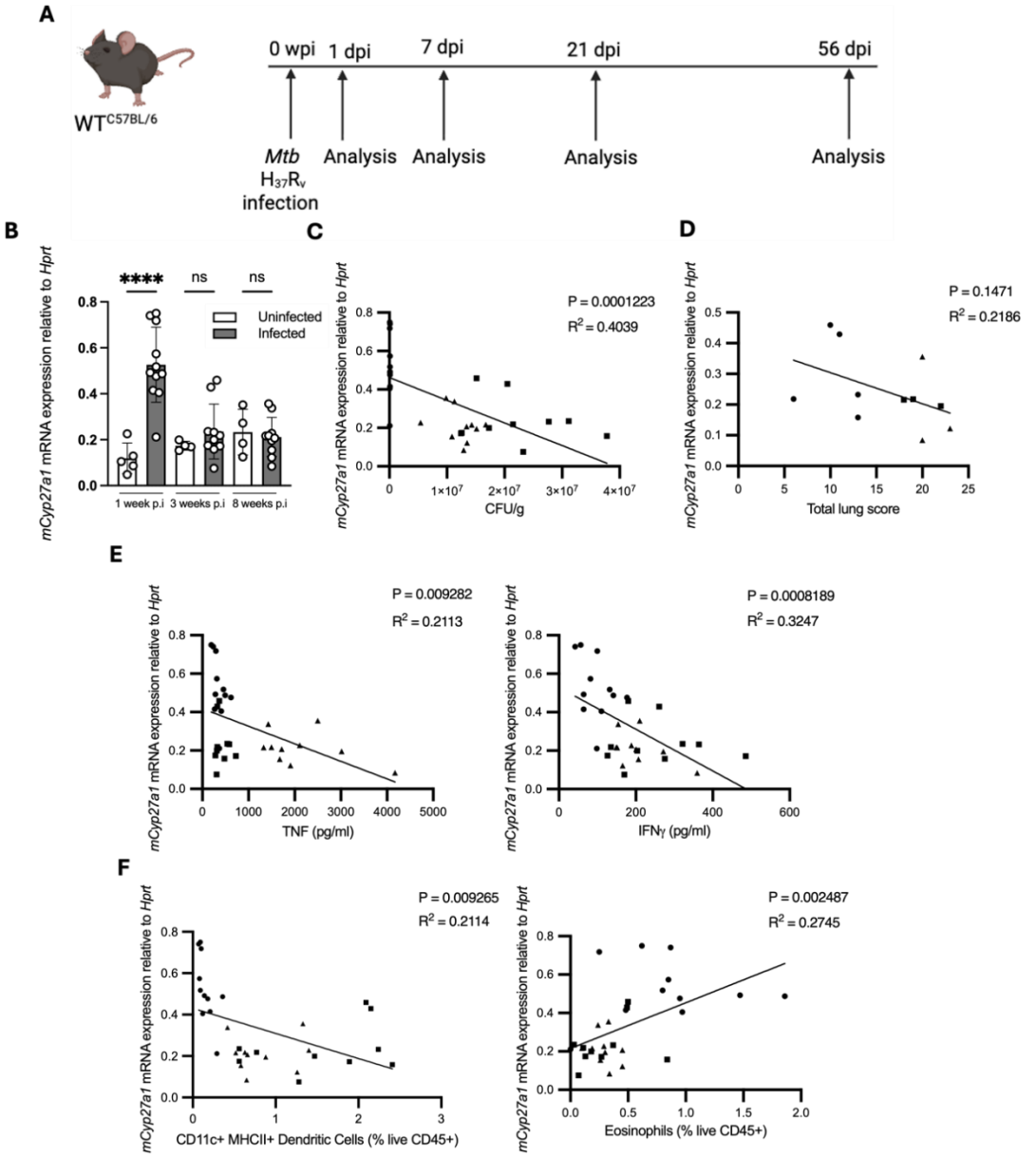

Figure 1. *Mycobacterium tuberculosis (Mtb)* infection leads to an upregulation of cytochrome p450 family 27 subfamily A member 1 (cyp27a1) in the lung. A) Experimental design: 8-week-old $WT^{C57BL/6J}$ mice were infected with 800 CFU of aerosol Mtb $H_{37}R_v$ and infection analysed at 1-, 3-, and 8-weeks post-infection (p.i). B) mRNA expression of *Cyp27a1* was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) at 1-, 3-, and 8-weeks p.i normalised to *Hprt.* Correlation between Cyp27a1 expression and (C) colony forming units (CFU), (D) total lung pathology score, (E) cytokine levels including tumour necrosis factor (TNF) and interferon-γ (IFNγ), (F) immune cell frequencies including total eosinophils and CD11c+ MHCII+ dendritic cells (DCs), 1-week p.i (n = . Data are presented as means $\pm$ SEM; not significant (ns) $P > 0.05$; * $P \leq 0.05$; **** $P \leq 0.0001$.

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Posters

DAYS 1 & 2

FUNCTIONAL LOSS OF A CYP450 CHOLESTEROL HYDROXYLASE IN THE GLYCOALKALOID PATHWAY ALTERS STEROL HOMEOSTASIS AND IMPAIRS PLANT DEVELOPMENT IN POTATO

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Potato is the most important non-grain food crop species in the world. Cholesterol is a sterol present at unusually high levels in potato plants, and serves as the primary precursor for synthesis of toxic steroidal glycoalkaloids (SGAs), mainly α-solanine and α-chaconine. While SGAs protect against herbivores and pathogens, their excessive accumulation compromises tuber quality and poses health risks to humans and animals.

SGA biosynthesis shares early steps with plant sterol metabolism before diverging into two branches: one producing essential sterols required for membrane integrity and growth, and the other leading to SGAs. Two CYP450 enzymes catalyze the initial cholesterol hydroxylation steps in SGA biosynthesis. Recent work has demonstrated that cholesterol is first glucuronidated before hydroxylation, a protective mechanism that drives metabolites into SGA biosynthesis while preventing a formation of free oxysterol intermediates.

To gain a deeper understanding of the SGA biosynthesis, we have generated CRISPR/Cas9 potato mutants targeting seven genes acting either upstream or downstream of cholesterol[1], including one of the CYP450 cholesterol hydroxylase enzymes. Among all the mutated genes, only *cyp450* full-KO mutants exhibited severe developmental defects throughout the entire life cycle, including dwarfism, impaired rooting, small leaves, low tuber yield and abnormal tuber morphology (Fig. 1). All other six pathway mutants, remained phenotypically normal[1], excluding SGA depletion as the cause of the pleiotropic effects.

The *cyp450* full-KO mutants also displayed a markedly altered sterol profile, with reduced cholesterol levels, increased levels of other phytosterols, and accumulation of hydroxycholesterol-like species. Transcriptomic analysis revealed altered expression of several SGA- and sterol-related genes, as well as downregulation of genes related to terpene biosynthesis, tracheary element differentiation, and cell wall modification, together indicating a broad disruption of sterol-dependent cellular processes. In line with this, previous work from our group has demonstrated that overexpression of mammalian cholesterol hydroxylases results in dwarf phenotypes and altered sterol composition in Arabidopsis transgenic plants[2].

Collectively these findings, suggest that loss of a controlled cholesterol hydroxylation - and not loss of SGA levels per se due to *CYP450* knockout in the SGA pathway, leads to abnormal hydroxysterol accumulation, impairing sterol homeostasis and plant growth and development.

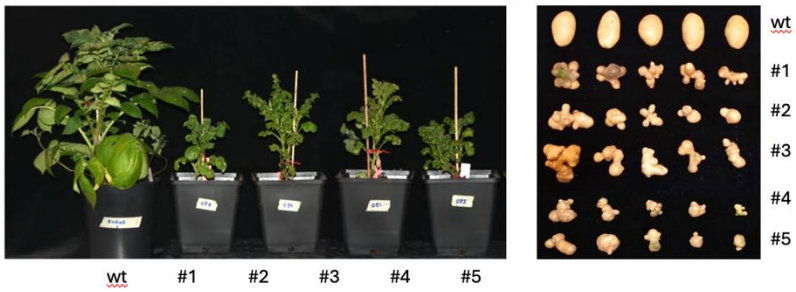

Figure 1. Representative potato grown-soil plants and tubers from the wild-type and cyp450 full-KO mutant lines.

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SARINGOSTEROL ENRICHMENT IN FUCOIDS USING NATURAL AND *LACTOBACILLUS* FERMENTATION.

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Phycosterols and oxysterols in fucoid brown seaweeds can influence lipid metabolism in human liver cells through nuclear receptor signalling. The oxy-phytosterol 24(S)-saringosterol is a liver X receptor (LXR) agonist with reported cardiometabolic and neuroprotective potential[1]. Conversion of the dominant fucoid phytosterol, fucosterol, to 24(S)-saringosterol has been reported under light exposure; however, fermentation-mediated conversion under dark conditions has not been investigated.

This study examined natural fermentation and lactobacillus enriched fermentation on saringosterol enrichment in the Australian fucoid Phyllospora comosa. Seaweed biomass samples collected from Victoria were cleaned of foreign material, shredded, and divided into two aliquots: natural fermentation (endogenous microbiota only) or E+C inoculation (*Aspergillus* cellulase with a co-culture of *L. plantarum* and *L. rhamnosus*). Aliquots were vacuum-packed to allow replicated (n = 5), non-destructive sampling at 0, 2, 10, and 60 days, and incubated at 30 °C in the dark. Saringosterol was quantified (µg mL^{-1}) using GC-MS [2].

Saringosterol increased with fermentation time under both treatments. From day 0 to day 60, concentrations rose from 56.77 to 115.62 µg mL^{-1} during the natural fermentation (2.04-fold) and from 52.25 to 116.88 µg mL^{-1} during the E+C fermentation (2.24-fold). These results indicate that fermentation enriches the oxy-phytosterol saringosterol in Phyllospora comosa in a time-dependent manner. Parallel studies on European fucoids are underway to compare conversion efficiency and enzymatic mechanisms across species.

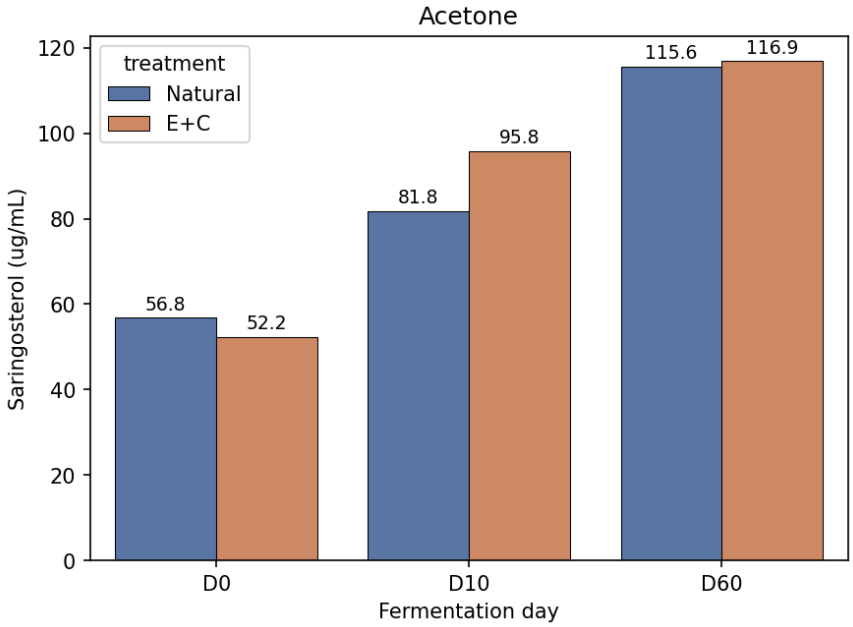

Figure: Saringosterol concentration (µg mL^{-1}) in *Phyllospora comosa* extracts during natural fermentation or lactobacillus enriched treatment (*Aspergillus* cellulase with *Lactobacillus plantarum* and *L. rhamnosus*), sampled at day 0, 10, and 60. Bars show mean values for acetone extracts.

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ISOTOPE DILUTION LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY ANALYSIS OF STEROLS, PHYTOSTEROLS AND BILE ACIDS IN NUTRITION AND HEALTH RESEARCH

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Our research focuses on cholesterol-derived steroids and related metabolites in mammalian systems, with particular interest in their roles in nutrition, health and disease. Cholesterol serves as the precursor for steroid hormones, oxysterols and bile acids, while structurally related phytosterols are obtained from dietary sources and have been associated with beneficial health effects, including reduced cancer risk [1-5]. However, the molecular mechanisms underlying these effects remain incompletely understood.

To address these questions, our laboratory has established isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) methodologies for the analysis of sterols, phytosterols, oxyphytosterols and bile acids in biological and food samples. Samples are processed using enzyme-assisted derivatisation for sterol analysis (EADSA) [5-6]. Briefly, samples are spiked with deuterated sterol internal standards, and the Avanti Bile Acid Splash mixture undergo ethanol extraction followed by C18 solid-phase fractionation. Sterols are subsequently oxidised with cholesterol oxidase and derivatised with Girard P reagent to enhance ionisation efficiency and enable improved structural characterisation. In contrast, bile acids are co-extracted during the same workflow but are not derivatised. Analyses are performed using an Agilent 6530 QTOF LC-MS/MS (CID-MS/MS), a Vanquish UHPLC–Orbitrap Fusion Lumos (ion trap MS^3 and HCD-Orbitrap MS/MS), and a Vanquish UHPLC–Q Exactive operating in PRM mode. Chromatographic separation is achieved on a Hypersil C18 column. Each sample is analysed in both positive and negative electrospray ionisation modes, enabling the profiling of Girard P-derivatised sterols and unconjugated and conjugated bile acids.

Using these platforms, we have identified oxyphytosterols in food products, including 7-keto-β-sitosterol, 7-ketostigmasterol and 7-ketocampesterol. We also report findings from a blinded study of brain tissue from control and GBA1 L444P mutant mice, a model of Parkinson's disease. To date, 17 bile acids and 10 sterols have been identified. Preliminary results indicate increased concentrations of taurocholic acid, deoxycholic acid and chenodeoxycholic acid, suggesting altered bile acid metabolism in GBA1-associated neurodegeneration.

We have investigated sterol profiles in plasma from 36 individuals comprising 9 healthy controls, 14 patients with sepsis on Day 1 and 13 patients with sepsis on Day 2. Preliminary findings demonstrate significantly increased concentrations of 24S-hydroxycholesterol and 27-hydroxycholesterol. In contrast, 25-hydroxycholesterol was not significantly altered. Marked increases 7-ketocholesterol and cholestane-3β,5α,6β-triol, were observed, consistent with enhanced oxidative stress during sepsis. These studies demonstrate the utility of LC–MS/MS approaches for characterising sterol and bile acid metabolism and identifying biomarkers relevant to nutrition, neurodegenerative disease and systemic inflammation.

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TARGETED UHPLC–MS/MS PROFILING OF SERUM OXYSTEROLS IN NEURODEGENERATION RESEARCH

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Oxysterols are bioactive oxygenated derivatives of cholesterol that regulate lipid metabolism, cellular signalling, cholesterol homeostasis, and immune responses[1,2]. In the nervous system, they contribute to neuronal development, neuroinflammatory regulation, and maintenance of cellular viability. Increasing evidence suggests that dysregulated oxysterol metabolism is associated with the pathogenesis of several disorders, including atherosclerosis, cancer, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis[3-7]. As neurodegenerative diseases currently affect millions of people worldwide and reliable early biomarkers remain unavailable, oxysterols represent promising candidates for improved diagnosis and disease monitoring. Certain oxysterols, such as 24(*S*)-hydroxycholesterol, can cross the blood–brain barrier and may therefore reflect pathological processes occurring within the central nervous system[8].

This work presents a sensitive and high-throughput targeted metabolomic method for the analysis of oxysterols in human blood serum using ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS). The developed analytical workflow employs efficient reversed-phase chromatographic separation and enables the simultaneous quantification of ten structurally related oxysterols from only a few microlitres of serum sample. The method combines minimal sample requirements with high sensitivity, robust detection, and reliable quantification, making it suitable for monitoring oxysterol dynamics under diverse physiological and pathological conditions.

Detailed profiling of systemic alterations in oxysterol levels using this approach may contribute to a deeper understanding of the biological roles of oxygenated cholesterol metabolites in health and disease. Furthermore, the proposed method may support the identification of novel biomarkers and expand current diagnostic and therapeutic strategies for neurodegenerative disorders associated with impaired oxysterol metabolism.
The work was supported from European Regional Development Fund-Project "Interdisciplinary Approaches for the Development and Application of New Materials in Medical Practice - New Omic Technologies" (No. CZ.02.01.01/00/23_021/0009224).

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UNDERSTANDING HOW PHYTOSTEROLS EXERT ANTI-CANCER EFFECTS USING CHEMICAL PROTEOMICS

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Phytosterols are plant-derived sterols that closely resemble the structure of mammalian cholesterol and play analogous roles in membrane structure and function. Phytosterols are broadly renowned for their positive health effects as dietary nutrients. Among them, β-sitosterol is the most abundant phytosterol in the diet, and has also been reported to exhibit anti-proliferative effects in several cancer cell lines and suppress multiple oncogenic pathways in breast and other cancers[1]. However, the molecular mechanisms underlying these effects, including the proteins that are directly targeted by β-sitosterol, remain largely unexplored[2].
This project will map the protein interactome of β-sitosterol in breast cancer cell lines and from primary human breast tumours through chemical proteomics. As an initial target validation step, molecular docking (Glide) and saturation transfer difference-NMR were employed to investigate the ligand interactions of CHOL and β-sitosterol with a known cholesterol-binding protein (RAR-related orphan receptor-alpha) and a putative β-sitosterol-binding protein (liver x receptor-alpha). In parallel, biotinylated cholesterol and β-sitosterol probes were synthesised, enabling streptavidin-based enrichment of cholesterol- and β-sitosterol-binding proteins and subsequent quantitative identification via LC-MS/MS[3]. Sulfonyl fluoride (SF) functionalised sterols form covalent bonds with target proteins in their binding pockets allowing identification of sitosterol-protein complexes to be identified via LC-MS/MS[4]. Cholesterol-SF and β-sitosterol-SF probes have been synthesised and will be used to identify the β-sitosterol-binding proteins in breast cancer cells. Together, these approaches aim to provide new insights into the biological activities and anti-proliferative effects of phytosterols.

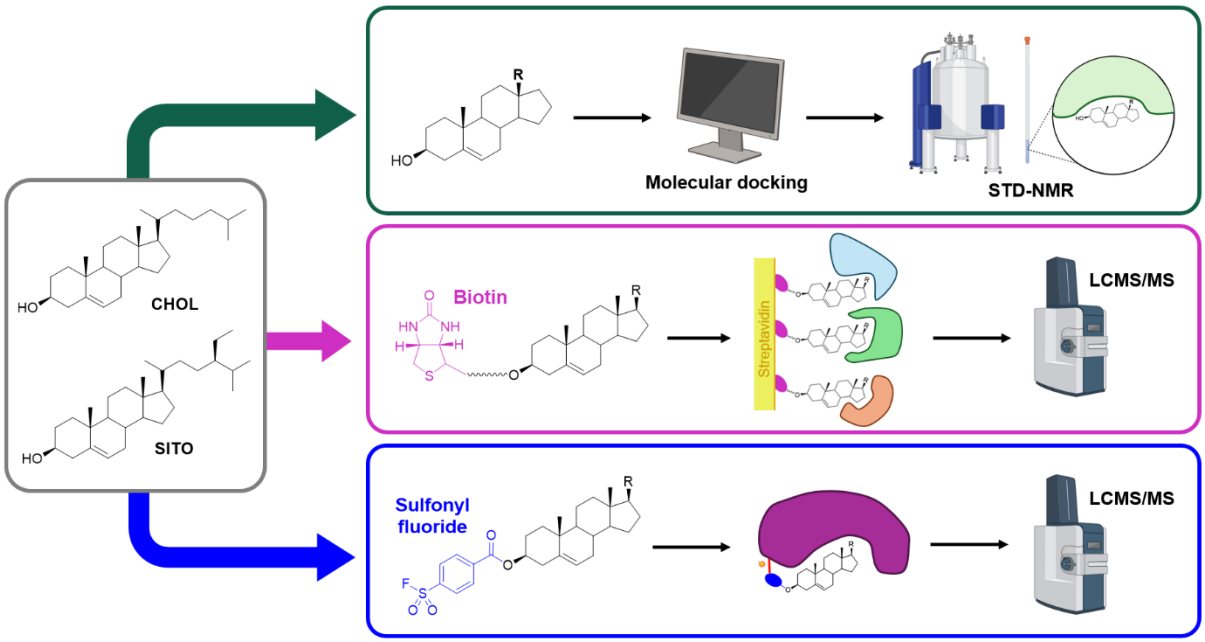

Figure: Schematic overview of the experimental approaches used to investigate β-sitosterol- and cholesterol-protein interactions. Molecular docking was used to identify putative binders of cholesterol and β-sitosterol which were further investigated with STD-NMR (green). Biotinylated cholesterol and β-sitosterol were synthesised to enable streptavidin enrichment of sterol-binding proteins, to be identified using LCMS/MS (pink). A panel of sulfonyl fluoride-based sterol probes were synthesised to facilitate covalent labelling of target

proteins, to be identified using LCMS/MS (blue). This multifaceted approach aims to discover previously unidentified β-sitosterol-binding proteins and elucidate the mechanisms of their anti-cancer properties.

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EXPLORING BIOREMEDIATION AND NANO-ENZYMATIC BIOTRANSFORMATION OF 7-KETOCHOLESTEROL

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Oxidation of cholesterol generates several oxysterols, including 7-ketocholesterol (7KC), which has been implicated in numerous age-related disorders. Current therapeutic strategies primarily rely on antioxidants and other natural or synthetic compounds to reduce 7KC-induced cytotoxicity. Here, we propose an alternative enzyme-based strategy termed medical bioremediation, in which microbial enzymes are used to degrade oxysterols *in vitro*. Initial screening identified *Pseudomonas aeruginosa* PseA and *Rhodococcus erythropolis* MTCC 3951 as promising strains capable of utilizing 7KC as the sole carbon source. Under optimized conditions, these strains degraded 88% of 7KC within 25 days and 91% within 15 days, respectively, from an initial concentration of 1 g/L (1000 ppm). Extracellular enzyme extracts also showed substantial 7KC degradation, confirming the presence of active enzymatic systems. Both strains produced cholesterol oxidase, lipase, dehydrogenase and reductase in response to 7KC, with cholesterol oxidase (ChOx) identified as the key enzyme in the degradation pathway. Several intermediates were detected, enabling prediction of the likely biodegradation route.
To improve catalytic stability and applicability, cholesterol oxidases from *P. aeruginosa* PseA (ChOxP), *R. erythropolis* MTCC 3951 (ChOxR), and a commercial *Streptomyces* sp. enzyme (ChOxS) were immobilized on functionalized magnetic iron oxide nanoparticles (MNPs) and silica nanoparticles (SNPs). In all cases, immobilized enzymes exhibited nearly twofold higher catalytic efficiency than free enzymes and showed enhanced stability across broad temperature (10–70°C) and pH (4.0–9.0) ranges, while maintaining an optimum at pH 7.5 and 30°C. The nanobioconjugates were reusable for up to 10 catalytic cycles. Successful immobilization was confirmed by FTIR, SEM, and TEM analyses. Biotransformation studies demonstrated conversion of cholesterol and 7KC into the pharmaceutically relevant products 4-cholesten-3-one and 4-cholesten-3,7-dione, respectively. These findings highlight the potential of microbial enzymes and nanoparticle-immobilized biocatalysts for both oxysterol remediation and value-added steroid biotransformation.

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SYSTEMIC DISTRIBUTION OF 25-HYDROXYDIHYDROLANOSTEROL IN HEPATOCYTE-SPECIFIC *CYP51* KNOCKOUT MICE

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25-hydroxydihydrolanosterol (25-OH-DHL) is an oxysterol that has not yet been analyzed *in vivo*. It is generated as a major product of CYP27A1-mediated oxidation of 24,25-dihydrolanosterol (DHL) *in vitro*, pointing to a potential shunt pathway for DHL metabolism. As an intermediate of the Kandutsch-Russell cholesterol biosynthesis pathway, DHL accumulates under conditions of impaired lanosterol 14α-demethylase (CYP51) activity. We therefore investigated the occurrence and tissue distribution of 25-OH-DHL in an inducible hepatocyte-specific *Cyp51* knockout (iLKO) mouse model[1].

Sterols were extracted from liver, plasma, bile, kidney and adipose tissue using liquid-liquid extraction with cyclohexane and quantified by targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS). As expected, lanosterol and DHL, the two *Cyp51* substrates, were markedly increased in iLKO liver and were also elevated in extrahepatic tissues, with the highest fold-change (iLKO/wild type) outside the liver observed in plasma. 25-OH-DHL was detected in iLKO liver (median: 0.35 ng/mg of tissue, IQR: 0.21–0.51 ng/mg), plasma (median: 0.69 pg/mL, IQR: 0.32–1.40 pg/mL) and bile (median: 9.46 pg/mL, IQR: 0.07–12.32 pg/mL). In contrast, 25-OH-DHL was below the limit of quantification in all wild type samples, while its presence in iLKO kidney and adipose tissue requires further validation.

Together, these findings provide evidence for the *in vivo* occurrence of 25-OH-DHL across multiple tissues following hepatocyte-specific *Cyp51* deletion and support further investigation of this previously unquantified oxysterol as a potential product of altered cholesterol metabolism.

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MAPPING ENDOGENOUS CORTICOSTEROIDS IN THE KIDNEY: A 3D MALDI-MSI WORKFLOW

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The regulation of sodium balance is central to blood pressure control, and dysregulation of corticosteroid action in the distal nephron contributes significantly to salt-sensitive hypertension. While aldosterone has long been viewed as the principal regulator of sodium reabsorption via activation of the mineralocorticoid receptor (MR), increasing evidence implicates glucocorticoids in modulating sodium transport in the aldosterone-sensitive distal nephron (ASDN)[1]. However, our understanding of corticosteroid action at the tissue level remains limited by the inability to resolve their spatial distribution within the kidney's intricate tubular architecture.

This project aims to establish a spatially-resolved workflow to map endogenous corticosteroids, such as corticosterone, 11-dehydrocorticosterone, and aldosterone, in male C57BL/6 mouse kidney with ethical approval using matrix-assisted laser desorption/ionisation mass spectrometry imaging (MALDI-MSI), with subsequent 3D reconstruction. The developed analytical pipeline involved cryosectioning at regular intervals aligned to histological landmarks, on-tissue derivatization with Girard T reagent to enhance ionisation[2], matrix application, and high-resolution detection using a 12T SolariX FT-ICR mass spectrometer. Resulting ion maps were co-registered with H&E-stained tissue sections and reconstructed in 3D using custom software developed in-house with AI assistance, allowing spatial alignment of steroid signals with defined kidney tubular architecture.

The results confirm the sensitivity of the method for localising corticosteroids within the kidney and the generation of 3D molecular maps. These promising results enable the investigation of hormone gradients in kidneys of experimental models of blood pressure control and kidney disease. Next, a similar workflow will be applied to a broader lipidomic study of the same tissue, with the ultimate goal of determining how local corticosteroid action and lipidomic markers are modulated under physiological versus pathophysiological conditions, and how these spatial dynamics may contribute to the understanding of the aetiology of hypertension.

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OXYSTEROLS AS DRIVERS OF ASTROCYTE REACTIVITY IN ALZHEIMER'S DISEASE: MOLECULAR MECHANISMS OF LIPOCALIN-2 INDUCTION

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Alzheimer's disease (AD) is the most common form of dementia and affects millions of people worldwide. It is characterized by the accumulation of extracellular amyloid-β plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein. Additional hallmarks of AD include neuronal loss, synaptic dysfunction, and the increased presence of reactive astrocytes. Astrocyte reactivity is associated with substantial changes in gene expression and cellular functions that correlate with cognitive decline and neurodegeneration. Although the mechanisms through which reactive astrocytes affect neuronal health remain unclear, lipocalin-2 (Lcn2) has emerged as a key mediator of neuroinflammation. Previous work from our laboratory demonstrated that Lcn2 released by reactive astrocytes impairs neurite complexity and reduces dendritic spine density in primary neuronal cultures (1).

Alterations in lipid metabolism, particularly cholesterol homeostasis, are increasingly recognized as important contributors to AD pathogenesis (2). Astrocytes are the primary source of cholesterol in the brain and play a crucial role in maintaining its balance. Excess cholesterol in the brain is oxidized into oxysterols, bioactive molecules involved in cholesterol homeostasis, inflammation, and immune responses. Notably, oxysterol accumulation correlates with AD progression. In advanced AD brains (Braak stages IV-VI), oxysterol levels are significantly increased in both the frontal and occipital cortex, with the exception of 24-hydroxycholesterol (24-OHC), whose levels are reduced (3). Moreover, exposure of primary astrocytes to an oxysterol mixture reproducing the relative proportions detected in late-stage AD brains induces a reactive phenotype characterized by marked morphological changes and increased expression of reactive astrocyte markers, including Lcn2, SerpinA3N, and several other inflammatory mediators (1).

The present study aimed to identify the molecular pathways responsible for oxysterol-induced Lcn2 upregulation in primary astrocytes. Astrocytes were isolated from 2-day-old CD1 mouse pups and treated with an oxysterol mixture (33.2% 24-OHC, 5.8% 27-OHC, 12.6% 7-KC, 5.3% 7α-OHC, 23.5% 7β-OHC, 4.8% α-EPOX, 13.9% β-EPOX, 0.9% 25-OHC) for different time points. Western blot analyses confirmed the upregulation of Lcn2 and SerpinA3N, while immunofluorescence studies showed the induction of a reactive astrocytic phenotype. To elucidate the molecular mechanisms underlying oxysterol-induced Lcn2 expression, we first evaluated the activation of ERK1/2 and STAT3 signalling pathways. Oxysterol treatment induced a time-dependent increase in ERK1/2 and STAT3 phosphorylation, as assessed by Western blotting. To determine whether these pathways contribute to Lcn2 induction, astrocytes were exposed to oxysterols in the presence or absence of the ERK1/2 inhibitor PD98059 or the STAT3 inhibitor Stattic. Pharmacological inhibition of either pathway significantly attenuated Lcn2 upregulation, supporting a role for ERK1/2 and STAT3 signalling in mediating the astrocytic response to oxysterols.

Overall, these findings provide mechanistic insight into how oxysterols contribute to astrocyte-mediated neuroinflammation in AD and highlight potential targets for therapeutic intervention.

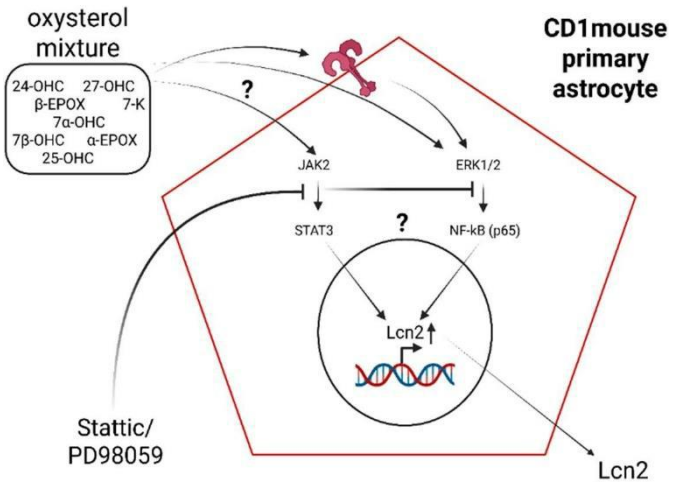

Figure. Signalling pathways involved in oxysterol-induced Lcn2 expression.

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HYDROXYTYROSOL MODULATES OXYSTEROL-INDUCED DYSFUNCTION OF CELLULAR CLEARANCE MECHANISMS IN ALZHEIMER'S DISEASE

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Growing evidence indicates that the dysfuntional proteostasis represents an early and critical event in Alzheimer's disease (AD), promoting the accumulation of aggregated proteins and organelle dysfunction **[1].** Sirtuin 1 (SIRT1), a NAD+-dependent deacetylase with neuroprotective properties, acts as a key regulator of autophagy and mitophagy involving PINK1, Parkin, and LC3 pathways **[2]**. Among the polyphenols of the Mediterranean diet, hydroxytyrosol (HXT), the most powerful polyphenol of olive oil, showed antioxidant and anti-inflammatory properties through SIRT1 activation **[3]**; however, its role in counteracting oxysterol-induced alterations of microglial function and, consequently, of the clearance mechanisms remains poorly understood.

The aim of this study was to evaluate the effects of HXT in preventing the alteration of autophagy, mitophagy, and phagocytosis induced by oxysterols, cholesterol oxidation products that accumulate in AD brains, in microglia cells. Human microglia cells HMC3 and wild type primary microglia cells were treated for 24h with a mixture of oxysterols, representative of that detected in brain samples from advanced AD patients, with or without HXT pretreatment (3h). The involvement of the SIRT1 pathway and key markers associated with autophagic and mitophagic processes were analyzed. In HMC3 cells, in both oxysterol and HXT+oxysterol cell treatment, SIRT1 and the major autophagic and mitophagic markers, PINK1, Parkin, and LC3-II/I, protein levels were increased suggesting an activation of cellular degradation pathways in response to oxysterol-induced stress. However, the levels of p62-SQSTM1, an autophagosome cargo protein, were significantly increased in cells incubated with the oxysterols alone whereas a significant reduction was observed in cells incubated with HXT+oxysterols, compared with controls. These findings suggest that oxysterols are able to trigger autophagy and mitophagy but these processes are inefficient or incomplete, resulting in cargo accumulation within the lysosomal compartment. In contrast, HXT pretreatment promotes a more efficient degradation of autophagic and mitophagic cargo. Confocal microscopy analyses was also performed to investigate the involvement of both autophagic-lysosomal and mitochondrial compartments in HMC3 and primary microglia cells. Moreover, in primary microglia cells, the oxysterol mixture impaired the microglial function compromising the phagocytic activity which was prevented by HXT cell pretreatment, suggesting a potential role of HXT in limiting aberrant phagocytic responses that might be associated with chronic inflammatory conditions.

Overall, the findings indicate that HXT could modulate the cellular clearance mechanisms altered by oxysterols, contributing to the microglial homeostasis and a more efficient handling of damaged cellular components. This point to HXT as a promising strategy for AD prevention.

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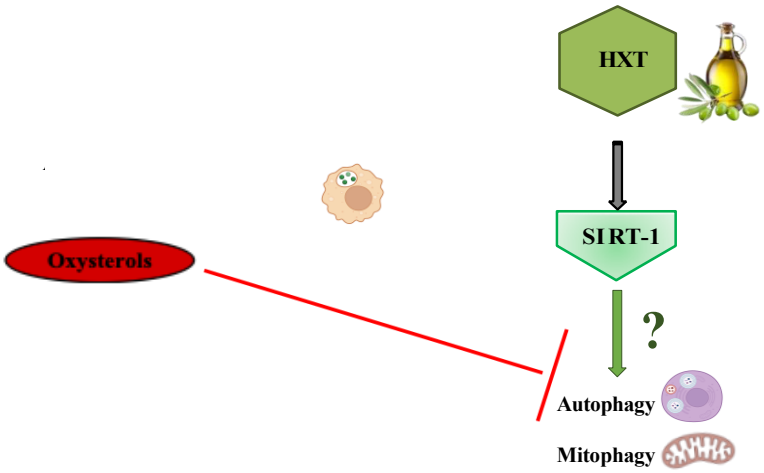

AGING AND MITOCHONDRIAL DYSFUNCTION IN MYELOID CELLS: A DRIVER OF ATHEROSCLEROSIS DEVELOPMENT

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Aging is a major and independent risk factor for atherosclerosis. Yet, the underlying mechanisms remain poorly understood[1]. Aging is associated with chronic low-grade inflammation ("inflammaging") and mitochondrial dysfunction, two hallmarks which are shared with atherosclerosis[1,2]. Oxysterols are key regulators of inflammatory signaling and mitochondrial homeostasis, and their levels are altered both within atherosclerotic plaques and during aging[3]. Myeloid cells, which play a central role in atherogenesis, undergo metabolic reprogramming that influences their activation state and functions, notably through the modulation of mitochondrial activity. However, no direct link between inflammatory responses, oxysterol metabolism, mitochondrial dysfunction, and aging in the context of atherosclerosis has been clearly established to date. These alterations may contribute to the development and progression of age-associated atherosclerosis. Therefore, our study aims to characterize these dysfunctions to better understand their role and to identify novel biomarkers and therapeutic targets of age-related atherosclerosis.

Mitochondrial dysfunction and myeloid cell phenotype were investigated *in vitro,* using primary human and murine macrophages; and *in vivo,* in *Ldlr*$^{-/-}$ and *ApoE*$^{-/-}$ mouse models. Mice were fed either a standard chow diet or an atherogenic diet, with or without supplementation with a pharmacological inducer of mitophagy. We also used a *Ldlr*$^{-/-}$ mouse model transplanted with bone marrow from young or aged *Pink1*$^{+/+}$ mice, or aged *Pink1*$^{-/-}$ mice (mitophagy-deficient). In parallel, a lipid profile from both atherosclerotic plaques and plasma was performed on a clinical cohort of 200 patients with atherosclerosis. The experimental approach relied on an integrative strategy combining multi-omics analyses (transcriptomics, metabolomics, and lipidomics), spectral flow cytometry, cytokine profiling, histopathology, and biochemical and functional assays. All data were integrated using the MOFA2 factorial model (Multi-Omics Factor Analysis v2).

In vivo investigations in *Ldlr*$^{-/-}$ mice aged 7 to 24 months allowed us, notably through the use of the MOFA2 model, to identify age as a major discriminating factor, confirming that ageing is associated with increased disease progression. Multi-omic integration of the clinical cohort data further revealed a correlation between specific oxysterols, notably 25-hydroxycholesterol and 27-hydroxycholesterol, and aged-associated molecular signatures in the context of atherosclerosis. In a second step, multi-omics analyses and *in vitro* investigations on primary murine and human macrophages revealed metabolic reprogramming and mitochondrial dysfunction in a context of atherosclerotic plaque. We further demonstrated that pharmacological induction of mitophagy reduces atherosclerotic plaque development across several mouse models, highlighting a protective role for mitophagy in age-associated atherosclerosis.

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SEX, AGE, AND LIPIDS: HOW *CYP46A1* OVEREXPRESSION AFFECTS ALZHEIMER'S DISEASE PATHOLOGY

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Introduction: Altered brain cholesterol metabolism has been evidenced across aging and neurodegenerative disorders, including Alzheimer's disease (AD). Postmortem analyses of AD brain tissue show reduction in 24(S)-hydroxycholesterol (24(S)-OH), decrease in cholesterol precursors, and downregulation of genes involved in cholesterol biosynthesis. 24(S)-OH is a product of CYP46A1 enzymatic activity and the key regulator of brain cholesterol turnover. Previously, we showed that *CYP46A1* overexpression in a mouse model confers neuroprotection in female mice during chronological and endocrine aging[1]. Here we describe how *CYP46A1* overexpression differentially affects AD pathology in male and female mice.

Methods: We crossed *CYP46A1* overexpressing mice (Cyp46 Tg) with $App^{NL\text{-}F/NL\text{-}F}$ and $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice, mouse models of amyloid-beta pathology harbouring familial Swedish, Beyreuther/Iberian ($App^{NL\text{-}F/NL\text{-}F}$); and additional Arctic mutation ($App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$), yielding aggressive neuropathological features. To scope different stages of pathology progression, males and females from these crossings (Cyp46 Tg $App^{NL\text{-}F/NL\text{-}F}$ and Cyp46 Tg $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$) were aged **to 6 (adult), 12 (middle-aged), and 20 (aged mice)** months. We quantified cholesterol, cholesterol precursors and oxysterol in the cortex and the serum using gas chromatography/mass spectrometry (GC/MS). Untargeted lipidomics by ultra-performance liquid chromatography/high resolution mass spectrometry was applied on the hippocampal tissue. Targets relevant to glia and lipid metabolism were investigated by western blotting, qPCR and immunofluorescence techniques.

Results: CYP46A1 overexpression affected glial, oxysterol, and lipidomic profile in a sex- and age- dependent manner. In adult Cyp46 Tg $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice, *CYP46A1* overexpression led to a decrease in protein levels of glial markers Iba1 and GFAP in both sexes. Lipidomic analysis revealed sex differences, with distinct lipid profile in CYP46A1 overexpressing females, featured by an increase in triglycerides and phospholipids. In contrast, both adult and middle-aged male mice largely mirrored the lipidome of their $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ controls, with significant increase in phosphoglycerides in both groups. Middle-aged Cyp46 Tg $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ females, however, had a relative decrease in triglyceride species, together with partial rescue in desmosterol and 24(S)-OH levels. Interestingly, we observed no change in 24(S)-OH levels in middle-aged males. Additionally, middle-aged females have increased *Cpt1c* expression, involved in β-oxidation and fatty acid transport. Image analysis further revealed reduced hippocampal microglia area occupancy in females, but not males.

Middle-aged Cyp46 Tg $App^{NL\text{-}F/NL\text{-}F}$ mice of both sexes exhibited decrease in Iba1 protein levels. By 20 months, aged males showed increased levels of GFAP, whereas females showed a reduction in Iba1 immunoreactivity. In line with observations seen in Cyp46 Tg $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ males, CYP46A1 overexpression had minimal effect on the lipid profile of the Cyp46 Tg $App^{NL\text{-}F/NL\text{-}F}$ male mice during aging, despite a few notable differences. In particular, aged males exhibited aggravated increase in triglycerides, together with increase in *Plin2* expression, gene encoding for perilipin 2, protein coating lipid droplets. Conversely, aged Cyp46 Tg $App^{NL\text{-}F/NL\text{-}F}$ females demonstrated higher levels of lysophosphatidylcholines and carnitine, along with increased *Cpt1c* expression.

Conclusions: CYP46A1 overexpression drives lipidome changes across different pathology stages, with females exhibiting a greater degree of lipid remodeling. Together with observed differences in expression of genes pertinent to metabolism and glial profile, our results may indicate different metabolic adaptations between males and females. Overall, we suggest potential sex-specific effects of CYP46A1 overexpression in regulating lipid and glial homeostasis in AD pathology, and prospect of CYP46A1 as a therapeutic target for women in early disease stages.

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EXPOSURE TO ELEVATED MATERNAL 27-HYDROXYCHOLESTEROL LEVELS DURING PREGNANCY AND LACTATION LEADS TO LATE ONSET MEMORY IMPAIRMENTS IN THE OFFSPRING

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BACKGROUND

Hypercholesterolemia, obesity, and diabetes are risk factors for Alzheimer's Disease (AD). Indeed, epidemiological studies have suggested a relationship between elevated levels of cholesterol in the bloodstream and a higher risk of developing dementia during midlife [1, 2]. This association has been reinforced by *in vivo* studies where mice fed with a high-fat/cholesterol diet exhibited dementia-related neuropathological features [3].

Besides, there is growing evidence suggesting that the mother's lifestyle and any health problems she may experience during pregnancy can influence the fetal development and impact the long-term health of the offspring. For instance, studies in mice showed that a high-fat diet during pregnancy had a long-term impact on the brain, affecting cortical blood vessels, microglial reactiveness and the offspring's behaviour [4, 5], as well as causing permanent changes in the periphery, such as neuroinflammation, oxidative stress, and neuroendocrine changes in the offspring [6, 7].

SCIENTIFIC QUESTION

Our main goal is to study how alterations of cholesterol metabolism during pregnancy increase offspring susceptibility to cognitive decline during ageing. As the fetus depends on maternal cholesterol in early pregnancy, any alterations in maternal lipid profiles may affect embryonic and fetal development, potentially impacting the offspring's health later in life [8, 9, 10].

STUDY MODEL

Hypercholesterolemia is a disorder known for an excess of cholesterol in the bloodstream. The blood-brain barrier is impermeable to plasma cholesterol, but permeable to its oxidized metabolites known as oxysterols. 27-hydroxycholesterol (27-OHC) is one of the most abundant oxysterols in the periphery, and it is produced from cholesterol hydroxylation by the enzyme sterol 27-hydroxylase (CYP27A1). In our study, hypercholesterolemia is mimicked using a specific transgenic mouse model called Cyp27Tg in which mice are expressing the human CYP27A1 ubiquitously, in addition to their own mouse CYP27A1 expression. These mice show a 3-5 times higher levels 27-OHC in the circulation and the tissues during their whole life [11]. Experiments were carried out on three groups of mice: 1) Cyp27Tg mice born from a Cyp27Tg dam, 2) non-transgenic littermate mice born from a Cyp27Tg dam and 3) wild-type mice born form a C57BL/6J dam.

RESULTS

Preliminary data from our group demonstrated that elevated 27-OHC during pregnancy and lactation (caused by maternal CYP27A1 overexpression) induce late onset cognitive impairments in offspring at 20-month-old. Given that embryonic and fetal development shapes future cognitive functions, we performed microarray-based transcriptomics followed by differential gene expression analysis in E19 embryonic forebrains from the offspring. It revealed significant alterations in metabolic signaling pathways and mitochondria function in the embryonic brain of the offspring exposed to maternal hypercholesterolemia. As oxidative phosphorylation was one of the most highly altered pathways, we evaluated mitochondria respiratory function in E19 embryonic forebrains from the offspring using the Seahorse apparatus. It revealed a reduction of approximately 50% in mitochondrial respiratory capacity in the offspring exposed to maternal hypercholesterolemia, in association with a significant reduction in mitochondrial complexes protein levels.

CONCLUSION AND RELEVANCE

In this context, we aim to highlight that disrupted maternal cholesterol metabolism – particularly reflected by elevated levels of circulating 27-hydroxycholesterol (27-OHC) – may lead not only to immediate pregnancy-related complications but also to long-term adverse effects on the cognitive health of the offspring. Our study suggests that maternal dyslipidemia may program cognitive vulnerability later in life.

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REVERSE TRANSLATION OF THE FINGER MULTIDOMAIN LIFESTYLE INTERVENTION MODULATES DEMENTIA-RELATED MECHANISMS ACROSS DISTINCT MOUSE MODELS OF DEMENTIA RISK

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Background: Multidomain lifestyle interventions targeting modifiable dementia risk factors have emerged as a promising strategy to delay cognitive decline and reduce dementia risk [1]. However, the biological mechanisms underlying their beneficial effects, and whether these differ across distinct dementia risk profiles, remain poorly understood. We reverse-translated the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) [2] into mice to investigate the molecular mechanisms underlying multimodal lifestyle intervention in different mouse models of dementia-related pathology.

Methods: The Mouse PAW (Prevention of Alzheimer's and Related Dementias by a Multimodal Lifestyle Protocol - Working-mechanisms for Memory Gains) study was conducted in female wild-type, App^{NL-G-F}, and CYP27A1-overexpressing mice, representing healthy aging, amyloid pathology, and cholesterol dysmetabolism, respectively [3, 4]. Mice underwent an eight-week multimodal lifestyle intervention combining voluntary exercise, Fortasyn Connect diet [5], and cognitive training in the IntelliCage system [6]. One group received atorvastatin and enalapril as pharmacological vascular prevention [7]. Behavioural performance, blood pressure, serum cholesterol and oxysterols, cortical amyloid pathology, and hippocampal proteomic changes were evaluated.

Results. The response to the intervention differed across mouse models. In wild-type mice, the multimodal intervention improved spatial working memory and activated hippocampal pathways related to synaptic function. In App^{NL-G-F} mice, it enhanced recognition memory and reduced insoluble cortical Aβ42/40 levels. In CYP27A1-overexpressing mice, both the PAW and pharmacological interventions modulated hippocampal pathways involved in proteostasis, inflammation, and immunity, although with distinct molecular signatures. While the PAW intervention additionally reduced circulating total cholesterol, 27-hydroxycholesterol, and 24S-hydroxycholesterol levels, neither intervention resulted in measurable cognitive improvement in this mouse model. The pharmacological intervention consistently lowered blood pressure across all models and reduced cortical insoluble Aβ42/40 in App^{NL-G-F} mice.

Conclusions: Reverse translation of the FINGER protocol demonstrates that multidomain lifestyle intervention exerts beneficial but model-dependent effects on cognition and molecular pathways associated with dementia. The reduction in circulating 27-hydroxycholesterol and the modulation of pathways related to synaptic plasticity and inflammation identify potential mechanisms through which lifestyle interventions may promote brain health and reduce dementia risk, warranting further investigation.

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THE ROLE OF OXYSTEROLS IN NEURODEGENERATIVE DISEASES: IMPACT OF SECOSTEROL-B ON ENDOPLASMIC RETICULUM STRESS

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Neurodegenerative disorders are characterized by altered cholesterol metabolism and accumulation of misfolded proteins and are therefore commonly known as proteinopathies. This accumulation process is tightly linked to endoplasmic reticulum (ER) stress and impaired protein quality-control mechanisms [1,2]. Although cholesterol itself cannot cross the blood–brain barrier, oxysterols can readily penetrate the central nervous system connecting peripheral metabolic dysfunction with neuronal diseases. Among these compounds, secosterol-B (SEC-B), a cholesterol autoxidation product, has been detected in atheromas and in the brains cortex of patients with Alzheimer's disease and Lewy body dementia [3]. In our previous studies we demonstrated that SEC-B induces ER stress in endothelial cells, leading to vascular dysfunction, yet its effects on neuronal stress pathways have not been clearly defined [4].
In this work, we want to investigate whether SEC-B induces endoplasmic reticulum stress and proteostasis alterations in HT22 mouse hippocampal neuronal cells, potentially contributing to neurodegenerative processes. Our findings indicate that SEC-B exposure induces oxidative stress and impairs neuronal cell-cycle progression without causing overt cell death. Our results further demonstrated that SEC-B activates unfolded protein response signaling pathways, including ATF4, ATF6, and XBP1s, associated with endoplasmic reticulum expansion, and accumulation of protein aggregates. Despite aggresome formation, proteasomal activity remained unchanged. However, we observed an alteration in the lysosomal compartment of HT22 cells induced by SEC-B suggesting an engagement of autophagic pathways.
Taken together, our findings identify SEC-B as a potential inducer of neuronal ER stress and proteostasis dysregulation, supporting the hypothesis that cholesterol oxidation products contribute to proteinopathies linking cardiovascular and neurodegenerative diseases. These findings provide further insight into the molecular interplay between peripheral lipid oxidation and neuronal dysfunction and may aid in the identification of novel therapeutic targets for neurodegenerative disorders.

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LYSOSOMAL DYSFUNCTION MEDIATED BY 25-HYDROXYCHOLESTEROL INDUCES NON-APOPTOTIC CELL DEATH IN MOTOR NEURON-LIKE CELLS

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Objective:
Amyotrophic lateral sclerosis (ALS) is characterized by progressive degeneration of motor neurons. Recent studies have reported elevated levels of 25-hydroxycholesterol (25-OHC) in both ALS patients and ALS model mice[1], although its effects on motor neurons remain incompletely elucidated. In this study, we investigated the impact of 25-OHC on murine motor neuron-like NSC-34 cells.

Methods & Results:
25-OHC induced non-apoptotic cell death in NSC-34 cells in a concentration-dependent manner. α-Tocotrienol (α-Toc3) suppressed 25-OHC-induced cell death. Lipid peroxidation predominantly occurred in lysosomes in 25-OHC-treated cells and was attenuated by co-treatment with α-Toc3. To evaluate lysosomal integrity, we assessed lysosomal membrane permeabilization (LMP) marker galectin-3 and observed its puncta formation in lysosome under 25-OHC treatment. 25-OHC treatment also caused pronounced lysosomal overacidification. Since lysosomes are essential for physiological autophagic flux, we examined autophagy markers LC3-II and p62. Both LC3-II and p62 levels were increased following 25-OHC treatment, indicating impaired autophagic flux due to lysosomal dysfunction. Notably, co-treatment with α-Toc3 prevented this autophagic impairment.
We further found that 25-OHC suppressed SREBP2 activation and reduced the expression of its downstream target, CYP51A1, at both the mRNA and protein levels. Notably, the expression of TMEM175 which was lysosomal proton channel was also decreased following 25-OHC treatment. CYP51A1 maintains the proper TMEM175 expression levels through playing an important role in cholesterol biosynthesis pathway[2]. Therefore, both the reduced expression of CYP51A1 and TMEM175 likely contribute to excessive lysosomal acidification.

Conclusion:
These findings indicate that 25-OHC induces lysosomal dysfunction through overacidification and lipid peroxidation, leading to impaired autophagic flux and non-apoptotic cell death in NSC-34 cells. α-Toc3 exerts a protective effect by suppressing lipid peroxidation and preserving lysosomal function. This study provides new insights into the molecular mechanisms underlying ALS and suggests potential therapeutic targets.

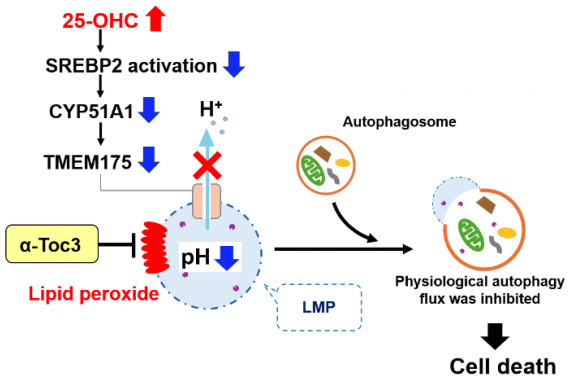

Figure: Proposed model of 25-OHC-induced motor neuron cell death

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ELEVATED 24-HYDROXYCHOLESTEROL LEVELS COUNTERACT OKADAIC ACID-INDUCED TAU HYPERPHOSPHORYLATION AND NEURONAL MORPHOLOGY IMPAIRMENT

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Multiple findings underline a link between altered brain cholesterol metabolism and Alzheimer's disease (AD) pathogenesis [1]. Excess brain cholesterol is mainly converted into 24-hydroxycholesterol (24-OHC) by the neuron-specific enzyme CYP46A1. We previously reported that both 24-OHC levels and CYP46A1 expression are reduced in advanced AD brains [2], suggesting a possible contribution to disease progression. In this study, we investigated whether maintaining elevated 24-OHC levels, via its exogenous administration or CYP46A1 overexpression, could counteract tau hyperphosphorylation and oligomerization. Using okadaic acid (OKA) to induce an AD-like tauopathy in primary neurons, we found that both 24-OHC treatment and CYP46A1 overexpression effectively prevented tau hyperphosphorylation and the accumulation of prefibrillar tau oligomers. Moreover, OKA-induced loss of dendritic arborization was significantly attenuated, preserving neuronal cytoskeletal integrity. While hypothesized underlying molecular mechanisms (GSK3β, CDK5, ERK1/2, and PP2A) seem not to be involved, elevated 24-OHC consistently exerted anti-neurodegenerative actions (Figure 1). Our results encourage more in-depth investigations into the cellular pathways implicated in tau propagation and further support that pharmacological enhancement of CYP46A1 activity may exert disease-modifying effects in AD.

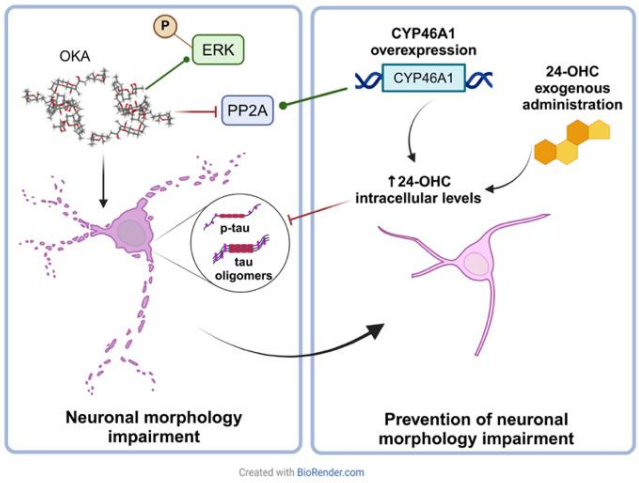

Figure 1. The beneficial effects of 24-OHC treatment and CYP46A1 overexpression on primary neurons are summarized (created with Biorender.com).

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PLASMA 24S-HYDROXYCHOLESTEROL IS REDUCED IN THE LACQ140 HUNTINGTON'S DISEASE MOUSE MODEL

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Huntington's disease (HD) is associated with altered cholesterol metabolism and oxysterol homeostasis [1,2]. The brain-derived oxysterol 24S-hydroxycholesterol (24S-HC) has been proposed as a blood-based biomarker of neuronal cholesterol turnover and HD disease progression [2]. In this study we investigated the LacQ140 mouse model of *mHtt* repression, which is designed as a mimic for *mHtt* lowering strategies [3]. Plasma samples from 9 month old wild-type (WT), LacQ140 (expression of *mHtt* for 9 months), LacQ140-6M (expression of *mHtt* for 6 months) and LacQ140-2M (expression of *mHtt* for 2 months) mice (n = 40) were analysed using liquid chromatography–mass spectrometry (LC–MS) to investigate changes in sterol metabolism [2].

Plasma 24S-HC concentrations were significantly reduced in LacQ140 and LacQ140-6M but not LacQ140-2M mouse groups compared with WT controls, with the largest effect observed in males. Campesterol, sitosterol and total cholesterol were also significantly decreased in male LacQ140 mice. These findings indicate sex-dependent alterations in cholesterol metabolism in the LacQ140 HD model and confirm reduced circulating 24S-HC as a marker of disease-associated sterol dysregulation in HD. The results support the use of plasma 24S-HC as a biomarker for monitoring HD progression in the LacQ140 mouse model and for evaluating therapeutic interventions.

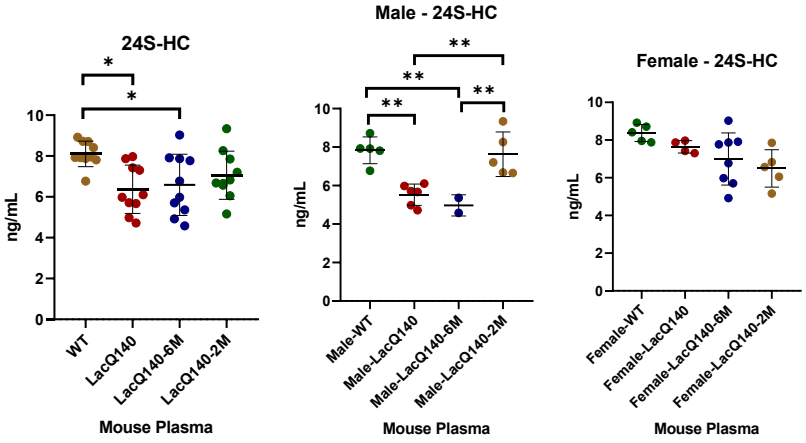

Figure. Plasma concentrations of 24S-hydroxycholesterol in WT and LacQ140 mice.

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INTEGRATED ANALYSIS OF OXYSTEROL-INDUCED PHENOTYPIC AND TRANSCRIPTOMIC CHANGES IN PANCREATIC CANCER MODELS

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Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest malignancies due to late diagnosis, molecular heterogeneity, and limited therapeutic options. Increasing evidence suggests that oxysterols influence tumor-associated signaling, metabolic adaptation, and treatment response, highlighting their potential as biomarkers and modulators of therapeutic sensitivity. In our previous breast cancer studies, we demonstrated that 7-ketocholesterol affects tamoxifen response and identified transcriptomic differences potentially explaining distinct effects in ER-positive and ER-negative cell lines [1,2].
In the present study, we extended this approach to PDAC and evaluated the effects of selected oxysterols on tumor-associated phenotypes and gemcitabine response using integrated functional and transcriptomic profiling. Nine oxysterols were tested in three PDAC cell lines differing in *KRAS* status and molecular background (Panc-1, Paca-44, and BxPC-3). Cell viability, oxysterol–gemcitabine interactions, migration, cell cycle distribution, and transcriptomic alterations were assessed using CellTiter-Blue assays, combination index analysis, xCELLigence monitoring, flow cytometry, and mRNA sequencing.

Oxysterols exerted pronounced cell line–specific effects across all assays. Several oxysterols showed synergistic or additive interactions with gemcitabine in BxPC-3 and Paca-44 cells, whereas predominantly additive or antagonistic effects were observed in Panc-1 cells, indicating strong context-dependent modulation of treatment response. Migration assays revealed reduced migratory capacity in BxPC-3 cells but predominantly pro-migratory effects in Paca-44 cells. Cell cycle analyses demonstrated reduced S-phase fractions and increased subG0 populations following oxysterol exposure.

Transcriptomic profiling identified marked differences in oxysterol-induced gene deregulation among PDAC models, with the strongest effects observed after 5β,6β-epoxycholesterol and 7-ketocholesterol treatment. Deregulated genes were primarily associated with stress response, lipid metabolism, unfolded protein response, and oxidative stress adaptation, including *DDIT3, CHAC1, SESN2, HERPUD1, ABCA1, GDF15*, and *TRIB3*.

Overall, our findings demonstrate that oxysterols substantially modulate PDAC-associated phenotypes and chemotherapy response in a highly context-dependent manner. Integration of functional and transcriptomic analyses may contribute to the identification of treatment-response biomarkers and novel combinatory therapeutic strategies in pancreatic cancer. This study was supported by the Czech Ministry of Education, Youth and Sports, INTER-ACTION LUAUS23164, the Ministry of Health of the Czech Republic in cooperation with the Czech Health Research Council under project no. NW24-03-00024 00281 and Czech National Institute of Public Health (institutional support to A.S.).

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ROLE OF LXRs IN IMMUNE RESPONSE TOWARDS PROSTATE CANCER: FOCUS ON DENDRITIC CELLS

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Immunotherapies have shown limited efficacy in Prostate Cancer (PCa), in which immunologically "cold" microenvironment is mainly composed of suppressive and dysfunctional immune cells (1). Previous results from our laboratory showed that LXRs double knock-out (LXRs DKO) induces an accumulation of non-functional myeloid cells and promotes neoplasia in prostate of castrated mice (2). LXRs are cholesterol-sensing nuclear receptors whose endogenous ligands are oxysterols. They play key roles in the regulation of immune responses and can be pharmacologically targeted by synthetic ligands to modulate genes involved in antitumor immunity (3, 4, 5, 6).

We therefore investigate the immune changes associated with LXRs loss in preclinical models of PCa. We found that LXRs DKO enhanced tumor growth, even when restricted to non-tumoral cells, in both genetically engineered ($Pten^{pc-/-}$) and RM-1 subcutaneous-graft model of prostate cancer. This phenotype was associated with profound alterations of tumor immune infiltrate, characterized by an enrichment in dendritic cells (DCs) in $Pten^{pc-/-}$ and RM-1 models (fig. a,b). DCs are professional antigen-presenting cells, essential for immune surveillance, but that can be subverted by tumor cells to promote tolerance and progression, making them key determinants for tumor growth. Mechanistically, splenic splenic DCs from LXRs DKO mice exhibited impaired migratory capacities towards lymph nodes, as evidenced by reduced migration in response to CCL19 in chemotaxis assays. Consistently, in $Pten^{pc-/-}$ model, pharmacological activation of LXRs using the agonist GW3965 reduced dorsolateral prostate tumor weight (fig.c). This effect was associated with a decrease in the proportion of CD11c+ DCs among CD45+ immune cells. Furthermore, GW3965 treatment decreased the expression of DC activation markers, including *CD11c, MHC-II*, and *CCR7* in tumor. In human THP-1 derived human DCs, GW3965 also reduces DC activation marker expression *Cd80*, *MhcII*, *Ccr7*, while treatment with an inverse agonist increased them. Further studies are ongoing to better define the role of LXRs in DC-mediated immune responses, both within the tumor microenvironment using DC-specific LXR-deficient mice in the RM-1 model and at the cellular level using LXRs-knockout THP-1 cells.

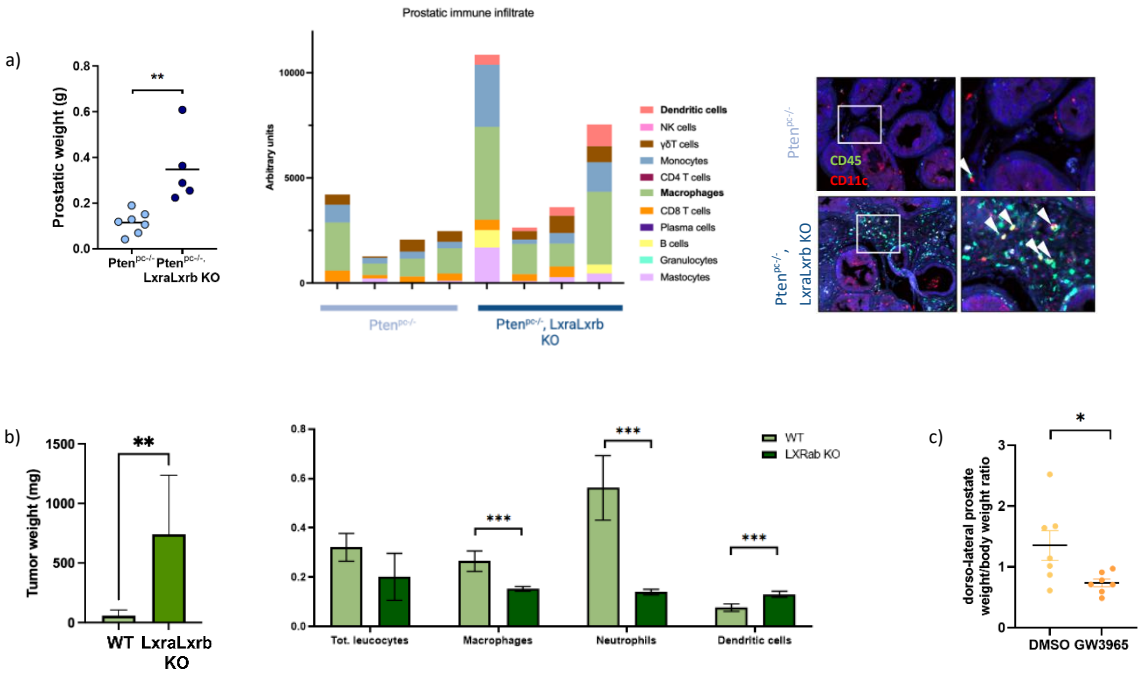

Figure: LXR loss significantly modifies immune infiltration and DCs accumulation, leading to enhanced tumor growth. (a) LXR DKO mice exhibit increased tumor weight in *Pten*$^{pc-/-}$ model. RNAseq data from tumors were analyzed by deconvolution using Cibersortx software to identify 11 immune cell population infiltrates. These data were confirmed by IF staining on *Pten*$^{pc-/-}$ model. (b) LXR DKO also induced higher tumor weight in syngeneic RM-1 subcutaneous graft model, associated with modifications of immune infiltrate. c) Targeting LXRs with GW3965 treatment successfully induced a reduction in the weight of dorsolateral prostates in *Pten* deficient mice.

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STEROL PROFILES COMPOSED OF 25-OH, 7A,25-DIOH AND NEUTRAL STEROLS IN PBMC FRACTIONS ARE ASSOCIATED WITH THE SPECIFIC IMMUNOLOGICAL PHENOTYPES OBESITY, AND/OR NASH/FIBROSIS AND/OR ASTHMA; AN EXPLORATIVE ANALYSIS

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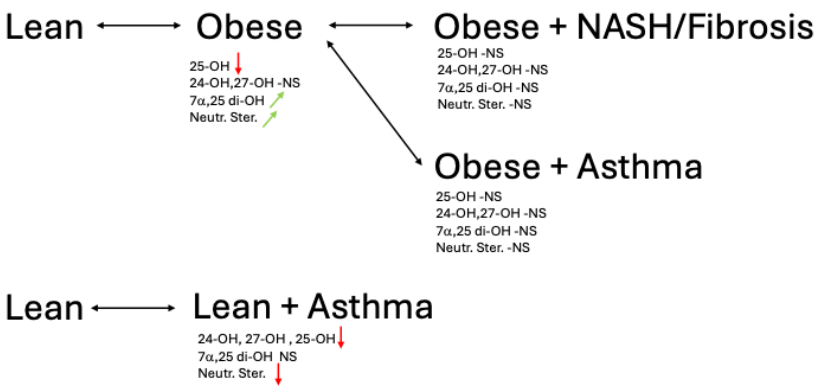
Objective: There is growing evidence that oxysterols, such as 25-OH and 7a,25-dihydroxycholesterol as well as non-cholesterol sterols such as desmosterol, can affect biological pathways and behavior of immune cells, especially in immunologically oriented diseases. However, there is no clear data regarding concentrations of these regulatory sterols in peripheral mononuclear blood cells of specific patient groups. Therefore, as an explorative study, we here investigated the concentrations of oxysterols and non-cholesterol sterols in different populations, e.g. lean vs obese, lean vs lean with asthma, obese vs obese with asthma and obese vs obese with NASH/fibrosis.

Methods: Blood was sampled from 15 women that were divided in 5 groups (n=3 per group), lean, lean & asthma, obese, obese & asthma or obese with NASH/fibrosis. PBMC's were isolated from EDTA blood of which one part was used for extraction of the sterol fractions and the other part for flow cytometry analysis. Concentrations of 24-OH, 25-OH, 27-OH and 7α,25-dihydroxy-cholesterol as well as non-cholesterol sterols were determined via GC-MSMS[1].

Results: In PBMC's from obese women, 25-OH concentrations were significantly lower whereas 7α,25 di-OH concentrations were higher as compared to lean women, while sitosterol, campesterol, desmosterol, lathosterol, cholestanol and cholesterol where also significantly higher. Next, when comparing PBMCs from obese to obese + NASH/fibrosis, 25-OH concentrations were clearly higher and 7α,25-diOH concentrations lower, however these differences did not reach statistical significance. However, when we compare obese to obese + asthma, 25-OH is comparable while 7α,25-OH was reduced to concentrations observed in lean women. This seems related to the asthma phenotype because the lean + asthma group, has significantly lower 25-OH concentrations as compared to the lean. Moreover in asthma patients all other oxysterols and non-cholesterol sterols were significantly lower as compared to leans, except for 7α,25-OH. Flow cytometry analysis of immune cells is ongoing and will be presented later.

Conclusion: Different inflammatory and immunological phenotypes may be associated with different oxysterol and non-cholesterol concentrations. Whether these differences are causal for immune cell behaviour deserves further study.

Figure:



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