ENOR 6th Symposium
September 29-30 2016
At Paris Descartes University
www.oxysterols.com
Dear participants, welcome to Paris!

This is the 6th international symposium of the European Network on Oxysterols Research (ENOR) (https://oxysterols.com/2013/10/24/5th-enor-symposium/). After a kick-off meeting in Munich in 2010, since then we met annually in Rome, Dijon, Swansea, Coimbra and Bonn.

According to our goals of the previous meetings, we continue:

- to provide an international forum for researchers to present their results of ongoing research (oral presentations, posters);
- to bring European oxysterol researchers together;
- to stimulate discussions and possible collaborations of the European Network on Oxysterol Research;
- to offer the possibility to submit manuscripts of ongoing research for peer-review process in high ranking biochemical journals.

It is a pleasure to have presentations of researchers outside of Europa: Belarus, Brazil, Canada, India, Lebanon, Morocco, Tunisia, USA.

It is a great honor and pleasure to welcome you and your co-workers to the University of Paris Descartes.

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Program ........................................................................................................................................ p 9

First plenary session ........................................................................................................................ p 15
  Jean Marc Lobaccaro. Prostate cancer, cholesterol and LXR: the impossible triad ...

Oral communications ....................................................................................................................... p 17

Session 1: Detection of oxysterols

Steven Ray Wilson. “Sub-chip” liquid chromatography-mass spectrometry for measuring oxysterols and related proteins. ............................................................................................ p 19

Sabrina Krautbauer. Relevance of appropriate internal standards for accurate quantification using LC-MS/MS – analysis of tauro-conjugated bile acids as an example................................................... p 20

Ruth Andrew. Derivatisation Strategies to Quantify Androgens and Estrogens and Enable Isomer Separation by Ion Mobility Mass Spectrometry (IMS-MS) ............................................ p 21

Yuqin Wang. Oxysterols generated during motor neuron differentiation differentially regulate the hedgehog signalling pathway .................................................................................................P 22

Silke Matysik. Analysis of steroid hormones – LC-MS vs. Immunoassay: a practical consideration...............................................................................................................................P 23

Session 2: Neuroscience

Nathalie Cartier. Targeting CYP46A1 to restore impaired cholesterol metabolism in Alzheimer disease. ............................................................................................................................... P 25

Betuing Sandrine. CYP46A1 expression is neuroprotective in Huntington’s disease mice. .................................................................................................................................................. P 26

Othman Ghribi. Regulation of α-synuclein levels by 27-hydroxycholesterol. Relevance to Synucleinopathies ................................................................................................................................. p 27

Ingemar Björkhem. What is the driving force for the flux of oxysterols across the blood-brain barrier? .................................................................................................................................P 28

Charbel Massaad. Dual role of LXR in myelination in the Central and Peripheral nervous system. ................................................................................................................................. P 29

Session 3: Nutrition and oxysterols biological activities

Meryam Debbabi. Evaluation of the effects of major compounds present in the Mediterranean diet (oleic acid, docosahexaenoic acid, α-tocopherol) and hydrogenated oils (elaidic acid) on 7-ketocholesterol-induced oxidative stress and cell death on microglial BV-2 cells. .............................................................P 33
Caroline Pot. Interleukin-27 induces oxysterols that regulate CD4 T cell response via LXR signaling ................................................................. p 34

Elodie Olivier. Role of P2X7 cell degenerescence receptor and granzyme B in 25-hydroxycholesterol-induced apoptosis and degenerative pathways in skin ....................... p 35

Ségoène Gambert. Cholesterol metabolism and glaucoma: modulation of Muller cells membrane organisation by 24S-hydroxycholesterol.......................................................... p 36

Second plenary session ........................................................................ p 39

Ned A. Porter. Perturbations of Sterol Homeostasis: The Formation of Toxic Oxysterols as a Consequence of Genetics or Small Molecule Exposures

Session 4: Cancer

Julie Leignadier. The oxysterol dendrogenin A: an alternative against breast cancers resistant to hormonotherapy .......................................................... p 41


Nada Zabaiou. Propolis has androgen receptor antagonist activity and affects prostate cancer survival .......................................................................................... p 43

Session 5: Energy metabolism and stress

Sergio Bydlowski. Outside-in, inside-out: Proteomic analysis in endothelial stress mediated by 7-ketocholesterol ........................................................................ p 45

Valerio Leoni. Evidences of an impairment of cholesterol metabolism associated to mitochondrial dysfunction. A metabolomic approach based on mass spectrometry .......... p 46

Vesa M. Olkkonen. The involvement of ORP2 in cellular energy metabolism. .................P 47

Gerd Schmitz. The plasma elimination rate of current lipoprotein apheresis methods differs for lipoprotein remnants, HDL-particles, oxysterols, cholesterol precursors and phytosterols .................................................................................. p 48

Session 6: Oxysterol metabolism and agonist of LXRs

Marc Poirot. Oxysterols and Cancer ......................................................................... p 51

Shubhrima Ghosh. Microbial degradation of cytotoxic 7-Ketocholesterol and mining therapeutic enzymes .................................................................................. p 52

Andrei A. Gilep. Identification of Mycobacterium tuberculosis enzymes involved in metabolism of immunoactive sterols. ..............................................................P 53

Posters .................................................................................................................. p 57

P01 Rola Abboud - Progestins and Corticoids Interaction with Human Serum Albumin ...... p 59

P02 Hélène Greige-Gerges - Effect of tetra- and penta-cyclic triterpenes on lipid bilayer fluidity by DSC, FTIR, Raman and fluorescence anisotropy ........................................ p 60
P03 Guadalupe Garcia-Llatas - Dietary phytochemicals in the protection against oxysterol-induced damage ................................................................. p 61

P04 Asmaa Badreddine - Cytoprotective effects of alimentary and cosmetic argan oils on 7-ketcholesterol - treated 158N murine oligodendrocytes ................................................ p 62

P05 Maryem Bezine - Evidence of K⁺ homeostasis disruption and potential involvement of Kv channels in cellular dysfunctions triggered by 7-ketcholesterol, 24S-hydroxycholesterol, and tetracosanoic acid on 158N oligodendrocytes ............... p 63

P06 Fiorella Biasi - Dietary oxysterols as stressors of intestinal mucosa in developing colorectal cancer ........................................................................... p 64

P07 Lydie Boussicault - Effect of CYP46A1 on lipid raft composition and excitotoxicity in the context of Huntington’s disease ......................................................................... p 65

P08 Maria Teresa Rodriguez-Estrada - Dietary effects of Raphanus sativus cv Sango on lipid accumulation and oxysterols in rat brain: a lipidomic study on a non-genetic obesity model ................................................................. p 66

P09 Isabelle Delton - Oxidatively modified HDL and HDL isolated from diabetic subjects show reduced ability to efflux oxysterols from THP-1 macrophages ....................... p 67

P10 Antonina Germano - In vitro mitotane cytotoxicity on adrenocortical carcinoma H295R cells may be mediated by oxysterols ................................................................. p 68

P11 William J. Griffiths - Identification of Immunoregulatory Sterol-Metabolites in the Infant Gut ........................................................................................................ p 69

P12 Philip Hofflinger - Induced pluripotent stem cell derived disease model of Hereditary Spastic Paraplegia Type 5 .............................................................................. p 70

P13 Maurice Konings - Development and validation of a new Isotope dilution method for combined oxyphyto/oxycholesterol analysis in a small plasma sample on a GC-QQQ-MS/MS ................................................................. p 71

P14 Francesca Luchetti - Sec-B induces endoplasmic reticulum stress and triggers autophagy in HUVEC cells .............................................................................. p 72

P15 Jo Mailleux - Liver X receptor activation in MS lesions .................................................. p 73

P16 Neura Bragagnolo - Impact of high hydrostatic pressure processing on the formation of cholesterol oxides in chicken meat and the use of tomato by-products as antioxidants .............................................................................. p 74

P17 Marc Poirot - Development of a method of dosage for the tumor suppressor Dendrogenin A ........................................................................................................ p 75

P18 Elodie Olivier - Effects of two plant extracts on two actors of skin photoaging: UV and 25-hydroxycholesterol .............................................................................. p 76

P19 Maria Manuel Silva - Enlarging the chemical diversity of oxysterols by means of MMPP mediated oxidations .............................................................................. p 77
P20 Lilian R Barros Mariutti - Combined effect of thermal treatment, storage and cumari pepper addition on the formation of cholesterol oxides in buffalo meat ........................................ p 78

P21 Marc Poirot - Reactivity of 5,6α-epoxycholesterol, 5,6β-epoxycholesterol, cholestan-3β,5α,6β-triol and 6-oxo-cholestan-3β,6β-diol towards trimethylsilylation, importance for their quantification by GC/MS. ........................................................................................................ P 78

P22 Gabriella Leonarduzzi - The oxysterols along the different stages of Alzheimer’s disease: their involvement in neuroinflammation .................................................................................................................. p 80

P23 F. Fermin Castro Navas - Side-Chain Modified Stigmasterol and Ergosterol Derivatives as Liver X Receptor Agonists ...................................................................................................................... p 81

P24 Hans-Frieder Schött - Determination of sterols by liquid chromatography high-resolution tandem mass spectrometry .................................................................................................................. p 82


P26 Anna Sandebring-Matton - Study of brain myelination in relation to risk factors for Alzheimer disease ............................................................................................................................... p 84

P27 Yuguang Lin - Formation of sitosterol and campesterol oxidation products in foods after cooking with margarines without and with added plant sterols ........................................ p 85

P28 Diny Knol - Stability of plant sterols to thermal oxidation and formation of plant sterol oxidation products in margarines and vegetables oils without and with added plant sterols ............................................................................ P 86

Participants .................................................................................................................................................. p 87

Sponsors ..................................................................................................................................................... p 91

Maps ......................................................................................................................................................... p 93
6th ENOR Symposium
29-30 September 2016
University Paris Descartes, Paris, France
45 rue des Saints-Pères, 75270 PARIS

September 29th 2016

8 h 30 - 9 h 00  Registration
9 h 00 - 9 h 15  Opening of the symposium: C. Massaad, L. Iuliano, G. Lizard
9 h 15 - 10 h 15  Plenary Session
   Jean Marc Lobaccaro (UMR CNRS6293-INSERM U1103 and CRNH - Auvergne, France) Prostate cancer, cholesterol and LXR: the impossible triad ...
10 h 15 - 10 h 45  Coffee break – poster session

Session 1: Detection of oxysterols

10 h 45 – 11 h 05  Steven Ray Wilson (Department of Chemistry, University of Oslo, Norway) “Sub-chip” liquid chromatography-mass spectrometry for measuring oxysterols and related proteins.
11 h 05 – 11 h 20  Sabrina Krautbauer (Regensburg University Hospital, Regensburg, Germany) Relevance of appropriate internal standards for accurate quantification using LC-MS/MS – analysis of tauro-conjugated bile acids as an example.
11 h 20 - 11 h 40  Ruth Andrew (University of Edinburgh, UK) Derivatisation strategies to quantify androgens and estrogens and enable isomer separation by ion mobility mass spectrometry (IMS-MS).
11 h 40 -12 h 10  Yuqin Wang (Swansea University Medical School, UK) Oxysterols generated during motor neuron differentiation differentially regulate the hedgehog signalling pathway.
12 h 10 – 12 h 30  Silke Matysik (Institute for Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg, Germany) Analysis of steroid hormones – LC-MS vs. immunoassay: a practical consideration.
12h30 – 14h15  Lunch and Poster session
**Session 2: Neuroscience**

14 h 15 - 14 h 45  **Nathalie Cartier** *(INSERM U1169 MIRCen CEA, University Paris Sud, France)* Targeting CYP46A1 to restore impaired cholesterol metabolism in Alzheimer disease.

14 h 45 - 15 h 05  **Sandrine Betuing** *(University Pierre et Marie Curie Paris 6, Paris, France)* CYP46A1 expression is neuroprotective in Huntington’s disease mice.

15 h 05 - 15 h 25  **Othman Ghribi** *(University of North Dakota, USA)* Regulation of α-synuclein levels by 27-hydroxycholesterol. Relevance to synucleinopathies.

15 h 25 - 15 h 45  **Ingemar Björkhem** *(Karolinska Institutet, Stockholm, Sweden)* What is the driving force for the flux of oxysterols across the blood-brain barrier?

15 h 45 - 16 h 05  **Charbel Massaad** *(University Paris Descartes, Paris France)* Dual role of LXR in myelination in the central and peripheral nervous system.

16 h 05 - 16 h 45  Coffee break and poster session

**Session 3: Nutrition and oxysterols biological activities**

16 h 45 - 17 h 05  **Meryam Debbabi** *(University Bourgogne Franche Comté / INSERM, Dijon, France)* Evaluation of the effects of major compounds present in the Mediterranean diet (oleic acid, docosahexaenoic acid, α-tocopherol) and hydrogenated oils (elaidic acid) on 7-ketocholesterol-induced oxidative stress and cell death on microglial BV-2 cells.

17 h 05 - 17 h 25  **Caroline Pot** *(Department of Clinical Neurosciences, Lausanne, Switzerland)* Interleukin-27 induces oxysterols that regulate CD4 T cell response via LXR signaling.

17 h 25 - 17 h 45  **Elodie Olivier** *(UMR CNRS 8638, Université Paris Descartes, Paris, France)* Role of P2X7 cell degenerescence receptor and granzyme B in 25-hydroxycholesterol-induced apoptosis and degenerative pathways in skin.

17 h 45 - 18 h 05  **Ségolène Gambert** *(Centre des Sciences du Goût et de l’Alimentation. UMR 1324 INRA, 6265 CNRS, Université Bourgogne Franche Comté, Dijon, France)* Cholesterol metabolism and glaucoma: modulation of Muller cells membrane organisation by 24S-hydroxycholesterol.

Event Dinner
September 30th 2016

9 h 30 - 10 h 30  Plenary Session
Ned A. Porter (Vanderbilt University, Nashville, USA) Perturbations of sterol omeostasis: the formation of toxic oxysterols as a consequence of genetics or small molecule exposures.

10 h 30 - 11 h 00  Coffee break and poster session

Session 4 : Cancer

11 h 00 - 11 h 30  Julie Leignadier (UMR-1037 INSERM-University of Toulouse, CRCT, Toulouse, France) The oxysterol dendrogenin A: an alternative against breast cancers resistant to hormonotherapy.

11 h 30 - 11 h 50  Hanne Røberg-Larsen (Department of chemistry, University of Oslo, Norway) 27-hydroxycholesterol in cancer exosomes – potential new separation method?

11 h 50 – 12 h 10  Nada Zabaiou (Université Clermont Auvergne, Université Blaise Pascal, France) Propolis has androgen receptor antagonist activity and affects prostate cancer survival.

12 h 10 - 14 h 00  Lunch and poster session

Session 5 : Energy metabolism and stress

14 h 00 - 14 h 20  Sergio Bydlowski (University of São Paulo Medical School, Sao Paulo, Brazil) Outside-in, inside-out: Proteomic analysis in endothelial stress mediated by 7-ketocholesterol.

14 h 20 - 14 h 40  Valerio Leoni (Hospital of Varese, Varese, Italy) Evidences of an impairment of cholesterol metabolism associated to mitochondrial dysfunction. A metabolomic approach based on mass spectrometry.

14 h 40 - 15 h 00  Vesa M. Olkkonen (Minerva Foundation Institute for Medical Research, Helsinki, Finland) The involvement of ORP2 in cellular energy metabolism.

15 h 00 – 15 h 20  Gerd Schmitz (Institute for Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg, Germany) The plasma elimination rate of current lipoprotein apheresis methods differs for lipoprotein remnants, HDL-particles, oxysterols, cholesterol precursors and phytosterols.

15 h 20 - 15 h 35  Coffee Break
### Session 6: Oxysterol metabolism and agonist of LXRσ

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Topic</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 h 35 – 16 h 05</td>
<td>Marc Poirot (UMR-1037 INSERM-University of Toulouse, CRCT, Toulouse, France)</td>
<td>Oxysterols and Cancer.</td>
</tr>
<tr>
<td>16 h 05 - 16 h 25</td>
<td>Shubhrima Ghosh (Indian Institute of Technology Delhi, Delhi, India)</td>
<td>Microbial degradation of cytotoxic 7-Ketocholesterol and mining therapeutic enzymes.</td>
</tr>
<tr>
<td>16 h 25 - 16 h 45</td>
<td>Andrei A. Gilep (Institute of Bioorganic Chemistry, Minsk, Belarus)</td>
<td>Identification of Mycobacterium tuberculosis enzymes involved in metabolism of immunoactive sterols.</td>
</tr>
<tr>
<td>16 h 45 - 17 h 00</td>
<td></td>
<td>Coffee Break</td>
</tr>
<tr>
<td>17 h 00 – 17 h 45</td>
<td></td>
<td>General assembly</td>
</tr>
<tr>
<td></td>
<td>Round table discussion of ENOR activities</td>
<td></td>
</tr>
<tr>
<td>17 h 45 – 18 h 00</td>
<td>Poster Awards and closing remarks (G. Lizard, L. Iuliano, C. Massaad)</td>
<td></td>
</tr>
</tbody>
</table>
Prostate cancer, cholesterol and LXRs: the impossible triad

Lobaccaro Jean-Marc A.,1,2,3,4 Trousson Amalia1,2,3,4, Morel Laurent1,2,3,4, Baron Silvère1,2,3,4

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Prostate cancer (PCa) is one of the most common malignancies, mainly affecting elders. Various risk factors have been involved including aging, ethnic origins, hormonal status and energy balance. As far as 1909 (White, J Pathol Bacteriol 1909), lipid, and more particularly cholesterol, excess has been associated to PCa (review in De Boussac et al. Am J Cancer Res 2013). This important structural as well as signaling molecule needs hence to have its homeostasis tightly regulated by the cell, in terms of uptake, de novo synthesis, storage, export and metabolism. Among the various transcription factors involved in this regulation, liver X receptors (LXRα, NR1H3, and LXRβ, NR1H2) have been shown to be activated by oxidized derivatives of cholesterol and to maintain a strict cellular concentration of cholesterol (Maqdasy et al. Molecular Aspects Med 2016).

Over the last years, we and others have been working on exploring how cholesterol disequilibrium could be correlated to the development of prostate tumor, and if LXRs could become interesting pharmacological targets in the treatment of PCa (Viennois et al. Expert Opin Ther Targets 2011).

In vitro as well as in vivo studies using LXR deficient animals, xenografted mice and mouse models for PCa led us to demonstrate that LXRs: i) downregulate survival signaling pathway and, once activated, induce apoptosis of prostate cancer cells (Pommier et al. Oncogene 2010); ii) control proliferation of epithelial cells of dorsal prostate (Dufour et al. PLoS ONE, 2013).

While LXRs could not be considered as oncosupressors, LXR deficient mice fed a high cholesterol diet develop prostatic intra-epithelial neoplasia (Pommier et al. PLoS Genet 2013), pointing a protective role of these nuclear receptors. Noteworthy, LXRα interact with the androgen signaling pathway and the androgen receptor (Viennois et al. Endocrinology 2012), these latest having a crucial role in the enhancement of PCa.

Besides, alteration of LXR signaling by genetic or pharmacological events (Fouache et al. submitted) could enhance tumor development. Likewise, in prostate cancer model, LXRs have a strong protective effect (Alioui et al. submitted).

Altogether, these data enlighten the role crucial of LXRs in prostate physiology and demonstrate that selectively modulating their transcriptional activity could be a promising pharmacological approach in PCa (Viennois et al. Mol Cell Endocrinol 2012).
COMMUNICATIONS
“Sub-chip” liquid chromatography-mass spectrometry for measuring oxysterols and related proteins

Steven Ray Wilson*¹, Tore Vehus¹, Ole Kristian Brandtzæg¹, Kristina Erikstad Sæterdal¹, Henriette Sjånes Berg¹, Tore Smetop¹, Hanne Røberg-Larsen¹, Elsa Lundanes¹

¹Department of chemistry, University of Oslo, Norway

A key focus of our group is developing high-sensitivity methods based on liquid chromatography and mass spectrometry (LC-MS). Our approach is largely based on using very narrow LC columns, e.g. nanoLC. NanoLC allows for significantly enhanced signals, as compounds are less diluted in the column prior to MS detection. We have developed a new nano LC column, which provides unprecedented sensitivity for “charge-tagged” oxysterols. The column is in open tubular format, meaning it is not filled with particles. This format allows for the columns to be extremely narrow (“sub-chip”, 10 micrometer inner diameter), resulting in a virtual absence of column-related dilution. Using this column, charge-tagged oxysterols can be detected in amounts as low as 25 attograms, which is about 100 times less than any previously reported method. The column functions well for biological samples (e.g. exosomes), and has a very good reproducibility. We will employ the columns for high sensitivity sterolomics.

In addition, novel approaches to mass spectrometry-based targeted proteomics will be presented, exemplified with highly selective measurements of CYP27A1 in cancer samples.
Relevance of appropriate internal standards for accurate quantification using LC-MS/MS – analysis of tauro-conjugated bile acids as an example

Sabrina Krautbauer, Christa Büchler§, Gerhard Liebisch

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§ Department of Internal Medicine I, Regensburg University Hospital, Regensburg, Germany

Introduction: It is well established that matrix effects (MEs) may detrimentally affect signal intensities in electrospray ionization (ESI) and thus may be considered as main culprit for quantitative LC-MS/MS. There exist strategies to evaluate and to reduce MEs mainly by sample purification and chromatography. However, quantification by LC-MS/MS usually requires internal standards (ISs). Stable isotope labeled (SIL) ISs are considered as gold standard because they closely resemble the properties of the analyte and show similar ionization efficiency and retention times. Since MEs may not be uniform and can vary substantially between different samples only ISs which coelute with the analyte can compensate for these variations accurately. Frequently, SIL-ISs are either not available or very expensive and alternative ISs are used for quantification. Here, we present a systematic comparison of ISs from a routine LC-MS/MS method for bile acid (BA) analysis with a focus on tauro-conjugated BAs including stable isotope labelled (SIL) D5-tauro BAs.

Materials and Methods: BA analysis was performed with a previously developed and validated LC-MS/MS with minor modifications. Taurine conjugated BAs (TBAs) calibration curves were generated using different ISs. The calibration curves were based on ratio analyze peak area to internal standard peak area versus analyte concentration. The obtained 15 internal standard curves were linear in the tested calibration range and used for quantification of the respective TBA in quality controls (QCs) and patient samples. Quantification was performed in serum of up to 76 different individuals and values below the limit of quantitation (LOQ) were excluded from the data analysis.

Results: Both human serum based QCs and human serum samples were quantified with a variety of SIL-ISs. As expected, matching SIL-ISs provided the highest data quality (precision, accuracy). We could not observe systematic correlations of data quality with chemical similarity or proximity in retention time to the analyte. However, both accuracy and precision of QCs and serum concentrations showed significant correlations. This provides evidence that calculation of matrix-based QC with various ISs could be applied for the selection of ISs whenever matching SILs are not available. Moreover, data calculated without ISs exhibited a poor data quality for both QCs and serum concentrations.

Conclusions: Here, we show that selection of ISs for quantification by LC-MS/MS is of enormous importance to achieve accurate and precise values in routine analysis of TBAs. Unsurprisingly, matching SILs show the best performance. Whenever SILs are not available, analyses of QCs based on sample matrix provide a tool to select ISs.
Derivatisation Strategies to Quantify Androgens and Estrogens and Enable Isomer Separation by Ion Mobility Mass Spectrometry (IMS-MS)

Shazia Khan\textsuperscript{1}, Emmanuelle Claude\textsuperscript{2}, Mark Towers\textsuperscript{2}, Diego F Cobice\textsuperscript{1}, Dawn EW Livingstone\textsuperscript{1}, Natalie ZM Homer\textsuperscript{1}, C. Logan Mackay\textsuperscript{3}, Brian R Walker\textsuperscript{1} and Ruth Andrew\textsuperscript{1}.

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Introduction: Steroid hormones circulate systemically but amounts of active hormones in tissues are regulated by local metabolism. Enzymes catalysing these reactions are pharmacological targets e.g. in breast, prostate cancers. Knowing the local hormonal environment may aid understanding of pathology and allow tailored treatment. Analysis of androgens and estrogens by MS in tissues is challenging through low abundance and poor propensity to ionise. Moreover, inert isomers must be distinguished from active steroid. Derivatisation was explored to improve sensitivity of analysis, and separate isomers by IMS, as an alternative to chromatography, with a view to imaging active hormones on tissue surfaces.

Materials and Methods: Analysis was performed using a Waters Synapt G2Si and Sciex QTrap 5500 MS. Two sets of isobaric steroids were derivatised; 17\textalpha-estradiol (17\textalpha E2; inert) and 17\textbeta-estradiol (17\textbeta E2; active), and epitestosterone (17\textalpha; inert), testosterone (17\textbeta; active) and DHEA (inert)). For MALDI, derivatised steroids were mixed 1:1 with 9-aminoacridine (5mg/mL, 4:1 ethanol:water). For DESI, spray conditions were 1.5µl/min, 98:2 MeOH:water with nebulising gas pressure (5bar). IMS cell pressure was 3.1 mBar, with a travelling wave height of 40V and velocity ramp from 1,000 to 300 m/s.

Results: Many charged derivatives increased sensitivity by electrospray but not all gave intense signals by MALDI. The greatest sensitivity gain for estrogens was with glycidyltrimethylammonium chloride (LOQ: 50fg on-column for 17\textbeta E2-GTMA, RSD<10%; linear range 5pg/mL-500ng/mL). Transitions were analyte specific (m/z 388.2/311.2), however isobaric steroids were not resolved by IMS. In contrast, derivatives formed using p-toluenesulfonyl isocyanate (PTSI) improved signal intensity, generated analyte-specific product ions upon collision but also allowed resolution of isomers by drift time; 17\textalpha and 17\textbeta E2 differed by 0.38ms. Epitestosterone-PTSI had the shortest drift time (3.53ms), suggestive of its being the most compact in gas phase, followed by testosterone-PTSI (4.01ms). DHEA-PTSI was separated less efficiently (4.29ms) but could still be distinguished by IMS. Interference from DHEA in the testosterone signal could be avoided by derivatising with (+)-O,O'-diacetyl-L-tartaric anhydride, which did not yield a signal for DHEA in positive mode, whereas separation of epitestosterone and testosterone (0.75ms) was achieved. Similar data were observed for steroids applied on or off-tissue.

Conclusions: Therefore, tailored novel derivatisation approaches have potential for tissue surface analysis of steroids and sterols using IMS, without chromatography. Challenges remain to optimise derivatisation methods on the tissue surface.

\textit{This work was performed as collaborative project with Waters Corp.}
Oxysterols generated during motor neuron differentiation differentially regulate the hedgehog signalling pathway

Yuqin Wang¹, Ian Gilmore¹, Monika Seidel¹, William J. Griffiths¹ and Meng Li²

1. Swansea University Medical School, Singleton Park, Swansea SA8 PP, UK
2. Cardiff University, UK

Introduction: Motor neuron disease (MND) is a neurodegenerative disorder, characterised by the selective death of motor neurons (MNs) in brain and spinal cord. Imbalanced cholesterol metabolism is linked to motor dysfunction. Our Previous study shows that the CYP27A1 mediated cholesterol metabolite 3β,7α-dihydroxycholest-5-enolic (3β,7α-diHCA) regulates MN survival via Liver X Receptors (LXRs). Cerebrotendinous xanthomatosis (CTX) patients, which show mutations in the cytochrome P450 (CYP) 27A1, display peripheral neuropathy. Whether CYP27A1 and its enzymatic products are important for motor neuron development is unknown. This study is to identify oxysterols which are produced during human motor neuron differentiation using induced pluripotent stem (iPS) cells.

Materials and Methods: iPS cells were generated from fibroblasts from healthy control and CTX patients, and were differentiated towards spinal MNs. At different stages during differentiation, oxysterols were extracted from cells and conditioned media. Samples were analysed using enzyme-assisted derivatisation for sterol analysis (EADSA) and LC-MSn.

Results: We found that iPS cells from both healthy control and CTX patients can be differentiated to MN with added Hedgehog signalling agonist. However, oxysterol profiles change significantly between fibroblasts, iPS cells, neuroepithelial cells, motor neuron progenitors and motor neurons. Both CYP27A1 and CH25H generated metabolites, i.e. 27-hydroxycholesterol (27-HC) and 25-HC were present in fibroblasts and iPS cells. 27-HC is most abundant in neuroepithelial cells then declined afterwards. The level 24-HC increased in motor neuron progenitors and onwards. Most importantly we found that 7-keto-27-HC is only detected in motor neuron progenitors and activates Hedgehog signalling while its downstream metabolites 3β-hydroxy7-ketocholest-5-enonic acid is an inverse agonist.

Conclusions: CYP27A medicated cholesterol metabolites are produced during motor neuron development and regulate the balance of Hedgehog signalling.

Analysis of steroid hormones - LC-MS vs. Immunoassay.  
A practical consideration.

Silke Matysik

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Introduction: Liquid chromatography-mass spectrometry is increasingly becoming the method of choice for steroid hormone measurements in clinical labs. High sensitivity, reliability and a broad panel of several compounds combined with small sample volumes are the main advantages of LC-MS based approaches. However, these methods are often laborious, automatization is limited and standardization is needed.

Materials and Methods: We determined steroid hormones in serum samples with LC-MS/High Resolution-MS and immunoassays. For LC-MS all samples underwent liquid-liquid extraction and MS analysis was carried out in batch mode by a QExactive system, Thermo Fisher Scientific. Immunoassays were performed with commercially available test systems (Siemens, IBL, Roche).

Results: We evaluated the complete analytical process under special consideration of accuracy, precision, and effort of time, consumables and personnel. Furthermore, steroid hormone concentrations of samples of children, men and women obtained by LC-MS and immunoassays were compared by Passing/Bablock regression.

Conclusions: The main limitations of immunoassays such as lack of antibody specificity due to cross reactivities and low sensitivity can be overcome by LC-MS methods. In contrast, matrix effects can be an important issue in LC-MS. These effects need to be investigated and defined in extensive method validation, especially in icteric and lipemic serum samples or in samples from intensive care units. Ion suppression can lead to profound signal loss.

However, from the practical point of view immunoassay autoanalyzers can generate values much faster, 24 hours a day, without any sample preparation, and do not need expert operator knowledge. In view of scarcity of resources diagnostic procedures in medicine should no longer be analysed purely in terms of their effectiveness, but also in terms of their cost-effectiveness-the ratio between the resources and the related effects. Advantages and disadvantages including costs of equipment, reagents and human resources have to be evaluated carefully.
Targeting CYP46A1 to restore impaired cholesterol metabolism in Alzheimer disease

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Introduction: Growing evidences indicate that brain cholesterol metabolism is impaired in neurodegenerative diseases, particularly Alzheimer’s disease (AD). Since the blood-brain-barrier (BBB) prevents peripheral cholesterol to enter the brain, excess of cholesterol cannot be directly eliminated. To be degraded, the cholesterol in excess must be exported to the peripheral circulation. The conversion to 24-hydroxycholesterol, (24-OHC) by the key neuronal enzyme cholesterol 24-hydroxylase (CYP46A1), represents the main mechanism by which cholesterol in excess is eliminated from the brain. We evaluated in different mouse models of the pathology if direct targeting of CYP46A1 into the brain could be a therapeutic strategy for AD.

Materials and Methods: CYP46A1 expression was measured in the brain of AD patients and mouse models of AD. Adeno-Associated Virus (AAV) vectors were used to target CYP46A1 directly into the brain of AD mice.

Results: We demonstrate that CYP46A1 is decreased in hippocampal biopsies from AD patients. In a therapeutic perspective we overexpressed CYP46A1 by mean of an AAV vector, which was demonstrated to efficiently transduce hippocampal neurons in mice. We then treated APP23 mice before the onset of plaque load and showed that AAV9-CYP46A1 injection increased hippocampal 24-OHC content, strongly prevented amyloid plaques deposition, and decreased Aß peptides and astrogliosis. Notably, this tissue remodeling was associated with recovery of memory deficits, as demonstrated in the Morris water maze. Strikingly, APP/PS1 knock-in mice having severe and established early AD pathology treated with AAV9-CYP46A1, not only showed reduced amyloid plaques load and reduced astrogliosis, but also complete rescue of impaired electrophysiological and spine defects. In addition, we demonstrate the efficacy, neuronal tropism and tolerability of the AAV9 vector in the non-human primate hippocampus.

Conclusions: Our data strongly suggest AAV-CYP46A1 brain delivery as a relevant therapy for AD, therefore opening new avenues for the treatment of early severely affected Alzheimer patients.
CYP46A1 expression is neuroprotective in Huntington’s disease mice


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Introduction: Huntington’s Disease (HD) is a dominant hereditary neurodegenerative disease, that typically emerge in adulthood and results in motor, cognitive and behavioural abnormalities. HD has a single genetic cause (abnormal expansion of a CAG trinucleotide repeat into the gene encoding huntingtin, HTT) and a well-defined striatal neuropathology. There is no available treatment for HD. Recent findings emphasize the involvement of cholesterol metabolism in Huntington’s disease (HD) pathogenesis. The concentration of the brain cholesterol metabolite, 24S-hydroxycholesterol, is decreased in the plasma of HD patients. Moreover cholesterol membrane accumulation has been shown in cellular and mouse models of HD. We thus investigated the role of CYP46A1, the enzyme responsible for the conversion of cholesterol into 24S-hydroxycholesterol, in HD pathogenesis.

Materials and Methods: Different protein extracts were analyzed (human, cell line and mouse extracts) to quantify CYP46A1 expression by western-blotting. cyp46A1 mRNA expression were quantified by RT-qPCR. HD mouse model (R6/2) was studied for behaviour tests and neuropathological endpoints. Sterols and oxysterol quantification in the striatum were performed using GC-MS (Gas chromatography Mass spectrometry). To deliver CYP46A1 in the striatum of R6/2 mice, we used associated adenovirus rh10 (AAVrh10) carrying cyp46A1-ha cDNA under the CAG promoter control.

Results: cyp46A1 mRNA expression is decreased into the striatum of the R6/2 mouse model from early stages (6 weeks) as well as in STHdh Q111, a HD cell line. Interestingly, CYP46A1 protein expression is also significantly decreased in HD patient putamen extracts. Furthermore, overexpression of CYP46A1 is neuroprotective in primary striatal neurons expressing polyQ-HTT. Restoration of CYP46A1 expression in the striatum of R6/2 HD mouse models improved locomotor behavior, decreased neuronal atrophy and mHTT aggregate formation. In the striatum of R6/2 mice, levels of lanosterol are dramatically down as compared to WT mice whereas cholesterol level is up. In R6/2 mice, CYP46A1 restored levels of cholesterol and lanosterol and increased levels of desmosterol in the striatum. Lanosterol and desmosterol were found to protect striatal neurons expressing polyQ-HTT from death.

Conclusions: Our findings reveal a previously unknown and unexpected role for CYP46A1 in Huntington’s disease pathogenesis. CYP46A1 prevented cholesterol accumulation in R6/2 mice and displayed neuroprotective effect via regulation of sterols, more specifically lanosterol and desmosterol. This study paves the way to restore CYP46A1 expression in the striatum/putamen and caudate of Huntington’s disease patients by direct injection of viral vector.
Regulation of α-synuclein levels by 27-hydroxycholesterol-Relevance to Synucleinopathies

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Introduction: Accumulation of α-synuclein (α-Syn) protein is a common hallmark of a group of brain disorders collectively known as synucleinopathies. These disorders include Parkinson’s disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and Alzheimer’s disease (AD). The causes of synucleinopathies are likely multi-factorials with several factors including environmental agents and genetic susceptibility potentially participating in the pathogenesis of these diseases. For the last few years, cholesterol dyshomeostasis has been under scrutiny as a risk factor for some type of synucleinopathies. However, the reported studies didn’t show consistency as to the potential role of cholesterol metabolism in the pathogenesis of these disorders. The discrepancies in all of the above studies may be explained by the following: fluctuations in cholesterol oxidation products (oxysterols), but not in cholesterol per se, may correlate better with the onset of synucleinopathies. In line with our hypothesis is studies showing increased levels of several oxysterols in cortex of PD brains and AD. Oxysterols were also shown to be elevated in the cerebral cortex of individuals with LBD where they are suggested to accelerate α-Syn aggregation. We tested the hypothesis that the cholesterol oxidation product 27-Hydroxycholesterol (27-OHC) increases α-synuclein transcription through over-activation of its cognate receptor, liver X receptor (LXR). Reducing over-activation of LXR can potentially prevent α-synuclein overproduction and slow or reverse synucleinopathy progression.

Materials and Methods: We treated human neuroblastoma cells, mouse dopaminergic neurons differentiated from embryonic stem cells, and human dopaminergic neurons differentiated from human normal dopaminergic neuronal precursor cells with 27-OHC and examined the effects of increased 27-OHC concentrations on the expression levels of α-synuclein.

Results: Our results show that 27-OHC dose-dependently regulates the transcription of α-synuclein through modulation of LXR in the different cell types. We also found that inhibiting LXR with specific antagonists or knocking down LXR opposes 27-OHC effects on α-Syn transcription and protein levels.

Conclusions: Despite extensive research, no disease-modifying therapy is currently available for synucleinopathies and the search for diagnostic tests and biomarkers are still under development. Identification of the oxysterol 27-OHC and the LXR as the underlying cellular mechanisms by which 27-OHC increases α-synuclein levels may help in designing therapeutic agents that can prevent, reverse, or stop the over-production of α-synuclein and ultimately may protect against synucleinopathies.
What is the driving force for the flux of oxysterols across the blood-brain barrier?

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In catheterization experiments we have shown that there is a flux of oxysterols from the brain into the circulation, 24S-hydroxycholesterol (about 6 mg/24h) and 7 alpha hydroxy-3-oxo-4-cholestenoic acid (about 2 mg/24h). One oxysterol, 27-hydroxycholesterol, fluxes from the circulation into the brain (about 5 mg/24h). The different fluxes are illustrated in the figure below.

The flux of 24S-hydroxycholesterol (24OH) from the brain into the circulation is likely to be concentration-driven. Thus the levels of 24OH are markedly higher in the brain than in the circulation. Also the flux of 27-hydroxycholesterol (27OH) into the brain is likely to be concentration-driven. Thus the levels of 27OH are normally markedly lower in the brain than in the circulation. The reason for the low levels of 27OH in the brain is the very high metabolism of this oxysterol into the steroid acid 7 alpha hydroxy-3-oxo-4-cholestenoic acid (7-Hoac). Patients with a lack of the critical enzyme CYP7B1 have very high levels of 27OH in cerebrospinal fluid, about 30-fold higher than normal. The levels of 27OH in the circulation are about 6-fold higher than normal in these patients. This means that the concentration difference between brain and the circulation is reduced by a factor of 5. If the concentration difference between the brain and the circulation is the driving force for the flux of 27OH between the circulation and the brain, this flux could be expected to be reduced in patients with SPG5. In accordance with this hypothesis, reduction of the levels of 27OH in the circulation of SPG5 patients had little or no effect on the levels of 27OH in CSF (unpublished pilot study).

The levels of the steroid acid 7-Hoac is higher in the circulation than in CSF (6-10 fold) and thus the flux from the brain into the circulation occurs against a concentration gradient. 7-Hoac has however a very high affinity to albumin, and most of it is bound to albumin both in the brain and in the circulation. The ratio between the steroid acid and albumin is about 4 ng/mg in the circulation but about 30 ng/mg in CSF. When exposing an albumin solution in a dialysis bag to CSF there is a significant flux of the steroid acid from the CSF to the albumin.

We conclude that both absolute concentration differences and differences in the degree of albumin binding are important for the flux of oxysterols across the blood-brain barrier.
Dual role of LXR in myelination in the Central and Peripheral nervous system

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Liver X receptors (LXRα and LXRβ) are nuclear receptors implicated in lipid metabolism; they are activated by natural oxysterol like 25-hydroxycholesterol (25-OH). Their role in myelination and remyelination processes is poorly understood. Here we show that oxysterols are present in the cerebellum and nerve, their biosynthetic enzymes are also expressed as well as their nuclear receptors (LXRα and LXRβ). We also demonstrate that LXRs play a crucial role in myelination of the cerebellum and the nerve. LXRα/β double knockout mice (LXR dKO) exhibited thinner myelin sheaths and reduced expression of myelin genes in the cerebellum. The administration of an LXR agonist (TO901317) stimulated expression levels of the two major central myelin genes, proteolipid protein (PLP) and myelin basic protein (MBP), in the cerebellum. We also showed that TO901317 or 25-OH stimulated promoter activity, mRNA and protein expression of PLP and MBP in primary cultures of oligodendrocytes as well as in the oligodendroglial cells. Finally, by using organotypic cultures of cerebellum slices, we showed that LXR activation by 25-OH or TO901317 stimulated myelin gene expression after lysolecithin-induced demyelination, and accelerated the remyelination process. Those effects were abolished in LXR dKO organotypic cultures of cerebellum slices. Our results indicate that LXRs are positive regulators of myelination, and might be pharmacological targets for remyelination therapies of the central nervous system.

Few data are available for the functions of LXR in the peripheral nervous system. Our aim was to study the influence of oxysterols on myelin gene expression and myelin sheath formation in peripheral nerves. We demonstrate that oxysterols inhibit peripheral myelin genes expression (MPZ, PMP22) in a Schwann cell line. This down-regulation is mediated either by LXRα or LXRβ, depending on the promoter context, as suggested by siRNA strategy and ChIP assays in Schwann cells and in the sciatic nerve of LXR knockout mice. Importantly, the knockout of LXR in mice results in thinner myelin sheaths surrounding the axons. Oxysterols repress myelin genes via two mechanisms: by binding of LXRs to myelin gene promoters and by inhibiting the Wnt/beta-catenin pathway that is crucial for the expression of myelin genes. The Wnt signaling components (Disheveled, TCF/LEF, beta-catenin) are strongly repressed by oxysterols. Furthermore, the recruitment of beta-catenin at the levels of the MPZ and PMP22 promoters is decreased.

We also show that the invalidation of LXR provoked locomotor and sensory defects and elicited an oxidative stress in the nerve and mitochondrial abnormalities. The consequence is the oxidation of lipids and carbonylation of proteins. This provoked the aggregation of PMP22.
Evaluation of the effects of major compounds present in the Mediterranean diet (oleic acid, docosahexaenoic acid, α-tocopherol) and hydrogenated oils (elaidic acid) on 7KC-induced oxidative stress and cell death on microglial BV-2 cells

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Introduction: 7-ketocholesterol (7KC) is often enhanced in the plasma and/or cerebrospinal fluid of patients with neurodegenerative diseases (Alzheimer’s disease, Multiple Sclerosis, X-ALD). The lipotoxicity of 7KC is characterized by cellular dysfunctions including oxidative stress and cell death induction. It is therefore important to identify molecules capable to cross the blood brain barrier which could attenuate or amplify its side effects. As olive oil and fish consumption is important in the Mediterranean diet, the aim of our study was to determine the impact of major olive oil compounds (oleic acid (OA: C18:1 cis n-9), α-tocopherol) and of docosahexaenoic acid (DHA, C22:6 n-3) present in fat fishes, such as sardines, to attenuate 7KC-induced side effects. As elaidic acid (EA: C18:1 trans n-9), the trans isomer of OA, can be found in hydrogenated cooking oils and fried foods, it was of interest to precise its effects on 7KC-induced cytotoxicity.

Materials and Methods: Olive oils profiles (Morocco, Spain, Tunisia) were determined by gas chromatography (GC: fatty acids), high pressure liquid chromatography (HPLC: tocopherols, polyphenols) and gas chromatography–flame ionisation detection (GC–FID: sterols). Olive oils contain high levels of OA and α-tocopherol, high phytosterol levels and low amounts of polyphenols. Murine microglial BV-2 cells, were treated with 7KC (25, 50 μM; 24 h), without or with olive oil (1% in ethanol), oleic acid (OA: 50-500 μM), elaidic acid (EA: 50-500 μM), DHA (12.5-100 μM), and α-tocopherol (400 μM). These compounds were introduced in the culture medium 2 h before 7KC or simultaneously. Microscopical, flow cytometric and biochemical methods were used to characterize their biological activities.

Results: On BV-2 cells, 7KC induces several side effects: inhibition of cell growth, loss of cell adhesion, enhancement of cytoplasmic membrane permeability, mitochondrial dysfunctions (decrease of succinate dehydrogenase activity, loss of ΔΨm), lysosomal membrane destabilization, reactive oxygen species overproduction, caspase-3 and PARP degradation, which are apoptotic criteria, and an increased ratio of LC3-II/LC3-I, which is an autophagic criteria. Olive oils attenuate 7KC-induced mitochondrial dysfunctions and/or cell growth inhibition evaluated with the MTT test. The different 7KC cytotoxic effects were also strongly attenuated by the major compounds of olive oils, OA and α-tocopherol, as well as by DHA. Protective effects were also observed with EA. Olive oils, OA and EA also favor the accumulation of numerous cytoplasmic lipid droplets revealed with Oil Red O staining. However, different effects on membrane fluidity were observed with OA and EA.

Conclusions: Major compounds of the Mediterranean diet (OA, DHA, α-tocopherol) are able to prevent 7KC-induced ROS overproduction and cell death on microglial cells. Protective effects were also observed with EA which is often supposed to have deleterious activities. It is suggested that cytoplasmic lipid droplets accumulation induced by OA and EA might have radical scavenger activities, and could contribute to prevent 7KC-induced cell death.
**Interleukin-27 induces oxysterols that regulate CD4 T cell response via LXR signaling**

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**Introduction:** Oxysterols have pleiotropic roles on the immune response aside from their involvement in lipid metabolism. The oxysterols 25-hydroxycholesterol (25-OHC) and 7α,25-dihydroxycholesterol (7α,25-OHC) regulate antiviral immunity and immune cell chemotaxis respectively. However their impact on the adaptive immune response in particular on CD4⁺ T lymphocytes is largely unknown. Adaptive immune homeostasis relies in part on orchestrated interactions among subsets of CD4⁺ T cells with effector or regulatory functions. Among regulatory CD4⁺ T cells, type 1 regulatory T (Tr1) cell, induced by the cytokine IL-27 and that produce the anti-inflammatory cytokine IL-10, are instrumental in the prevention of autoimmune diseases.

**Materials and Methods:** CD4⁺ T lymphocytes were differentiated *in vitro* into different Thelper subsets; Ch25h expression and 25-OHC production were analyzed by real-time PCR and mass spectrometry respectively. Tr1 cells were then generated *in vitro* in the presence of IL-27 from Ch25h⁻/⁻ or wild-type control mice and the effect of 25-OHC and LXR agonists were tested. Cytokine secretions, in particular IL-10, were evaluated by ELISA and flow cytometry. The expression of downstream transcription factors was assessed by real-time PCR and Western Blot. Finally, we conducted repeated treatments with anti-CD3 to induce IL-10⁺ regulatory T cells *in vivo*.

**Results:** We observed that Ch25h and 25-OHC were expressed at high level by IL-27-induced Tr1 cells but not by other subsets of CD4⁺ Thelper cells. Tr1 cells derived from Ch25h⁻/⁻ mice featured increased IL-10 secretion both *in vitro* and *in vivo* while external addition of 25-OHC on Tr1 cells inhibited IL-10 production. At the mechanistic level, we observed that 25-OHC fine-tuned IL-10 production by down-regulating the transcription factor B lymphocyte-induced maturation protein 1 (Blimp1). We further describe that LXR agonists decreased IL-10 secretion in the same range than 25-OHC and that combination of 25-OHC to LXR activators depicted additive effects on cytokines secretion. Finally, the administration of anti-CD3 to wild-type mice resulted in an induction of IL-10⁺ T cells in the mesenteric lymph nodes that was significantly increased in Ch25h⁻/⁻ mice.

**Conclusions:** Together, our findings suggest that 25-OHC control Tr1 cell differentiation and negatively regulate IL-10 production. Not only these findings unravel novel molecular mechanisms accounting for the generation of Tr1 cells, but they also provide oxysterols as critical players to regulate the adaptive immune response, in particular regulatory CD4⁺ T cell differentiation.
Role of P2X7 cell degenerescence receptor and granzyme B in 25-hydroxycholesterol-induced apoptosis and degenerative pathways in skin

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Introduction: Oxysterols have been associated to several degenerative diseases in the brain, the eye and the blood circulation. Skin can also be affected by degenerative disorders such as photoaging or vitiligo, and some oxysterols like 25-hydroxycholesterol (25-OH) have recently been associated to these processes. The connection between skin cells and oxysterols has received little attention while oxysterols are closely related to degenerative diseases and cutaneous degenerative process can be debilitating. Consequently, our aim is to study the skin/oxysterols relationship focusing on two characteristics of degenerative disorders: apoptosis and inflammation.

Materials and Methods: 25-OH effects on apoptosis and inflammation were studied on human keratinocytes (HaCaT cell line) using microplate cytometry and immunoenzymatic assays. Apoptosis was evaluated through chromatin condensation, caspases 3 and 8 activation, P2X7 cell degenerescence receptor activation and the release in cell supernatants of Granzyme B. Inflammation was measured through IL-6, IL-8, IL-1α and MIF pro-inflammatory cytokines release.

Results: 25-OH induced caspase 3 and 8 activation after a 48-hour incubation time on keratinocytes. At high concentrations, 25-OH stimulated P2X7 cell degenerescence receptor activation and granzyme B release. IL-6, IL-8, IL-1α and MIF release all increased after 25-OH incubation.

Conclusions: 25-OH, which we previously associated to skin aging and retinal degenerative process, plays a key role in inflammation and apoptosis. P2X7 cell degenerescence receptor activation and granzyme B release highlight that this oxysterol appears more and more as a key factor in skin degenerative disorders.
Cholesterol metabolism and glaucoma: modulation of Muller cells membrane organisation by 24S-hydroxycholesterol

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Introduction: Glaucoma is a severe and irreversible ocular disease characterized by progressive optic neuropathy with the loss of retinal ganglion cells (RGC). One of the established mechanism associated with RGC apoptosis in glaucoma is Müller Glial Cell (MGC) activation, so-called retinal gliosis. MGC are the most numerous glial cells in the retina and one of their roles is to sustain cholesterol homeostasis. A lack or an excess of cholesterol in neurons can cause neuro-degeneration. One of the elimination forms of cholesterol in the retina is 24S-hydroxycholesterol (24S-OHC), among others such as 27-hydroxycholesterol or cholestenoic acid. 24S-OHC is produced by 24S-hydroxylase (CYP46A1) in RGC, and overexpressed during glaucoma. The objective of this study was to evaluate whether 24S-OHC triggers MGC membrane dynamics involving lipid rafts and participates to gliosis.

Materials and Methods: MGC were grown in vitro from retinas of young rats. They were triggered with 24S-OHC for 2 min or 6 h. Lipid-raft of MGC membrane’s were obtained after 1% Lubrol lysis and 20h-ultracentrifugation at 180,000g in a sucrose gradient. The expression of GFAP, Vimentin, Nestin, Connexin43, Kir4.1, Aquaporine 1, Crystallin alpha B, phosphorylated and non-phosphorylated p38 and p42-44 MAPK was analysed by Western-blotting. High performance liquid chromatography coupled with mass spectrometry (LC-MS) was used to semi-quantify the proportion of ganglioside GM3 (monosialodihexosylganglioside) in raft and non-raft fractions from MGC after 24S-OHC treatment. MGC’s membrane fluidity was evaluated using an anisotropy probe (TMA-DPH) on in vitro MGC.

Results: 24S-OHC treatment strongly increased GFAP in non raft fractions, and Kir4.1 in all cell fractions after 2 min incubation, and decreased anisotropy of MGC, suggesting raft-independent stiffening of MGC membrane and glial activation. Activation of the proliferation pathways was also suggested by the induction of p42-44 MAPK phosphorylation after 6 h incubation with 24S-OHC.

Conclusions: Our data reported early changes in membrane dynamics induced by 24S-OHC that were partially independent on lipid raft formation, but were associated to changes in K+ conductance, and gliosis.
Perturbations of Sterol Homeostasis: The Formation of Toxic Oxysterols as a Consequence of Genetics or Small Molecule Exposures.

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Severe mutations of the enzymes in the pathway from lanosterol to cholesterol are frequently embryonically lethal while the phenotype of mildly-affected individuals includes intellectual disabilities and developmental disorders. For example, 7-Dehydrocholesterol (7-DHC) accumulates in tissues and fluids of patients with Smith-Lemli-Opitz syndrome (SLOS), a recessive neurodevelopmental disorder caused by mutations in the gene encoding 3-hydroxysterol-reductase (DHCR7; EC 1.3.1.21). Although the exact mechanism of the syndrome is not understood, 7-DHC is the most oxidizable lipid known and its derived oxysterols may be critical to the pathobiology of SLOS. 7-DHC derived oxysterols have been isolated from brain, liver and serum from the Dhcr7- null mouse that has been used extensively to study the phenotype and pathogenesis of SLOS and these oxysterols have significant biological activities. Studies in cell culture described in this lecture have identified dozens of small molecules, many of which are in the US and European Pharmacopoeias, that inhibit specific biosynthetic transformations in the cholesterol biosynthesis pathway. The consequence of exposure of cells to an “active” compound is a perturbation of sterol homeostasis similar to the change in the sterol profile found for a known genetic disorder. Risperidone, aripiprazole, trazodone and fluoxetine, for example, inhibit the conversion of 7-DHC to cholesterol, the consequence of exposure being the accumulation of cholesterol biosynthetic precursors and their toxic oxysterols in a way that parallels SLOS, the genetic disorder.
The oxysterol dendrogenin A: an alternative against breast cancers resistant to hormonotherapy.

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Introduction: Breast cancer (BC) remains the most frequent cause of death from cancer among women worldwide despite the development of targeted therapies such as Tamoxifen (Tam) for treating tumors expressing the estrogen receptor (ER), or agents that target the overexpressed growth factor receptor HER2. These failures are explained by the fact that many BC do not respond to these therapies or develop resistance, and there are currently no effective targeted therapies to treat tumors expressing neither ER nor HER2. So there is an urgent need to characterize the molecular players involved in the etiology of breast cancers, progression and resistance in order to develop new therapeutic alternatives to improve the prognosis of patients. We have discovered a new oxysterol, named dendrogenin A or DDA, in different tissues and fluids in mammals and found that the level of DDA is decreased in breast tumors of patients compared to normal breast tissue, indicating a deregulation of DDA metabolism in BC. In addition, DDA exhibits a potent anti-cancer activity in vitro and in vivo against various tumor cells including BC cells and presents a pharmacology distinct from Tam since it is not a ligand of the ER as Tam (de Medina et al, Nature Commun, 2013; Dalenc F et al, Curr Med Chem, 2015). In different tumor models, we showed that DDA had an impact on cells of the microenvironment and activates a lethal autophagy in tumor cells.

Aim: The aim of the present work was to study the efficacy of DDA in BC cells sensitive and resistant to Tam and its mechanism of action based on our previous results.

Materials and Methods: DDA was synthesized as described before (de Medina et al, Nature Commun, 2013). The efficacy of DDA to mediate cell death and an autophagic flux was measured in various ER(+) and ER(-) BC cell lines and in presence or not of autophagic inhibitors. The efficacy of DDA on tumor growth and mice survival was studied on established ER(+) and ER(-) BC tumors implanted into mice. The impact of DDA on cells of the tumor microenvironment was studied by flux cytometry.

Results: DDA was very efficient against BC cells sensitive or resistant to Tam in vitro and in vivo and improved mice survival. Cell death mediated by DDA in both cell models involved a lethal autophagy. In addition, DDA showed a significant impact on different cells of the microenvironment that contribute to its anti-tumor activity.

Conclusions: We show that DDA is very efficient against BC with intrinsic resistance to Tam, in vitro and in vivo, with a significant impact on tumor growth and mice survival. We identified an original mechanism of action by showing that the anti-tumor effect of DDA involved a dual action on the tumor and on the microenvironment. Our data indicate that DDA may offer new perspectives for the treatment of very aggressive and to date untreatable BC.
27-hydroxycholesterol in cancer exosomes – potential new separation method?

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Exosomes are small extracellular vesicles (30 -100 nm) secreted from cells. These vesicles are heavily involved in extracellular communication and can cargo different cellular molecules such as oncogenic metabolites, RNA and proteins. In general, cancer cells releases a greater number of exosomes compared to healthy cells.

Oxidized metabolites of cholesterols, oxysterols, are important in numerous biological processes and pose multiple roles in the body. A variety of isomers exist, with different biological roles, e.g. 27-hydroxycholesterol (27-OHC) is a selective estrogen receptor modulator. It can promote proliferation of estrogen receptor (ER) positive breast cancer by binding to ER and metastasis by binding to liver X receptor. We have previously reported enrichment of 27-OHC in exosomes derived from ER+ breast cancer cells and are currently exploring the link further.

Traditionally, separations of the oxysterols are performed by gas chromatography (GC) or liquid chromatography (LC) after derivatization, before detection with mass spectrometry (MS). Nevertheless, good separation of the close eluting oxysterols can be difficult. In this study we explore potential new separation techniques for the better separation of the oxysterol isomers, by using capillary electrophoresis mass spectrometry (CE-MS). The method will be compared to our established method using capillary LC-MS. The best separation technique will be used to further explore oxysterol isomers in cancer cell derived exosomes.
Propolis has androgen receptor antagonist activity and affects prostate cancer survival

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\textbf{Introduction.} During the pharmacological treatment of prostate cancer, most of the patients respond to anti-androgens. However, over the time, a resistance occurs in 100% of the cases. It seems thus indispensable to develop new efficient molecules that could be accessible to the majority of the patients, whatever their income, and that bypass this androgen resistance. Natural products are an interesting source of new therapeutics, especially for cancer therapy as 70% of them have botany origin. Based on ethnobotany, we evaluated the effect of extracts of propolis on prostate cancer cell survival. Propolis or bee glue is a resinous mixture that honey bees collect from tree buds, sap flows, or other botanical sources. It is used as a sealant for unwanted open spaces in the hive and is enriched in flavonoids.

\textbf{Materials and Methods.} LNCaP, an androgen responsive cell line with a low metastatic potential derived from a lymph node metastasis, and PC-3, an androgen insensitive cell line with a high metastatic potential derived from bone metastasis, were used for the \textit{ex vivo} studies. Aqueous extracts of propolis from the region of Jijel (North of Algeria) were studied by LC/MS and CG/MS (ICCF-Clermont-Ferrand). For biological activity, extracts were diluted in ethanol and used at various concentrations.

\textbf{Results} show that:

i) The various percentages of polyphenols have been characterized.

ii) Propolis reduces the survival of LNCaP (androgen sensitive) and PC3 (androgen resistant) cells at IC50 of 0.04 mg/ml and 0.2 mg/ml, respectively, after 48 hrs of treatment. Molecular analyses suggest the induction of apoptosis and the blocking of the cell cycle at G0/G1 phase.

iii) In LNCaP cells, propolis induces a significant decrease of the accumulation of the androgen receptor (AR) and of its target PSA after 4 hrs of treatment. This diminished effect of the transcriptional activity of AR is also observed on target genes such as \textit{PSA}, \textit{FKBP5} and \textit{SGK}.

iv) Transient transfections of human AR and the reporter gene ARE-tk-Luc point out a clear antagonistic activity of propolis vs. R1881 (a synthetic androgen, 1000 nM) and DHT (a natural androgen, 10 nM) on AR.

\textbf{Conclusions.} Altogether, these results highlight the potential pharmacological effects of propolis in the treatment of prostate cancer. They need to be confirmed \textit{in vivo} on animal models of prostate cancer.

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Outside-in, inside-out: Proteomic analysis in endothelial stress mediated by 7-ketocholesterol

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Introduction: Oxysterols are cholesterol oxidation products formed through enzymatic or autoxidation mechanisms. 7-ketocholesterol (7KC) is one of most abundant oxysterols found in atherosclerotic lesions. Its role in atherosclerosis pathogenesis has been broadly studied in a variety of models. The arterial microenvironment is a multicellular dynamic compartment that is continuously stimulated, among other systemic factors, by 7KC. Endothelial cells have a key role on that environment, being in intimate contact with both, blood stream and vessel wall, the disease starting site. 7KC has been shown to promote endothelial cell death and/or dysfunction, depending on the concentration studied. However, its contribution to the cell microenvironment through cell stimulation has not received much attention. Here we applied mass spectrometry-based proteomics followed by bioinformatics workflow to analyze the effect of non-toxic 7KC concentration on endothelial cell protein expression in vitro.

Materials and Methods: HUVEC endothelial cells secretome and total cell pellet were collected after 24h exposure to 7KC. Samples were reduced and alkylated, followed by trypsin digestion and then cleaned up. All samples were analyzed by high resolution and high accuracy nano-LC MS/MS. After database search and statistical analysis, differential expressed proteins were selected for further studies.

Results: Our workflow identified 1805 secreted proteins and 2203 intracellular proteins, 40 and 55 being regulated, respectively. Gene ontology cellular component analysis showed that secretome protein enrichment was accomplished with majority of regulated proteins being assigned to extracellular region, exosome or membrane-bounded vesicle. On the contrary, regulated proteins extracted from HUVEC cell pellets were associated to intracellular non-membrane-bounded organelle, cytosol and intracellular organelle part cellular components. Analyses of canonical pathways involved in secretome proteins revealed 5 regulated proteins involved in signaling by Rho family GTPases, which have been associated with activation of smooth muscle cells contraction. Another main pathway regulated was blood coagulation through downregulation of Tissue Factor Pathway Inhibitor (TFPI), an antithrombotic agent found to be 2 times downregulated in coronary disease.

Conclusions: Our study shows differential endothelial protein regulation and secretion upon 7KC exposure in non-toxic conditions for short time period. Herewith, data support the role of 7KC in atherosclerosis pathophysiology and reinforce the deleterious effect of endothelial cells stress in the artery microenvironment.
Evidences of an impairment of cholesterol metabolism associated to mitochondrial dysfunction. 
A metabolomic approach based on mass spectrometry

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Introduction: Cholesterol and fatty acid (FAs) esters as phospholipids and sphingolipids are structural elements of cellular membrane. In the central nervous system, cholesterol is involved in synaptogenesis, maintenance, turnover, stabilisation and restore of synapses. Within the brain, about 70% of cholesterol is present in myelin sheath formed by sections of oligodendrocyte plasma membrane repeatedly wrapped around an axon, with the extrusion of virtually all of the cytoplasm. Changes in lipid composition of plasma and brain were reported in neurological disorders (NDs) such as Parkinson disease, Huntington Disease (HD) and Multiple Sclerosis, and were proposed as predictors of disease progression. Abnormalities of the plasma phospholipids and of the cholesterol metabolism biomarkers were also reported in aging, obesity, T2D, metabolic syndrome, dyslipidemia, which are, altogether, risk factors for AD and NDs. Alterations of the lipid composition of neuronal membranes enhance formation of Aβ. Lipid metabolism is also an emerging therapeutic target in NDs.

Mitochondria with tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS) are the production site of energy and substrates for cellular lipid biosynthesis. FAs and cholesterol are synthesized from acetyl-CoA originating from mitochondrial citric acid transported in the cytosol and cleaved by ATP-citrate lyase. Abnormalities in mitochondrial dynamics and functions were observed in NDs, aging and age-related diseases.

Materials and Methods: Sterols, oxysterols, fatty acids and metabolic organic acid were measured by isotope dilution gas chromatography-mass spectrometry after ethanolic saponification, liquid to liquid and cartridge separation and TMS-derivatisation in cultured cells exposed to oxysterols, rifampicine, metformin with a pro-apoptotic and autophagic stimulation. In parallel also mitochondrial function was measured. In brain samples collected from rodent models of NDs lipids were also measured.

Results: In presence of pro-apoptotic stimulation, morphologic and functional abnormalities of mitochondria were observed. The transmembrane mitochondrial potential (ΔΨm) was reduced, the cellular amount of lactate was higher while pyruvate, citrate, fumarate, succinate (TCA intermediates) were significantly reduced, suggesting that an impairment of mitochondrial respiratory function lead to an increase of lactate production and a reduced amount of ATP, NAD and Acetyl-CoA available for the anabolic pathways. Sterol precursors (lathosterol, lanosterol and desmosterol) and saturated and unsaturated long chain fatty acids (C16 - C18, structural elements of membrane phospholipids) were reduced. Changes in oxysterol composition were observed. In brain samples were observed a significant reduction of sterol precursors, 24OHC and 27OHC and changes in fatty acids profile.

Conclusions: Mitochondrial alterations are associated to TCA and OXPHOS dysfunction which result into a reduced production of ATP, NADPH and Acety-CoA. These dysfunctions are able to induce changes to the lipid composition of the cellular membranes as consequence of an anabolic impairment. It is likely that this mechanism might contribute to the process of neurodegeneration.
The involvement of ORP2 in cellular energy metabolism


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Introduction: ORP2/OSBPL2 is a member of the oxysterol-binding protein family implicated in lipid transport and signaling at membrane contact sites. By interaction with the ER anchor protein VAP, ORP2 localizes to the interfaces of the ER and lipid droplets (LDs). Our earlier work demonstrated that this localization is sensitive to signal inputs from the high-affinity ligand 22(R)OHC, which induces translocation of ORP2 from LDs to cytosol or plasma membrane. Moreover, reduction of ORP2 or ORP2/VAP complexes with RNAi modifies the cellular metabolism of triglycerides (TGs). In this work we aimed to elucidate in detail the role of ORP2 in lipid and carbohydrate metabolism, to be able to position it in the global networks that coordinate cellular bioenergetics.

Materials and Methods: We created a complete ORP2/OSBPL2 gene knock-out (ORP2-KO) in HuH7 hepatoma cells by CRISPR/Cas9 genome editing. The lipidome of the cells was analysed by TLC and MS approaches. Glucose uptake and glycogen synthesis were measured with radioisotope methods, and signalling components by Western blotting.

Results: We discovered a significant (-40%) reduction of the total cellular TGs as a major phenotype of the ORP2-KO cells. Interestingly, the cellular content of both free and esterified cholesterol, oxysterols (25OHC, 27OHC, 4βOHC, 7αOHC, 7βOHC, 7KC) or phospholipids (PLs) was unaffected, except for LDL-loaded cells in which free cholesterol was mildly reduced. These findings suggest that the major function of endogenous ORP2 does not involve cholesterol or PL metabolism but rather TGs and possibly other aspects of energy metabolism. In anabolic state, the energy and carbon of excess glucose are mainly stored as glycogen and TGs. Therefore we determined the impacts of ORP2-KO on HuH7 cell glucose uptake and glycogen synthesis, as well as on key signaling mediators that control these events. In ORP2-KO cells both glucose uptake and glycogen synthesis were significantly reduced (by -30% and -50%). Most interestingly, ORP2-KO cells displayed dampened phosphorylation of Akt(Ser473) and GSK-3β(Ser9), consistent with the reduced glycogen synthesis phenotype.

Conclusions: The present study suggests that ORP2 is, in addition to sterols, sensitive to signals controlling energy metabolism. The data also reveal that the function of ORP2 is not limited to TG metabolism: The impact of ORP2 on bioenergetics may be exerted already at the level of glucose metabolism, resulting in an extensive contribution of ORP2 to the cellular anabolic processes.
The plasma elimination rate of current lipoprotein apheresis methods differs for lipoprotein remnants, HDL-particles, oxysterols, cholesterol precursors and phytosterols

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Introduction: Lipoprotein apheresis is a procedure that allows the elimination of lipoproteins from plasma or whole blood. It is a therapeutic option in high-risk patients for atherosclerosis, who do not respond satisfactorily to drug therapy. Functional principles include (i) filtration (MDF, MONET), (ii) adsorption (Liposorber D, Therasorb and DALI) and a (iii) precipitation based approach using heparin (HELP). Currently no data on a method specific modulation of the plasma lipidome, proteome and epigenome (e.g. miRNAs) are available.

Materials and Methods: Aim of this study was to perform lipidomic, proteomic and miRNA analysis before and after apheresis and identify differences between the different apheresis approaches. 79 patients with elevated LDLc or Lp(a) were enrolled at the TU Dresden. Each patient was randomized to one of six different treatment groups. Lipidomic and miRNA analysis was performed in Regensburg and proteomic analysis in Victoria. Additionally routine lipid parameters were determined.

Results: LDLc was lowered effectively by all apheresis methods with a mean decrease of 70% (110mg/dl to 34mg/dl). Lp(a) levels decreased on average 76% (55mg/dl to 16mg/dl). Triglycerides showed an average reduction rate of 54% with the highest reduction of 67% by MONET. VLDL-cholesterol decreased on average 68%, with the highest efficacy of 75% by MONET, while sdLDL decreased on average 86% with highest elimination through adsorption (Liposorber D, Therasorb). Apolipoprotein apoB100 and apoB48 decreased on average 69% and 57% respectively, while apo CII and apo CIII decreased 41% vs. 37% with the highest elimination by MONET. HDLc, LCAT-mass, apoAI and PLTP were most affected by the filtration methods MDF and MONET. Therasorb led to a stronger reduction in ApoD than all other methods.

Lipidomic analysis revealed an average reduction of 58% for total cholesterol with highest efficacy of 67% by MONET and DALI. Among the oxysterols 24-OHC decreased on average by 45% and most efficiently by Therasorb (57%) and MONET (52%), while 27-OHC decreased on average by 30% and most efficiently by MONET (57%). In contrast, 7-Ketocholesterol decreased on average 12%, with borderline significance, while MONET was significantly efficient with 32%. However, more detailed analysis revealed, that the majority of >90% of 7-Ketocholesterol is bound to red blood cells (RBC) and related extracellular vesicles (RBC-EVs) and not to lipoproteins. Therefore, the MONET-data may rather indicate RBC-EV and not lipoprotein particle elimination. Cholesterol precursors were eliminated on average by 46% for lanosterol, 34% for lathosterol, and 19% for desmosterol, but only elimination with DALI reached significance. Among the phytosterols sitosterol and campesterol were not significantly reduced. The glycerophospholipids PC, PC ether, LysPC and PE-species showed a slight decrease with the strongest reduction for PC and PE induced by MONET. PE plasmalogens, PI and ceramides were lowered equally by all methods. Currently analysis of the elimination rate of microparticle bound proteins and miRNAs is performed. First evidence indicates that the apheresis
methods differ in their efficacy to eliminate EV an non-EV components, and that miR451a, miR574-5p, miR923, miR940 and miRNA1281 are significantly altered during apheresis.

**Conclusion:** All techniques lowered standard lipid parameters comparably. There are method specific differences in the impact on individual lipid species (e.g. oxysterols, PC, PE) and lipoprotein subclasses (e.g. remnants, HDL-particles), showing that the methods clearly differ in their therapeutic efficacy.
Oxysterols and Cancer

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Even if great strides have been made in the treatment of cancers, these diseases remain a major public health issue. Researchers are actively seeking for early biomarkers and for the identification of new mechanisms involved in the etiology of cancers. This challenging area could certainly give new opportunities to improve ongoing treatments and to develop new therapeutics. The sex steroid hormones like estrogens and androgens are known since a long time to promote several types of cancers. This led to the development of anticancer therapies targeting steroid hormone receptors or steroid biosynthetic enzymes. Cholesterol, which is the precursor of steroid hormones, has been proposed as a possible etiologic factor in certain cancers. This raised questions related to the possible implication of hypercholesterolemia, obesity, lifestyle, and diet in the occurrence and the development of certain cancers. Epidemiological studies or clinical trials with hypocholesterolemic drugs gave conflicting or unconclusive results deserving further investigations. Cholesterol by itself is devoid of tumor-promoting or tumor-suppressing activities in cellular and animal models. Interestingly, recent data from the literature showed that cholesterol metabolites including certain oxysterols (oxygenation products of sterols) could play a dynamic role in cancers at least on model systems. This opens up new perspectives in our understanding of these diseases and offers new tracks for the development of therapeutic strategies. This talk will present a survey of recent data from the literature.
Microbial degradation of cytotoxic 7-Ketocholesterol and mining therapeutic enzymes

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Introduction: Many age-related disorders such as Alzheimer’s disease, atherosclerosis and age-related macular degeneration are associated with the accumulation of cholesterol oxidation products, a major content of which is 7-Ketocholesterol (7KC). An alternative to using natural or synthetic molecules to reduce their cytotoxicity is the application of enzymes from microbial sources to degrade oxysterols in vitro and in vivo, a novel approach termed as “medical bioremediation”. Through the present work, we aim to assess the potential of bacteria in degrading 7KC and mining candidate enzymes involved for further application as therapeutics.

Materials and Methods: The bacterial strains were grown in minimal salt media with 7KC (1g/l) as the carbon source. Equal amount of samples were collected at different time periods and extracted with hexane:isopropanol (3:2 v/v). They were analyzed in HPLC using methanol:water:acenonitrile (85:10:5 v/v/v) as the solvent in a C18 column with a flow rate of 1ml/min and UV-detector set at 230nm. The area obtained in HPLC were converted to percentage of control set. Process variables such as the percentage of surfactant used, inoculum size, pH, temperature, nitrogen source and presence of solvents were optimized for maximum degradation. Morphological changes in the cell structure were studied in a Carl Zeiss EVO Scanning Electron Microscope. The activity of cholesterol oxidase was assayed using standard protocol with o-dianisidine and horseradish peroxidase. ESI-MS and GC-MS were used to determine the possible intermediates and the degradation pathway.

Results: Pseudomonas aeruginosa PseA and Rhodococcus erythropolis MTCC 3591 have been identified as potential degraders of 7KC. Presence of surfactants, pH and inoculum size are found to be important parameters for the degradation process. Under optimized conditions, P. aeruginosa PseA and R. erythropolis MTCC 3591 are able to degrade 88% and 93% of an initial concentration of 1g/l 7KC respectively. The cells show typical morphology in 7KC, while in its absence, the cells undergo breakage in low-nutrient condition. Studies with cell-free supernatant has shown degradation of the compound, thus reinforcing the occurrence of suitable enzymatic systems involved in the process. 4-cholesten-3,7-dione is an important intermediate and cholesterol oxidase has been identified as one of the enzymes involved in the degradation process.

Conclusions: The current study presents the novel strategy of producing and using relevant microbial enzymes to biodegrade cytotoxic compounds, thus providing a remedial approach. Cholesterol oxidase has been identified in P. aeruginosa PseA as well as R. erythropolis MTCC 3591 as a potential enzyme for degradation of 7KC. This would help in further application of the enzyme in food systems and as a therapeutic for the mitigation of harmful effects of 7KC and other cholesterol oxidation products.
Identification of *Mycobacterium tuberculosis* enzymes involved in metabolism of immunoactive sterols.

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**Introduction**: Problems associated with treatment of XDR forms of tuberculosis are well known. Identification of new molecular drug targets is of particular importance to combat the disease. Members of the cytochrome P450 family (CYP) considered as potential candidates being involved in metabolism of biologically important molecules in the host organism. *M. tuberculosis* has unusually high number of different cytochrome P450s (20 genes). Despite intensive research in this field 75% of these enzymes remain orphans. Our research is focused on investigation of mycobacterial P450 that might be involved in metabolism of bioactive sterols.

**Materials and Methods**: We apply protein family based approach in our experiments to study the panel of CYPs using methods of biotechnology, enzymology, structural biology, bioinformatics as well as experimental and technological practices in synthesis of compounds with desired properties.

**Results**: In present work we carried out molecular cloning, heterologous expression and purification of 8 cytochrome P450s from XDR strains of *Mycobacterium tuberculosis*. The substrate specificity of these enzymes was studied using various steroid and vitamin D derivatives. We identified mycobacterial cytochrome P450 that shows hydroxylation activity toward vitamin D and provitamin D3 *in vitro*. We also found that mycobacterial P450s can metabolize bioactive oxysterols.

**Conclusions**: We have discovered that pathogenic mycobacteria are involved in metabolism of immunoactive sterols. Based on these results we suggest that modulation of oxysterol and secosteroid biosynthesis or metabolism could be an adaptation mechanism of mycobacteria infection for human immune system.
POSTERS
Progestins and Corticoids Interaction with Human Serum Albumin

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Introduction: Triterpenes (TTPs) constitute a large family of endogenous and exogenous molecules. They regulate many biological functions such as metabolism, reproduction, immunity...Literature data focusing on plasma protein-TTPs interactions are controversial; some studies suggested a crucial role of albumin in TTP distribution while others neglected the importance of such interaction. Human serum albumin (HSA) is the major plasma protein and binds various ligands, affecting their diffusion and thus their biological effects. Here, two series of TTPs molecules, corticoids and progestins, were studied for their interaction with human serum albumin in vitro at pH 7.4.

Materials and Methods: The glucocorticoid series includes cortisol (Co), cortisone (Con), prednisolone (PD), prednisone (Pn), 9-fluorocortisol acetate (9-FA), and 6-methylprednisolone (6-MP); that of progestogens includes progesterone (PG), 17-hydroxyprogesterone (17-OHPG), 21-hydroxyprogesterone (21-OHPG), medroxyprogesterone (MP), medroxyprogesterone acetate (MPA) and dydrogesterone (DYG). Fluorescence and FTIR spectroscopies were used to study the drug-protein interaction. The binding parameters were calculated and the binding site was identified by competitive displacement study.

Results: The emission spectra of HSA were obtained upon excitation of the single Trp residue located in site I. At molar ratios TTP/HSA varying from 0 to 10, all the studied molecules quenched the Trp fluorescence. Besides, the binding constant of bilirubin, a marker of site I, to albumin was not affected by the presence of TTPs at TTP/HSA molar ratio of 1. Furthermore, for all the investigated TTPs, the peak positions of amide I and amide II bands of HSA were shifted and a negative feature was also observed in amide I region.

Conclusions: The binding constant values of the studied TTPs with albumin were in the range of 10^4 M^-1. TTPs did not affect the binding of bilirubin to albumin suggesting that they did not bind to the same pocket as bilirubin. FTIR results suggested the formation of hydrogen bonding between albumin and TTPs, indicating a partial alteration of the albumin secondary structure.
Effect of tetra- and penta-cyclic triterpenes on lipid bilayer fluidity by DSC, FTIR, Raman and fluorescence anisotropy

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Introduction: Triterpenoids (TTPs) are a large group of active molecules derived from squalene. They possess a non-genomic and a genomic mechanism of action. The non-genomic mechanism may involve changes in lipid membrane properties. Our research aimed to understand the molecular interactions of three series of TTPs with synthetic membranes. Glucocorticoids, progestogens, oleanolic and ursolic acid derivatives are included. The studies are conducted in vitro and liposomes are used as model membrane.

Materials and Methods: The glucocorticoids series include cortisol (Co), prednisolone (PD) and 9-fluorocortisol acetate (9-FA), that of progestogens include progesterone (PG), 17-hydroxyprogesterone (17-OHPG), 21-hydroxyprogesterone (21-OHPG), medroxyprogesterone (MP), medroxyprogesterone acetate (MPA) and dydrogesterone (DYG), while erythrodiol (ER) and uvaol (UV) represent the pentacyclic TTPs.

Liposomes are prepared in the absence and presence of TTPs at various molar ratios TTP/lipids by the thin film hydration method. The obtained batches are characterized by differential scanning calorimetry (DSC), raman spectroscopy, fourier transform infrared spectroscopy (FTIR) and fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH).

Results: The DSC results showed the disappearance of the pre-transition peak and a decrease of the main transition temperature for TPP-loaded liposomes. Compared to blank liposomes, the Raman and FTIR results proved a modification of the intensity of the peak at 715 cm\(^{-1}\) and 1230 cm\(^{-1}\) for TTP-loaded liposomes, respectively. Incorporation of TTP molecules into DPPC liposomes caused also an increase in the Raman height intensity ratios of the peaks \(I_{2935}/I_{2880}\), \(I_{2844}/I_{2880}\) and \(I_{1090}/I_{1130}\), and in the frequencies of infrared absorption bands arising from the symmetric stretching vibrations of the methylene groups on lipid hydrocarbon chains (2800-3000 cm\(^{-1}\)) giving information about the ratio disorder/order of the alkyl chains. An increase of the bandwidth of the CH\(_2\) stretching bands measured at 50 % of the peaks height by FTIR was observed. Furthermore and compared to blank vesicles, the presence of TTPs in vesicles reduced the anisotropy values at 25, 41 and 50°C.

Conclusions: The results demonstrated the interaction of TTPs with the polar head groups of phospholipids and with the alkyl chains. Moreover, the studied molecules fluidized the liposomal membrane at 25, 41 and 50°C. Pentacyclic TTPs, being more hydrophobic than tetracyclic ones, demonstrated higher fluidizing effect than tetracyclic TTPs in the liquid crystalline phase, suggesting a deeper incorporation in the lipid bilayer. The presence of a free polar group at the ring D seems to be the main factor favoring the destabilization of lipid membranes.
P03

Dietary phytochemicals in the protection against oxysterol-induced damage

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The intake of fruits and vegetables is associated with reduced incidence of many chronic diseases. These foods contain phytochemicals that often possess antioxidant and free radical scavenging capacity and show anti-inflammatory action, which are also the basis of other bioactivities and health benefits, such as anticancer, anti-aging, and protective action for cardiovascular diseases, diabetes mellitus, obesity and neurodegenerative disorders. Many factors can be included in the etiopathogenesis of all of these multifactorial diseases that involve oxidative stress, inflammation and/or cell death processes, oxysterols, i.e. cholesterol oxidation products (COPs) as well as phytosterol oxidation products (POPs), among others. These oxidized lipids result from either spontaneous and/or enzymatic oxidation of cholesterol/phytosterols on the steroid nucleus or on the side chain and their critical roles in the pathophysiology of the abovementioned diseases has become increasingly evident. In this context, many studies investigated the potential of dietary phytochemicals (polyphenols, carotenoids and vitamins C and E, among others) to protect against oxysterol toxicity in various cell models mimicking pathophysiological conditions. A literature survey has been done, summarizing the mechanisms involved in the chemopreventive effect of phytochemicals against the injury by oxysterols, may constitute a step forward to consider the importance of preventive strategies on a nutritional point of view to decrease the burden of many age-related chronic diseases.

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Cytoprotective effects of alimentary and cosmetic argan oils on 7-ketocholesterol - treated 158N murine oligodendrocytes

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Introduction: The argan oil, extracted from argan-tree fruits, has been used in traditional medicine as a natural remedy for several centuries. Traditionally, argan oil is well known for its cardioprotective properties and it is also used in the treatment of skin infections. Our objective was to study the cytoprotective effects of various types of argan oils used in diet (Agadir-Berkane, Morocco) and cosmetic (Berkane, Morocco).

Materials and Methods: The effects of argan oils were evaluated on a cellular model mimicking an environmental oxidative stress in a context of neurodegeneration. To this end, 158N murine oligodendrocytes were treated with 7-ketocholesterol (7KC: 25 µM (10 µg/mL)) for 24 h without or with argan oils (1% final volume; pre-treatment 2 h). 7KC has been shown to induce a complex mode of cell death on various cell types: oxiapoptophagy (OXIdation, APOPTOsis, and autoPHAGY). Argan oils profiles were determined by gas chromatography (GC: fatty acids), high pressure liquid chromatography (HPLC: tocopherols, polyphenols) and gas chromatography–flame ionisation detection (GC–FID: sterols). Various complementary microscopical, flow cytometric and biochemical methods were used to characterize the biological activities of argan oils.

Results: All argan oils were mainly rich in fatty acids (C18:1 n-9 > C18:2 n-6 > C16:0), and phytosterols (spinasterol, schotenol, β-amyrin, cycloartenol, Δ7-stigmasterol, Δ7-avenasterol, 24-methylene cycloartenol and/or citrostadienol). Very low traces of polyphenols were identified. On 158N murine oligodendrocytes, 7KC induces several side effects characteristic of oxiapoptophagy: a decrease of adherent cells and an increase of floating cells observed under phase contrast microscopy and by crystal violet staining, an increased number of dead cells observed by trypan blue and propidium iodide, an overproduction of reactive oxygen species (ROS) revealed by dihydroethidium staining, a loss of transmembrane mitochondrial potential measured with DiOC6(3), a lysosomal destabilization evaluated with acridine orange. 7KC also induces condensation and/or fragmentation of the nuclei which is a typical morphological criteria of apoptosis. It also promotes conversion of LC3-I to LC3-II which is a criteria of autophagy. These different effects were counteracted by argan oils.

Conclusion: The ability of argan oils to attenuate 7KC-induced ROS overproduction, apoptosis and autophagy on oligodendrocytes reveals new biological activities of argan oil on nerve cells. Since several compounds of argan oils are able to pass the blood brain barrier, these properties could be of interest to prevent cell death of oligodendrocytes in neurodegenerative diseases associated with oxidative stress, and lipid peroxidation processes leading to increased 7KC levels.
**Evidence of K⁺ homeostasis disruption and potential involvement of Kv channels in cellular dysfunctions triggered by 7-ketocholesterol, 24S-hydroxycholesterol, and tetracosanoic acid on 158N oligodendrocytes**

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**Introduction:** Imbalance in the homeostasis of K⁺ ions has been reported to contribute in the pathogenesis of various neurodegenerative diseases. 7-ketocholesterol (7KC), 24S-hydroxycholesterol (24SOHC), and/or tetracosanoic acid (C24:0) are often found at increased levels in the plasma, cerebrospinal fluid and/or brain of patients with Alzheimer’s disease, Multiple Sclerosis and X-ALD. These lipids are supposed to favor numerous dysfunctions on nerve cells of the central nervous system. However, their effects still remain unclear. We therefore studied the impact of 7KC, 24SOHC, and C24:0 on oligodendrocyte cells, and we attempt to precise the part taken by K⁺ in their ability to trigger cellular dysfunctions.

**Materials and Methods:** 158N murine oligodendrocytes were treated with 7KC (2.5–25 µM), 24SOHC (2.5–25 µM) or C24:0 (1–20 µM) for 1–24 h. The effects of 7KC, 24SOHC and C24:0 on lipid membrane organization and membrane potential were examined from 1 to 24 h with the lipophilic dye merocyanine 540 (MC540) and the anionic dye bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (DiSBAC2(3)), respectively. Intracellular K⁺ concentration ([K⁺]) was measured with the flam photometer method and the ratiometric approach using the PBF-I–AM fluorescence indicator. To determine whether the lipotoxicity was associated with an involvement of voltage gated potassium channels (Kv channels), 158N cells were pre-treated with an universal Kv channel blocker, 4-Aminopyridine (4-AP) (2-4 mM, 1 h) without or with 7KC, 24SOHC or C24:0. Cell adhesion, cell growth, mitochondrial depolarisation, cytoplasmic membrane integrity, cell cycle and morphological aspect of the nuclei were determined with various microscopical, flow cytometric and biochemical methods.

**Results:** 7KC, 24SOHC and C24:0 induce important changes in lipid content and polarisation of the cytoplasmic membrane. These events were associated with increased [K⁺]. Blocking of Kv channels with 4-AP provides evidences for the involvement of Kv channels in 7KC-, 24SOHC- and C24:0-induced cellular dysfunctions. Thus, 4-AP strengthens loss of cell adhesion and cell growth, and potentializes mitochondrial depolarization and cytoplasmic membrane damages. With 7KC and 24SOHC, in the presence of 4-AP, higher percentages of cells in the sub-G1 peak, and with condensed and/or fragmented nuclei (which are apoptotic criteria) were observed.

**Conclusion:** Our data show i) that 7KC, 24SOHC and C24:0 favor intracellular K⁺ accumulation which could be a consequence of cytoplasmic membrane damages, and ii) support that lipid compounds, including major oxysterols and C24:0, could affect the transmission of nerve influx through the disruption of K⁺ homeostasia involving Kv channels.
Dietary oxysterols as stressors of intestinal mucosa in developing colorectal cancer.

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Introduction - Intestinal epithelial barrier derangement is recognized as having an important effect on the risk of cancer. The association between high animal fat consumption in the diet and cancer development is supported by solid epidemiologic data. Oxysterols from dietary origin have been demonstrated to contribute to development and progression of human chronic diseases associated with inflammation including inflammatory bowel diseases, which are considered preneoplastic diseases. However, the role of oxysterols in the multistep process of colorectal carcinogenesis, in particular mechanistic information regarding their possible action on gut barrier impairment associated with tumor progression, has not been clarified yet.

Materials and Methods - Differentiated CaCo-2 colorectal cancer cells were treated with a 60µM mixture of dietary oxysterols consisting in 7-ketocholesterol (42.96 %), 5α,6α-epoxycholesterol (32.3%), 5β,6β-epoxycholesterol (5.76%), 7α-hydroxycholesterol (7α-OH) (4.26%), and 7β-hydroxycholesterol(14.71%), for 24, 48 and 72 hours. Trans-Epithelial Electrical Resistance was assessed to measure epithelial layer integrity. Tight Junction (TJ) molecules were evaluated in cell medium by ELISA and immunofluorescence at different times of incubation. Activation of metalloproteinases (MMP)-2 and -9 was evaluated by zymography.

Results – A mixture of principal oxysterols present in the diet induced the activation of MMP-2/9 in enterocyte-like CaCo-2 cells, and affected cellular distribution and synthesis of TJ proteins. Specific phenolic compounds with well-known anti-inflammatory and anti-oxidant properties were able to protect cell layer from both increased permeability and MMPs activation. These results were in parallel validated by using specific inhibitors of NADPH oxidase and MMPs.

Conclusions - Pro-oxidant and pro-inflammatory properties of dietary oxysterols suggest them as molecules able to maintain an active status of intestinal inflammation, which favors MMPs activation, extracellular matrix degradation and mucosal layer destabilization, thus leading to a leaky intestinal barrier, and predisposing enterocytes to cancerous phenotype.
Effect of CYP46A1 on lipid raft composition and excitotoxicity in the context of Huntington’s disease

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Introduction: Huntington’s disease (HD) is a genetic neurodegenerative disease caused by an abnormal expansion of poly-CAG repeats in the gene encoding for the protein Huntingtin (Htt). The main clinical manifestations are motor, cognitive and psychiatric symptoms. Recent evidences indicate that cholesterol homeostasis is altered in HD patients and mouse models of the disease. Although sterol biosynthesis is reduced in the brain of several HD mouse models, elevated cholesterol content in the caudate of HD patients and mice has also been reported (Karasinska and Hayden, 2011). We showed that CYP46A1, the rate-limiting enzyme for the degradation of cholesterol in neurons, is decreased in the brain of HD patient and in the striatum of a transgenic mouse model of HD. CYP46A1 restoration, by injection of an AAVrh10 vector in the striatum of R6/2 mice, restored cholesterol homeostasis and improved motor behaviour (Boussicault et al., 2016). Increased cholesterol in HD neurons leads to an accumulation of glutamate receptors (NMDAR) in the lipid raft that induces excitotoxicity (Del Toro et al., 2010). We hypothesized that by restoring CYP46A1 activity, we would increase membrane fluidity and concentrate NMDAR outside lipid rafts thus protecting neurons from NMDA-induced excitotoxicity.

Materials and Methods: We used both biochemical and super-resolution microscopy to assess lipid raft composition and NR2b localization in neurons. We isolated Detergent Resistant Membrane (DRM) domain from primary striatal neuron cultures infected with AAV-rh2-Htt-18Q or Htt-82Q +/- AVVrh10-CYP46A1. We performed cholesterol assays and western blots to determined NR2b localization in DRM. In addition we used live Fluorescent Correlation Spectroscopy (FCS) to assess NR2b sub-localization in lipid rafts of primary neurons.

Results: We showed that mutated Htt dramatically increased cholesterol content and NR2b localization in DRM fractions. CYP46A1 overexpression decreased cholesterol in DRM fractions but had no effect on NR2b sub-localization. Co-infection of CYP46A1 with mutated Htt did not correct the effects of mutated Htt on cholesterol content or NR2b localization. Finally, FCS experiments confirmed that CYP46A1 had an impact on lipid rafts properties.

Conclusions: In primary neurons, mutated Htt increased cholesterol and NR2b contents of lipid rafts. However decreasing cholesterol by overexpressing CYP46A1 was not sufficient to counterbalance the effects of mutated Htt.
Dietary effects of *Raphanus sativus* cv Sango on lipid accumulation and oxysterols in rat brain: a lipidomic study on a non-genetic obesity model

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**Introduction:** Obesity is recognized as a leading global health concern, associated with an increased risk for several chronic diseases. The brain plays a key role in energy homeostasis as the master regulator of food intake balance and energy expenditure, controlling the adipose mass gain responding to hormonal and nutrient-related signals. Promising weight-management strategies include the use of vegetables rich in bioactive compounds, to improve the antioxidant status and to stimulate lipid catabolism. The aim of this study was to evaluate the dietary effects of *Raphanus sativus* cv Sango on lipid accumulation and oxysterols in brain of obese rats.

**Materials and Methods:** Four-day old *Raphanus sativus* cv Sango sprouts (SSJ) were squeezed to obtain a juice, which was collected and used for the experiments. Thirty-five male Sprague Dawley rats aged 9 weeks (270–300 g), after 10-days adaptation, were split randomly into five groups (7 animals per group) and fed specific diets: RD-RD (regular diet for 14 weeks); HFD-HFD (high fat diet for 14 weeks); HFD-RD (HFD for 10 weeks and then RD for 4 weeks); HFD-RD 75 (HFD for 10 weeks and then RD and 75 mg/kg b.w. SSJ for 4 weeks); HFD-HFD 75 (HFD for 10 weeks and then HFD and 75 mg/kg b.w. SSJ for 4 weeks). After 14 weeks, rats’ brains were isolated and their lipid matter was subjected to the analysis of total fatty acids, sterols and oxysterols composition.

**Results:** The lipid content, fatty acid composition, cholesterol and its oxidation products (COPs) content were significantly affected. The HFD-HFD group showed a fat accumulation in the brain, with a significant reduction of linoleic acid. Although the lowest cholesterol content was detected on HFD-HFD group, the latter generated the highest COPs amount (63.098 μg/g brain). The main COPs found were 7α/β-hydroxycholesterol, α/β-epoxycholesterols, 7-ketocholesterol, cholestanetriol and 24-hydroxycholesterol. The Principal Component Analysis revealed that all COPs were highly correlated to HFD-HFD diet, except for 24-hydroxycholesterol which resulted highly correlated to linoleic acid content.

**Conclusions:** The high fat diet increased lipid infiltration in rat brain, however, it reduced the amount of cholesterol with a significant increase of COPs, except for 24-hydroxycholesterol, thus confirming the inflammatory status. However, the *Raphanus sativus* cv Sango sprouts supplementation was not able to counteract the oxidative stress in rat brain since the lipidomic profile was not significantly affected.
Oxidatively modified HDL and HDL isolated from diabetic subjects show reduced ability to efflux oxysterols from THP-1 macrophages


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Introduction: Oxysterols, the products of oxidation of cholesterol, are accumulated in subendothelial macrophages during atherogenesis process by massive uptake of oxidized low density lipoproteins (oxLDL). Some oxysterols are involved in the regulation of cholesterol homeostasis, cellular oxidative stress or cytotoxicity. We recently reported that oxysterols were produced intracellularly in macrophages exposed to oxLDL and were efficiently released by HDL but not apoA1. Diabetic status is generally correlated with increased oxidative stress. We aimed to characterize oxysterol composition of lipoproteins isolated from healthy controls and type 2 diabetic patients and to evaluate the impact of oxidative modification of HDL on their ability to efflux oxysterols.

Materials and Methods: THP-1 macrophages were labeled for 18h with [3H]cholesterol or [3H]7-ketocholesterol to follow cellular sterol efflux or incubated with [3H]cholesterol-oxidized LDL to follow LDL-derived sterol efflux. Efflux was stimulated by incubation with native, oxidized, glycated, glycoxidized HDL or HDL from diabetic subjects for 6h.

Results: Both LDL and HDL of diabetic patients exhibit increased levels of oxysterols, in particular 7-derivatives 7α/β-hydroxycholesterol and 7-ketocholesterol. After oxidative and glycoxidative modification, HDL were less efficient to efflux oxysterols compared to intact HDL. This effect was observed for both cellular and LDL-derived oxysterols. In contrast, glycation alone had no impact on oxysterols efflux. Cholesterol efflux was not or only barely affected regardless the nature of HDL modification. A decrease of oxysterol efflux was also observed when the macrophages were challenged with HDL isolated from diabetic patients compared to HDL of healthy controls.

Conclusions: These results indicate that circulating lipoproteins LDL and HDL undergo cholesterol oxidation mostly by non-enzymatic routes. We confirm that HDL plays a major role in efflux of oxysterols in THP1 macrophages. In addition, HDL functionality with respect to oxysterol efflux is significantly impaired after oxidative modification as recovered in oxidized, glycoxidized HDL and HDL of diabetic patients.
**P10**

**In vitro mitotane cytotoxicity on adrenocortical carcinoma H295R cells may be mediated by oxysterols**

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**Introduction:** Mitotane is the reference therapy for adrenocortical carcinoma (ACC). Several studies showed a marked increase of plasma cholesterol in ACC patients treated with mitotane. Oxysterols are oxygenated products in catabolism of cholesterol. Because of the well-known role of oxysterols in several diseases, including atherosclerosis, neurodegenerative diseases and tumor progression, we studied a possible involvement of these compounds in the cytotoxic activity induced by mitotane. H295R cell lines were used as an “in vitro” ACC model to evaluate possible effects of oxysterols.

**Materials and Methods:** H295R ACC cells were incubated with mitotane at concentrations corresponding to therapeutic doses and sterols levels were measured in cellular pellets by gas chromatography-mass spectrometry (GC-MS). Compounds found at relevant intracellular concentrations were tested on ACC cells to assay effects on cell viability.

**Results:** A modification in the lipidomic pattern was observed in H295R cells after a 48-h exposure to 10 μM mitotane. In particular, 27-hydroxycholesterol (27 OHC) and lanosterol were significantly higher than in basal conditions (<0.05 for both). These compounds exhibited a marked cytotoxic effect when added to H295R cells (27-OHC IC50 = 11.7 μM; Lanosterol: IC50 = 26.8μM).

**Conclusions:** The present preliminary data suggest that specific sterols derived from cholesterol metabolism could contribute to the antineoplastic effect of mitotane. Further studies on this topic may shed light on mechanism of action of mitotane, linking the drug-induced sharp increase in cholesterol with the cytotoxic effect of defined intermediates of cholesterol metabolism.
Identification of Immunoregulatory Sterol-Metabolites in the Infant Gut

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Introduction: The incidence of asthma in young adults rose steeply (to 10% of the young age population) in developed countries between the years 1955 and 1995. Some studies suggest that certain microbial exposures in early childhood limit the development of asthma and there are strong correlations between antibiotic receipt in young children and the likelihood of a diagnosis of asthma at 15 years of age. Administration of antibiotics disrupts the commensal bacteria in the gut, known as the gut microbiota, and a myriad of evidence suggest that gut microbiota play an important role in regulating host immune response. The functional interaction between the gut microbiota and the host is via bioactive molecules released by the microbiota. The aim of this study is to investigate the link between antibiotics, gut microbiota and immunoregulatory sterol-metabolites produced in the gut of infants. Here we report a liquid-chromatography - mass spectrometry (LC-MS) method to analyse sterols including oxysterols and bile acids, i.e. potential immunoregulatory molecules, from faeces samples of infants.

Materials and Methods: Sterols and bile acids were extracted in ethanol from faeces of infants (4 – 24 mo) and fractionated on a reversed-phase C18 column. Sterols including oxysterols were derivatised using enzyme-assisted derivatisation for sterol analysis (EADSA) to give Girard P (GP) derivatives [1] which were analysed by LC-MS. Bile acids were analysed in the absence of derivatisation by LC-MS in the negative-ion mode.

Results: In total we profiled for 294 sterol metabolites. Of particular interest are metabolites of 24S-hydroxycholesterol, with many of the intermediates in its conversion to bile acids being observed. Also noteworthy are the high levels of mono-, di- and tri-hydroxyoxocholanoic acids.

Conclusions: LC-MS with and without EADSA can be exploited to analyse almost 300 sterol metabolites in infant faeces samples

Induced pluripotent stem cell derived disease model of Hereditary Spastic Paraplegia Type 5

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Introduction: Hereditary spastic paraplegia (HSP) is a rare monogenetic neurodegenerative disorder characterized by progressive lower limb spasticity due to axonal degeneration of the corticospinal tract. Spastic paraplegia gene type 5 (SPG5) is an autosomal recessive subtype of HSP caused by mutations in CYP7B1, an enzyme essential for the liver-specific alternative pathway in bile acid synthesis. Mutations within CYP7B1 lead to a decreased enzyme activity and consecutively to an accumulation of oxysterol substrates (e.g. 27-hydroxycholesterol) in plasma and cerebrospinal fluid (CSF) of patients.

Materials and Methods: In order to study the cellular effects of accumulating oxysterols patient-derived induced pluripotent stem cells (iPSCs) were generated. We used non-integrating episomal plasmids to reprogram primary fibroblasts of five SPG5 patients. Furthermore, iPSCs were differentiated into hepatocyte-like cells (HLC) and cortical neurons (CN) to develop a disease model for SPG5.

Results: Pluripotency marker expression, the ability to differentiate into all three germ layers, and genomic stability declare our iPSCs as ‘bona fide’. HLCs were characterized by expression of hepatic markers (e.g. Albumin) and are morphologically similar to primary human hepatocytes. Additionally, we could show an increased secretion of 27-hydroxycholesterol (27-HC) compared to control HLCs. CNs predominantly express markers of the layers V and VI (CTIP2, TBR1) and treatment with 27-HC as well as cholestenoic acid leads to a dose-dependent neurotoxic effect.

Conclusions: The established cell types provide useful tools for further studies. HLCs are a useful platform to identify the composition of secreted ‘toxic’ lipids as well as for drug screening studies, whereas the mechanism of toxicity will be investigated with the use of CNs.
P13

Development and validation of a new Isotope dilution method for combined oxyphyto/oxycholesterol analysis in a small plasma sample on a GC-QQQ-MS/MS

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Introduction: Several methods to measure oxyphytosterols and oxycholesterol simultaneously have been described for different platforms, however these assays mostly need plasma volumes of ± 500µl. This might hamper retrospective analysis in studies that have limited access to plasma samples. Therefore, there is a need for a method to determine oxyphytosterols and oxycholesteroles in smaller, i.e. 50µl EDTA plasma samples. After development, optimisation and validation, we measured these oxidation products in a randomized controlled trial where subjects received antibiotics treatment for 1 week using this method on a GC-QQQ-MS.

Materials and Methods: After saponification at room temperature of 50µl butylated hydroxytoluene (BHT)-enriched EDTA plasma, deuterated ISTD’s oxysterols were extracted, separated from their substrates (i.e. campesterol, sitosterol and cholesterol) by solid phase extraction and subsequently derivatised to their trimethylsilyl-ethers prior to analysis on the GC-MSMS. The following oxidation products were measured: 7α-hydroxy(OH)-, 7β-OH- and 7-keto-campesterol, -sitosterol and -cholesterol, as well as 24-OH-cholesterol and 27-OH-cholesterol. Validation was performed according to the International conference on Harmonisation Guidelines and additionally results of extracting 500 µl vs 50 µl EDTA plasma samples were compared. The human intervention study was a double blind randomized controlled trial where 55 impaired glucose tolerant subjects received 7 days 3 times a day either Amoxicillin 500mg (broad spectrum antibiotic), or Vancomycin 500mg (antibiotic against gram-positive bacteria) or Placebo. Oxyphytosterol and oxycholesterol concentrations were determined at baseline and at the end of each intervention period and changes were statistically compared by an ANCOVA with baseline concentration as a covariate.

Results: Limit of detection (LOD) and limit of quantitation (LOQ) as well as linearity and the recovery of each component was determined. In addition, results of extracting 50µl EDTA plasma samples were validated against 500µl samples. In the context of the described intervention study, baseline plasma oxyphytosterol and oxycholesterol concentrations were comparable between the three intervention groups. Despite an overall decrease in almost all individual oxyphytosterols and oxysterols, average plasma oxyphytosterol and oxycholesterol concentrations were not significantly changed after 1-week Amoxicillin or Vancomycin treatment.

Conclusions: We here present a method that has been proven accurate and useful for measuring oxy-(phyto)cholesterols in small amounts (50µl) EDTA plasma. This method was used in the first study to assess the effect of antibiotic treatment on circulating plasma oxyphytosterol and oxycholesterol concentrations. Even though oxy(phyto)sterol concentrations were not changed, there was an overall decrease in plasma concentrations and it would be interesting to assess the role of microbiota in oxy(phyto)sterol formation in more detail.
Sec-B induces endoplasmic reticulum stress and triggers autophagy in HUVEC cells.

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Introduction: Oxysterols, a family of oxidation products of cholesterol, are increasingly drawing attention of researchers due to their multifaceted biochemical properties, several of them being of clear relevance to human pathophysiology. The possibility that oxysterols may have a potent impact in vascular pathology was initially prompted by the observation that they are concentrated in atherosclerotic lesions. However, despite several studies have been performed on their role in vascular health and disease, equivocal results have been obtained until now. Recently, different research groups have begun to shed light on the mechanisms and in vivo relevance of endoplasmic reticulum (ER) stress–driven atherosclerosis. ER stress is caused by accumulation of misfolded proteins in the ER. One of the mechanism to remove these misfolded proteins, involves their ubiquitination and retrotranslocation to the cytoplasm, where they are degraded by the proteasome. Moreover, emerging data indicate that ER stress is a potent inducer of autophagy; indeed when the amount of unfolded or misfolded proteins exceeds, autophagy is triggered to remove these otherwise toxic products.

In this scenario, the present study was designed to test the hypothesis that sec-B could perturbs the ER status in HUVEC, thus inducing a condition of ER stress which may ultimately lead to cell damage and death.

Materials and Methods: HUVEC were treated with different concentrations of sec-B (1, 5, 20 µM f.c.) for different times up to 24h. The production of ROS was evaluated by flow cytometry using 2 µM of DCFDA. The ER was labelled with 100nM of ER-tracker. Autophagy was detected by measuring the aggregation of LC3B protein coupled to green fluorescence protein and by labelling with 5 µM of MDC. For electron transmission microscopy, cells were centrifuged at 200 x g and the pellets were fixed in 2% glutaraldehyde and then post fixed in 2% osmium tetroxide solution. For western blot analyses, HUVEC were directly lysed in SDS buffer or submitted to nuclear-cytoplasmic subcellular fractionation Lysates were submitted to SDS-PAGE and western immunoblotting using antibodies against ubiquitin, IkBα, NF-kB p65, actin.

Results and Conclusions. Our results show that sec-B induces an evident increase in ROS production up to 5-fold over the basal level and as soon as after 1 hr of treatment. In particular the dose-response study reveals that the effect of sec-B on ER becomes evident at the threshold of 1 µM. At this concentration an ER expansion, as highlighted by flow cytometry and deeply detailed by electron microscopy, was observed. When the treatment was extended to 24 h cisternae appeared fragmentized and mislocated. At 2h incubation the transcription factor NF-kB was activated, as determined by p65 nuclear translocation which was not accompanied by a significant degradation of the inhibitor IkBα. The activation was dose-dependent, but transient; at 4h nuclear levels of p65 already declined, in some cases under the basal level. Accumulation of ubiquitin-conjugated proteins was evident at later times, suggesting that the ubiquitin/proteasome system could become congested and/or impaired. Consistently, MDC-labelled vacuoles and the LC3B aggregates doubled, indicating an activation of the autophagic pathways.

In conclusion, our results demonstrate that sec-B induces ER stress. Cells try to cope with the stress on one hand by activating NF-kB, whose target genes should attenuate ROS and promote cell survival, and on the other by targeting ER misfolded proteins to ubiquitin-mediated protein degradation and, ultimately, to the autophagic machinery.
Liver X receptor activation in MS lesions

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Background: Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS). It is characterized by infiltrating macrophages that actively phagocytose myelin, the protective insulating layer around axons. Myelin contains high amounts cholesterol. Clearance of myelin debris by macrophages leads to processing and accumulation of cholesterol, turning macrophages into foam cells. Liver X receptors α and β (LXRs) are sterol sensors controlling cholesterol homeostasis. Previous work from our institute has demonstrated that myelin-derived lipids activate LXRs in primary murine macrophages. Therefore, we hypothesize that LXRs are activated in phagocytes that contain myelin in active MS lesions.

Methods: In this study we used real-time quantitative PCR (qPCR) and immunohistochemistry (IHC) to determine the expression of LXRs and their response genes ABCA1, ABCG1 and APOE in human phagocytes after myelin phagocytosis and in MS lesions. In addition, we used gas chromatographic/mass spectrometric analysis (GC/MS) to determine LXR-activating oxysterols present and formed in myelin and myelin-incubated cells respectively.

Results: Myelin ingestion induced the LXR response genes ABCA1 and ABCG1 in human monocyte-derived macrophages, demonstrating myelin activates LXRs in human phagocytes. In active MS lesions, we found that both ABCA1 and APOE gene expression and protein levels are highly upregulated in MHCII-positive macrophages and microglia, indicating LXRs are activated. Moreover, we found that the LXR ligand 27OHCholesterol is significantly increased in human monocyte-derived macrophages after five days of myelin uptake.

Conclusions: We demonstrated that LXRs are activated in phagocytes active MS lesions. In addition, we have shown that the LXR ligand 27OH-cholesterol is generated in human monocyte-derived macrophages after myelin ingestion, suggesting that this oxysterol is in part responsible for LXR activation in macrophages in MS lesions.
Impact of high hydrostatic pressure processing on the formation of cholesterol oxides in chicken meat and the use of tomato by-products as antioxidants

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Introduction: High-pressure processing technology is able to inactivate microorganisms causing only minor alterations in food taste and flavor. However, it can also result in undesired changes in food color, texture and structure. Moreover, lipid oxidation is induced in high-pressure processed meats depending on the pressure level and time of exposure. The aim of the present work was to verify the effects of high-pressure processing on the formation of cholesterol oxides in chicken meat with and without the addition of tomato by-products during chill storage for 14 days.

Materials and Methods: The tomato by-products were collected during the industrial production of concentrated tomato pulp in four points: (1) in natura, (2) finishing, after enzymatic inactivation (hot break), (3) final, after sterilization and (4) waste, process waste (peel + seeds). The tomato by-products were lyophilized and added to the chicken meat in the following concentrations: 0.1% in natura, finishing and final and 0.3% waste. The chicken meat without addition of tomato by-product was the control sample. The chicken meat was submitted to high hydrostatic pressure of 800 MPa for 10 min, stored in the dark at 5°C for 14 days. Cholesterol and cholesterol oxides were analyzed by HPLC-UV-RI-MS/MS at days 0 and 14.

Results: The major cholesterol oxide found in all samples was 7-ketocholesterol. 7β-Hydroxycholesterol was found in small amounts in both non-pressurized and pressurized samples, while 7α-hydroxycholesterol was found only in pressurized samples. At day 0, all samples pressurized at 800 MPa showed a decrease in the content of total cholesterol oxides when compared to the raw unpressurized sample, suggesting that the high pressure exerted an effect on the degradation of the cholesterol oxides originally present in the raw meat. After 14 days of storage, an increase in the total cholesterol content was observed in the pressurized samples when compared with day 0. All tomato by-products were able to inhibit cholesterol oxidation, showing lower total cholesterol contents than the control, being the tomato in natura the most effective, (the lowest total cholesterol oxides formation), followed by waste, finishing and final tomato by-products.

Conclusions: All tomato by-products inhibited the formation of cholesterol oxides in pressurized chicken meat probably due to the presence of phenolic compounds (quercetin, rutin, naringin) and carotenoids (lycopene and β-carotene), which could have acted synergistically.
Development of a method of dosage for the tumor suppressor Dendrogenin A

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Introduction: Dendrogenin A (DDA) is a cholesterol metabolite recently identified in mammals and which present tumor suppressor and neurostimulating properties (Dalenc et al, Current Med Chem, 2015). Importantly, we found that DDA levels were decreased in breast tumors compared to normal matched tissues suggesting that a metabolic deregulation on DDA production in breast cancers occurred. DDA is steroidal alkaloid which cationized in acidic conditions. We previously used a manual three steps analytical method: 1. Purification of DDA from a Bligh and Dyer extract by RP-HPLC using a 25 cm column using TFA as a counter-ion. 2. evaporation in vacuo of the 18–21 min fractions. 3. analyses of the fraction by nano-electrospray ionization MS fragmentation up to MS4 which enabled us to distinguish DDA from its geometric isomer C17 (de Medina et al, Nature commun, 2013). In order to study DDA levels in larger cohorts of patients, a new method of quantification is required. We report here the development of a new method using LC coupled to mass spectrometry.

Material and Methods: DDA and its geometric isomer C17 were synthesized as described before (de Medina et al, Nature commun, 2013). Compounds were tested on RP-18W TLC (818146) and TLC Plates were revealed by ninhydrine impregnation and 5 min heating at 110°C. Nucleoshell RP18 5µm, 100 x 2.0 mm from Macherey Nagel, Accucore 2.6 µm RP MS 100 x 2.1mm by Thermo Fisher and normal phase Acquity UPLC 2.1 x 50 mm, 1.7µm from Waters were tested.

Results: The development of a LC/MS method required the change of TFA by an ion compatible with electrospray MS, the shortening of the column and the resolution of the two isomers to avoid MS4 analyses too. We first observed a high carry-over of DDA associated with a loss of resolution of the peak due to the ion exchange of DDA with the chromatographic support. We then studied its chromatographic comportment through TLC which made possible an optical visualization of DDA on the TLC plate after ninhydrin staining. We found that acidic conditions, and choosing the good anion for ion paring of DDA, helped DDA desorption and solved carryover problems as well as peak resolution. We took these parameters into account and tested different elution conditions with different columns.

Conclusions: We obtained a more powerful method that the previously published one. The peak resolution was high (3 sec), the carry-over was weak (<0.1%). DDA and C17 get separated with this method, the run duration was 10 min (instead of 60 min in our previous method), the limit of detection (LOD) was in the pg/µl range. The robustness was very good with a coefficient of variation (CV) < 0.05%. This method will be useful to measure DDA levels in larger cohorts of patients.
P18
Effects of two plant extracts on two actors of skin photoaging: UV and 25-hydroxycholesterol

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Introduction: Oxysterols are oxidized lipids that have been associated to skin degenerative disorders such as photoaging or vitiligo. More precisely, we previously demonstrated the role of 25-hydroxycholesterol (25-OH) in UV-induced photoaging. We selected Calophyllum inophyllum and Gardenia florida plant extracts known to be antioxidant to modulate the effects of UV and 25-OH which are involved in photoaging process, on human keratinocytes.

Materials and Methods: Calophyllum inophyllum and Gardenia florida extracts cytotoxicity was evaluated using Alamar Blue assay. The protective effects of Calophyllum inophyllum and Gardenia florida extracts were studied on keratinocytes (HaCaT cell line) after UV irradiation and 25-OH incubation. Microplate cytometry was used to evaluate P2X7 cell degenerescence receptor activation (YO-PRO-1 assay) and reactive oxygen species production (H2DCF-DA assay).

Results: Calophyllum inophyllum and Gardenia florida extracts didn’t induce any cytotoxicity on HaCaT cells. The two formulations both reduced UV-induced oxidative stress. Calophyllum inophyllum oil induced a higher decrease in oxidative stress than Gardenia florida extract. 25-OH induced P2X7 cell degenerescence receptor activation was decreased by the two formulations.

Conclusions: The two plant extract formulations we selected seem to be promising to protect keratinocytes from UV-induced oxidative stress and 25-OH-induced P2X7 cell degenerescence receptor activation.

Enlarging the chemical diversity of oxysterols by means of MMPP mediated oxidations

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Oxysterols are oxygenated derivatives of cholesterol that exhibit a variety of interesting biological activities, including selective toxicity towards cancer cells [1-3]. Endogenous and synthetic oxysterols have been the focus of our interest. Slight modifications in the oxysterols structure induce significant changes in their biological activities. Therefore, the enlargement of the chemical space, with the synthesis, characterization and biological evaluation of novel molecules of this large family, continues to be important, not only to find new drug candidates, but also to gain insights into their cellular targets.

Diverse oxidative transformations are required to afford natural and synthetic oxysterols. We found that magnesium monoperoxyphthalate (MMPP), a cheap, safe and commercially available alternative to m-CPBA, is a quite convenient reagent for oxidative conversions of steroids. The hydrophilicity of MMPP and the lipophilicity of the cholestane substrates led us to use hydrophobic reaction solvents, rendering the oxidant in suspension in the reaction media, which makes the work-up easier. Different oxidative reactions are presented in this communication, the reaction conditions and outcomes are discussed.

The compounds synthesized exhibit antiproliferative activity in low micromolar range, displaying preferential toxicity against cancer cells. Structure-activity correlations are discussed.


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**P20**

**Combined effect of thermal treatment, storage and cumari pepper addition on the formation of cholesterol oxides in buffalo meat**

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**Introduction:** Buffalo meat has similar cholesterol content than beef meat; however, it has a higher amount of unsaturated fatty acids, being more prone to oxidation. The aim of this work was to evaluate the formation of cholesterol oxides in buffalo meat (Murrah breed) during frozen storage and subsequent grilling. Moreover, the effect of the addition of 0.2% lyophilized cumari pepper (*C. bacccatum var praetermissum*) on the inhibition of cholesterol oxidation was also verified.

**Materials and Methods:** Buffalo meat patties (80 ± 2 g, *longissimus dorsi* muscle) were prepared with and without cumari pepper addition. Raw patties were stored at -18°C during 90 days and samples were analyzed raw and after grilling (180°C for 6 min) at days 0, 15, 30, 60 and 90. The phytochemical composition of cumari pepper was determined. Cholesterol and cholesterol oxides were determined by HPLC-UV-RI and identification was carried out by LC-MS.

**Results:** Cumari pepper presented 49.8 mg/g total phenolic compounds, 85.0 µg/g total capsaicinoids, 57.4 µg/g capsaicin, 27.6 µg/g dihydrocapsaicin, 5.7 µg/g total chlorophyll, 3.8 µg/g chlorophyll a and 1.9 µg/g chlorophyll b. At day 0, cholesterol content was 352 mg/100g (d.w.) in raw meat and 282 mg/100g (d.w.) in grilled meat, while the content of total cholesterol oxides was 13.9 µg/g (d.w.) in raw meat and 15.7 µg/g (d.w.) after grilling. Similar behavior was noticed during the entire storage period, i.e. raw meat always presented higher contents of cholesterol and lower contents of cholesterol oxides than grilled meat, indicating that the temperature and time conditions applied in this study was a determining factor on the formation of cholesterol oxides. During storage, the cholesterol content decreased (14%) and cholesterol oxides content increased (21%) both in the samples with and without cumari pepper addition, showing that the addition of 0.2% cumari pepper was not effective in protecting cholesterol against oxidation.

**Conclusions:** Although cumari pepper possess compounds with recognised antioxidant capacity in its composition, the addition of such pepper at 0.2% level did not inhibit the formation of cholesterol oxides during thermal treatment and frozen storage.
Reactivity of 5,6α-epoxycholesterol, 5,6β-epoxycholesterol, cholestane-3β,5α,6β-triol and 6-oxo-cholestan-3β,6β-diol towards trimethylsilylation, importance for their quantification by GC/MS.

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Introduction: 5,6α-epoxycholesterol (5,6α-EC), 5,6β-epoxycholesterol (5,6β-EC) were shown to contribute to the anticancer pharmacology of the widely used antitumor drug Tamoxifen. 5,6-ECs are metabolized into cholestane-3β,5α,6β-triol (CT) by the cholesterol-5,6-epoxide hydrolase (ChEH) and CT is metabolized by a yet uncharacterized enzyme into 6-oxo-cholestan-3β,6β-diol (OCDO). We recently published a feasibility study showing that these oxysterols (OS) might represent surrogate markers of tamoxifen activity in patients with breast cancer under endocrine therapy (Dalenc F et al, J Steroid Biochem Mol Biol, 2016). These OS were quantified by gas-liquid chromatography coupled to mass spectrometry (GC/MS) using an isotope dilution methodology using corresponding deuterated OS. This method is a relative quantitative one as far as all deuterated OS were used as standards assuming that these OS have the same susceptibility to extraction, pre-purification by solid phase extraction (SPE) and trimethylsilylation. Because we need a true quantitative method, we investigated in the present study the reactivity of 5,6-ECs, CT and OCDO towards trimethylsilylation.

Materials and Methods: 5,6α-EC, 5,6β-EC, CT and OCDO were synthesized in their 14C labeled form from 14C-labelled cholesterol. Trimethylsilylation was performed on pure OS or in OS incubated with fetal bovine serum extracted and submitted or not to SPE before trimethylsilylation. Reactants and products of the reactions were separated on normal phase TLC, and the yields of the reactions were determined.

Results: The yield of trimethylsilylation of 5,6-ECs, CT and OCDO were different. the trimethylsilylation of purified standards was found quantitative only for CT and not for 5,6-ECs and OCDO in the different tested conditions.

Conclusions: Our data showed that 5,6-ECs, CT and OCDO displayed different susceptibilities to trimethylsilylation in standard conditions. This means that the use of the corresponding deuterated standards of these OS is absolutely required for quantification by GC/MS. In these conditions, the quantification of OS is relative and not absolute. Further studies are required to improve the yield of trimethylsilylation of 5,6α-EC, 5,6β-EC, and OCDO for quantification by GC/MS.
The oxysterols along the different stages of Alzheimer’s disease: their involvement in neuroinflammation

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Introduction: Alzheimer’s disease (AD) is a slowly devastating neurodegenerative disorder that leads to dementia. The molecular mechanisms underlying AD are still not clear, and at present no reliable biomarkers for AD early diagnosis are available. In the last years, together with oxidative stress and neuroinflammation, the altered cholesterol metabolism in the brain is becoming increasingly involved in AD development. Considerable evidence indicates that cholesterol oxidized products, named oxysterols, are the driving force behind the development of AD. The oxysterols most widely considered to be potentially implicated in AD pathogenesis are 24-hydroxycholesterol (24-OH) and 27-hydroxycholesterol (27-OH), deriving from cholesterol oxidation by CYP46A1 and CYP27A1, respectively. In addition, other oxysterols have been found in AD brains deriving from cholesterol autooxidation such as 7-ketocholesterol (7-K) and 7β-hydroxycholesterol (7β-OH).

Materials and Methods: The brains were classified basing on Braak staging system of neurofibrillary pathology (early AD: stage 1 and 2; late AD: stage 4 to 6). In the control brains the presence of senile plaques and tau pathology was excluded. Oxysterols were measured by isotope dilution mass spectrometry. The inflammatory molecule expression was analyzed by qRT-PCR.

Results: A variety of oxysterols, both of enzymatic and non-enzymatic origin, was identified and quantified in AD brains taking into account the different Braak stages of AD. The enhancement of some inflammatory mediator and the proteolitic enzyme matrix metalloproteinase-9 (MMP-9) expression was also demonstrated in the brains in agreement with the progression of the disease. Conversely, a marked reduction of sirtuin 1 (SIRT-1) expression, an enzyme which regulates several pathways involved in the anti-inflammatory response, was observed with the progression of AD.

Conclusions: Taken together, these results strongly support the association between changes in oxysterol levels and Alzheimer’s disease progression. The pathogenic association between inflammatory molecule and SIRT-1 loss and oxysterol level trend along the evolution of AD was also highlighted. Oxysterol levels in peripheral circulation or in cerebrospinal fluid could represent novel biomarkers for the early diagnosis of AD, since they reflect the number of metabolically active neurons in the brain.
Over the past two decades the number of reports about the physiological roles of mammalian oxysterols, as well as about their contribution to the pathogenesis of different diseases has been exponentially increased. The identification of a specific subset of oxysterols, such as 24(S)-hydroxycholesterol (1), 22(R)-hydroxycholesterol (2), and 24(S),25-epoxycholesterol (3), as endogenous ligands of the Liver X Receptor (LXR), has represented a major breakthrough in the oxysterol research.

LXR belongs to the superfamily of nuclear receptors and exists in the two α and β isoforms, endowed with a different tissue distribution and a high sequence identity. By acting LXR as a whole-body cholesterol sensor and a key regulator of lipogenesis, the roles of oxysterols in lipid metabolism and glucose homeostasis were the first ones to be evidenced. Several reports have then shown that LXR and its ligands suppress inflammatory responses by activating genes encoding anti-inflammatory proteins or by the suppression of genes that are under the control of proinflammatory transcription factors. Intrigued by the ability of some phytosterols, including stigmasterol, to interfere with cholesterol homeostasis, by acting as analogs of endogenous oxysterols, and in consideration of the scarcity of biological data on LXR for the class of phytosterol analogs, we engaged ourselves in a project aimed to develop stigmasterol and ergosterol derivatives characterized by the presence of side-chain oxygenated functions, structural features known to be crucial for LXR activation. The results of the preliminary biological evaluation of the synthesized compounds will be presented. Briefly, we have thus identified several compounds able to potently bind LXR⁵ and at lesser extent LXR⁶, as evaluated by LXR luciferase-based reporter assays. These compounds also activated LXR target genes, such as ABCA1 and inhibited the expression of the pro-inflammatory gene MCP-1.

Determination of sterols by liquid chromatography high-resolution tandem mass spectrometry

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A comprehensive analytical method based on liquid chromatography and high-resolution tandem mass spectrometry was developed and applied for the simultaneous quantification of cholesterol precursors, oxysterols, and phytosterols. The described LC-HR-MS method is a reliable, rapid, and sensitive tool to quantify different kinds of sterols in biological liquids. The novelty of our method lies in the fact that it allows reproducible and accurate determination of relevant sterols because of short run time and automatable sample preparation steps. The samples were treated by alkaline hydrolysis and the sterol species were extracted by an organic solvent. To enhance the signal of the target compounds in electrospray ionization (ESI) derivatization of the free hydroxyl group of the molecules has been performed with N,N-dimethylglycine (DMG). The high selectivity of the method was provided by the high-resolution mass spectrometer which is a powerful tool to resolve quasi-isobaric interferences. High resolving power is needed to resolve closely spaced mass multiplets in complex mixtures like human blood samples. The presence of unresolved multiplets requires improved mass resolution to separate the overlapping peaks. In the present method high specificity was provided by the hybrid quadrupole-orbitrap mass spectrometer. The applied resolution (35,000 at m/z 200) and mass tolerance of ion extractions (±10 ppm) ensured appropriate separation of quasi-isobaric peaks in many cases. The method has a run time of less than 10 min and was validated according to FDA and EMA guidelines. With the developed method sterol species were measured in different kinds of samples such as human plasma and feces.
P25


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Hypercholesterolemia is associated with cognitively deteriorated states. Here, we show that excess 27-hydroxycholesterol (27-OH), a cholesterol metabolite passing from the circulation into the brain, reduced in vivo brain glucose uptake, GLUT4 expression and spatial memory. Furthermore, patients exhibiting higher 27-OH levels have reduced ¹⁸F-FDG uptake. This interplay between 27-OH and glucose uptake revealed the engagement of the insulin-regulated aminopeptidase (IRAP). 27-OH increased the levels and activity of IRAP, countered the IRAP antagonist angiotensin IV (AngIV) mediated glucose uptake, and enhanced the levels of the AngIV degrading enzyme, aminopeptidase N (AP-N). These effects were mediated by liver X receptors. Our results reveal a molecular link between cholesterol, brain glucose and the brain renin-angiotensin system, all of which are affected in some neurodegenerative diseases. Thus, reducing 27-OH levels or inhibiting AP-N maybe a useful strategy in the prevention of the altered glucose metabolism and memory decline in these disorders.
Study of brain myelination in relation to risk factors for Alzheimer disease

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Myelination of the human brain underlies its unique complexity but also to its susceptibility to develop neuropsychiatric disorders. Myelin production by oligodendrocytes is critical for circuit function and connectivity but is a vulnerable layer. Lesions in the white matter (WML) is an early phenomenon in Alzheimer disease (AD) however the disease mechanisms linking myelination to AD has not yet been elucidated. AD is the most common neurodegenerative disorder characterized by progressive memory failure and additional cognitive deficits. The strongest risk factor for AD is to be carrier of the apolipoprotein isoform E4 (ApoE4). On the contrary, the ApoE2 isoform has protective properties concerning cognitive decline. High fat- and cholesterol diet in combination with the ApoE4 isoform is additive for acquiring AD. Cholesterol does not pass the blood-brain-barrier, but 27-hydroxycholesterol (27-OH) readily does and increased levels of this metabolite indeed correlate to cognitive decline.

In the current study we examine how ApoE4 in combination with high-fat or high-carbohydrate diet in mice affects myelination and further if the cholesterol metabolite 27-hydroxycholesterol (27-OH) influence the myelin sheet. The aim is to determine if and how risk factors for AD alters the pattern of myelination. We show that oligodendrocytes are sensitive to 27-OH treatment in vitro and that CYP27 mice exhibit altered myelination. Further, we measure components of the myelin sheet in cerebrospinal fluid from memory clinic patients and in mice exposed to different ApoE isoforms in combination with diets. Overall, our data support the notion that myelination may be affected by risk factors for AD and hence take part in the early phase of dementia. We are currently examining the underlying disease mechanisms in more detail.
Formation of sitosterol and campesterol oxidation products in foods after cooking with margarines without and with added plant sterols

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Introduction: Plant sterols (PS) in foods are susceptible to oxidation leading to the formation of PS oxidation products (POP). This study aimed to measure the concentrations of POP in cooked foods by specifically determining the profiles of common POP derived from sitosterol vs. campesterol.

Materials and Methods: Foods (n=15) including vegetables, potato, meats, fish and egg were prepared in pentuplicate by typical household cooking methods (including stir- and shallow-frying, stewing, roasting and microwave cooking) using liquid margarines containing 66% of fat without (control) and with 7.5% added PS (as 12.5% PS-esters, referred to as PS-margarine). POP analyses were carried out using GC-MS method.

Results: Median POP content per typical portion size of cooked foods was 0.23 mg (range 0.06-0.89 mg) with control margarine and 1.62 mg (range 0.08-13.20 mg) with PS-margarine. Shallow-fried potatoes had the highest POP contents among the cooked foods (0.89 mg and 13.20 mg per portion with control and PS-margarine, respectively). Among the different cooking methods, microwave-cooking led to the lowest POP formation. In foods cooked with control margarine, the relative abundances of individual POP were 5,6-epoxy- (37%), 7-keto- (36%), 7-OH- (21%), triols (6%), while in foods cooked with PS-margarine, the abundances of individual POP were 5,6-epoxy- (42%), 7-OH- (30%), 7-keto- (24%), triols (3%). The profile of individual POP derived from sitosterol was similar to that of POP derived from campesterol in each food prepared with control or PS margarine.

Conclusions: Compared to control margarine, using a PS-added margarine for cooking increased POP contents in the foods by less than 12 mg (median 1.4 mg) per portion, which was associated with a decrease in the proportional amount of 7-keto and an increase in 7-OH-derivatives of sitosterol and campesterol.
Stability of plant sterols to thermal oxidation and formation of plant sterol oxidation products in margarines and vegetables oils without and with added plant sterols

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Introduction: Plant sterols (PS) occur naturally in all plant-derived foods such as vegetable oils. PS are susceptible to oxidation especially when exposed to heat treatment leading to the formation of PS oxidation products (POP). POP formation might be influenced by various factors including the initial content of PS. The present study assessed the kinetic of POP formation in a variety of fat-based products.

Materials and Methods: Six different fat-based products (sunflower and rapeseed oil, brick and liquid margarines without and without PS) were heated in a Petri-dish at temperatures of 150, 180 and 210°C for 8, 12 and 16 min. For each experimental condition, three independent experiments were performed. PS analyses were performed with GC-FID (Mackay DS et al. J Chromat B 2014) and POP analyses were performed with a GC-MS-SIM method (Husche et al. CPL 2011). Susceptibility of PS to oxidation was expressed as PS oxidation rate (ORP) which is defined as POP content divided by the PS content at baseline x 100%.

Results: Before heating (initial values) POP contents were low in the six fat products ranging between 1.4 mg (rapeseed oil) and 13.1 mg POP/100 g fat (PS-added margarine). After heating the total amount of POP (defined as sum of POP derived from sitosterol and campesterol) ranged between 1.3 mg (brick margarine; 8 min heating at 150°C) and 150.2 mg POP per 100 g fat (PS-added liquid margarine; 16 min at 210°C). Increasing the temperature and the heating duration led to higher POP formation in all tested fat-based products. Both margarines with added PS had higher POP contents compared to the margarines without added PS under all experimental heating conditions. ORP values were higher in vegetable oils than in the margarines under all the heating conditions. The susceptibility to oxidation for sitosterol was about 20% lower than that of campesterol. During heating, the relative abundance of 7-Keto-PS (% of total POP) decreased in all fat-based products, while the relative abundances of 7-OH-PS and 5,6-epoxy-PS increased or remained stable.

Conclusions: Heating temperature and time, and their interaction increased POP formation and changed POP profiles in the fat-based products. Under these experimental conditions adding PS to margarines increased POP formation, while it did not change POP profiles. The susceptibility to oxidation of PS, i.e. ORP were higher in the two vegetables oils than in the margarines.
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