SCIENCE BEHIND REFSUM DISEASE
Today’s Hosts & Presenters

Hosts

Kristie DeMarco
President and Founder at Global DARE Foundation

Susan Kuranoff
Secretary at Global DARE Foundation

Presenters

Ronald JA Wanders, PhD
Professor Emeritus Clinical Enzymology of Metabolic Diseases at the Amsterdam UMC

Sacha Ferdinandussa, PhD
Clinical Laboratory Geneticist at the Laboratory Genetic Metabolic Diseases, Amsterdam UMC
Today’s Agenda

- Global DARE Foundation’s Mission
- Presentation on the Science behind Refsum Disease
- Question & Answer Session
Webinar Housekeeping Details

• All participants are in listen only mode
• How to ask a question during the Q&A:
  – Participants following on Zoom can type their questions in the Q&A box at any time during the presentation or by raising their hand at the end to ask a question live.
  – Participants joining by phone can press *9 on their phone to raise their hand.
• Questions will be answered in the following order:
  – Q&A box in Zoom
  – Dial in participants
  – Online participants
• Today’s session will be recorded for later viewing on Global DARE Foundation Website (www.defeatadulttrefsumeverywhere.com)
Global DARE Foundation's mission is to promote world-wide awareness and better quality of life for all who are diagnosed with Adult Refsum Disease.
PHYTANIC ACID IN HEALTH AND DISEASE

Ronald JA Wanders
Laboratory Genetic Metabolic Diseases
Department of Pediatrics & Clinical Chemistry
Academic Medical Center, University of Amsterdam
Amsterdam, The Netherlands
METABOLISM IN A NUTSHELL

CARBOHYDRATES

SIMPLE SUGARS
  glycogen
  Synthesis to complex sugars

PROTEINS

AMINO ACIDS
  De novo protein synthesis

FAT

FATTY ACIDS
  De Novo synthesis of various lipids

ACETYL-CoA

Citric acid cycle

OXPHOS
  ADP + P_i

Oxygen (O₂)

CO₂ + H₂O

ATP = ENERGY
METABOLISM IN A NUTSHELL

CARBOHYDRATES

PROTEINS

FATTY ACIDS

SIMPLE SUGARS

AMINO ACIDS

De novo synthesis of various lipids

ACETYL-CoA

O2

ADP + P_i

CO2 + H2O

Citric acid cycle

OXPHOS

ATP = ENERGY

De novo protein synthesis

glycogen

Synthesis to complex sugars

PHYTANIC ACID = FATTY ACID
FATTY ACIDS

General structure:

\[
\text{CH}_3-(\text{CH}_2)_n-\text{C}-\text{OH}
\]

- Fatty acids occur in all kinds of different forms:
  - Short-, medium-, and long-chain
  - Saturated (no double bonds) / unsaturated (double bonds)
    
    Example: linoleic acid, oleic acid
  - Branched-chain (2-methyl, 3-methyl, etc.)
  - As FREE fatty acids (FFA) or coupled to other metabolites (e.g. in lipids)
- **PHYTANIC ACID = A LONG-CHAIN SATURATED, BRANCHED CHAIN FATTY ACID**

Backbone = palmitic acid (C16) plus 4 methyl branches
Most fatty acids are broken down in mitochondria via a process called beta-oxidation which cuts the fatty acids in small pieces.

Example: palmitic acid
WHY IS PHYTANIC ACID NOT BROKEN DOWN IN REFSUM DISEASE?

CONSEQUENCE:
Phytanic acid cannot be broken down by beta-oxidation and requires a different system to be broken down called:

**ALPHA-OXIDATION**
Phytanic acid

1. Phytanoyl CoA synthetase

2. Phytanoyl CoA hydroxylase

3. 2-OH-phytanoyl CoA lyase

4. Pristanal dehydrogenase

5. Pristanoyl-CoA synthetase

Wanders et al. 2010 BBA
APLHA-OXIDATION

Phytanoyl CoA synthetase

Phytanoyl CoA → phytanoyl CoA hydroxylase

2-OH-phytanoyl CoA → 2-OH-phytanoyl CoA lyase

Pristanoyl-CoA synthetase

Pristanoyl-CoA → pristanal dehydrogenase

Formyl-CoA

CO₂

Janssen et al. 1997
Nature Genetics 17 190-193
WHAT ARE THE SOURCES OF PHYTANIC ACID IN HUMANS?

• PHYTANIC ACID itself, either as such as free phytanic acid or in some conjugated bound-form as present in various phytanic acid-containing foodstuffs like butter, meat, certain fishes, etc.

• PHYTANIC ACID precursors especially phytol and its derivatives notably phytyl fatty acid esters.
ESSENTIAL FEATURES OF PHYTOL

Structure phytol

Compare Structure phytanic acid
Phytol is the direct product of chlorophyll as present in plants and is released from chlorophyll by ruminants - but NOT HUMANS! – to give rise to free phytol which is then either converted to phytanic acid or coupled to other metabolites notably fatty acids as in phytyl-fatty acid esters.

In short:

\[ \text{CHLOROPHYLL} \rightarrow \text{PHYTOL} \rightarrow \text{PHYTANIC ACID} \rightarrow \text{PHYTYL ESTERS} \]
PHYTOL CAN BE COUPLED TO VARIOUS METABOLITES INCLUDING FATTY ACIDS (PHYTYL-FATTY ACID ESTERS)

Fig 3. Schematic metabolism of PFAE in the human body with cleavage of PFAE (a) into free trans-phytol (b), which is then further metabolized by oxidation into phytanic acid (c) and reduction of the double bond into phytanic acid (d) [6,30].
PHYTYL-FATTY ACID ESTERS ARE PRESENT IN DIFFERENT VEGETABLES AND FRUITS.
PHYTYL-FATTY ACID ESTERS ARE PRESENT IN DIFFERENT VEGETABLES AND FRUITS....... AND ARE MOST LIKELY DIGESTED IN HUMANS

Table 1. Composition of the digestion fluids.

<table>
<thead>
<tr>
<th></th>
<th>Saliva</th>
<th>Gastric juice</th>
<th>Intestinal juice</th>
<th>Bile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inorganic solution</strong></td>
<td>2 mL KCl (22.4 g/L)</td>
<td>3.7 mL KCl (22.4 g/L)</td>
<td>10 mL NaHCO₃ (33.9 g/L)</td>
<td>8.54 mL NaHCO₃ (33.9 g/L)</td>
</tr>
<tr>
<td></td>
<td>1 mL KSCN (10.0 g/L)</td>
<td>3.1 mL NaCl (87.7 g/L)</td>
<td>8 mL NaCl (87.7 g/L)</td>
<td>3 mL NaCl (87.7 g/L)</td>
</tr>
<tr>
<td></td>
<td>1 mL Na₂SO₄ (28.5 g/L)</td>
<td>1.8 mL CaCl₂·2H₂O (22.2 g/L)</td>
<td>2 mL MgCl₂·6H₂O (11.5 g/L)</td>
<td>0.01 mL HCl (32%)</td>
</tr>
<tr>
<td></td>
<td>0.17 mL NaCl (87.7 g/L)</td>
<td>0.65 mL HCl (32%)</td>
<td>2 mL KH₂PO₄ (4.0 g/L)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.09 mL NaOH (1 M)</td>
<td>0.6 mL NaH₂PO₄·H₂O (51.1 g/L)</td>
<td>0.018 mL HCl (32%)</td>
<td></td>
</tr>
<tr>
<td><strong>to 25 mL H₂O</strong></td>
<td></td>
<td>to 50 mL H₂O</td>
<td>to 50 mL H₂O</td>
<td>to 25 mL H₂O</td>
</tr>
<tr>
<td><strong>Organic solution</strong></td>
<td>1.6 mL urea (6.25 g/L)</td>
<td>2 mL glucose (32.5 g/L)</td>
<td>0.4 mL urea (6.25 g/L)</td>
<td>2 mL urea (6.25 g/L)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mL glucuronic acid (1.0 g/L)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>2 mL glucosamine-HCl (16.5 g/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.36 mL urea (6.25 g/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>to 25 mL H₂O</strong></td>
<td></td>
<td>to 50 mL H₂O</td>
<td>to 50 mL H₂O</td>
<td>to 25 mL H₂O</td>
</tr>
<tr>
<td><strong>Additional substances</strong></td>
<td>7.25 mg α-amylase</td>
<td>0.1 g BSA</td>
<td>0.9 mL CaCl₂·2H₂O (22.2 g/L)</td>
<td>0.5 mL CaCl₂·2H₂O (22.2 g/L)</td>
</tr>
<tr>
<td></td>
<td>0.75 mg uric acid</td>
<td>0.1 g pepsin</td>
<td>0.1 g BSA</td>
<td>0.09 g BSA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 g pancreatin</td>
<td>0.3 g bile salts</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>0.5 g lipase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>~ 6.5</td>
<td>~ 1.0</td>
<td>~ 7.5–8.0</td>
<td>~ 8.0</td>
</tr>
</tbody>
</table>
CONCLUSION:
It would be wise to check as many food components as possible not only with respect to phytanic acid but also phytol especially phytyl- fatty acid esters.
REFSUM DISEASE: THE FUTURE

1. Re-evaluate the contents of phytanic acid and phytol (free and esterified) in food components;
2. Device methods to circumvent and/or correct the enzyme defect in Refsum disease

HOW?

3. Gene therapy
4. Enzyme replacement therapy
5. Upregulation of residual activity of the defective enzyme phytanoyl-CoA hydroxylase
6. Alternative route of phytanic acid breakdown: omega-oxidation
OMEGA-OXIDATION OF PHYTANIC ACID AS AN ALTERNATIVE THERAPY FOR REFSUM DISEASE

- **Cytochrome P450**
  - NADPH, $O_2$
  - NADP, $H_2O$

- **Alcohol dehydrogenase**
  - $CH_3OH$

- **Aldehyde dehydrogenase**
  - $CHO$

**Deficient in Refsum disease**

- **Beta-oxidation from the omega-end**
  - 3-Methyl adipic acid
Metabolism of phytanic acid and 3-methyl-adipic acid excretion in patients with adult Refsum disease

Anthony S. Wierzbicki, Phillip D. Mayne, Matthew D. Lloyd, David Burston, Guin Meik, Margaret C. Sidey, Michael D. Feher, and Brian Gibberd

Department of Chemical Pathology and Refsum Disease Clinic, Chelsea & Westminster Hospital, 369 Fulham Road, London, United Kingdom; and Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, United Kingdom


1. Plasma PA concentration in response to fasting in five patients with ARD assuming first-order kinetics.
OMEGA-OXIDATION OF PHYTANIC ACID AS AN ALTERNATIVE THERAPY FOR REFSUM DISEASE

Deficient in Refsum disease

CYP4F2
CYP4F3A
CYP4F3B
CYP4A11

NADPH, O₂

NADP, H₂O

Cytochrome P450

Aldehyde dehydrogenase

Alcohol dehydrogenase

Beta-oxidation from the omega-end

3-Methyl adipic acid
ACKNOWLEDGEMENTS

Amsterdam Refsum Team

Sacha Ferdinandusse
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Clara van Karnebeek
Arthur Bergen
A mouse model for Refsum disease

Laboratory Genetic Metabolic Disease, Amsterdam UMC, The Netherlands

Sacha Ferdinandusse
Pedro Brites
Ronald Wanders
Generation Refsum mouse model (Phyh-/-)

- Disruption of Phyh gene

- No mRNA, protein expression or Phyh enzyme activity

  ![Image of genotyping](image)

- +/+ = wild type = control
- +/- = heterozygote = carrier
- -/- = homozygote = Refsum disease

- Phyh-/- mice are viable and fertile
### Experimental setup (1)

- No phytanic acid in normal mouse chow
- To generate good model mice must be fed with phytol

<table>
<thead>
<tr>
<th></th>
<th>Phyh +/-</th>
<th>Phyh -/-</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>0.1% Phytol</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>0.25% Phytol</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
Experimental setup (2)

Start of experiment

• SHIRPA: Systematic and objective protocol for phenotype analysis

• Catwalk: Automated gait analysis

End of experiment

• SHIRPA

• Catwalk

• Isolation of tissues
  - Biochemistry
  - Histology/Histochemistry
WT+/+ (+phytol)  Phyh-/- (+phytol)

• Phyh-/- weigh less and loose weight on phytol diet
Phytanic acid levels in plasma and tissues

• Phyh-/- mice accumulate phytanic acid on phytol diet

• Highest level in liver followed by kidney, testis and cerebellum

• In plasma levels up to 1 mM

![Graph showing phytanic acid levels in different tissues and plasma]

**Conclusion:** A good biochemical model for Refsum disease
SHIRPA

- Systematic and objective protocol for phenotype analysis
- 24 tests were done before and after phytol diet
Response to turning

- Phyh-/- on phytol diet showed abnormal tests:
  - Increased pawslips
  - Absent trunk curl
  - Abnormal movement

- Conclusion: abnormal neuromuscular function in Phyh-/- mice after phytol diet
What is a catwalk?

Closed box with light source
Paw
Glass plate
Bright spot
High resolution camera
Signal to P.C.
Digital output

- Every pawprint must be labeled by hand (fore paw etc) to allow data analysis

- Result: Label, time and light- (pressure-) intensity per paw.

- Resulting calculated information:
  - step sequence (pattern)
  - regularity index
  - swing stance phase
  - base of support
  - paw print area
  - total contact area
  - paw pressure
  - overall velocity
Data acquisition example
Results catwalk analysis

Phyh -/- mice after the diet period reveal:

• A decreased base of support for the hind paws
• An abnormal and decreased paw print area of both the fore- and the hind paws
• An abnormal wind off both of fore- and hind paws

Conclusion: Phyh-/- mice show gait abnormalities pointing to neuropathy
Motor nerve conduction velocities (MNCV)

- MNCV are decreased in Phyh-/- revealing a peripheral neuropathy
- Myelination was normal
Purkinje cells

- Neurons in the cerebellum (part of the hind brain) – means little brain in Latin
- The cerebellum is important for movement and motor learning
Abnormalities in central nervous system in Phyh-/- mice
Abnormalities in central nervous system in Phyh-/- mice

**Conclusion:**
Reduced number of Purkinje cells in Phyh-/- mice on phytol
Conclusions

Phyh-/ mice on phytol:

• Accumulate phytanic acid in plasma and tissues

• Develop a peripheral neuropathy

• Show cerebellar ataxia accompanied by a loss of Purkinje cells in the cerebellum

• Potentially a good model to study therapeutic strategies
Q&A

For more information contact:

- Global DARE Foundation
- info@globaldarefoundation.org
- www.defeatadultrefsumeverywhere.org
UPCOMING REFSUM WEBINARS

Global DARE Foundation will be holding additional webinars throughout the summer. Registration can be accessed through our website at https://www.defeatadultrefsumeverywhere.org/dare-events

7/24/20, 7:00AM EST
**Refsum Diet Overview & Discussion**
Eleanor Baldwin & Sarah Firman, the clinical dietitians at Guy's & St. Thomas Hospital in London will be providing an overview of the specialized diet for Refsum Disease

8/7/20, 8:00 AM EST
**Gene Therapy - A Potential Therapy for Refsum Disease**
Ryan Butler, PhD from UT Southwestern will provide an overview of Gene Therapy as a potential future therapy for Refsum Disease