Diagnosing Inborn Errors of Plasmalogen Biosynthesis by Liquid Chromatography Tandem Mass Spectrometry

Irene De Biase, MD, PhD, FACMG
Professor of Pathology, University of Utah
Medical Director, Biochemical Genetics, ARUP Laboratories

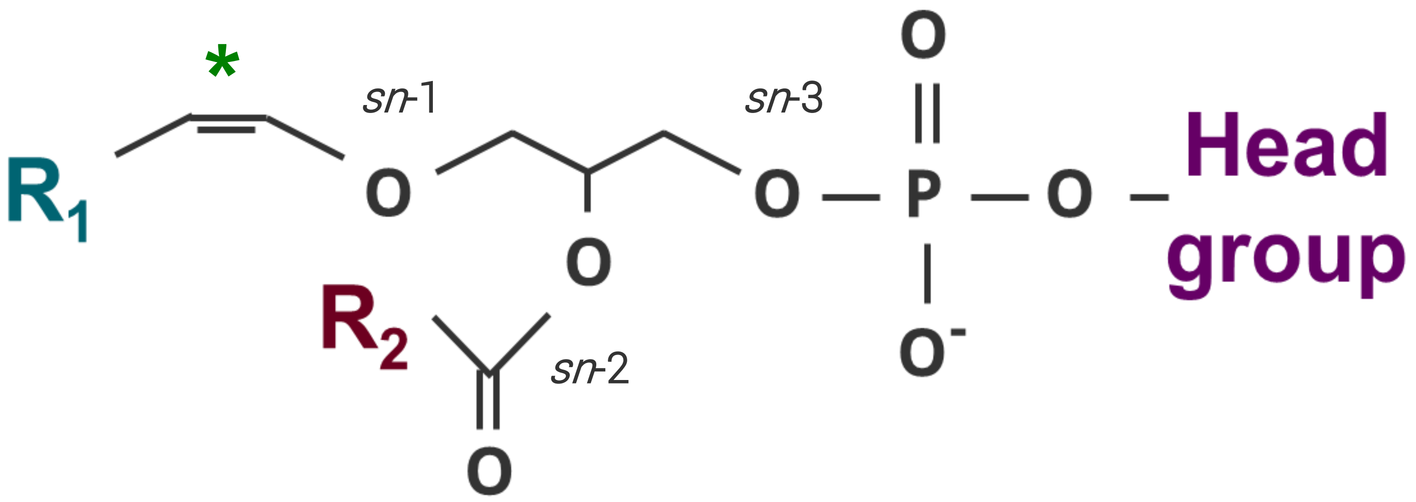
Learning objectives

1. Review plasmalogen structure & function
2. Define inborn errors of plasmalogen biosynthesis
3. Describe the key features of plasmalogen clinical testing
4. Explore the challenges in developing and validating testing to diagnose plasmalogen deficiency by LC-MS/MS
5. Demonstrate clinical utility of the LC-MS/MS method in diagnosing peroxisome biogenesis disorders

Lipids are an heterogenous group of organic compounds performing essential cell functions

- Lipids
  - Complex Lipids
    - Glycolipids
    - Phospholipids
      - Sphingomyelins
      - Glycerophospholipids » Plasmalogens
  - Simple Lipids

Plasmalogens are a unique class of membrane glycerophospholipids



R_1 = fatty alcohol; C16:0 (palmitoyl), C18:0 (stearoyl) or C18:1 (oleoyl)

R_2 = polyunsaturated fatty acid; most abundant: docosahexaenoic acid (DHA; 22:6ω3) or arachidonic acid (20:4ω6)

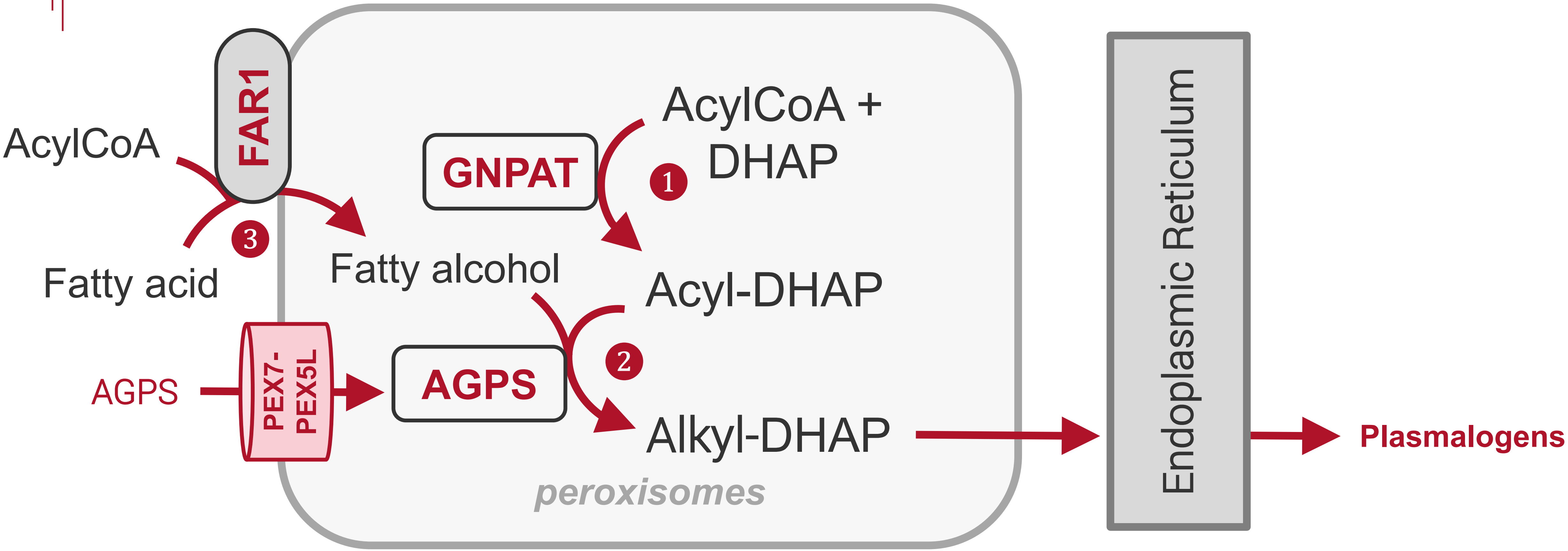
Head group, most commonly ethanolamine

(*) Vinyl ether linkage

Plasmalogens constitute ~20% of total phospholipids in cell membranes

- Major structural components of cell membranes
  - Decrease membrane fluidity
    - Particularly abundant in myelin
- Natural antioxidants
- Signaling molecules
- Reservoir of polyunsaturated fatty acids

The first two steps of plasmalogen biosynthesis occur in peroxisomes



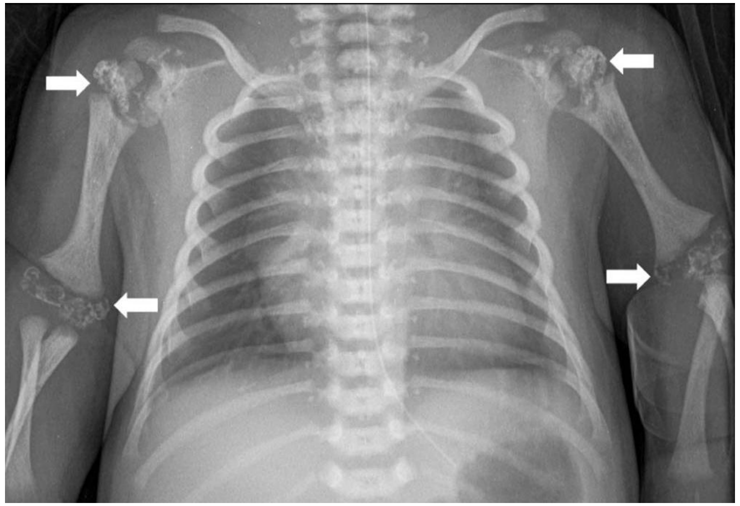
GNPAT: glyceronephosphate-O-acyltransferase; AGPS: alkylglycerone-phosphate synthase; FAR1: fatty acyl-CoA reductase 1

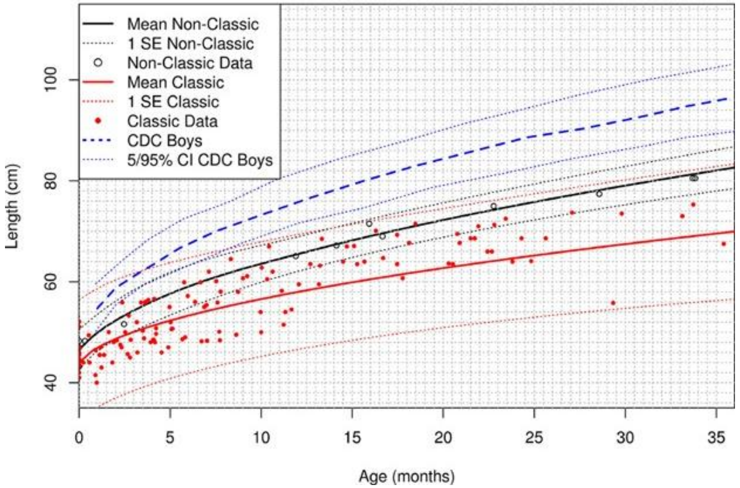
Inherited defects in plasmalogen biosynthesis cause rhizomelic chondrodysplasia punctata

Phenotype	OMIM	Gene
Rhizomelic Chondrodysplasia (RCDP) Type 1	601757	PEX7
Glyceronephosphate-O-acyltransferase (GNPAT) deficiency (RCDP Type 2)	222765	GNPAT
Alkylglycerone-phosphate synthase (AGPS) deficiency (RCDP Type 3)	600121	AGPS
Fatty acyl-CoA reductase-1 (FAR1) deficiency (RCDP Type 4)	616154	FAR1
Rhizomelic Chondrodysplasia (RCDP) Type 5 (*) only variants affecting PEX5 binding to PEX7	616716	PEX5 (long form)*

Patients with RCDP are not expected to survive past their third decade of life

- Characterized by rhizomelic shortening of the long bones and abnormal mineralization of the growth plates (chondrodysplasia punctata), dysmorphic features, congenital cataracts, profound growth impairment and intellectual disability, cardiac malformations, seizures
  - » Death often due to respiratory complications
  - » Milder (non-classic) phenotypes described





J Pediatr. 2018; 201:296
American J of Med Genetics Pt A. 2016; 173(1):108-113

Patients with RCDP exhibit markedly reduced plasmalogens

- Disease severity correlates with plasmalogen levels, not with RCDP type
  - The most severe patients have less than 30% residual levels; while mildly affected patients typically have 40 – 50% of residual plasmalogens

Plasmalogen deficiency can be caused by reduced number of functioning peroxisomes

- Impaired plasmalogen biosynthesis is also a prominent feature in peroxisome biogenesis disorders of the Zellweger spectrum (ZSD)
  - » Other metabolites are markedly abnormal in these patients
  - » Plasmalogens can be normal in ZSD patients

Plasmalogen deficiency is traditionally detected in red blood cells (RBCs) by GC-MS

- Requires derivatization to the dimethyl-acetal derivative of the corresponding aldehyde (DMA)
  - Relative abundance is assessed as ratio of DMAs to fatty acid: 16:0 DMA/C16:0 and 18:0 DMA/C18:0
  - Plasmalogen deficiency ≤60% of control median

Plasmalogen testing performed at Kennedy Krieger Institute on a patient with severe RCDP type 1

Analyte	Result	Reference Intervals
C16:0 DMA/ C16:0 Fatty Acid	0.012	0.079 - 0.128
C18:0 DMA/ C18:0 Fatty Acid	0.015	0.199 - 0.284

Ratios are markedly reduced Approximatively 12% and 6% of normal median, respectively

Limitations of measuring RBC plasmalogens by GC-MS

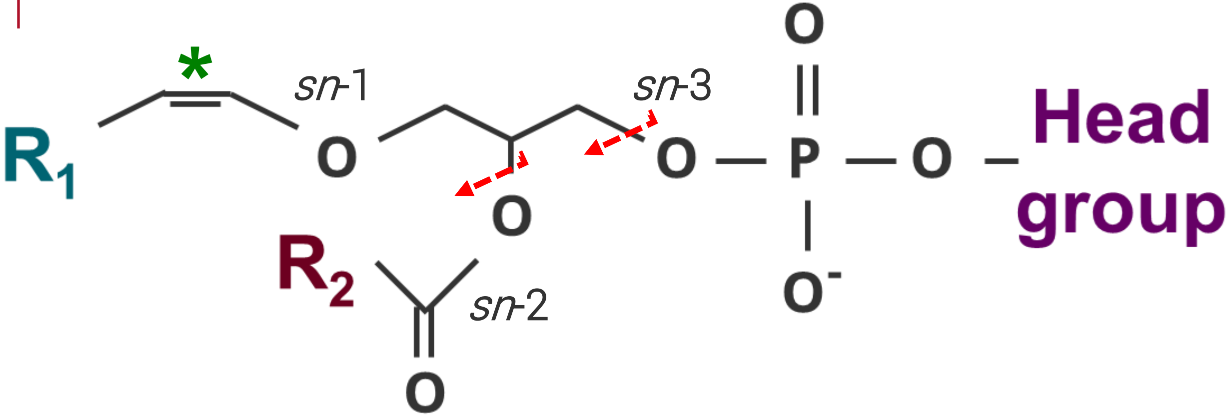
- Loss of specie-specific information
  - » GC-MS methods cannot distinguish individual plasmalogen species unlike methods using LC-MS/MS
- Lengthy sample preparation and long run time
- Requires CG-MS instrument that uses chemical ionization

Can we develop a clinical test
to quantify plasmalogens using LC-MS/MS?

Challenges in developing plasmalogen testing using LC-MS/MS

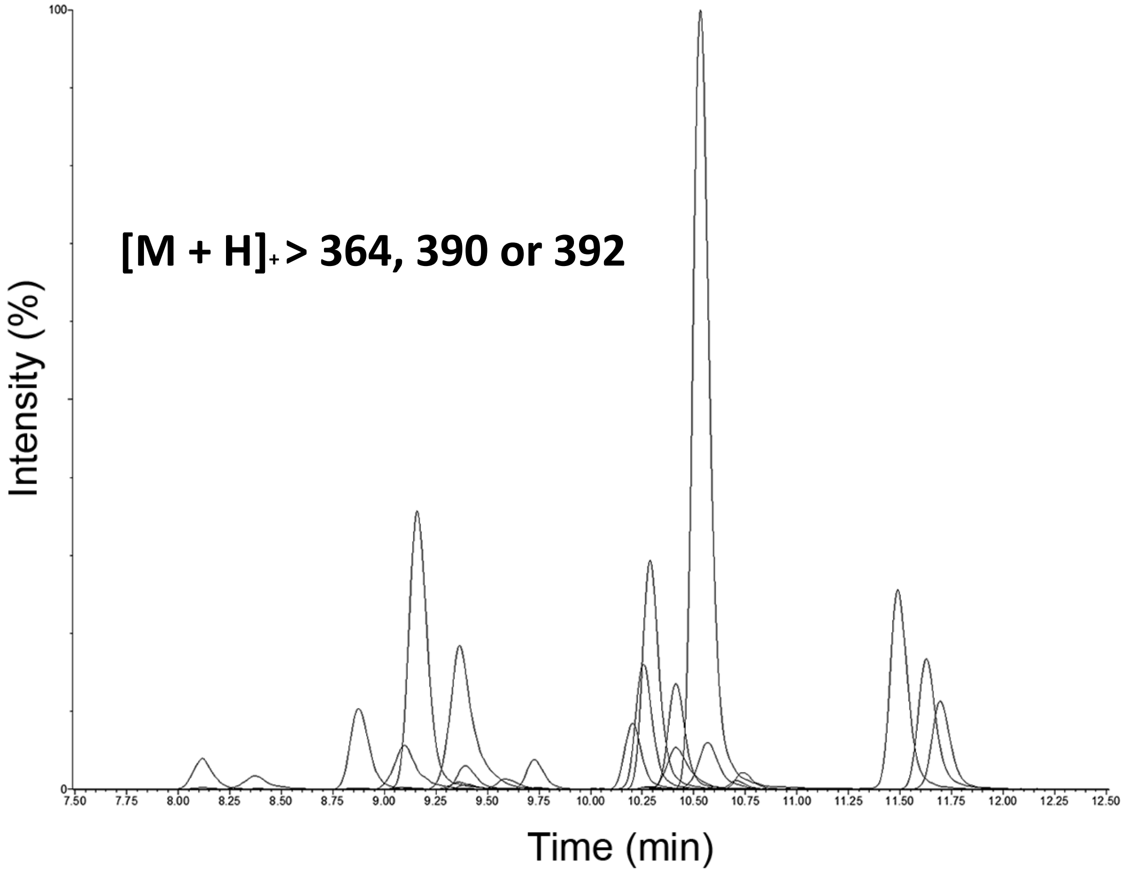
- Limited data available on individual plasmalogen specie distribution and relative abundance in RBCs
  - » LC-MS/MS mostly used in lipidomic studies employing plasma as sample type
  - » Reference intervals in RBC have not been established
- Confirming clinical utility in diagnosing inborn errors of plasmalogen synthesis
  - » Difficult to source patient RBC samples
  - » Does fatty acid content influence plasmalogen levels?

We have developed a LC-MS/MS method to quantify phosphoethanolamine plasmalogens



- Based on the collision-induced dissociation (CID) of protonated plasmalogens (red arrows)
  - Fragment ions of *m/z* 364, 390 and 392 are used to quantify 16:0, 18:1 and 18:0 species, respectively

Extracted ion traces in a matrix sample



De Biase et al. 2023 *Clin Chim Acta*

This method uses a simple and fast extraction

- RBC plasmalogens were extracted in methanol containing internal standards (one hour with shaking). Supernatant is further diluted in methanol before injection
- Chromatographic separation was performed using an Acquity Premier BEH C_{18} UPLC column and a binary gradient of 5 mM ammonium acetate in water:methanol (15:85) and 5 mM ammonium acetate in methanol
- Analysis was performed using a XEVO TQ-XS Mass Spectrometer with Ultra-High Performance Liquid Chromatography (Waters) in multiple reaction monitoring mode (run time: 18 minutes/sample)

De Biase et al. 2023 *Clin Chim Acta*

This method allows for accurate quantitation of the most abundant RBC plasmalogen species

	Analyte	Standard	IS	Molecular formula	*sn-1*	*sn-2*
16:0 species	16:0(plasm)-18:1-PE	16:0(plasm)-16:0-PE	16:0(plasm)-16:0(d_9)-PE	$C_{39}H_{76}NO_7P$	16:0	18:1ω9
	16:0(plasm)-18:2-PE	16:0(plasm)-16:0-PE	16:0(plasm)-16:0(d_9)-PE	$C_{39}H_{74}NO_7P$		18:2ω6
	16:0(plasm)-20:4-PE	16:0(plasm)-16:0-PE	16:0(plasm)-16:0(d_9)-PE	$C_{41}H_{74}NO_7P$		20:4ω6
	16:0(plasm)-22:4-PE	16:0(plasm)-16:0-PE	16:0(plasm)-16:0(d_9)-PE	$C_{43}H_{78}NO_7P$		22:4ω6
	16:0(plasm)-20:5-PE	16:0(plasm)-16:0-PE	16:0(plasm)-16:0(d_9)-PE	$C_{41}H_{72}NO_7P$		20:5ω3
	16:0(plasm)-22:6-PE	16:0(plasm)-16:0-PE	16:0(plasm)-16:0(d_9)-PE	$C_{43}H_{74}NO_7P$		22:6ω3
18:0 species	18:0(plasm)-18:1-PE	18:0(plasm)-18:1-PE	18:0(plasm)-18:1(d_9)-PE	$C_{41}H_{80}NO_7P$	18:0	18:1ω9
	18:0(plasm)-18:2-PE	18:0(plasm)-18:1-PE	18:0(plasm)-18:1(d_9)-PE	$C_{41}H_{78}NO_7P$		18:2ω6
	18:0(plasm)-20:4-PE	18:0(plasm)-20:4-PE	18:0(plasm)-18:1(d_9)-PE	$C_{43}H_{78}NO_7P$		20:4ω6
	18:0(plasm)-22:4-PE	18:0(plasm)-22:6-PE	18:0(plasm)-18:1(d_9)-PE	$C_{45}H_{82}NO_7P$		22:4ω6
	18:0(plasm)-20:5-PE	18:0(plasm)-20:4-PE	18:0(plasm)-18:1(d_9)-PE	$C_{43}H_{76}NO_7P$		20:5ω3
	18:0(plasm)-22:6-PE	18:0(plasm)-22:6-PE	18:0(plasm)-18:1(d_9)-PE	$C_{45}H_{74}NO_7P$		22:6ω3
18:1 species	18:1(plasm)-18:1-PE	18:0(plasm)-18:1-PE	18:0(plasm)-18:1(d_9)-PE	$C_{41}H_{78}NO_7P$	18:1	18:1ω9
	18:1(plasm)-18:2-PE	18:0(plasm)-18:1-PE	18:0(plasm)-18:1(d_9)-PE	$C_{41}H_{76}NO_7P$		18:2ω6
	18:1(plasm)-20:4-PE	18:0(plasm)-20:4-PE	18:0(plasm)-18:1(d_9)-PE	$C_{43}H_{76}NO_7P$		20:4ω6
	18:1(plasm)-22:4-PE	18:0(plasm)-22:6-PE	18:0(plasm)-18:1(d_9)-PE	$C_{45}H_{80}NO_7P$		22:4ω6
	18:1(plasm)-20:5-PE	18:0(plasm)-20:4-PE	18:0(plasm)-18:1(d_9)-PE	$C_{43}H_{74}NO_7P$		20:5ω3
	18:1(plasm)-22:6-PE	18:0(plasm)-22:6-PE	18:0(plasm)-18:1(d_9)-PE	$C_{45}H_{76}NO_7P$		22:6ω3

- Oleic ac.
- Linoleic ac.
- Arachidonic ac.
- Docosatetraenoic ac. (DTA)
- Eicosapentaenoic ac. (EPA)
- Docosahexaenoic ac. (DHA)

De Biase et al. 2023 *Clin Chim Acta*

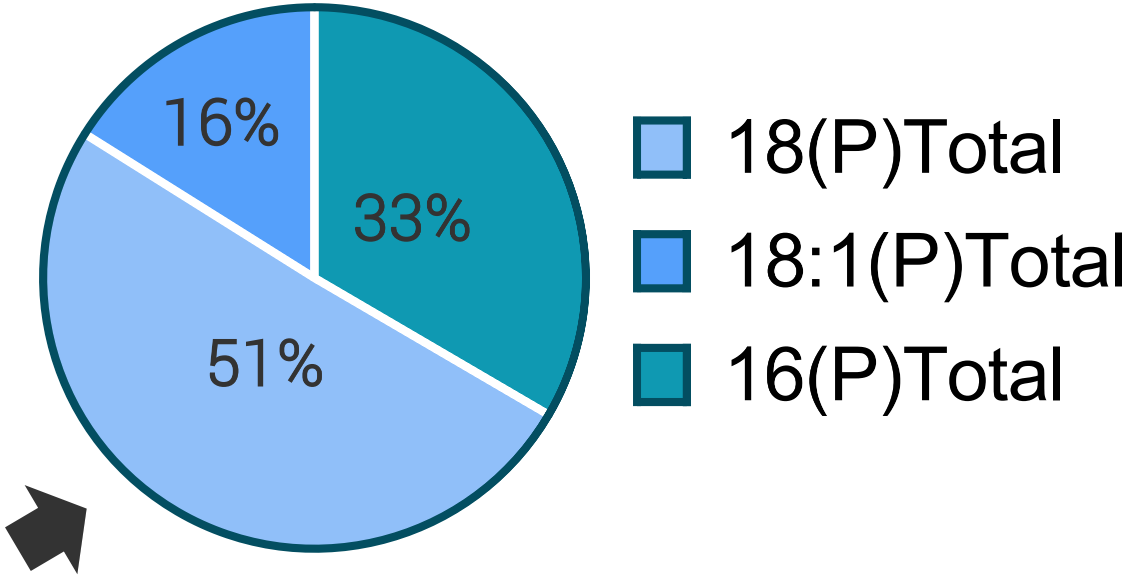
Performance characteristics were evaluated for all species included in the assay

- Method was linear throughout the calibration curve (r^2>0.99), with mean accuracy 97.2-102% of target and coefficient of variation (CV) ranging 2.2% to 6.4% (n=20)
- Intra-assay (n=6 replicates on 2 runs) and inter-assay variability (n=10 runs) in three donor RBCs was <10%
- Analyte recoveries ranged from 85.2 to 106%
- We confirmed equivalence among RBCs isolated from EDTA (preferred sample type), sodium heparin, PPACK or ACD whole blood, and storage stability in different conditions

De Biase et al. 2023 *Clin Chim Acta*

Similar species distribution based on *sn*-1 position to previously described by GC-MS

- On average, plasmalogens carrying 18:0 (stearoyl) at *sn*-1 represented half of total plasmalogens



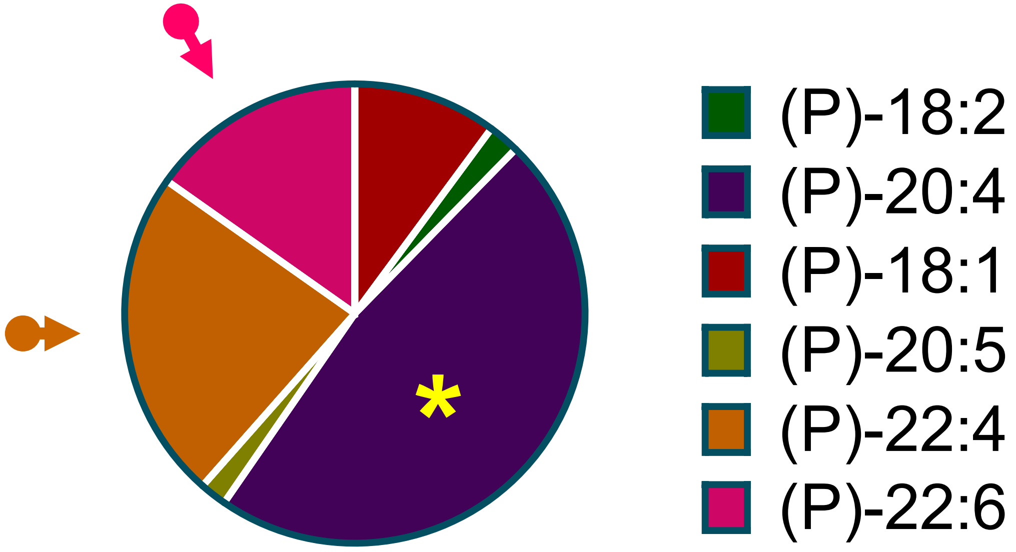
» 441 RBCs

» Age range birth to 88 years

De Biase, unpublished

GC-MS data reviewed in Braverman & Moser 2012 *Biochim Biophys Acta*

Our data is consistent with published relative abundance of intact species



- Omega-6 arachidonic acid (20:4ω6) is the most abundant fatty acid incorporated at *sn*-2
- Species containing omega-3 docosahexaenoic acid (DHA; 22:6ω3) and omega-6 docosatetraenoic acid (DTA; 22:4ω6) were also abundant

- 441 RBCs
- Age range birth to 88 years

De Biase, unpublished
Relative abundance expressed as a percentage of total phospholipids; published in Leidl et al. 2008 *Biochim Biophys Acta*

What is normal?
Establishing normal reference intervals in RBCs

Study Design

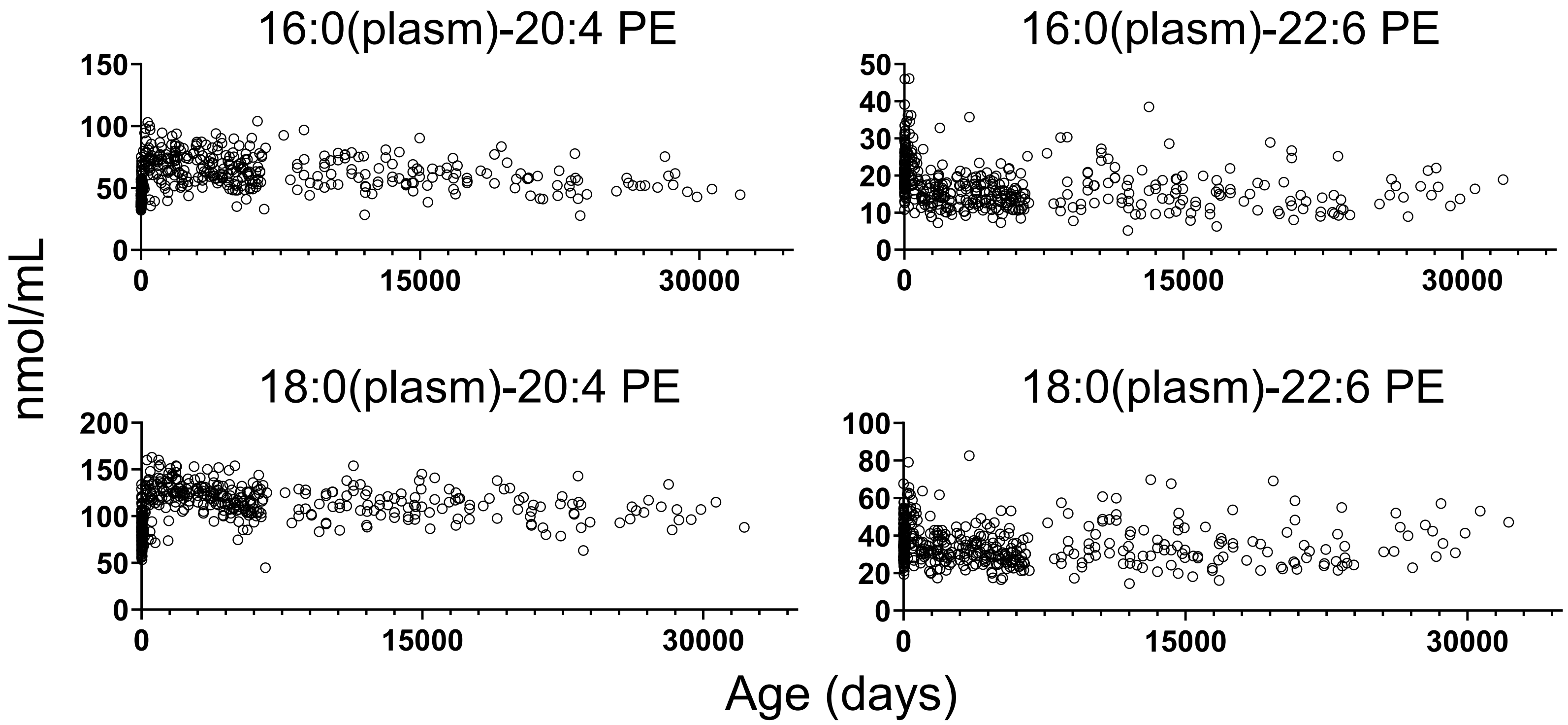
- Plasmalogens were measured in RBCs from:
  - » 294 healthy individuals (age range: 4 days to 76 years; average age 16 years; 133 males, 161 females)
  - » 147 residual clinical samples (age range: 0 to 88 years; average age 17 years; 90 males, 57 females). Those samples were initially submitted for testing unrelated to peroxisomal disorders and reported as normal

RI for individual plasmalogen species and their total values based on *sn*-1 and *sn*-2 position, and total plasmalogens

De Biase, unpublished

As expected, plasmalogen species display age-dependent changes

- Changes in concentrations (nmol/mL) over time (age in days)



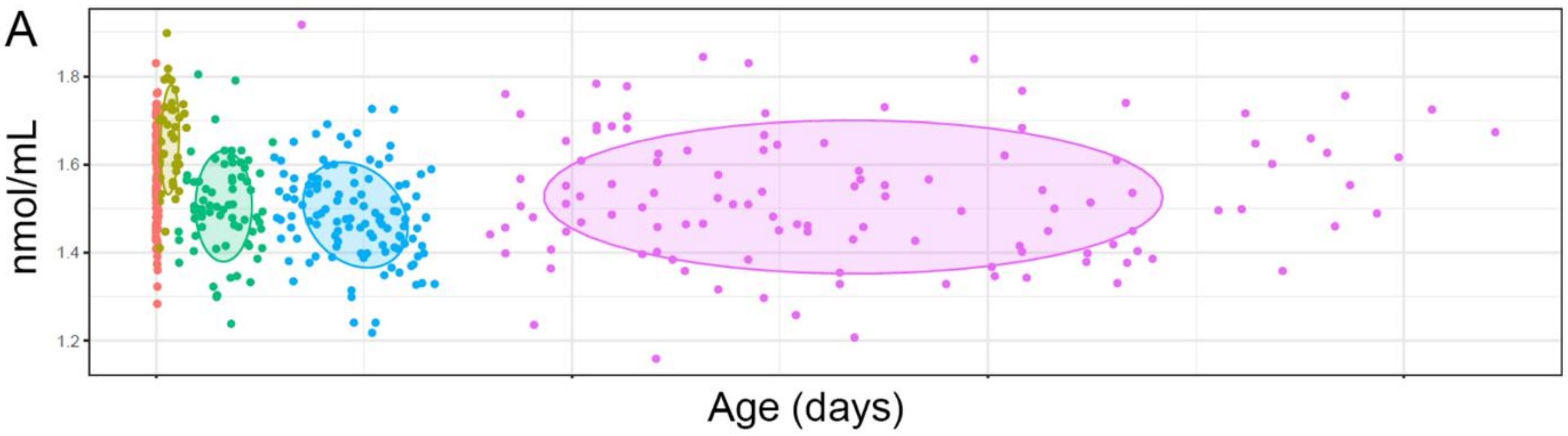
PE: phosphoethanolamine; species at *sn-1*: 16:0 (palmitoyl) and 18:0 (stearoyl); species at *sn-2*: 20:4ω6 and 22:6ω3 (Docosahexaenoic ac.; DHA)

De Biase, unpublished

Model-based clustering algorithm was applied to discover age-related clusters within dataset

18:0(plasm)-22:6 PE

A



B

Cluster boundaries (days)	Initial age groups (days)	Adjusted age groups (days)
0 - 59	0 - 59	0 - 60
77 - 715	60 - 715	61 - 730
551 - 2634	716 - 2634	731 - 2634
2914 - 6601	2935 - 6601	2635 - 6574
8085 - 30552	6602 - 30552	> 6574

- Cluster boundaries calculated based on the lower 1st and upper 99th age percentiles Unique clusters are color-coded
- Cluster borders were defined from the end of one cluster to the end of another

De Biase, unpublished

Once age groups were identified, RI were established for all species and their totals

- Statistically different groups (difference in means ≥10%) were selected (iterative application of Harris-Boyd method)
- Data was analyzed using parametric or non-parametric statistics to determine final reference intervals (95%, with 90% confidence intervals)

De Biase, unpublished

Most species are significantly lower in neonates compared to children and adults

- Consistent with what has been seen for GC-MS

Analyte	Age	Lower limit	Upper limit	n	Median
16:0(plasm)-PE Total	0 - 3 months	89.1	165	100	118
	4 months - 18 years	105	206	220	155
	> 18 years	89.7	189	117	138
18:0(plasm)-PE Total	0 - 2 months	125	252	96	176
	> 2 months	170	282	341	228
18:1(plasm)-PE Total	0 - 2 months	45.4	111	96	60.7
	3 months - 2 years	51.2	166	42	83.3
	> 2 years	50.7	97.4	299	67.7
Total Plasmalogens	0 - 2 months	275	484	96	360
	> 2 months	339	576	341	446

De Biase, unpublished

Based on these RI, can we discriminate between RCDP patients and controls?

Phenotype	OMIM	Gene
Rhizomelic Chondrodysplasia (RCDP) Type 1		PEX7
Glyceronephosphate-O-acyltransferase (GNPAT) deficiency (RCDP Type 2)		GNPAT
Alkylglycerone-phosphate synthase (AGPS) deficiency (RCDP Type 3)		AGPS
Fatty acyl-CoA reductase-1 (FAR1) deficiency (RCDP Type 4)		FAR1
Rhizomelic Chondrodysplasia (RCDP) Type 5 (*) only variants affecting PEX5 binding to PEX7	616716	PEX5 (long form)*

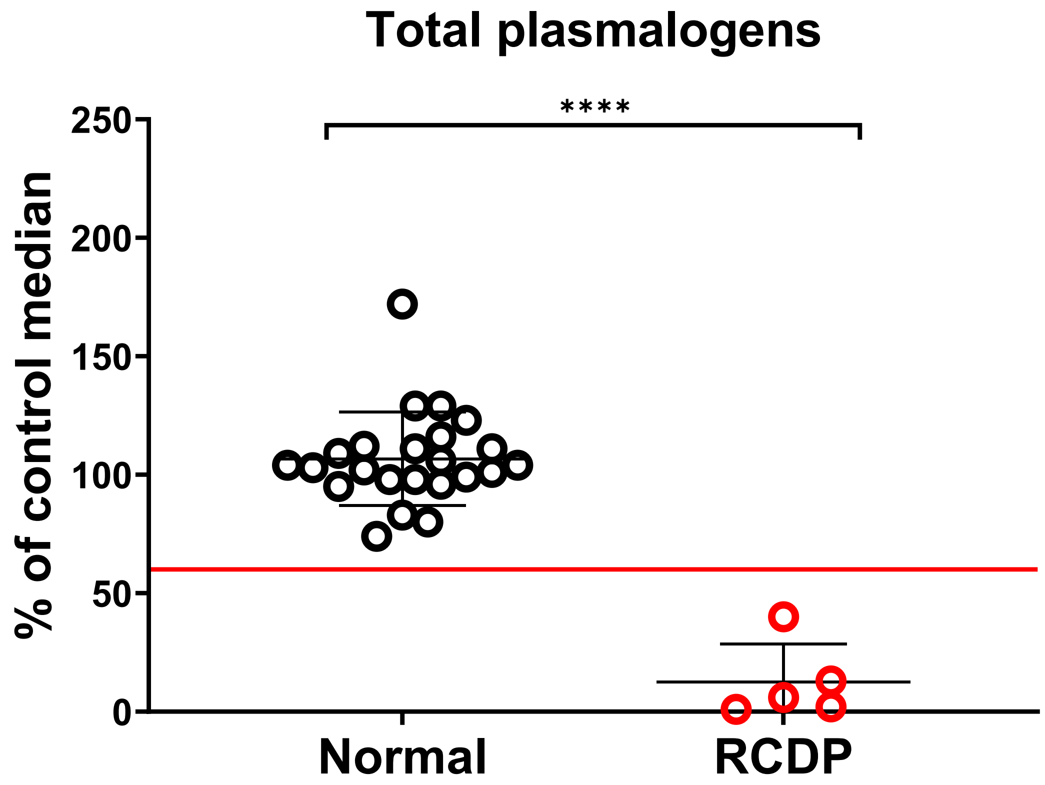
4 patients with RCDP type I

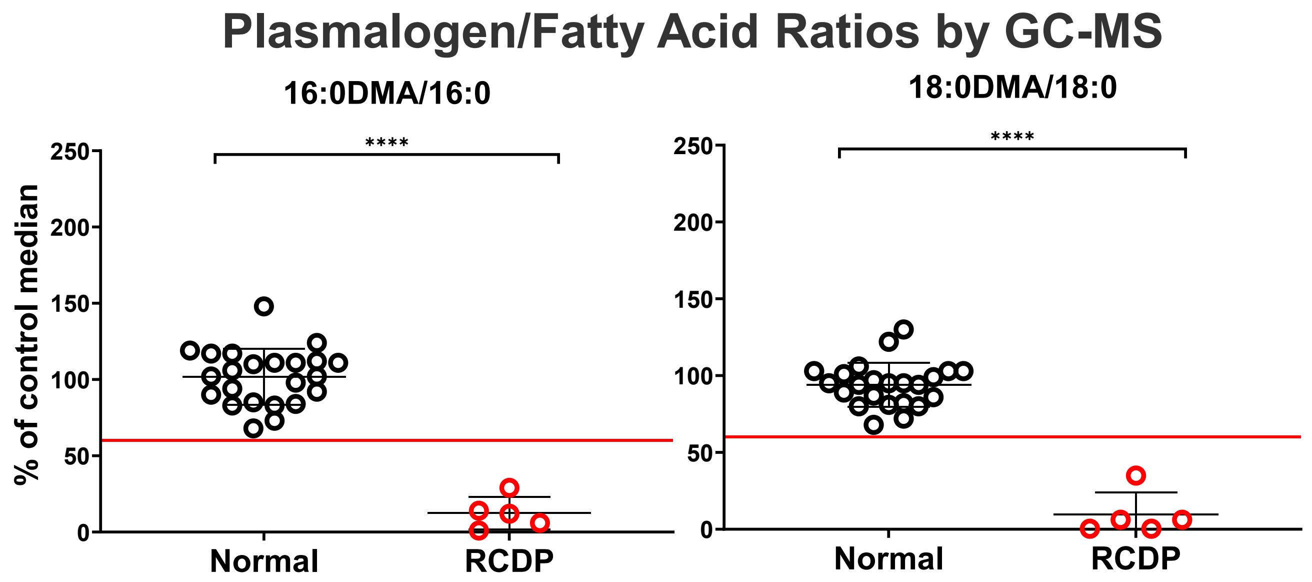
1 patient with RCDP type II

vs. 23 healthy controls

Based on these RI, can we discriminate between RCDP patients and controls?

- Plasmalogen deficiency ≤60% of control median



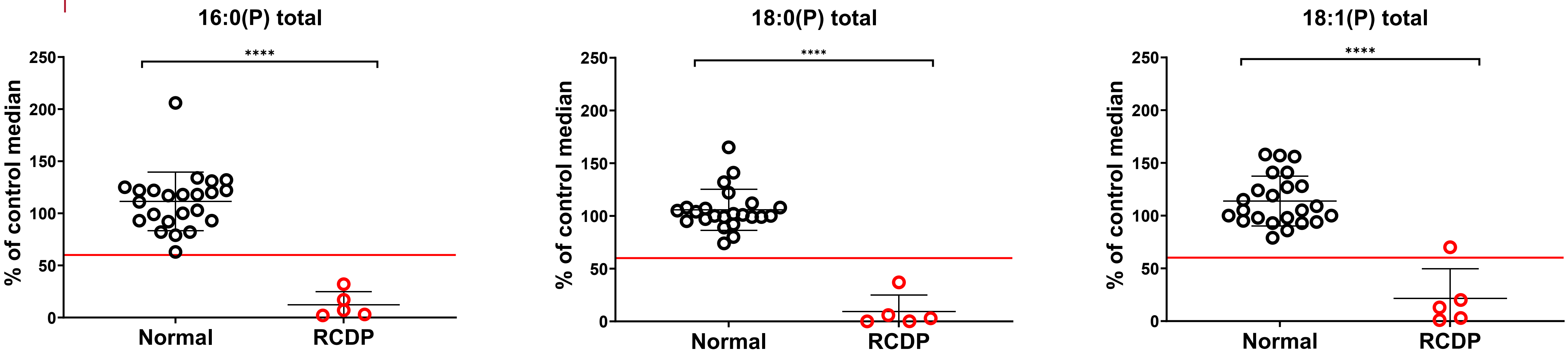


Testing performed at Kennedy Krieger Institute

» All RCDP patients have plasmalogen levels below 60% of control median (red line), as well as low ratios

De Biase et al. 2023 *Clin Chim Acta*

RCDP exhibit markedly reduced levels of all plasmalogen species



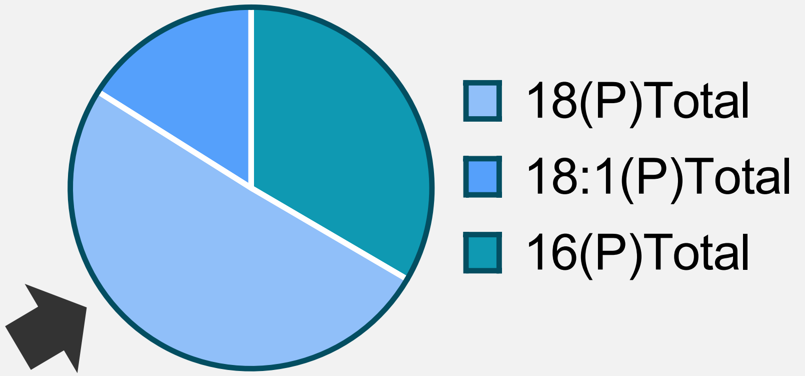
- 18:1 plasmalogen species seemed to be less useful in discriminating patients from controls
  - Effect of diet?

De Biase et al. 2023 *Clin Chim Acta*

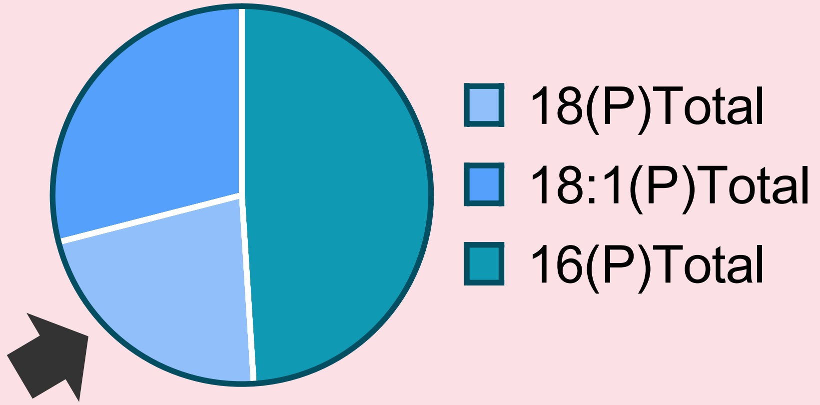
RCDP also exhibit differences in plasmalogen species composition

- Plasmalogens carrying 18:0 (stearoyl) at *sn*-1 represented half of total plasmalogens in controls but NOT in RCDP

Controls



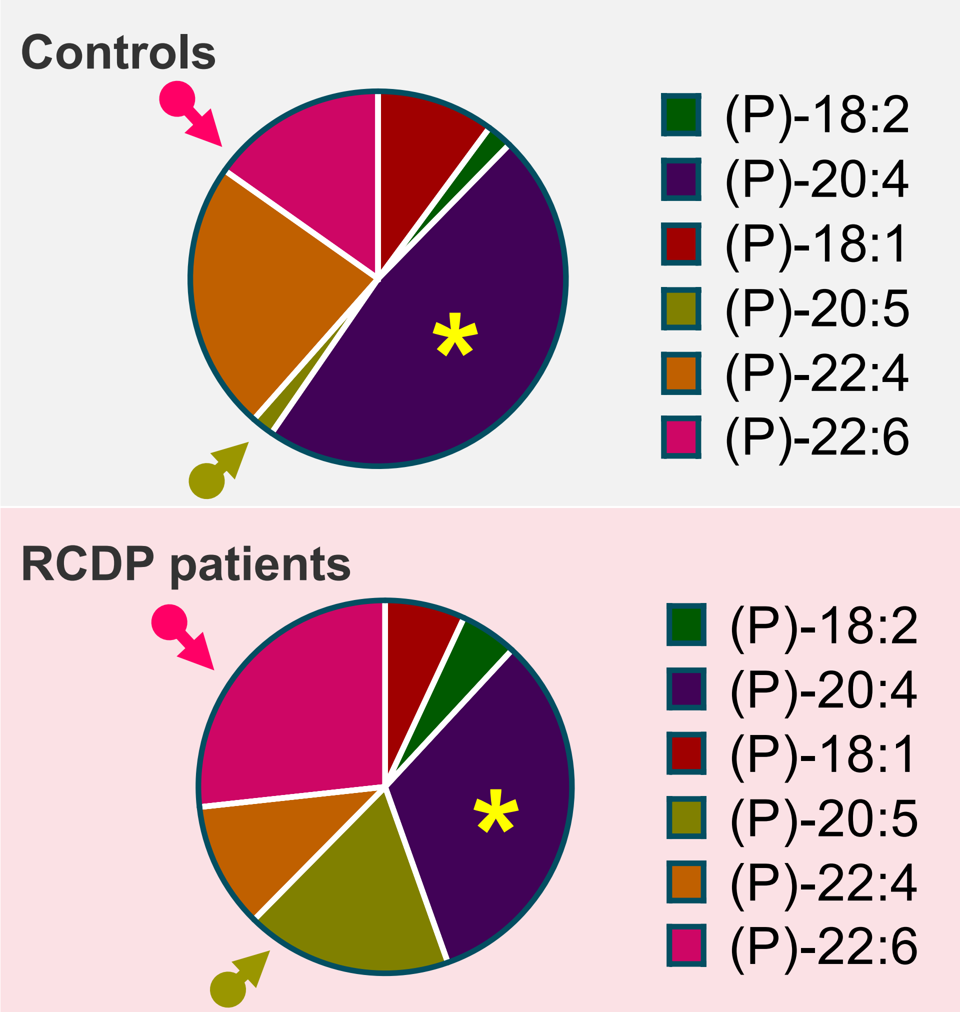
RCDP patients



De Biase, unpublished

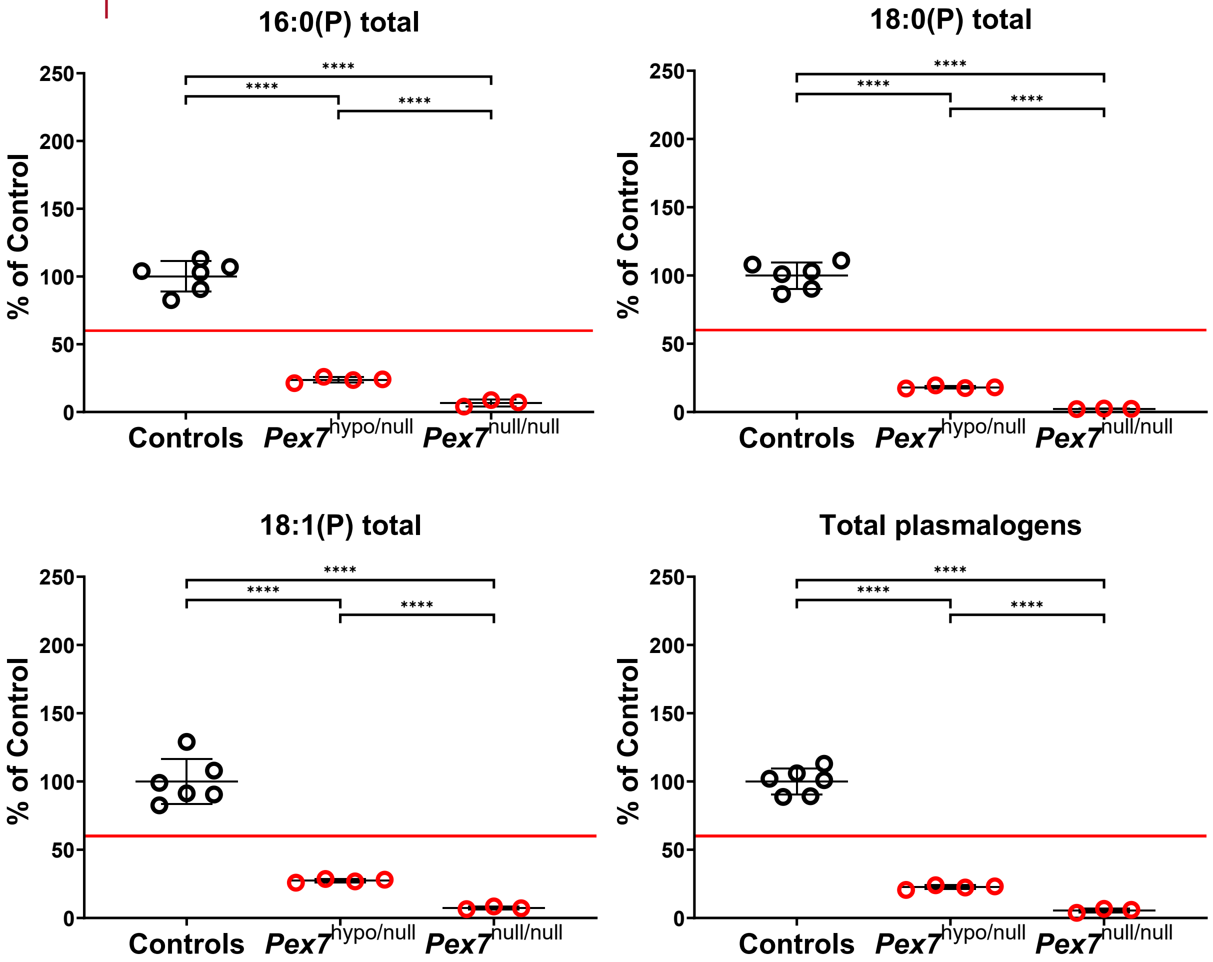
Species containing arachidonic acid were the most abundant in controls and RCDP patients

- RCDP patients displayed higher levels of species containing DHA (22:6ω3) and EPA (20:5 ω3) at *sn*-2 position



De Biase, unpublished

Clinical utility was confirmed in Pex7 deficient mouse model

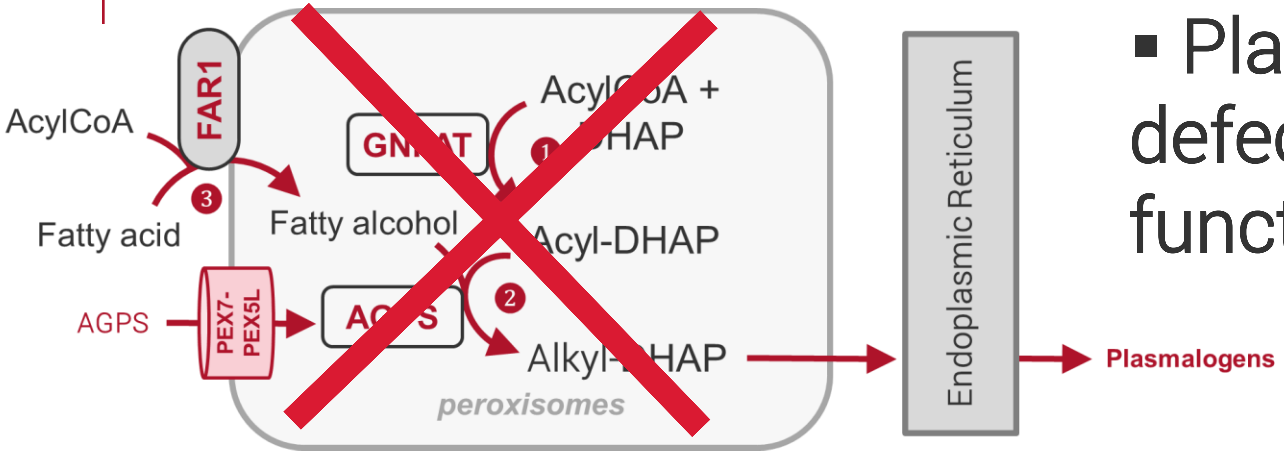


- Resembling severe (*Pex7*$^{\text{null/null}}$) and milder (*Pex7*$^{\text{hypo/null}}$) RCDP type 1 phenotypes

- Red line indicates 60% of the control median

De Biase et al. 2023 *Clin Chim Acta*

What about ZSD?

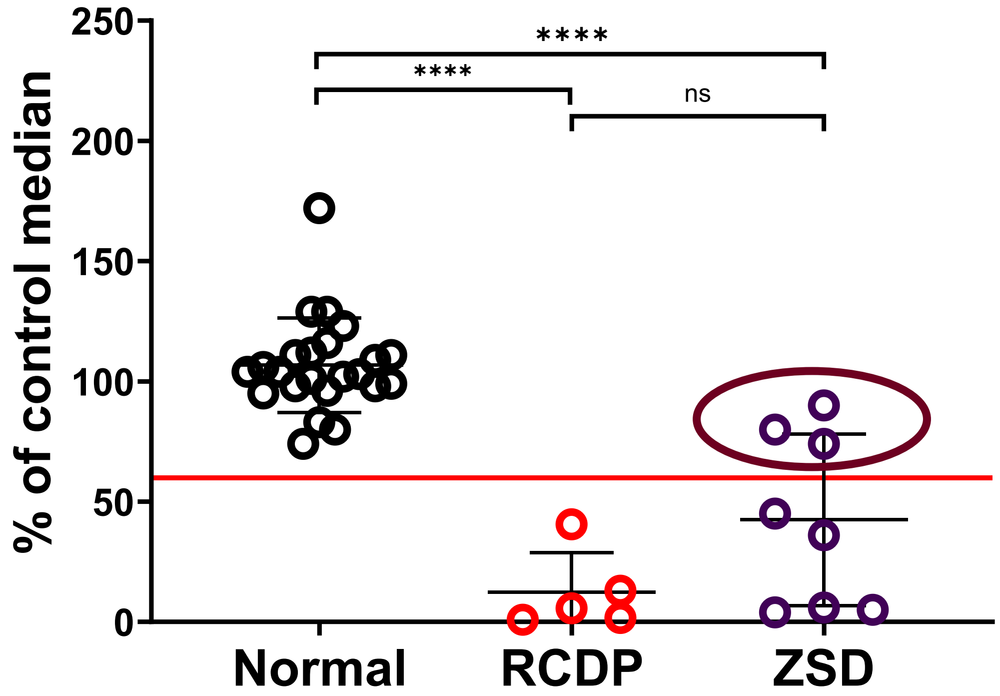


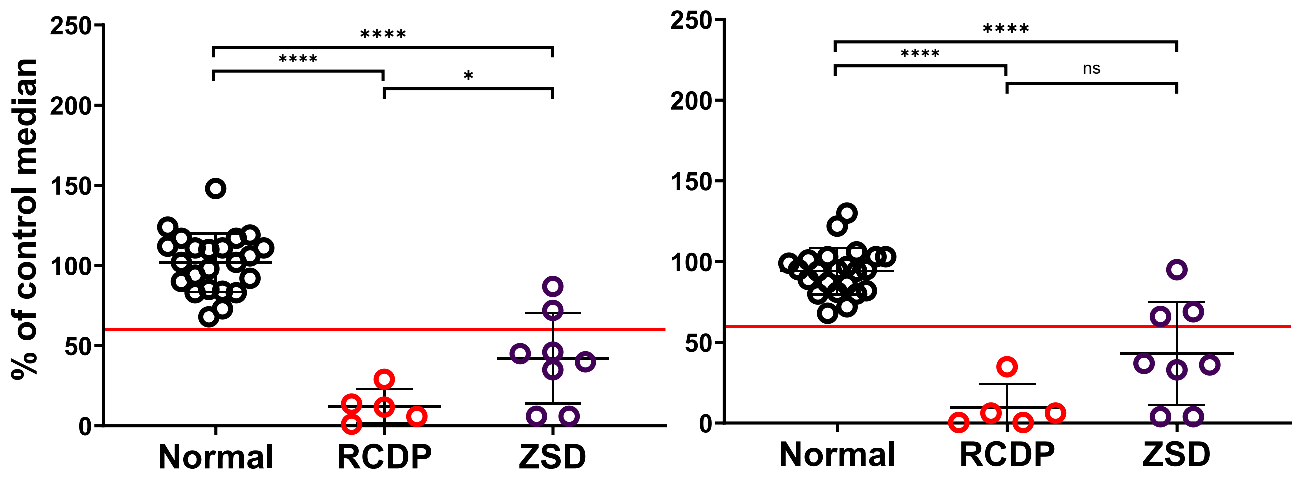
- Plasmalogen biosynthesis is defective in ZSD due to lack of functioning peroxisomes

» Plasmalogens were quantified in 8 RBCs from ZSD patients
- Age: 4 days to 9 years
- Deficient in PEX1, PEX6 or PEX12
- Four ZSD patients were older and with a less severe phenotype

Plasmalogens levels can be normal in ZSD patients

- Plasmalogen deficiency ≤60% of control median





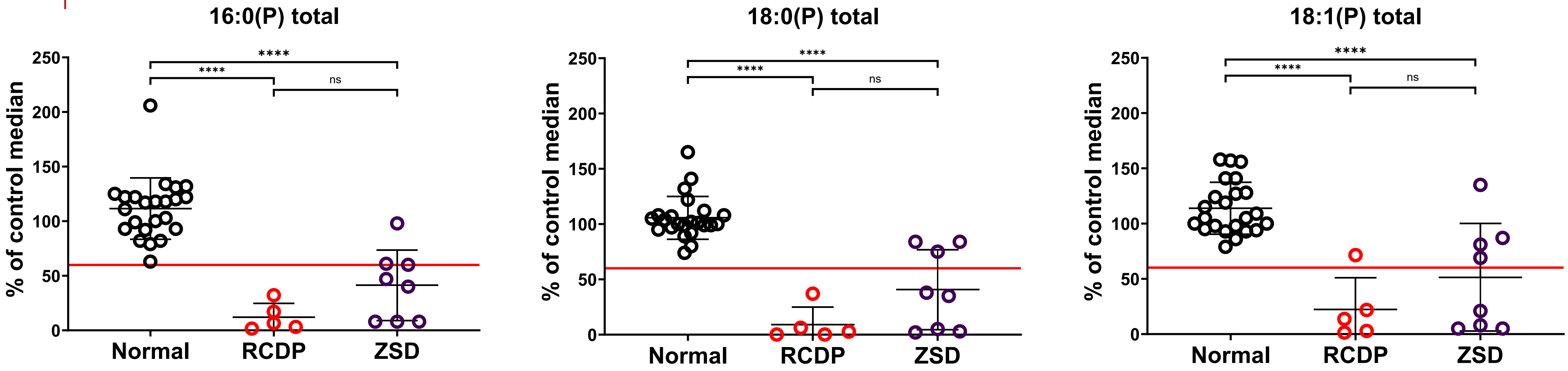
Testing performed at Kennedy Krieger Institute

» RBCs from 23 controls & 5 RCDP patients and 8 ZSD

De Biase, unpublished

De Biase et al. 2023 *Clin Chim Acta*

Severely affected ZSD exhibit reduced levels of all plasmalogen species



- As for RCDP, 18:1 plasmalogen species seemed to be less useful
- Interestingly, species carrying DHA (22:4ω6) were deficient in all samples regardless of disease severity

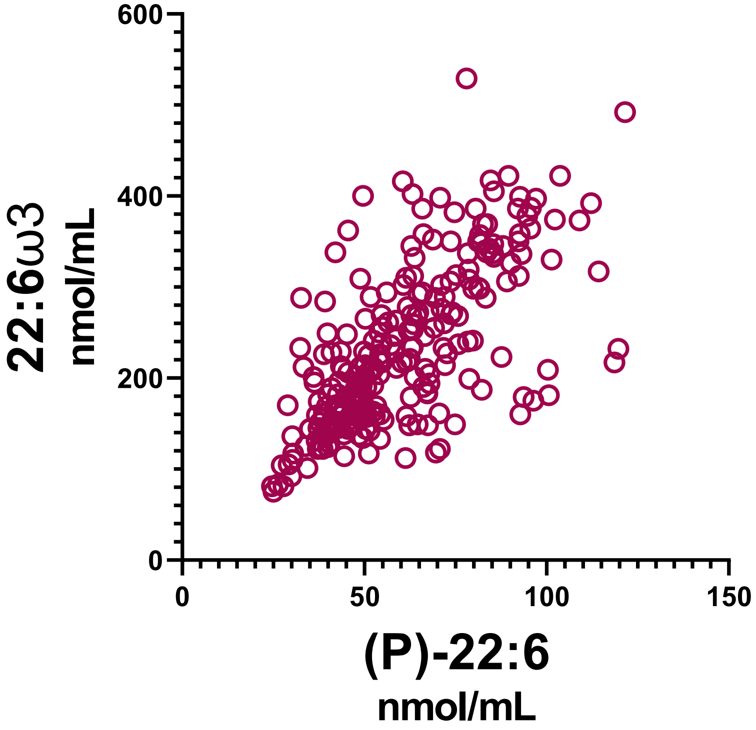
De Biase, unpublished
De Biase et al. 2023 *Clin Chim Acta*

Does fatty acid content influence plasmalogen levels?

- Since plasmalogen are composed of fatty acids, fatty acid content could potentially alter plasmalogen levels and affect the results of clinical testing
  - Fatty acid deficiency could cause low plasmalogens
  - Supplementation could increase plasmalogen levels

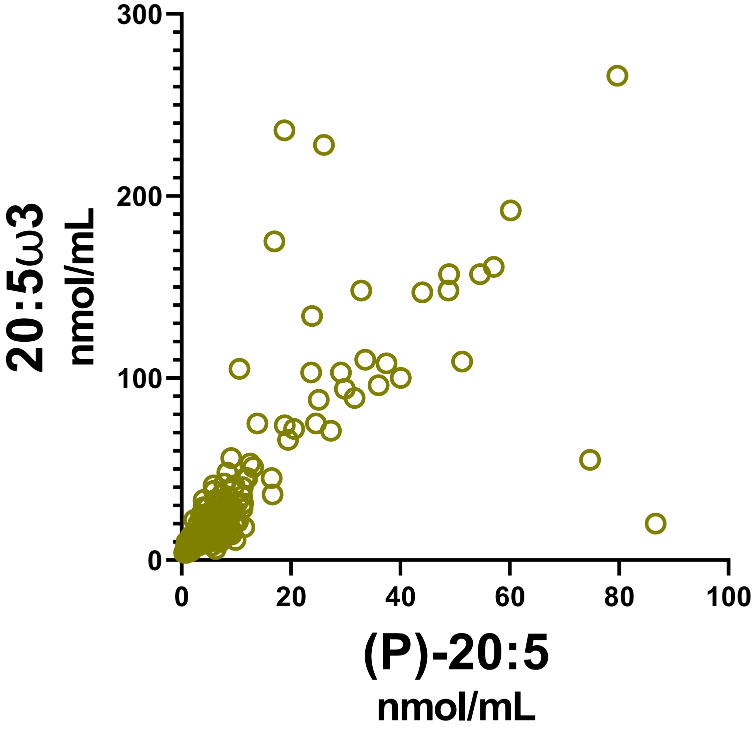
Some species correlate with the corresponding RBC fatty acid

Docosahexaenoic acid (DHA)



$r_s = 0.6803$
$p < 0.0001$

Eicosapentaenoic acid (EPA)



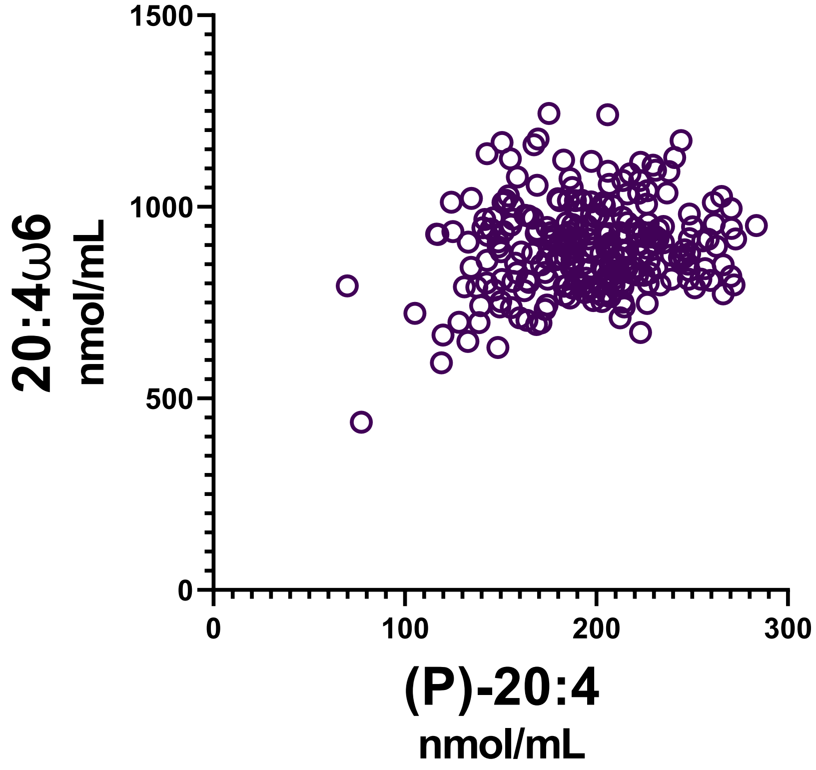
$r_s = 0.7974$
$p < 0.0001$

De Biase, unpublished

While other species do not correlate

- Plasmalogens carrying displayed low inter-individual variability and no correlation to ARA
  - » Inter-individual variability ≤ 20%

Arachidonic acid

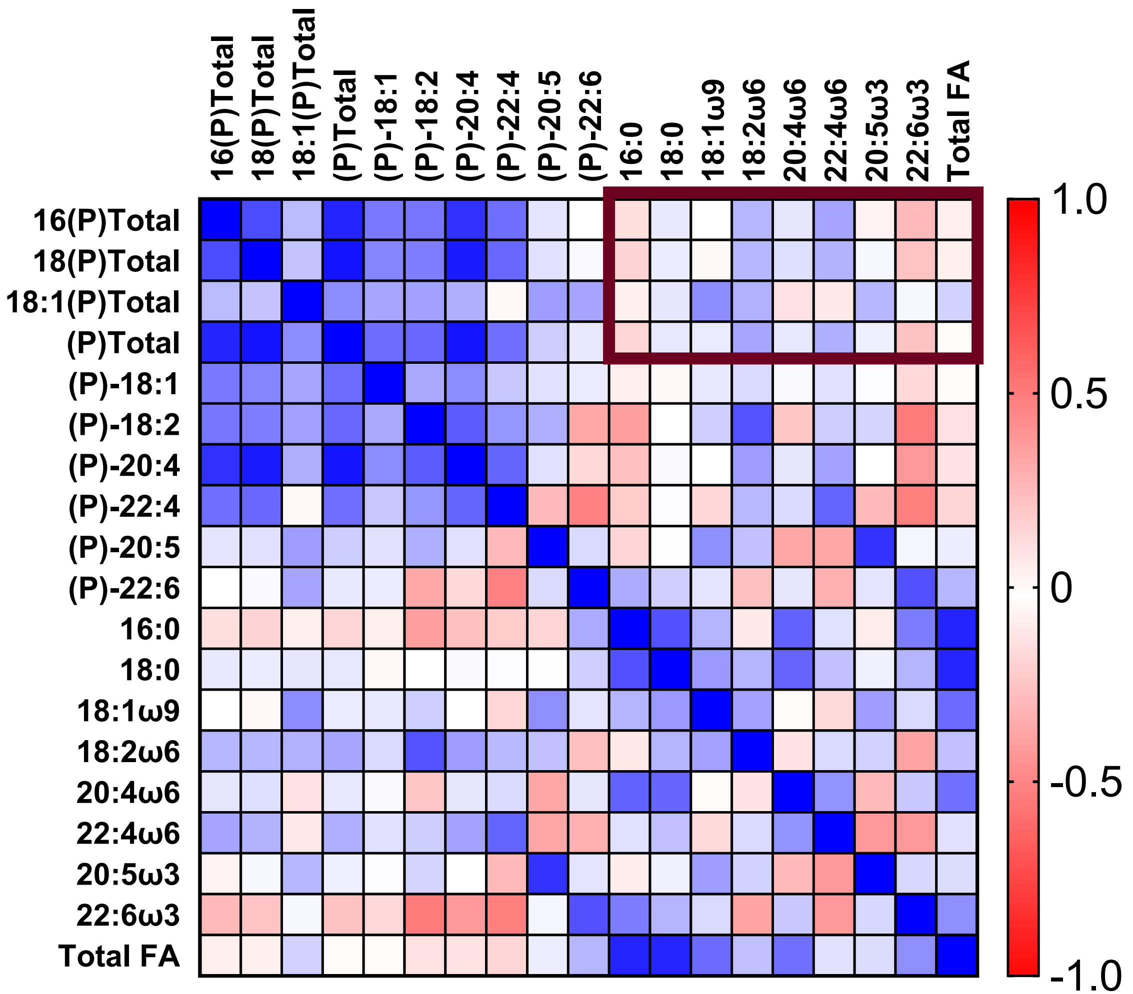


r_s = 0.0956
p = 0.1318

De Biase, unpublished

However, overall cumulative values do not correlate with fatty acids

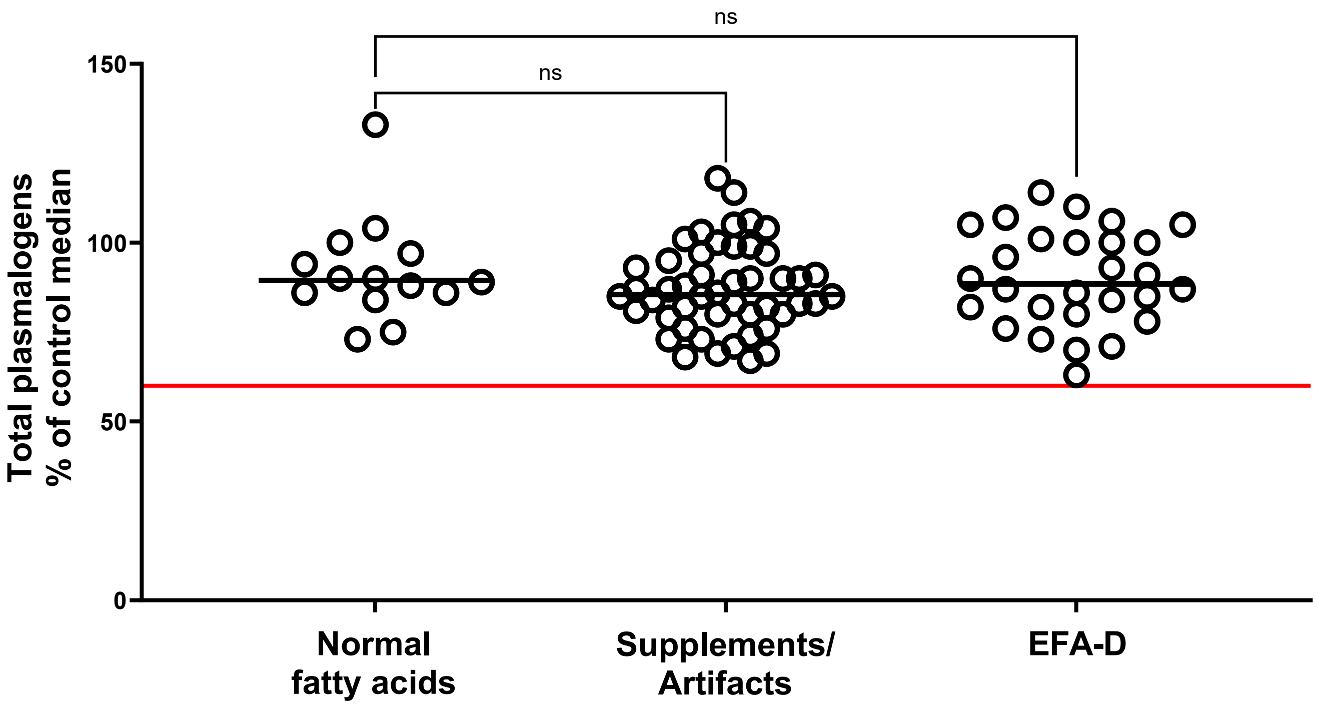
- Nonparametric Spearman rank correlations ranged -02772 to 0.4461, with most showing no correlation
  - » Total C18:1 plasmalogens showed weak positive correlation (r_s=0.4461) to oleic acid (18:1ω9)



De Biase, unpublished

Plasmalogens are not significantly lower in patients with essential fatty acid deficiency

- Plasmalogens and fatty acids (FA) were measured in RBCs patients referred for clinical FA testing



» Red line indicates 60% of the control median

» Significance determined using non-parametric Kruskal-Wallis test; *p*-values adjusted for multiple comparisons

De Biase, unpublished

Conclusions

- Plasmalogens are a unique class of glycerophospholipids that play an essential role in cell membranes
- Impaired plasmalogen biosynthesis is characteristic of rhizomelic chondrodysplasia punctata, but also a prominent feature in Zellweger spectrum disorders
- Plasmalogen deficiency is traditionally detected in red blood cells by gas chromatography mass spectrometry

Conclusions

- We have developed a robust and easy-to-implement method to quantify intact ethanolamine plasmalogens in red blood cells, and established age-specific reference intervals
- We have demonstrated clinical utility of our method in diagnosing inborn errors of plasmalogen synthesis using RBCs from patients and from an animal model of RCDP
- Fatty acid levels impact the abundance of some individual plasmalogen species. However, RBC plasmalogens by LC-MS/MS were not significantly affected by EFA deficiency

Acknowledgements

- Ann B. Moser

Kennedy Krieger Institute, Baltimore, MD, USA

- Nancy E. Braverman
- Wei Cui

Research Institute of the McGill University, Montreal, Quebec, Canada

- Tatiana Yuzyuk
- Lorenzo D. Botto
- Stacey L. Cole
- Malgorzata Miller

University of Utah School of Medicine, Salt Lake City, UT, USA

- Lauren M. Zuromski

ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT, USA

ARUP LABORATORIES

HEALTH
UNIVERSITY OF UTAH

ARUP is a nonprofit enterprise of the University of Utah and its Department of Pathology.

Questions?

irene.de-biase@aruplab.com