DON FREDRICKSON

LIPID RESEARCH CONFERENCE

September 3-5, 2025

# Fredrickson Lipid Research Conference

Milwaukee, WI

Institutional Host:

**Medical College of Wisconsin**

**Versiti Blood Research Institute**

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# DON FREDRICKSON
# LIPID RESEARCH CONFERENCE

## *Lipid Metabolism, Lipoproteins, and Atherosclerosis*

**September 3-5, 2025**

**Medical College of Wisconsin**
**Versiti Blood Research Institute**
**8701 Watertown Plank Rd**
**Milwaukee, WI**

**Sessions in *Bolger Auditorium, HRC Building, H1400***
**Welcome Reception, Poster Session and**
**Lunch in *Alumni Center, MEB Building, M1060***

## Wednesday, September 3

**3:00 – 4:00 pm**

- **Bus loops from Residence Inn Milwaukee West to Conference**
- **Registration | *Health Research Center Lobby***

**4:00 pm** **Welcome**
**Alan Mast, MD, PhD, Ze Zheng, PhD and Mary Sorci Thomas, PhD**
Medical College of Wisconsin & Versiti Blood Research Institute

**4:15 pm** **Session 1**

**Genetic determinants of lipids in CVD**

**Chair: Alison Kohan, PhD** | University of Pittsburg

4:15 pm **Iftikhar J. Kullo, MD |** Mayo Clinic
*Investigating biomarkers of cardiovascular risk*

4:45 pm **Judith Simcox, PhD |** University of Wisconsin Madison
*Inter-organ communication through lipids in varied populations*

***Selected Abstract Presentations***

5:15 pm **Karthickeyan Chella Krishnan, PhD** | University of Cincinnati
*Genetic regulation of hepatocyte inter-organelle crosstalk through APOE Isoforms in MASLD*

5:30 pm **Meredith Campbell** | University of Kentucky
*The impact of highly effective modulator therapies (HEMTs) on the ABCG5 ABCG8 sterol transporter*

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## Wednesday, September 3, continued

**5:45 pm** **Break**

**6:00 pm** **Welcome Reception and Judged Poster Session**

- **Sponsored by Medical College of Wisconsin Endocrinology Division and Cardiovascular Research Center**

  Odd number posters judged from 6:00 – 7:00 pm
  Even number posters judged from 7:00 – 8:00 pm

**8:00 – 8:45 pm**

- **Bus loops from Medical College of Wisconsin to Residence Inn Milwaukee West**

## Thursday, September 4

**7:00 – 8:00 am**

- **Breakfast Meet and Greet | *Hotel Lobby***

**8:00 – 8:30 am**

- **Bus loops from Residence Inn Milwaukee West to Conference**

**8:30 am** **Session 2**

**Lipoprotein structure and omics**

**Chair: Sean Davidson, PhD |** University of Cincinnati

8:30 am **Anna Schwendeman, PhD** | University of Michigan
*Phospholipid composition of HDL*

9:00 am **Chieko Mineo, PhD** | University of Texas Southwestern Medical Center
*SR-BI in cardiometabolic health and disease*

***Selected Abstract Presentations***

9:30 am **Lingshuang Wu** | Stony Brook University
*A spastic paraplegia-related DDHD2 functions as a lipase and transacylase that can remodel the acyl-chains of triglycerides*

9:45 am **Cazza Czerniak** | Medical College of Wisconsin/Marquette University
*The modulatory role of lipoproteins on magnesium biocorrosion*

**10:00 am** **Break**

# Thursday, September 4, Continued

## 10:15 am Session 3

### Larry Rudel Award Lectures

**Chairs: Ryan Temel, PhD** | University of Kentucky
**Mike Widlansky, MD, MPH** | Medical College of Wisconsin

10:15 am **Isabella James** | University of Wisconsin
*Plasma ceramides regulate energy expenditure in brown adipose tissue*

10:30 am **Clairity Voy** | University of Kentucky
*Lipoproteins are susceptible to proteolytic modifications that may alter their atherogenicity*

10:45 am **Allison Paschack** | Cleveland Clinic
*Acute ethanol exposure improves cholestatic liver injury in the humanized Cyp2c70-/- mouse model: serendipity uncovers novel pathways promoting fibrosis resolution*

## 11:00 Virgil Brown Lecture

**Chair: Roy Silverstein, MD** | Medical College of Wisconsin

**David Ginsburg, MD** | University of Michigan
*Cargo receptors in the ER: from clotting factors to cholesterol regulation*

## 11:45 am Lunch

12:00 pm Special Guest Presentation

**Introductions: Gissette Reyes-Soffer, MD** | Columbia University
**Alan Daugherty, PhD, DSc** | University of Kentucky
**Henry Ginsberg, MD** | Columbia University
*Honoring Lifetime Achievements*

## 12:45 Break

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# Thursday, September 4, Continued

## 1:00 pm Session 4

## Immune cell lineage/immunity and lipids in CVD

**Chair: Edward Benjamin Thorp, PhD** | Northwestern University

1:00 pm **Jesse Williams, PhD |** University of Minnesota
*Tissue specific functions of lipid associated macrophages (LAMs)*

1:30 pm **Sean Davies, PhD** | Vanderbilt University
*The role of NAPE-PLD in the regulation of macrophage function and atherosclerosis*

***Selected Abstract Presentations***

2:00 pm **Garrett Anspach** | University of Kentucky
*Liver-specific deletion of carnitine palmitoyltransferase 1a promotes tumorigenesis in a mouse model of obesity-driven hepatocellular carcinoma*

2:15 pm **Anahita Ataran, MD** | Washington University in St. Louis
*Lysosomal acid lipase regulates mitochondrial metabolism and cardiac growth response*

## 2:30 pm Break

## 2:45 pm Session 5

## Microvascular, adipose and lipids

**Chair: Irena Levitan, PhD** | University of Illinois, Chicago

2:45 pm **Rana Gupta, PhD |** Duke University
*Adipose tissue expansion and remodeling in cardiometabolic diseases*

3:15 pm **Yuwei Jiang, PhD** | University of Illinois Chicago
*Adipose stem cells in obesity and comorbidities*

***Selected Abstract Presentations***

3:45 pm **Elizabeth Poad** | University of Wisconsin-Madison
*The cobalamin trafficking protein Mmadhc is a modifier of Pparg-driven adipocyte differentiation*

4:00 pm **Sei Higuchi, PhD** | St. John's University
*16a-hydroxylation of bile acid enhances fatty acid oxidation and decreases lipid accumulation in the hepatocytes through PPARa activation*

## 4:15 pm Break

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# Thursday, September 4, continued

**4:30 pm Session 6**

**Novel perspectives in lipid research**

**Chair: Ada Weinstock, PhD** | Chicago University

4:30 pm **Jaume Amengual, PhD |** University of Illinois Champagne Urbana
*Carotenoids and vitamin A in lipid metabolism*

5:00 pm **Bishuang Cai, PhD |** UCLA
*Endocytic membrane trafficking in lipid metabolism*

5:30 pm **Alan Attie, PhD** | University of Wisconsin Madison
*Genetic Drivers of diet-dependent lipid and mRNA Splicing Phenotypes*

**6:15 pm – 8:30 pm - Lakefront Brewery Networking Dinner**

- **Sponsored by University of Kentucky Saha Cardiovascular Research Center**
- **Busses depart at 6:15 from in front of HRC and return to Residence Inn Milwaukee West**

# Friday, September 5

**8:00 – 8:30 am Bus loops from Residence Inn Milwaukee West to Conference**

**8:30 am Session 7**

**Lipids in atherothrombosis and hemostasis**

**Chair: Tessa Barrett, PhD | New York University**

8:30 am **Sidney W. Whiteheart, PhD** | University of Kentucky
*Platelet membrane trafficking and lipids*

9:00am **Woosuk Steve Hur, PhD** | University of North Carolina at Chapel Hill
*Plasminogen Activation System in the Pathogenesis of Obesity and Metabolic Syndrome*

***Selected Abstract Presentations***

9:30 am **Melissa Jauquet, PhD** | University of Kentucky
*When vWF Is Stressed, Lipoproteins and Protein S Are Never the Same*

9:45 am **Ziyu Zhang, MD** | Versiti Blood Research Institute
*FVIII-associated LDL circulates longer and participates in blood clot formation*

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# Friday, September 5, continued

**10:00 am** **Break**

**10:15am** **Keynote Address**

**Ira Tabas, MD, PhD** | Columbia University

*Efferocytosis and inflammation resolution in cardiometabolic diseases*

**11:00 am** **Trainee Research Blitz**

**Chair: Ze Zheng | Versiti Blood Research Center**

**Mary Sorci Thomas | Medical College of Wisconsin**

**Bethany Coleman**
Davidson Lab | University of Cincinnati

**Amparo Heliodora Blanco Cirer**
Amengual Lab | University of Illinois

**Sumita Dutta**
Brown Lab | Cleveland Clinic

**Alexander Rocksvold**
Sorci-Thomas Lab | Medical College of Wisconsin

**Nour Mouannes**
Brown Lab | Cleveland Clinic

**Alexis Smith**
Zheng Lab | Versiti Blood Research Center

**Grace Hamilton**
Brown Lab | Cleveland Clinic

**Wafa'a Hajeer**
Amengual Lab | University of Illinois

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# Friday, September 5, continued

**Mirza Beg**
Sorci-Thomas Lab | Medical College of Wisconsin

**Hortense Triniac**
Zheng Lab | Versiti Blood Research Center

**Olivia Hage**
Gordon Lab | University of Kentucky

**Maya Rodriguez**
Zheng Lab | Medical College of Wisconsin

**Donald Molina**
Amengual Lab | University of Illinois

**Mindy Kim**
Zheng Lab | Versiti Blood Research Center

**Minzhi Yu**
Schwendeman Lab | University of Michigan

**Afsin Malik**
Higuchi Lab | St. John's University

**Kaihua Liu**
Biddinger Lab | Harvard Medical School

**11:30 am Awards Ceremony**

- **Presentation Awards provided by MCW Graduate School**
- **Abstract Achievement Awards provided by Versiti Blood Research Institute**

**11:45 am – Closing Remarks**

# Event Sponsors

MEDICAL COLLEGE OF WISCONSIN

Cardiovascular Center

MEDICAL COLLEGE® OF WISCONSIN

DEPARTMENT OF MEDICINE

ENDOCRINOLOGY AND MOLECULAR MEDICINE

versiti™ Blood Research Institute

MCW GRADUATE SCHOOL

# Event Sponsors


National Heart, Lung, and Blood Institute

Saha Cardiovascular Research Center

Frontiers in Physiology

A journal by frontiers

# Event Organizers

## *2025 Conference Organizers*

Ze Zheng (Co-chair)

Mary Sorci-Thomas (Co-chair)

Daisy Sahoo

Yiliang Chen

Wen Dai

Gage Stuttgen

# Networking Opportunities

The Fredrickson Lipid Research Conference is known for its focus on trainees. The following events are built into the schedule to ensure all trainees have the ample networking opportunities.

**Wednesday, September 3**

**6:00 pm**

**Welcome Reception and Poster Session**

*Alumni Center*

**Thursday, September 4**

**7:00 am**

**Breakfast Meet and Greet**

*Hotel Lobby*

**6:15 pm**

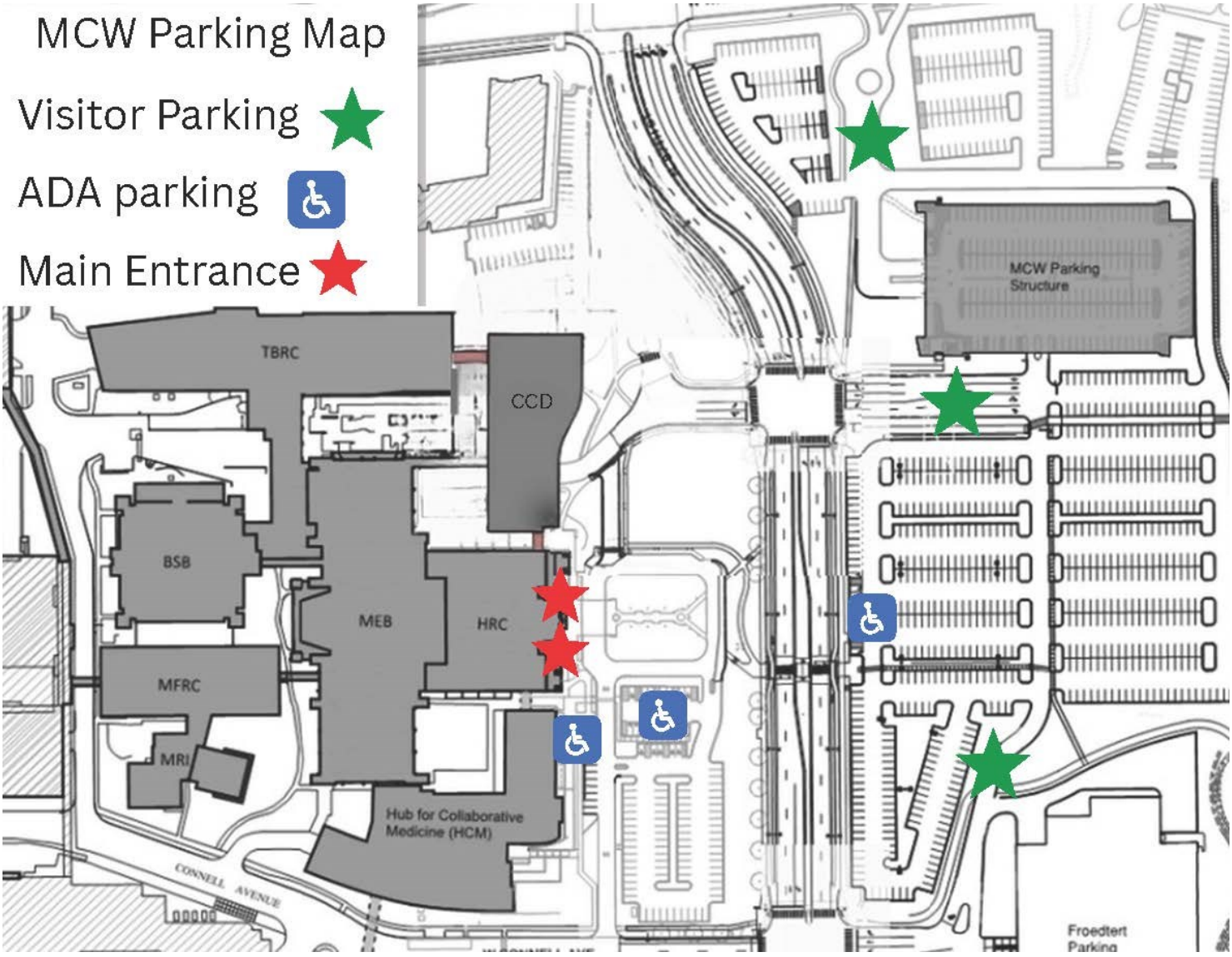
**Lakefront Brewery Networking Dinner**

*Busses depart from in front of Health Research Center*

# Parking Map

Detailed information on parking can be found at

https://www.mcw.edu/about-mcw/maps-directions



# Featured Speakers

## *Virgil Brown Lecture*

## David Ginsburg, MD

James V Neel Distinguished University Professor of Internal Medicine and Human Genetics
Warner-Lambert/Parke-Davis Professor of Medicine
Professor of Internal Medicine
Professor of Human Genetics
Professor of Pediatrics
Research Professor, Life Sciences Institute
University of Michigan

## *Fredrickson Keynote Address*

## Ira Tabas, MD, PhD

Richard J. Stock Professor
and Vice-Chair of Research
Department of Medicine
Professor of Pathology & Cell Biology (in Physiology and Cellular Biophysics)
Columbia University Medical Center

## *Lunchtime Speaker*

## Henry Ginsberg, MD

Herbert and Florence Irving Professor of Medicine
Director Emeritus, Irving Institute for Clinical & Translational Research
Columbia University

# Invited Speakers

**Jaume Amengual, PhD** | University of Illinois Champagne Urbana

**Alan Attie, PhD** | University of Wisconsin Madison

**Bishuang Cai, PhD** | UCLA

**Sean Davies** | Vanderbilt University

**Rana Gupta, PhD** | Duke University

**Woosuk Steve Hur, PhD** | University of North Carolina at Chapel Hill

**Yuwei Jiang, PhD** | University of Illinois Chicago

**Iftikhar J. Kullo, MD** | Mayo Clinic

**Chieko Mineo, PhD** | UT Southwestern Medical Center

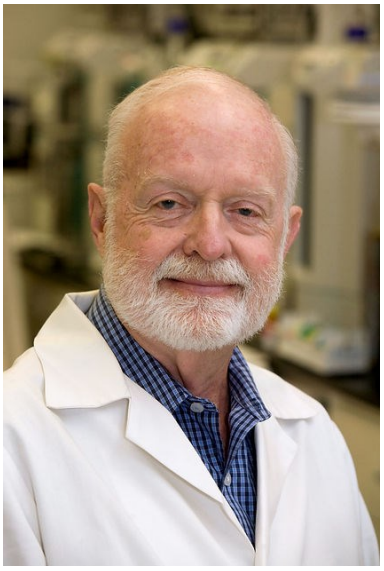
**Anna Schwendeman, PhD** | University of Michigan

**Judith Simcox, PhD** | University of Wisconsin Madison

**Sidney W. Whiteheart, PhD** | University of Kentucky

**Jesse Williams, PhD** | University of Minnesota

# Larry Rudel Award



Lawrence 'Larry' L. Rudel, PhD (1941-2019) was a renowned lipid scientist and biochemist who during his 50-year career published 243 peer-reviewed manuscripts, 19 book chapters, and delivered 164 scientific presentations. He joined the faculty at Wake Forest University School of Medicine (WFUSM) in 1973 and retired from the same institution in 2018 as Professor of Internal Medicine-Section on Molecular Medicine and Biochemistry. Larry had a distinguished record of service to the scientific community which included serving on the Editorial Board of the Journal of Lipid Research for 36 years, as Chair of the Metabolism Study Section for the National Institutes of Health (NIH), and as Chair of the Arteriosclerosis, Thrombosis and Vascular Biology (ATVB) Council of the American Heart Association (AHA). Most importantly he directly mentored seventeen PhD students, three MS students, and twenty-nine postdoctoral fellows, and indirectly impacted countless trainees and faculty by fostering an extremely rich learning environment.

To honor his legacy of trainee mentoring and development, the Larry Rudel Lipid Sciences Award Fund has been established to support a national award program that recognizes trainee excellence in lipid research. The Lipid Sciences Award is given annually at the Fredrickson Lipid Research Conference ( to an eligible graduate student or post-doctoral scholar with the best abstract presentation. The Saha Cardiovascular Research Center at The University of Kentucky is the administrative home for both the conference and the award.

## Larry Rudel Award Lectures

**Isabella James** | University of Wisconsin
*Plasma ceramides regulate energy expenditure in brown adipose tissue*

**Clairity Voy** | University of Kentucky
*Lipoproteins are susceptible to proteolytic modifications that may alter their atherogenicity*

**Allison Paschack** | Cleveland Clinic
*Acute ethanol exposure improves cholestatic liver injury in the humanized Cyp2c70-/- mouse model: serendipity uncovers novel pathways promoting fibrosis resolution*

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*Genetic regulation of hepatocyte inter-organelle crosstalk through APOE Isoforms in MASLD*

**Meredith Campbell |** University of Kentucky
*The impact of highly effective modulator therapies (HEMTs) on the ABCG5 ABCG8 sterol transporter*

**Lingshuang Wu** | Stony Brook University*A spastic paraplegia-related DDHD2 functions as a lipase and transacylasethat can remodel the acyl-chains of triglycerides*

**Cazza Czerniak** | Medical College of Wisconsin/Marquette University
T*he modulatory role of lipoproteins on magnesium biocorrosion*

**Garrett Anspach** | University of Kentucky
*Liver-specific deletion of carnitine palmitoyltransferase 1a promotes tumorigenesis in a mouse model of obesity-driven hepatocellular carcinoma*

**Anahita Ataran, MD** | Washington University in St. Louis
*Lysosomal acid lipase regulates mitochondrial metabolism and cardiac growth response*

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S**ei Higuchi, PhD** | St. John's University
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*When vWF Is Stressed, Lipoproteins and Protein S Are Never the Same*

**Ziyu Zhang, MD** | Versiti Blood Research Institute
*FVIII-associated LDL circulates longer and participates in blood clot formation*

# DON FREDRICKSON LIPID RESEARCH CONFERENCE

We are very excited to be hosting the 2025 Fredrickson Lipid Research Conference in Milwaukee! To prepare, we've started making decorations for the meeting. Since this will be in the fall, we have created a lipopumpkin!

Can you help us decorate it? We have already added the name of the conference. ***See if you can add the rest of these relevant abbreviations, terms, names, etc. to the lipopumpkin grid. When you are done, the lighted squares (read top-to-bottom) will tell you what we were thinking about when sending out this puzzle.***

**3-LETTERS**
HDL
IDL
NMR
VWF

**4-LETTERS**
APO(A)
APO B
APO E
ATVB
BEER
CETP
LDLR
MTTP
PCSK
VLDL

**5-LETTERS**
LIVER
OX. LDL
SREBP

**6-LETTERS**
ANGPTL
HMG-COA
LEPTIN
LIPASE
MYELIN
STATIN

**7-LETTERS**
ADIPOSE
DENSITY
GHRELIN
INSULIN
OBESITY

**8-LETTERS**
FIBRATES
FOAM CELL
FOIE GRAS
GLUCAGON
KARE BERG

**9-LETTERS**
EZETIMIBE
FATTY ACID
STEATOSIS

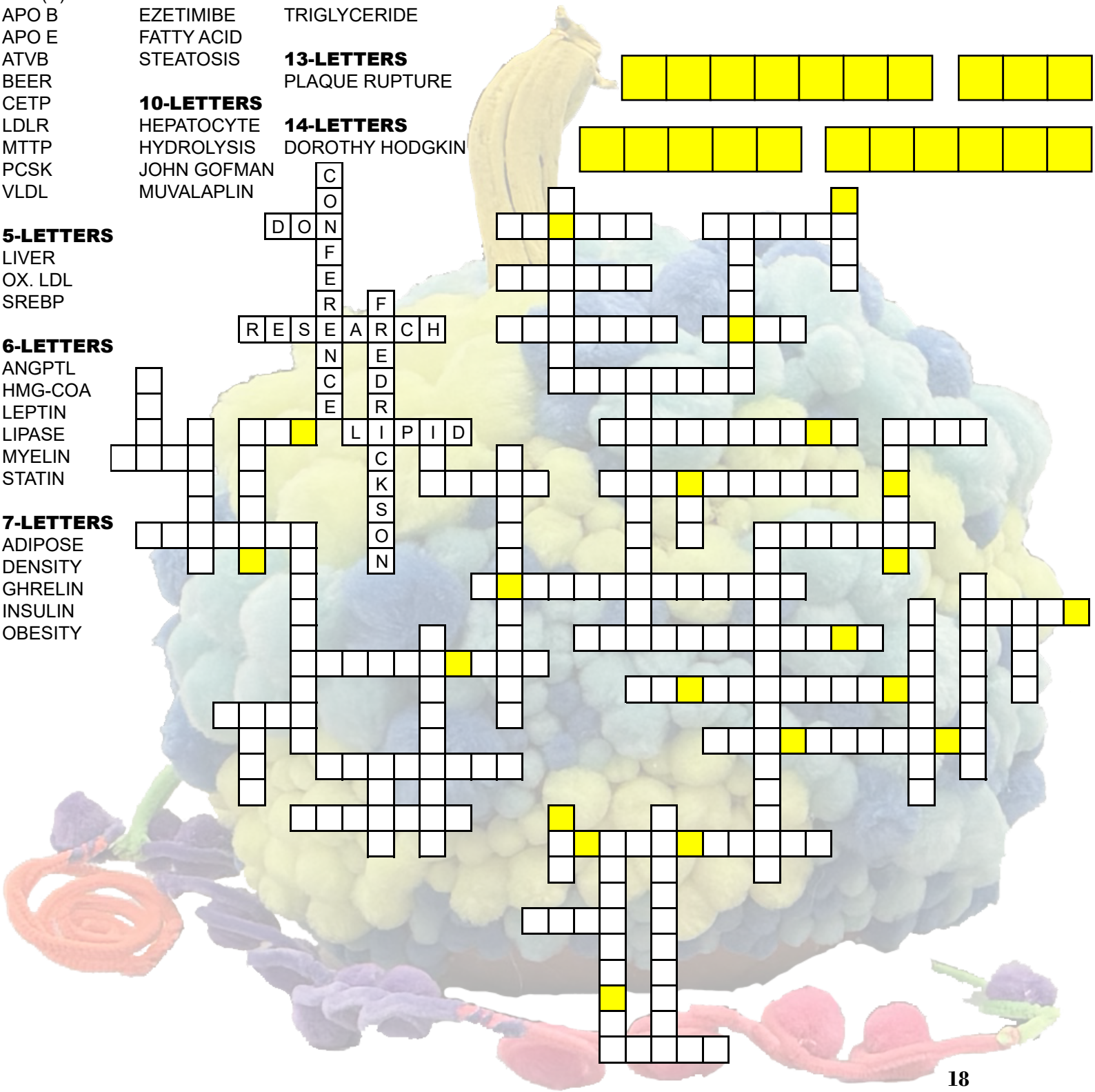
**10-LETTERS**
HEPATOCYTE
HYDROLYSIS
JOHN GOFMAN
MUVALAPLIN

**11-LETTERS**
CHOLESTEROL
CHYLOMICRON
SEMAGLUTIDE
WESTERN DIET

**12-LETTERS**
PANCREATITIS
TRIGLYCERIDE

**13-LETTERS**
PLAQUE RUPTURE

**14-LETTERS**
DOROTHY HODGKIN



# Poster Judges

***Thank you to the faculty that served as our poster judges.***

**Jaime Amengual**
**Sudha Biddinger**
**Bishuang Cai**
**Yiliang Chen**
**Alan Daugherty**
**Sean Davidson**
**Brandon Davies**
**Henry Ginsberg**
**David Ginsburg**
**Scott Gordon**
**Greg Graf**
**Rana Gupta**
**Nate Helsley**
**Steve Hur**
**Jay Jerome**
**Alison Kohan**
**Irena Levitan**
**Diego Lucero**
**Chieko Mineo**
**Joshua Muia**
**Deb Newman**
**Anna Schwendeman**
**Daisy Sahoo**
**Daochun Sun**
**Ira Tabas**
**Ryan Temel**
**Ed Thorp**
**Ada Weinstock**
**Sidney Whiteheart**
**Jeremy Wood**
**Laura Woollett**
**Xu Xiao**

# Poster Presentations

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**Michelle Zhu**
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## Lysosomal acid lipase regulates mitochondrial metabolism and cardiac growth response

Anahita Ataran[1], Ahmed Diab[1], Ken Bedi[2], J. Eduardo Rame[3], Marco Sardiello[1], Clair Crewe[1], Kenneth B. Margulies[2], Irfan J. Lodhi[1], Ali Javaheri[1,4]

1. Washington University School of Medicine, St. Louis, MO, USA
2. Cardiovascular Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
3. Jefferson University, Philadelphia, PA
4. John Cochran Veterans Affairs Hospital, St. Louis, MO

### Abstract

**Introduction:** Heart failure is characterized by profound depletion of bioenergetic lipid substrates. Lysosomal acid lipase (LAL) is essential for lipophagy, a process crucial for the breakdown of lipid droplets (LD)s in lysosomes. Herein, we employed a multi-model approach combining human tissue analysis, in vitro cardiomyocyte experiments, and murine models of cardiac injury to identify the homeostatic role of LAL in cardiac growth and metabolism.

**Methods:** We analyzed human myocardial tissues from patients with and without heart failure (NICM vs Donor) to assess LAL expression, lipidomic changes, and LD homeostasis. Neonatal rat cardiomyocytes (NRCMs) were treated with Lalistat-2, a LAL inhibitor, to assess effects on mitochondrial respiratory function via Seahorse XF analysis. Utilizing whole-body ($LAL^{KO}$) and cardiomyocyte-specific LAL knockout mice ($LAL^{CMKO}$), we characterized cardiac mass and function after hypertrophic (transverse aortic constriction, TAC) and atrophic (doxorubicin, Dox) stressors.

**Results:** In human NICM, LAL expression was increased in cardiomyocytes, accompanied by widespread reductions in lipid species and LD proteins. Inhibition of LAL with Lalistat-2 in NRCMs caused a significant, dose-dependent reduction in mitochondrial uncoupled respiration. $LAL^{KO}$ mice developed lipid accumulation and cardiac atrophy, with reductions in heart weight, LV mass, and cardiomyocyte size, and failed to mount a hypertrophic response to TAC, with impaired systolic function and increased myocardial fibrosis. Both $LAL^{KO}$ and $LAL^{CMKO}$ hearts were more vulnerable to Dox-induced atrophy, associated with diminished mTOR phosphorylation and increased nuclear localization of transcription factor EB (TFEB). In vitro studies confirmed that LAL inhibition prompted nuclear translocation of TFEB. In wild-type mice, HFD (vs. standard chow) improved survival after TAC, improved EF and LV mass, and decreased cardiac fibrosis with Dox treatment. However, in $LAL^{KO}$ mice, HFD failed to mitigate Dox-induced cardiotoxicity and fibrosis.

**Conclusion:** Our findings identify LAL as a key metabolic regulator of lipid homeostasis, mitochondrial bioenergetics, and cardiomyocyte growth under stress. In models of heart failure, LAL facilitates the adaptive use of lipid substrates via lipophagy. We show that exogenous dietary lipids improve outcomes in heart failure models. In turn, without LAL, dietary lipids fail to confer protection, underscoring the enzyme's essential role in nutrient-stress adaptation.

# Tissue-type plasminogen activator enhances apolipoprotein B degradation in hepatocytes, further promoting fibrinolysis

*Hortense Triniac[1,2], Wen Dai[1,2], Ze Zheng[1,2]*

*[1] Versiti Blood Research Institute, Milwaukee, WI*
*[2] Department of Medicine, Medical College of Wisconsin, Milwaukee, WI*

Background: The development of atherosclerosis is an established risk factor for ischemic stroke and involves the arterial accumulation of apolipoprotein B (apoB)-containing lipoproteins. The main source of atherogenic apoB are hepatocytes via the secretion of very-low-density lipoproteins (VLDL) into the bloodstream, where it is then hydrolyzed into low-density lipoproteins (LDL). Hepatocytes also synthesize tissue-type plasminogen activator (tPA) (Zheng et al., 2019) and contribute to fibrinolysis, the primary mechanism breaking down blood clots. tPA exists under two isoforms: it is synthesized under its single-chain form (sc-tPA) and can be cleaved by other proteases into its two-chain form (tc-tPA). Our group demonstrated that tPA directly binds to apoB in hepatocyte endoplasmic reticulum (ER), thereby blocking its lipidation and VLDL assembly (Dai et al., 2023). As degradative mechanisms play an important role in maintaining lipid homeostasis, excessive apoB and/or poorly lipidated VLDL will undergo intracellular degradation through lysosomal or ER-associated degradation (ERAD) (Conlon et al., 2016; Liao et al., 2003; Tiwari and Siddiqi, 2012). Moreover, the *N*-terminal region of apoB directly binds to the lysine-binding site (LBS) located on tPA's Kringle 2 domain. tPA associates with fibrin through the same LBS, therefore we hypothesize that apoB represents a potential competitive mechanism with fibrinolysis in the vascular compartment. However, the role of hepatocyte tPA on the fate of un-fully lipidated apoB and non-secreted lipids are unknown.

Aim: To investigate the role of hepatocyte tPA in intracellular apoB degradation and lipid accumulation in the livers, and the consequences of tPA-apoB interaction on fibrinolysis in the circulation.

Material and methods: We used mouse models with silenced or overexpressed tPA in hepatocytes fed with a normal diet (chow diet, CD), a high-cholesterol, high-fat diet (western diet, WD), or a high-fat diet (diet-induced obesity, DIO). Additionally, human primary hepatocytes with silenced tPA expression and human purified LDL were studied. We evaluated triglyceride level and performed histology analysis to determine steatosis in mouse livers. The expression of lysosomal trafficking- and autophagy-related proteins were assessed by western blot. Solid-phase binding assay was used to explore tPA binding affinity with LDL.

Results:

Despite an inhibitory effect on VLDL lipidation, overexpression of tPA did not lead to lipid accumulation, evidenced by unchanged liver triglyceride levels. Additionally, no significant changes in steatosis observed in hepatic tPA-silencing mouse livers.

The Golgi-lysosome trafficking receptor Sortilin 1 (SORT1), known for targeting apoB to lysosome for degradation, was upregulated in mouse livers overexpressing tPA using AAV8-TBG-tPA. Consistently, silencing tPA in human primary hepatocytes decreased SORT1 expression. Furthermore, silencing hepatocyte tPA altered liver autophagy in both WD and CD-fed mice. This blockage is characterized by the absence of changes in autophagosome formation, reflected by light-chain 3-II (LC3-II), and a decreased autophagosome degradation, evidenced by the accumulation of the cargo protein p62. In hepatocytes overexpressing tPA, p62 was the most abundant tPA interacting protein identified by mass spectrometry. These results suggest that tPA enhances apoB lysosomal degradation via an autophagic mechanism.

Moreover, we determine that the main isoform present in human primary hepatocytes is sc-tPA, whereas human plasma contains mostly tc-tPA. The tc-tPA isoform binds to LDL with a higher affinity than sc-tPA, suggesting an effective binding of the most abundant circulating form of tPA, tc-tPA, to the most abundant lipoprotein in the bloodstream, LDL. This interaction represents a potential new role for apoB-lipoproteins as inhibitors of fibrinolysis by competing with the tPA-fibrin interaction. Therefore, hepatocyte-derived tPA potentially improve fibrinolysis by inhibiting VLDL secretion and increasing the bioavailability of plasma tPA.

Conclusion: These data reveal a new role for hepatocyte tPA which reduces plasma LDL levels by enhancing autophagy-mediated apoB degradation before its secretion, subsequently leading to reduced circulating LDL and further improves fibrinolysis due to apoB's potential in sequestering bioavailable tPA. This multi-level interplay represents a new protective function for hepatocyte tPA in atherosclerotic cardiovascular diseases, such as ischemic stroke, through reducing LDL and improving fibrinolysis.

# The Hepatocyte Lysosomal Response to Dietary Stress

**Authors**: *Kaihua Liu*[1], Stuart Adamson[1], Alison Sears[1], George Pengo[1], Alex Panov[2], Wade Harper[2], Ryan Temel[3], Pamela Tuma[4], Gregory Graf[3], Sudha Biddinger[1]

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Lysosomes help maintain cellular homeostasis by degrading and assembling both extracellular and intracellular cargo to provide substrates for anabolic metabolism; however, when cargo load exceeds the lysosome's ability to handle it, lysosomal dysfunction can occur. Here, we aimed to understand how lysosomes in the hepatocyte respond to nutrient stress from a western diet. Although hepatocytes are the major cell type in the liver, they only contribute to approximately 60% of the liver total lysosomal pool. By taking advantage of the Lyso-Tag mice, in which hepatocyte lysosomes are genetically tagged, we were able to rapidly isolate hepatocyte lysosomes for the first time. Through metabolomic, lipidomic and proteomic analysis, we found that (1) western diet led to a ~3-fold increase in lysosome-associated cholesterol despite only a ~2-fold increase in total hepatic cholesterol; (2) the cholesterol efflux transporters ABCG5/ABCG8 were present in the lysosome and were upregulated with western diet. Furthermore, confocal microscopy showed that lysosomes, which were dispersed throughout the hepatocytes of chow-fed mice, were concentrated near canalicular membrane with western diet. The canalicular membrane is the site at which cholesterol is excreted into the bile. Taken together, these data suggest a model in which lysosomes offload excess cholesterol in the hepatocyte by shuttling both cholesterol and the cholesterol efflux transporters to the canalicular membrane, promoting the disposal of excess cholesterol into the bile.

**Title: A spastic paraplegia-related DDHD2 functions as a lipase and transacylase that can remodel the acyl-chains of triglycerides**

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**Objective**: Hereditary spastic paraplegia subtype SPG54 is a genetic neurological disorder caused by the mutations in the DDHD2 gene. Excessive lipid droplet accumulation is observed in the brains of SPG54 patients and DDHD2 knockout mice, consistent with DDHD2's reported neutral lipase activity. This study aims to elucidate the substrate specificity of DDHD2 as a lipase towards phospholipids and neutral lipids, and to define its molecular mechanisms to enhance our understanding of this enzyme's function.

**Methods**: We employed protein purification, enzyme activity assays, liposome co-sedimentation assays and liquid chromatography-mass spectrometry (LC-MS) to investigate DDHD2's substrate specificity and enzymatic mechanisms. We also used fluorescence microscopy to visualize the localization of DDHD2 and its variants in difference cell lines.

**Results**: We find recombinant human DDHD2 preferentially hydrolyzes diacylglycerol (DAG) over phospholipids, with a slight preference for DAG over triacylglycerol (TAG). DDHD2 also exhibits transacylase activity, which enables transfer of acyl chains from

triacylglycerols to diacylglycerols and monoacylglycerols to remodel the acyl chains of triglycerides in vitro. A predicted hydrophobic amphipathic helix on DDHD2 is essential for lipid droplet binding in vitro and in cells, and lack of it reduces the enzymatic activity and triglyceride acyl chain remodeling. Adipose triglyceride lipase (ATGL), but not hormone sensitive lipase (HSL), also has transacylation activity and can remodel triglyceride acyl chains, but to a lesser extent than DDHD2. Taken together, this provides evidence that DDHD2 is neutral lipid lipase and transacylase whose broad specificity enables triglyceride acyl-chain remodeling in vitro.

This work was supported by the NIH grant R35GM128666 (M.V.A.), a Sloan Research Fellowship (M.V.A.), a Carol Baldwin award (D.C.), startup funds from the Stony Brook Cancer Center (D.C.), and an American Heart Association Fellowship 23PRE1019634 (L.W.).

# Maintaining Higher Plasma Cholesterol Levels May Contribute to Obesity-Mediated Protection in Sepsis

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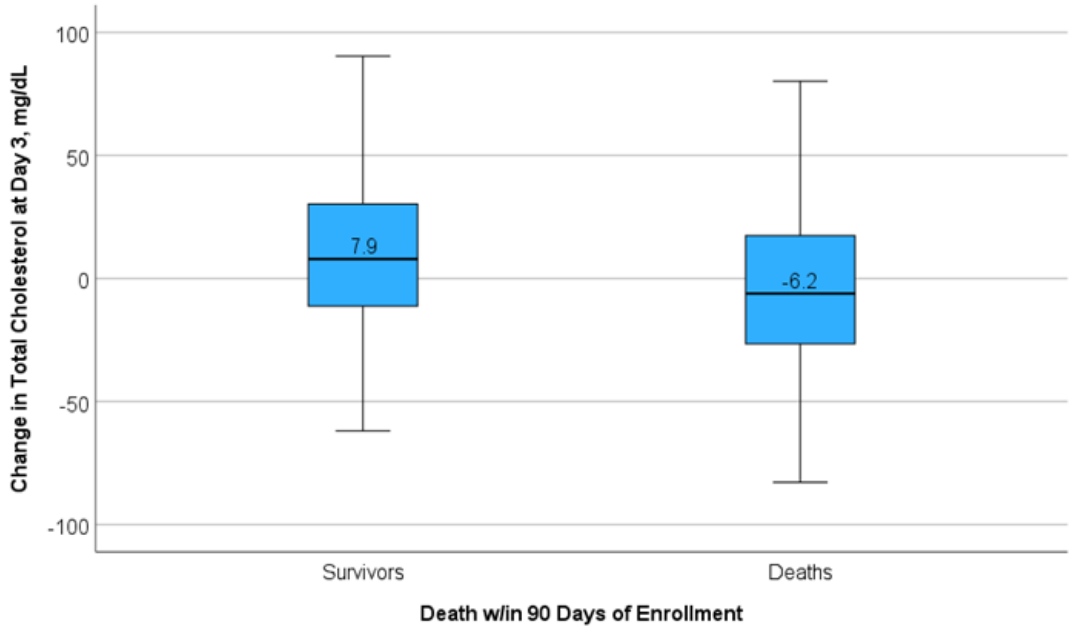
Background: Clinical data indicate that overweight or moderately obese patients have higher sepsis survival rates than those with a normal BMI, a phenomenon known as the "obesity paradox." Hyperlipidemia is believed to contribute to obesity-mediated protection in sepsis; however, limited data is available to support this theory. The objective of this study was to explore the association between obesity status, circulating lipid levels, and their impact on sepsis survival.

Methods: Clinical data and plasma samples from the multicenter "Acute Respiratory Distress Network Studies - Statins for Acutely Injured Lungs from Sepsis" trial were used to conduct a secondary analysis of the effect of obesity on plasma lipid levels and sepsis survival (N=467). One arm of the trial included enteral treatment with the lipid-lowering statin, rosuvastatin. Obesity categories are defined as normal (BMI 18.5 – 25 kg/m$^2$), obese (BMI 30.0 – 39.9 kg/m$^2$), and morbidly obese (BMI ≥ 40 kg/m$^2$). Plasma samples were evaluated for triglycerides (TG), non-esterified fatty acids (NEFA), free glycerol (FG), total cholesterol (TC), and beta-hydroxybutyrate (BOH) at enrollment (Day 0, n=467) and at Day 3 in surviving patients (n=417). The associations between BMI group, lipid levels, change in lipid levels at day 3, and 90-day survival were evaluated by non-parametric Kruskal Wallis test of rank distributions and multivariable Cox Survival Regression analysis.

Results: At enrollment, mean TG, FG, and TC levels were significantly higher with increasing BMI ($p<0.001$, 0.006, and 0.002, respectively). NEFAs showed a trend toward increased levels with higher BMI ($p=0.06$), while BOH levels were not different across BMI groups ($p=0.910$). None of the baseline lipid levels were predictive of survival. However, the change in TC levels from Day 0 to Day 3 strongly predicted death ($p<0.001$) after adjustment for Apache 3 Score, age, obesity class and treatment; with only survivors having an increase at Day 3 (Figure). Rosuvastatin treatment decreased TC levels at day 3 compared to Day 0 ($p<0.001$), independent of obesity status, and this was associated with increased risk of 90-day mortality compared to placebo across BMI groups. Overall, the risk for 90-day mortality was lower in the obese compared to normal BMI (Hazard Ratio 0.82, p=.376), while morbidly obese showed a higher odds ratio for death (1.17, p=.538), although neither reached significance.

Conclusions: Obese patients maintain higher levels of cholesterol during sepsis and moderate obesity improved survival in patients with sepsis, although significance was not achieved in this study that was not powered for this analysis. This study further indicates that maintaining higher cholesterol levels may be advantageous in the setting of sepsis and may account for the survival benefit observed in obese patients.



**Estrogenic Effects on Cardiometabolic Risk in Androgen-Deprived Males Receiving 17-Beta Estradiol**

Ariel Thorson
Molecular Physiology and Biophysics
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**Abstract:**
Estrogens were utilized as an effective adjuvant therapy in the treatment of prostate cancer (PC) in the United States from the 1940s through the late 1980s, until elevated CVD risk led to the discontinuation of this effective adjuvant treatment. Exploiting beneficial estrogenic pathways to improve prostate cancer survival can only be possible once the cause of increased CVD harm is understood, making the study of estrogenic changes to CVD risk factors in males a critical area of research. We sought to characterize estrogenic changes that may be promoting dysglycemia and dyslipidemia in hypogonadal males given estradiol. Using a novel mouse model, we examined glucose metabolism. We constructed this mouse model via gonadectomy and delayed-release estradiol (E2) pellet placement.

Estrogen treatment in hypogonadal XY mice improves insulin sensitivity and glucose tolerance relative to placebo XX mice. XY + E2 mice exhibited similar glucose sensitivity following oral gavage to XX + E2 mice and were significantly improved from that of placebo XX mice. Glucose excursion following oral glucose gavage was significantly higher in placebo males than in estrogen-treated males. XY+E2 mice had significantly lower circulating plasma glucose 15-minutes post-gavage than placebo males. E2-treated XX females had significantly lower insulin following glucose challenge than both XY groups. Hyperglycemic clamp revealed similarity in insulin production between XY and XX E2-treated mice, but differences in glucose infusion rates. Biological sex differences in glucose infusion rates were observed between XY and XX mice, with reduced infusion rates in XY mice.

Glucose uptake by adipose tissue was quantified during the hyperinsulinemic-euglycemic clamp, providing insight into preferential depots for storage of excess energy. Glucose uptake by subcutaneous fat was significantly increased in estrogenized females versus placebo females (Fig 4j). In estrogenized males compared to placebo males, there was a trend toward increased glucose uptake with E2.

Our results indicate that estrogens in XY + E2 mice alter glucose metabolism pathways by altering insulin sensitivity, which can be overcome by increased insulin secretion in response to a glucose challenge. Deposition of excess glucose into adipose depots remains higher in estrogenized XX mice than in XY mice, resulting in reduced benefit of estrogens in improving efficient energy storage in biological males. Further research is necessary to determine the molecular mechanisms by which estrogens alter glucose metabolism differently in biological males and females.

The findings of this foundational work could further our understanding of mechanisms mediating estrogen-induced cardiovascular harm in androgen-deprived men. We hope that future studies will build upon this to identify a target of harmful effects to allow estrogens to work as a highly effective cancer treatment without contributing to CVD mortality.

**VWF Unfolding Alters Protein S Distribution on Lipoproteins and Impairs Anticoagulant Function**

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Background: Accumulating evidence indicates that lipoproteins play a role in thrombosis, including through the direct regulation of procoagulant and anticoagulant activity, though the mechanisms of this effect under physiologic and pathologic conditions are unclear. Von Willebrand factor (vWF) is a circulating mechanosensor, which unfolds under pathologic shear stress, self-associates, and exposes protein binding sites, including for both low-density lipoprotein (LDL) and high-density lipoprotein (HDL). We recently showed that unfolded vWF binds the anticoagulant protein S (ProS) and inhibits ProS activity, and that this effect is amplified by an unknown plasma component. We hypothesize that unfolded vWF sequesters ProS onto circulating lipoprotein particles, impairing ProS function and promoting thrombosis.

Results: Human citrated plasma samples were either vortexed (30 seconds) to mimic shear and induce vWF unfolding, or else left untreated, and plasma lipoprotein particles were isolated by fast protein liquid chromatography (FPLC). Vortexing resulted in a consistent redistribution of cholesterol, marked by a 30% increase in the presence of VLDL-like particles. The absence of changes in ApoB or ApoA1 distribution suggests that the observed increase in VLDL level is likely derived from aggregated LDL. We hypothesized that this aggregation was vWF-mediated. Consistent with this hypothesis, no redistribution occurred when plasma from a patient with Type III von Willebrand Disease, who has no detectable vWF, was subjected to the same procedure.

We next assessed the effect of vWF unfolding on ProS. In the absence of shear, ProS was observed in all lipoprotein fractions, with a majority in the LDL, HDL and free protein ranges. On average, after vortexing, ProS decreased by 16% in HDL and free protein FPLC fractions and increased by 15% and 50% in the LDL and VLDL fractions respectively, although the magnitude of this change varied among individuals. By contrast, protein C, an anticoagulant that functions with ProS, was only detectable as free protein, and showed no redistribution after vortexing. Similarly, total protein distribution (primarily albumin), as measured by A280, remained unchanged, supporting the conclusion that shear-induced redistribution is specific to ProS. Finally, preliminary data indicate that redistribution of ProS is associated with loss of anticoagulant function in plasma thrombin generation assays, with VLDL-associated ProS exhibiting approximately 19% anticoagulant activity relative to its HDL-associated counterpart.

Conclusions: In summary, our data indicate that exposure of human plasma to shear forces, such as those that may occur in stenotic or tortuous vessels or in venous valves, unfolds vWF. This unfolding alters lipoprotein particles, leading to an increased presence of VLDL-like particles. ProS, bound to unfolded vWF, localizes to these VLDL-like particles, and anticoagulant activity is impaired. We propose this mechanism as a direct causal association between abnormal shear forces, ApoA1-containing lipoparticles, and increased thrombotic risk.

## VAMP8 Deficiency Attenuates Atherosclerosis Through Impaired Platelet Secretion and Hyperlipidemia-Driven Reprogramming

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**Background and Objective:** Hyperlipidemia and platelet hyperactivity are major risk factors for atherosclerosis. Activated platelets promote vascular inflammation and atheroprogression through the release of granule-stored bioactive cargo. Hyperlipidemia enhances platelet reactivity, yet the mechanistic link between lipid-driven granule secretion by platelets and atherosclerosis remains unclear. Here, we investigated how hyperlipidemia alters platelet programming and the role of VAMP8, the primary v-SNARE required for platelet granule exocytosis, in this context.

**Methods and Results:** Hypercholesterolemia was induced in VAMP8$^{-/-}$ and WT mice via PCSK9-AAV injection followed by 12 weeks of Western diet. Arterial thrombosis was assessed using ferric chloride-induced carotid injury with laser speckle contrast imaging. Hypercholesterolemia accelerated stable thrombus formation in WT mice, whereas VAMP8 deficiency resulted in unstable thrombi despite lipid elevation, suggesting that hypercholesterolemia primes the vasculature for acute thrombotic occlusion. Further, atherosclerotic burden, measured by *en face* staining and aortic root serial sectioning, was significantly reduced in VAMP8$^{-/-}$ mice. This protection occurred despite reduced platelet counts, increased platelet integrin activation, and markedly diminished platelet-neutrophil aggregates but unchanged P-selectin expression. Total plasma cholesterol levels were significantly reduced in VAMP8$^{-/-}$ mice compared to WT, with size-exclusion chromatography revealing marked reductions in both LDL and HDL fractions. Livers were collected and used for histological and lipid analysis**.** Liver bulk RNA-seq revealed dysregulation of lipogenic pathways in VAMP8$^{-/-}$ mice. Platelet RNA-seq from hypercholesterolemic vs normolipidemic mice also indicated dysregulation of lipid metabolism. Overall, our data suggests that VAMP8 deficiency alters systemic lipid metabolism, likely through hepatic regulatory pathways, and that hypercholesterolemia transcriptionally reprograms platelets during thrombopoiesis. Ongoing work is evaluating the effect of hypercholesterolemia on megakaryocytes.

**Conclusion:** VAMP8-mediated platelet secretion promotes thrombo-inflammatory responses and atherogenesis under hypercholesterolemic conditions. Modulating platelet exocytosis may represent a novel therapeutic strategy to reduce cardiovascular risk in metabolic disease.

**Title**: **Fibrinogen associates with LDL fractions, but not free protein fractions, in both human and mouse plasma**

Alexis N. Smith[1,2], Ziyu Zhang[1,2], Wen Dai[1,2], Hortense Triniac[1,2], Mindy Kim[1,2], and Ze Zheng[1,2]

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**Background**: Lipoproteins are spherical particles that circulate in the blood and transport insoluble lipids and other cargos, including proteins, in the body. Proteomic studies have shown that some of the proteins associated with lipoproteins are involved in blood clotting, such as fibrinogen. To understand the role of lipoproteins in blood clotting, we examined the association of lipoproteins with coagulation factors, starting with fibrinogen. Fibrinogen is of interest because it is an abundant plasma protein produced exclusively by hepatocytes, where most lipoproteins are assembled, and plays a critical role in blood clotting, as well as platelet activation and aggregation.

**Objective**: To investigate the interactions of lipoproteins with the coagulation factor I, fibrinogen.

**Methods**: We used size-exclusion Fast Protein Liquid Chromatography (FPLC) to separate fractions based on particle size in both human and mouse plasma. Total fibrinogen levels in all fractions were assessed using an ELISA assay. Human plasma was pooled from 10 healthy blood donors (5 males and 5 females, all adults aged under 40). Mouse plasma was pooled from wild-type C57BL/6 mice (7 female, all adults on normal chow diet). Cholesterol, triglyceride and total protein levels were assessed to determine lipoprotein and free protein fractions.

**Results**: Fibrinogen in pooled human plasma had a major peak at fractions (elution volume 24-27 mL) which are in the range of the LDL fractions, without any significant level detected in other lipoprotein fractions nor free protein fractions. We also examined the fibrinogen distribution in pooled mouse plasma fractions and viewed a consistent peak at fractions (elution volume 24-28 mL) in the range of LDL fractions, without any significant level detected in other lipoprotein fractions nor free protein fractions. The highest fibrinogen concentration in pooled human plasma was observed at fraction of elution volume 26 mL with a concentration of 0.255 mg/mL. The highest fibrinogen concentration in pooled mouse plasma was observed at fraction of elution volume 26 mL with a concentration of 0.360 mg/mL. These results suggest that fibrinogen potentially interacts with LDL.

**Conclusions**: This study suggests that fibrinogen was mostly found in the LDL fractions, but not in free protein fractions in both human and mouse plasma. These data fill gaps in our knowledge of the association of LDL fractions with coagulation factor I, fibrinogen, with future directions in understanding how hemostasis and thrombosis is regulated in dyslipidemic conditions.

# The modulatory role of lipoproteins on magnesium biocorrosion

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**Introduction:** Percutaneous intervention with bare metal or drug-eluting stents (DES) is the standard of care for patients with severe atherosclerosis, but the permanent nature of these vascular implants can lead to long-term complications such as neoatherosclerosis, late thrombosis, and restenosis due to neointima hyperplasia. Bioresorbable metallic stents (BRS) have emerged as the next possible generation of vascular implants, as their ability to degrade mitigates the long-term risks of DES. Magnesium (Mg) based BRS have been studied clinically and approved in European markets, but there is mounting evidence that devices made from the same material experience unpredictable corrosion when deployed in heterogeneous patient populations. We hypothesize that this could be caused by interactions between serum lipids and the Mg alloy. In this study, we utilize a well characterized hypertriglycerdemic transgenic mouse model (ApoE -/-) and a disease-specific in vitro corrosion methodology to evaluate the response of Mg materials in lipid-laden environments.

**Methods:** WE43 (4 wt.% yttrium – Y, 3 wt.% neodymium-Nd) 100 µm wires were immersed in DMEM or DMEM supplemented with varied levels of human low-density lipoprotein (LDL) or human high-density lipoprotein (HDL); wires were removed after 1 day of incubation, then examined with metallographic analysis to evaluate corrosion response. WE43 wires were implanted into the abdominal aorta of ApoE-/- and C57BL/6J mice for 10 days (n = 4 per group). Implants were excised and examined with histology and metallographic analysis.

**Results:**

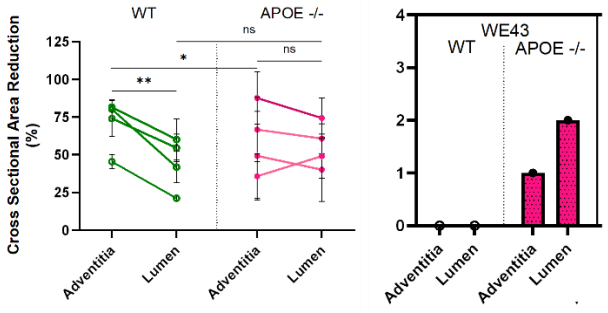


*Figure 1: In vivo corrosion behavior of WE43 in ApoE-/- and C57 female mice for 10 days based on vessel compartment (left), and categorical counting of wire breakages (right).*

WE43 wires implanted into WT animals had predictable corrosion, with a characteristic increase in cross sectional area reduction in sections of the wire that were implanted into the adventitia compared to those presenting in the lumen. Interestingly, when implanted into ApoE-/- animals, the implants exhibited inhomogeneous corrosion and lost the tissue compartment-based response seen in WT. Additionally, 75% of the implants analyzed from ApoE -/- animals experienced mechanical failure, while none of the implants failed in WT animals (Fig 1).

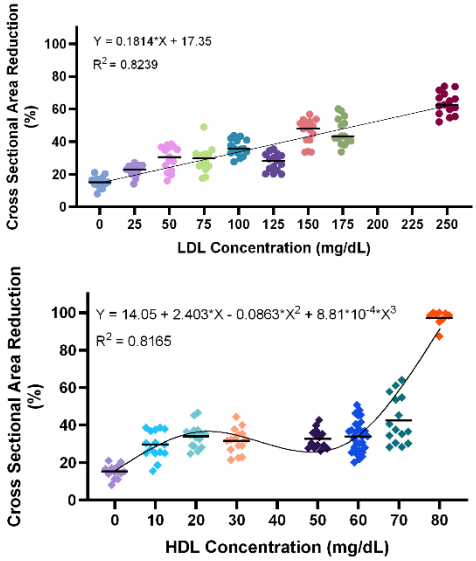


*Figure 2: In vitro corrosion of WE43 wires immersed in varied levels of LDL fit with linear regression (top) or HDL fit with a spline (bottom).*

When immersed in DMEM for 24 hours, WE43 wires had an average cross sectional area reduction of 15.28 ± 3.29%, which increased linearly with the addition of increasing amounts of human LDL. In the physiologic range of 50 to 100 mg/dL of LDL, the cross-sectional area reduction increases by 1.9, 2.1, and 2.4-fold for 50, 75, and 100 mg/dL respectively, and up to 4.16-fold in the supraphysiologic condition of 250 mg/dL. Similarly, supplementing DMEM with human HDL results in an increase in WE43 corrosion, but the relationship between HDL concentration and cross-sectional area reduction is non-linear, with most conditions (10 to 60 mg/dL) exhibiting a corrosion increase of nearly 2-fold compared to immersion in DMEM. Wires corroded in high levels of HDL (80 mg/dL) exhibited a 6.3-fold increase in cross-sectional area reduction (Fig 2).

**Conclusions:** The lipid-laden environment has a distinct impact on the corrosion and material performance of WE43 wires *in vivo* and *in vitro*. The data presented demonstrates that the vascular microenvironment of ApoE-/- mice increases the corrosion heterogeneity and results in mechanical failure of the wires after short-term implantation (Fig 1), and that lipoprotein supplementation increases Mg alloy corrosion in a dose and lipoprotein species-dependent manner (Fig 2). Ongoing and future work will further delineate the role lipoproteins play in Mg biocorrosion modulation by identifying target biomolecules that interact with the material and create a predictive model of Mg biocorrosion based on specific lipid profiles using *in vitro* and *in vivo* data collected from multiple dyslipidemia mouse models.

# Synthetic High-Density Lipoproteins Modulate Platelet Function Through Phospholipase $A_2$ Products

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High-density lipoprotein (HDL) exhibits diverse cardioprotective properties beyond cholesterol efflux, with its functionality closely linked to its lipid composition. This study investigates the impact of lipid composition on synthetic HDL (sHDL) interactions with platelets. We synthesized sHDL particles using ApoA1 mimetic peptide 22A complexed with various lipids (DMPC, POPC, DSPC, DPPC, and SM) and evaluated their effects on platelet aggregation, cholesterol removal capacity, and cellular uptake. DMPC sHDL showed superior inhibition of platelet aggregation, despite lower cellular uptake compared to POPC sHDL. Notably, all formulations exhibited similar cholesterol removal abilities. Further investigation revealed the involvement of phospholipase $A_2$ ($PLA_2$) enzymes in DMPC sHDL's potent antiplatelet effects. Inhibition of cytosolic $PLA_2$ ($cPLA_2$) and lipoprotein-associated $PLA_2$ (Lp-$PLA_2$) significantly reduced DMPC sHDL's antiplatelet activity. We demonstrated $PLA_2$-mediated hydrolysis of DMPC sHDL, resulting in bioactive lipid metabolites, lysophosphatidylcholine (LPC) 14:0 and myristic acid, both *in vitro* and *in vivo*. These metabolites directly modulated various platelet functions *in vitro*. Our findings elucidate a novel mechanism by which sHDL composition influences its antiplatelet properties, offering insights for developing targeted cardiovascular therapies.

# DENND5B is a Microtubule-associated Protein that is Required for Triglyceride-Rich Lipoprotein Secretion from Human Enterocytes and Hepatocytes

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**Background:** Hypertriglyceridemia is an atherosclerosis risk factor and is regulated by dietary triacylglyceride (TAG) absorption via the small intestine and endogenous production by the liver. Our laboratory reported that *Dennd5b*-deficient mice are protected from diet-induced hepatic steatosis, hyperlipidemia, and atherosclerosis. Electron microscopy imaging of intestinal epithelium of *Dennd5b*$^{-/-}$ mice revealed significant lipid accumulation in intracellular chylomicron secretory vesicles, suggesting a post-Golgi chylomicron secretion defect. Since *DENND5B* is expressed by both triglyceride-rich lipoprotein (TRL)-secreting organs (liver and small intestine) in Humans, we hypothesized that *DENND5B* disruption attenuates TRL secretion in both of these tissues.

**Methods and Results:** To determine if *DENND5B* disruption impairs TRL secretion in human cells, we generated *DENND5B*$^{-/-}$ intestinal epithelial (Caco-2) and hepatocyte (HepG2) cell lines using CRISPR. Western blots verified absence of DENND5B protein in *DENND5B*$^{-/-}$ cells. Cellular and secreted TAG was measured from *DENND5B*$^{+/+}$ and *DENND5B*$^{-/-}$ cells in growth media and after oleic acid loading. *DENND5B* disruption significantly reduced TAG secretion in both Caco-2 and HepG2 cells compared to *DENND5B*$^{+/+}$ controls (Caco-2: -67.8%, $p<0.0001$; HepG2: -91.0%, $p<0.0001$). In both cell types, *DENND5B*$^{-/-}$ reduced TAG secretion without affecting cellular TAG content. To define the mechanism by which DENND5B influences TRL secretion, multiple tagged DENND5B plasmids were transfected into HepG2 cells for subcellular visualization and immunoprecipitation to reveal potential binding partners. Super-resolution microscopy of HepG2 cells showed distinctive localization of DENND5B along Beta-Tubulin structures accompanied by APOB puncta associated with the filamentous tubulin network. Co-immunoprecipitation of DENND5B supports this finding with enrichment of motor protein-binding and microtubule-membrane tethering proteins. Ongoing studies investigate the impact of the DENND5B genotype on cellular lipid metabolism. Preliminary findings of RNA-seq analyses of *DENND5B*$^{+/+}$ and *DENND5B*$^{-/-}$ HepG2 cells maintained in normal and oleic acid-enriched media support altered expression of lipid metabolism genes with *DENND5B* disruption.

**Conclusions:** These data support the hypothesis that DENND5B plays a role in TRL secretion from human enterocytes and hepatocytes. Our findings suggest a mechanism where DENND5B facilitates vesicular transport of post-Golgi TRLs along the cytoskeleton to the membrane for secretion. Understanding the mechanistic details of this process may provide novel targets for regulation of plasma TAG and APOB concentrations.

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# Enterocyte tPA interacts with apoB-48 and limits chylomicron production

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**Introduction:** Non-fasting lipid levels, including those derived from enterocytes in the form of chylomicrons, provide a better risk assessment for cardiovascular disease (CVD), including ischemic heart disease and ischemic stroke than fasting lipid levels, which primarily reflect contributions from hepatocytes. Clinical studies showed inhibiting chylomicron production regresses carotid atherosclerosis plaque burden in humans. These findings underscore the significant role of chylomicrons in the development atherosclerosis and CVD. However, the regulatory processes governing chylomicron production are not fully understood. Chylomicrons are assembled in enterocytes by incorporating lipids onto apolipoprotein B-48 (apoB-48). This process is analogous to the production of very-low-density lipoprotein (VLDL) in hepatocytes, which involves lipid transfer to apolipoprotein B-100 (apoB-100). Our recent study revealed that tissue-type plasminogen activator (tPA), a key fibrinolytic protein, interacts with the N-terminus of apoB-100, where the microsomal triglyceride transfer protein (MTP) lipidates apoB-100, thereby reducing VLDL assembly in hepatocytes. Conversely, plasminogen activator inhibitor-1 (PAI-1) sequesters tPA away from apoB-100, promoting apoB-100 lipidation and VLDL production. Given that the MTP-interacting region of apoB-100 is retained in apoB-48, which comprises the N-terminal 48% of apoB-100, and that enterocytes also synthesize tPA, it is plausible that tPA may also interact with apoB-48 within enterocytes.
**Objective:** To test the hypothesis that tPA interacts with apoB-48 and limits chylomicron production in enterocytes.
**Methods and Results:** We engineered a genetic mouse model with tamoxifen-inducible, enterocyte-specific deletion of tPA (e-tPA-KO) by breeding *Plat* $^{fl/fl}$ mice with Vil1-ERT2-Cre mice. Intestinal chylomicron production can be assessed by measuring the rise in plasma triglyceride levels following an oral lipid challenge. Compared to control mice, e-tPA-KO mice exhibited plasma triglyceride levels that were 46% and 40% higher at 1 and 2 hours post oral gavage of olive oil, respectively. Chylomicrons were isolated from plasma collected 2 hours post-gavage by ultracentrifugation. Analysis of the chylomicron fraction by immunoblot revealed increased apoB-48 levels in e-tPA-KO mice relative to controls. These findings above indicate that enterocyte-specific tPA deficiency enhances intestinal chylomicron production. Tamoxifen-inducible, enterocyte-specific PAI-1 knockout (e-PAI-1-KO) mice were generated by crossing *Serpine1* $^{fl/fl}$ mice with Vil1-ERT2-Cre mice. In contrast to controls, e-PAI-1-KO mice exhibited increased free tPA protein levels in enterocytes and a reduced plasma triglyceride rise following oral olive oil gavage. In solid-phase binding assays, recombinant tPA bound dose-dependently to immobilized chylomicrons. This interaction was inhibited by the 1D1 antibody, which targets the N-terminus of apoB-48, as well as by an antibody that blocks the Kringle 2 domain of tPA. Furthermore, in rat hepatocyte McA-RH7777 cells transfected with plasmids encoding truncated forms of apoB, tPA co-eluted with apoB-9, apoB-15, and apoB-18, corresponding to the N-terminal 9%, 15%, and 18% of apoB-100, respectively. These findings further support the interaction between tPA and the N-terminus of apoB.
**Conclusion:** Our findings suggest a previously unrecognized role for enterocyte tPA in limiting chylomicron production through interactions with apoB-48.

# Hepatocyte tPA limits lipoprotein (a) assembly by blocking apo(a) binding to apoB-100

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**Introduction:** Lipoprotein(a) [Lp(a)] is an independent, causal risk factor for cardiovascular diseases. Lp(a) consists of apolipoprotein B-100 (apoB-100) linked to apolipoprotein(a) [apo(a)]. The interaction between lysine-binding sites (LBS) in the Kringle IV type 5–8 domains of apo(a) and lysine residues in the N-terminal domain of apoB-100 is critical for Lp(a) particle assembly. We recently demonstrated that tissue plasminogen activator (tPA), via its LBS in the Kringle 2 domain, directly binds to the N-terminus of apoB-100. The structural similarity between the LBS in tPA Kringle 2 and those in apo(a) Kringle IV raises the possibility that tPA may compete with apo(a) for binding to apoB-100.
**Objectives:** To test the hypothesis that tPA binding to apoB-100 interferes with the apoB–apo(a) interaction, thereby limiting Lp(a) assembly.
**Methods:** Human primary hepatocytes and HepG2 cells transduced with an apo(a)-expressing plasmid were used. Lp(a) levels were measured by ELISA, immunoblotting, and imaging flow cytometry. The ELISA employed an anti-apo(a) capture antibody and an anti-apoB detector antibody. Imaging flow cytometry used fluorescently labeled anti-apoB and anti-apo(a) antibodies to identify Lp(a) particles.
**Results:** In human primary hepatocytes, siRNA-mediated silencing of tPA increased Lp(a) levels in serum-free medium, as determined by ELISA, immunoblotting, and imaging flow cytometry, without altering apoB or apo(a) mRNA levels. To validate these findings, we generated an Lp(a)-expressing HepG2 cell line via transduction with an apo(a)-expressing plasmid. Consistently, tPA silencing in these cells led to increased Lp(a) secretion. In McA-RH7777 cells transfected with truncated apoB constructs, tPA co-eluted with apoB-9, apoB-15, and apoB-18 (representing the N-terminal 9%, 15%, and 18% of apoB-100, respectively). The lysine analog tranexamic acid (TXA) partially inhibited the tPA–apoB interaction, indicating lysine residue–mediated binding. Plasminogen activator inhibitor-1 (PAI-1) is the endogenous inhibitor of tPA. We previously found that PAI-1, by forming a complex with tPA, sequesters tPA away from apoB-100 in hepatocytes. Consistent with this, silencing PAI-1 in human primary hepatocytes and in Lp(a)-expressing HepG2 cells increased free tPA levels and reduced Lp(a) concentrations in the culture medium. Furthermore, plasma from individuals with a homozygous null mutation in *SERPINE1*, which encodes PAI-1, exhibited lower Lp(a) levels compared with age-, sex-, and BMI-matched controls from the same community.
**Conclusion:** Plasma Lp(a) levels were thought to be mostly determined by genetic variants in the *LPA* gene, which encodes apo(a). Our findings reveal a previously unrecognized regulatory mechanism, independent of *LPA* genetic variation, whereby tPA limits Lp(a) assembly by blocking the binding of apo(a) to apoB-100.

The leading cause of death in the US is heart disease, with an estimated 18 million American adults having atherosclerotic cardiovascular disease (ASCVD). High levels of very low-density lipoproteins (VLDL) are a risk factor for development of ASCVD and increase the risk of adverse events. We and others showed that hepatic VLDL secretion is controlled in part by the transcription factor SREBP2 via miR-33. The miR-183/96/182 cluster (miRc) is another transcriptional target of SREBP2, but its role in VLDL metabolism has not been explored. To address this question, we developed a novel mouse model with a hepatocyte specific miRc knockout. On chow diet, knockout mice have increased serum triglycerides and unchanged cholesterol with no significant difference in weight compared to floxed controls. When challenged with a 60% kcal fat diet, knockout mice show reduced weight gain over a 12 week period and retain high serum triglycerides. NMR body composition data showed knockout mice have a lower percentage of fat mass, and livers from these animals had significantly less lipid content than floxed counterparts. Finally, experiments in cultured primary hepatocytes revealed an inverse relationship between miRc expression and secretion of triglycerides and APOB. Taken together, these experiments identify a previously undescribed mechanism for decreasing VLDL secretion via the miR-183/96/182 cluster.

# Elucidating The Apolipoprotein A1 Structure in Reconstituted and Plasma High Density Lipoproteins Using 20 Antibody Fragments

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Apolipoprotein A1 (APOA1) is the most abundant protein on high density lipoproteins (HDL) and its structure likely dictates the subspeciation and functionality of HDL. Cryo-electron microscopy is a powerful tool for elucidating the structure of macromolecular complexes such as HDL. However, these symmetrical particles typically need fiduciary markers for image alignment during data analysis. Human APOA1 was incorporated into reconstituted HDL (rHDL) to form discoidal particles with diameters of ~10 nm. These 'discs' were screened against a phage display library of antibody fragments (fabs) to identify APOA1 binders. 20 such fabs have been expressed and purified in our lab. Current work is ongoing to map the APOA1 epitopes and characterize the affinity to APOA1 in various states such as, lipid free, rHDL and plasma HDL. All fabs bind to rHDL but only one binds lipid free APOA1 which highlights the major structural changes in the protein when lipidated. Using immunoaffinity chromatography, we isolated APOA1 containing HDL particles that lack apolipoprotein A2, LPA1, from human plasma. These form two distinct sized particles, large and small. Several fabs that bind strongly to rHDL particles completely lacked affinity for LPA1 or only had affinity for certain sizes of LPA1. We have set out to use a collection of fabs to solve the structure of APOA1 on rHDL as well as LPA1 small. Fab 2 and Fab16 have allowed us to get <5A information for their epitopes on rHDL as well as 6A data for the whole particle. At this resolution, we can visualize alpha helical density ¾ of the way around the particles. Using molecular dynamic flexible fitting, we modeled in a large portion of APOA1 into the density. Ongoing work will utilize other fab APOA1 rHDL complexes to gain higher resolution information for epitopes across all of APOA1. In addition, chemical crosslinking mass spectroscopy will be used to validate our molecular dynamics models. The structures we have obtained so far represent the highest resolution experimentally derived models for APOA1 in both reconstituted and human plasma HDL. This information will provide a high resolution platform for understanding how numerous other HDL modifying factors and enzymes interact with the particles.

# Non-Enzymatic Remodeling of Synthetic High-Density Lipoprotein Nanoparticles with Endogenous Lipoproteins

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**Introduction:** With structural and functional resemblance with endogenous pre-beta high-density lipoprotein (HDL), synthetic high-density lipoprotein (sHDL) nanodiscs have been proposed to treat varieties of diseases. sHDL nanoparticles are intrinsically dynamic due to their capacity to engage in lipoprotein remodeling process, which involves continuous apolipoprotein exchange, lipid transfer, enzymatic modification, and structural reorganization of lipoprotein nanoparticles. The dynamic lipoprotein remodeling process has been recently suggested as the key *in vivo* mechanisms of action of sHDL nanoparticles. Despite the significant impact of the remodeling process, the mechanisms and kinetics of sHDL remodeling have not been fully elucidated due to the complicated non-enzymatic and enzymatic processes. In the present study, we aim to the non-enzymatic remodeling process between sHDL and endogenous lipoproteins with a special focus on the translocation of lipid components.

**Method:** sHDL nanoparticles composed of different phospholipids and lipid-like fluorescent dyes were prepared and characterized. Particle integrity and lipid exchange kinetics between sHDL nanoparticles were investigated using intensity-based FRET analysis. To investigate the redistribution of lipid components to endogenous lipoproteins, fluorescent labeled sHDL nanoparticles were incubated with isolated human lipoproteins *ex vivo*. At different times, the particle size changes as well as the distribution of lipid-like dyes were determined by size exclusion chromatography.

**Results:** Lipid exchange between sHDL nanoparticles are collision mediated which are affected by phospholipid components and temperature. sHDL composed of phospholipids with higher phase transition temperature showed minimal lipid migration during incubation with endogenous lipoproteins, while significant lipid relocation was observed in sHDL with lipids of low phase transition temperature. Interestingly, new particle population with different particle sizes different than either endogenous HDL or sHDLs were observed in some cases.

**Conclusion:** The non-enzymatic lipid remodeling between sHDL nanoparticle and other lipoprotein species is affected by phospholipid components of sHDLs. The remodeling process may lead to the formation of new lipoprotein particle populations.

**Title:** Plin2 Deficiency Does Not Alter Foam Cell Formation or Cholesterol Efflux in vitro

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**Abstract:**

Perilipin 2 (PLIN2) is a lipid droplet–associated protein implicated in intracellular lipid storage, metabolism, and signaling. While PLIN2 is enriched in lipid-laden macrophages within atherosclerotic plaques, its specific role in foam cell biology remains unclear. In this study, bone marrow-derived macrophages (BMDMs) were generated from CX3CR1$^{Cre}$ Plin2$^{F/F}$ or control mice to investigate the role of PLIN2 in cholesterol loaded foam cells. Foam cell formation was induced by exposure to soluble cholesterol or DiI-labeled oxidized low-density lipoprotein (DiI-oxLDL). Lipid uptake was assessed by fluorescence microscopy, flow cytometry, and Oil Red O staining. Cholesterol efflux was measured following cholesterol loading and incubation with lipid acceptors. Our results indicate that Plin2 deficiency did not significantly affect lipid uptake or foam cell formation in BMDMs following treatment with either soluble cholesterol or DiI-oxLDL. Similarly, cholesterol efflux capacity was unchanged in Plin2-deficient BMDMs compared with controls. Despite its enrichment in lipid droplet–containing macrophages, Plin2 seems dispensable for foam cell lipid accumulation and cholesterol efflux in vitro. These findings suggest that Plin2 may not be a critical determinant of foam cell function in BMDMs under these conditions.

**AAV-CRISPR/Cas9-apoa2 knockout system disrupts apoa2, and efficiently decreases the number and size of HDL particles and HDL-Cholesterol**

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Heart disease is the number one killer in the US with ~700,000 deaths in 2020. Circulating lipids carried on lipoproteins play critical roles in the development (or absence) of atherosclerosis which underlies much of cardiovascular disease (CVD) pathology. It is becoming clear that circulating triglycerides (TG) carried in TG-rich lipoproteins (TGRL) and their remnants are also causally associated with CVD. Preliminary experiments had shown that injection of human APOA2 increased post-prandial TG in mice gavaged with a high fat meal. Furthermore, enrichment of human plasma VLDL with APOA2 reduced LPL mediated hydrolysis of those particles in vitro implying a possible involvement of APOA2 in circulating TG. Chronic knock-down of APOA2 in mice performed in the 80's showed dramatic reductions in HDL-cholesterol levels despite the fact that APOA2 only comprises about 12% of HDL protein mass. We hypothesized that a lack of APOA2 resulted in extensive remodeling of HDL, potentially via unchecked lipase activity. We tested the AAV-CRISPR/Cas9 system to deliver four gRNAs targeting the *apoA2* gene (Cr-A2KO) in the liver of C57BL/6J mice. Two of these showed efficient disruption of *apoA2 (by Inference of CRISPR Edits-ICE)*, resulting in a 46-56% reduction in plasma cholesterol levels within 7 days and persisting to 28 days corresponding with nearly complete loss of circulating APOA2 (detected by silver stained SDS PAGE analysis). FPLC analysis showed a 48-60% decrease in HDL cholesterol and 48-56% decrease in HDL-PL by day 7 with a clear decrease in HDL particle size. Ongoing work will characterize the proteomic remodeling of circulating lipoproteins as well as their susceptibility to lipase activity. This model will be useful for studying the functional roles of mouse APOA1 in an acute knock down system.

# Synthetic High-Density Lipoproteins Modulate Platelet Function Through Phospholipase $A_2$ Products

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High-density lipoprotein (HDL) exhibits diverse cardioprotective properties beyond cholesterol efflux, with its functionality closely linked to its lipid composition. This study investigates the impact of lipid composition on synthetic HDL (sHDL) interactions with platelets. We synthesized sHDL particles using ApoA1 mimetic peptide 22A complexed with various lipids (DMPC, POPC, DSPC, DPPC, and SM) and evaluated their effects on platelet aggregation, cholesterol removal capacity, and cellular uptake. DMPC sHDL showed superior inhibition of platelet aggregation, despite lower cellular uptake compared to POPC sHDL. Notably, all formulations exhibited similar cholesterol removal abilities. Further investigation revealed the involvement of phospholipase $A_2$ ($PLA_2$) enzymes in DMPC sHDL's potent antiplatelet effects. Inhibition of cytosolic $PLA_2$ ($cPLA_2$) and lipoprotein-associated $PLA_2$ (Lp-$PLA_2$) significantly reduced DMPC sHDL's antiplatelet activity. We demonstrated $PLA_2$-mediated hydrolysis of DMPC sHDL, resulting in bioactive lipid metabolites, lysophosphatidylcholine (LPC) 14:0 and myristic acid, both *in vitro* and *in vivo*. These metabolites directly modulated various platelet functions *in vitro*. Our findings elucidate a novel mechanism by which sHDL composition influences its antiplatelet properties, offering insights for developing targeted cardiovascular therapies.

**Development of Lipid Nanodiscs to Characterize Structural Details of CD36**

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Cardiovascular disease (CVD) is the leading cause of mortality worldwide. Atherosclerosis, the main cause of CVD, results from the buildup of cholesterol-filled plaques in artery walls. Accumulation of cholesterol from oxidized low-density lipoprotein (oxLDL) leads to the formation of macrophage foam cells, a process that is mediated by CD36, a scavenger receptor that serves as the primary receptor for oxLDL. The structure of full-length CD36 has not been resolved, nor have the details or role of quaternary structure been fully elucidated. Studies have shown that CD36 can form homodimers and multimers in some cell types. However, the role of CD36 oligomerization in mediating CD36 function remains unknown. We hypothesize that CD36 oligomerizes in macrophages to facilitate oxLDL uptake. To begin testing this hypothesis, we harvested elicited peritoneal macrophages from wild-type (WT) or CD36 knockout (CD36-KO) mice. We utilized Native-PAGE and PFO-PAGE electrophoresis to provide evidence of higher-order complexes in WT, but not in CD36-KO macrophages. To further validate our studies, we purified human full-length CD36 using an Sf9 insect cell system and demonstrated that pure CD36 forms homo-oligomers by PFO-PAGE. To begin evaluating CD36 oligomerization in a physiologically relevant lipid system, we reconstituted CD36 into phospholipid bilayer nanodiscs. We verified nanodisc formation by size exclusion chromatography and negative staining electron microscopy. Through a collaboration with Pacific Northwest National Laboratories, we are optimizing grid conditions to ultimately obtain the first full-length cryo-electron microscopy (CryoEM) images of CD36. With this novel tool in hand, we now have the ability to characterize the structural details of CD36, including its quaternary structure. The outcomes of our studies will provide structural insight into how we can target CD36 to prevent foam cell formation and atherosclerosis.

# PIM1 regulates lipid metabolism and foam cell formation in macrophages via CD36

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**Abstract**

**Background:** Atherosclerosis is defined by the accumulation of lipid-laden fibrous plaques that lead to arterial wall thickening and reduced elasticity, in which macrophages (Mφs) play a significant contributing role through their scavenger receptor CD36. The proviral integration site for Moloney murine leukemia virus 1 (PIM1) demonstrates immune modulatory properties through its regulation of lipid metabolism, yet its contribution to foam cell formation and atherogenesis has not been defined.

**Objective:** This study aims to investigate the regulatory relationship between PIM1 and CD36 expression in Mφs during foam cell formation and atherogenesis.

**Methods:** Peritoneal Mφs from PIM1$^{-/-}$ or wild-type (WT) mice were treated with or without oxidized low-density lipoprotein (oxLDL). CD36, PIM1, and plaque formation-associated protein and mRNA expressions were assessed by western blot, flow cytometry, and RNA-sequencing in Mφs. Foam cell formation, and oxLDL uptake and binding were quantified using Oil Red O staining and Dil-labeled oxLDL. Additionally, peritoneal Mφs were isolated from WT mice treated with PIM inhibitor on an ApoE$^{-/-}$ background after 6 weeks of high-fat diet (HFD) to assess foam cell formation and cholesterol accumulation.

**Results:** Upon oxLDL stimulation, deletion of PIM1 in Mφs significantly reduced CD36 protein expression by up to 96.7% compared to WT controls, resulting in a marked decrease in foam cell formation and intracellular cholesterol accumulation (reduced to 49.6% and 25.5%, respectively). Moreover, pharmacological inhibition of PIM activity in WT Mφs resulted in a comparable downregulation of CD36 (up to 35.6%), accompanied by impaired oxLDL handling, as evidenced by a reduction in binding to 64.5% and uptake to 57.9% of control levels. Inhibition of PIM activity in Mφs also led to substantial reductions in foam cell formation and cholesterol accumulation *in vivo* (reduction to 36.9% and 16.2%). RNA-seq and western analysis indicated that PIM1-induced CD36 downregulation was mediated by

peroxisome proliferator-activated receptor gamma (PPAR-γ) and its downstream signaling pathways, which were restored by rosiglitazone (PPAR-γ agonist).

**Conclusion:** Our findings support PIM1 as a key upstream regulator of CD36 expression in Mφs, driving oxLDL binding, uptake, and foam cell formation. Targeting the PIM1/PPAR-γ/CD36 pathway may offer novel strategies to modulate Mφ lipid metabolism and protect against atherosclerotic plaque progression.

**Impact of CD36-mediated endothelial stiffening on lesion formation in hypercholesterolemic mice**

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**Objective:** To determine the impact of endothelial stiffening induced by CD36 (cluster of differentiation 36)-mediated lipid uptake on endothelial barrier disruption and the development of atherosclerosis in hypercholesterolemic mice.

**Approach and Results:** Endothelial stiffening of intact *en face* monolayers, as assessed by atomic force microscopy, reveal the hyperlipidemic mouse model of atherosclerosis, $LDLR^{-/-}$ (low density lipoprotein receptor knockout) is significantly stiffer, especially in the atheroprone aortic arch, as compared to wild type controls (n=4 male mice with 8-15 tissue measurement sites per condition per mouse, $p<0.05$). Furthermore, there was a differential response in endothelial stiffness in males and females, with males having a greater endothelial stiffening. This difference correlated to a significantly greater plaque development, as analyzed using Oil O Red staining for lipids, in male HFD-fed $LDLR^{-/-}$ mice, as compared to age-matched females (n=5-6, $p<0.05$).

Specific downregulation of endothelial CD36 in hypercholesterolemic $Cdh5.CreERT2CD36^{fl/fl}LDLR^{-/-}$ mice led to a significant decrease in endothelial stiffening (n=5 mice, $p<0.05$), both on chow and on HFD. Our data further reveal that prevention of HFD-induced endothelial stiffening by downregulation of CD36 is associated with a protective effect against endothelial barrier disruption in the hypercholesterolemic mouse model, as assessed by the penetration of Evan's Blue Dye into the aortic wall. Evidence of the importance of CD36 is also demonstrated in atherosclerotic lesion formation, which is shown to be CD36-dependent (n=5-6 mice, $p<0.05$). Furthermore, a linear regression analysis of endothelial stiffness against percent plaque area across the two sexes, diet, and CD36 expression show a significant correlation ($r = 0.84$).

**Conclusions:** This data suggests that endothelial CD36-dependent endothelial stiffening in intact aortas result in endothelial barrier disruption and promote atherosclerotic plaque formation.

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**Title**: Evaluating the Contribution of CD36 to Carotenoid Homeostasis in Mice

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**Abstract**

Carotenoids are bioactive lipids present in foods and vegetables with antioxidant and provitamin A properties. Elevated serum carotenoid levels in tissues are associated with a reduced incidence of cardiometabolic diseases; however, the mechanisms responsible for carotenoid uptake remain largely unexplored. Cell culture studies suggest that the scavenger receptor CD36 participates in carotenoid uptake, both in the intestine and other tissues such as the adipose tissue and the eye, but whether these findings are reproducible in mammals has not been evaluated to date. We hypothesize that CD36 regulates carotenoid uptake in a "humanized" model for carotenoid accumulation. To mimic the accumulation of carotenoids in humans, we use mice lacking the two carotenoid-cleaving enzymes: β-carotene oxygenase 1 (BCO1) and 2 (BCO2). To examine the role of CD36, we cross-bred *Bco1*$^{-/-}$ and *Bco2*$^{-/-}$ with *Cd36*$^{-/-}$ mice to generate compound mutants. We compared *Bco1*$^{-/-}$*Bco2*$^{-/-}$ and *Bco1*$^{-/-}$*Bco2*$^{-/-}$*Cd36*$^{-/-}$ mice fed purified diets containing physiological doses of β-carotene and lutein, two major carotenoids in the human diet. After four weeks on diet, we quantified tissue carotenoid levels via HPLC following established protocols in our lab. To our surprise, mice lacking CD36 exhibited greater levels of carotenoids in plasma, liver, adipose tissues, and eyes. Because *Cd36*$^{-/-}$ mice displayed increased gut permeability, we also performed single gavage experiments to quantify the passage of carotenoids across the gut. Our data confirm that, in the absence of CD36, carotenoids are absorbed at higher rates, challenging the long-standing assumption that CD36 serves as a major carotenoid transporter.

**Lipoproteins are susceptible to proteolytic modifications that may alter their atherogenicity**

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**Introduction:** It is increasingly recognized that inflammation drives atherosclerotic cardiovascular disease (CVD). Consequently, recent CVD clinical trials focus on targeting atherogenic cytokines released by macrophages and neutrophils. However, inflammation *also* triggers these same cells to release excess proteases which may accelerate plaque development. For example, neutrophil elastase (NE) is a serine protease secreted by neutrophils and macrophages. Elastase is enriched in atheromatous plaques, and plasma levels can rise up to ten-fold in inflammation. *In vitro*, elastase modifies the protein components of low-density lipoprotein (LDL) and high-density lipoprotein (HDL), and these elastase-modified lipoproteins are rapidly internalized by macrophages. These data point to a proatherogenic role of NE through its ability to modify lipoproteins and amplify foam cell formation. It is unknown, however, how these modified lipoproteins are metabolized in plasma or how they affect plaque development *in vivo*.

**Hypothesis:** Alpha-1-antitrypsin (AAT) is the endogenous inhibitor of neutrophil elastase. Here, we aim to determine the effects of elastolytic activity on lipoprotein metabolism and atherosclerosis using a two-pronged approach: (1) acute administration of elastase to lipoproteins and (2) use of an AAT-deficient (*Serpina1*$^{-/-}$) mouse as a model for a long-term increase in elastase activity. We hypothesize that elastase cleavage of lipoproteins in plasma will affect LDL and HDL metabolism and will increase atherosclerotic plaque burden.

**Results:** We first characterized the impact of neutrophil elastase treatment on lipoproteins in whole plasma *ex vivo*. Western blotting revealed dose-dependent degradation of apolipoproteins in plasma from wild type and *Serpina1*$^{-/-}$ (AAT-deficient) mice. *Serpina1*$^{-/-}$ plasma proteins were more susceptible to NE treatment than wild type. A similar degradation pattern was observed when NE was injected *in vivo* into *Serpina1*$^{-/-}$ mice. Interestingly, elastase-modified particles were rapidly cleared from circulation in these mice. In human plasma, *ex vivo* elastase treatment resulted in an HDL shift towards larger fractions, suggesting elastase may cause HDL aggregation. Lipoprotein profile analysis in *Serpina1*$^{-/-}$ mice on chow diet revealed an altered lipoprotein profile characterized by increased HDL-cholesterol, similar to that observed in AATD human patients. We then conducted an atherosclerosis study with eight weeks of Western diet feeding and *Ldlr*-ASO injections. En face analysis demonstrated that *Serpina1*$^{-/-}$ mice exhibit ~45% higher plaque lesion area in the aortic arch compared to wild type mice.

**Conclusions:** Neutrophil elastase modifies lipoproteins *ex vivo* and *in vivo* which may affect their metabolism and functions relevant to atherosclerosis pathophysiology. AAT-deficient (*Serpina1*$^{-/-}$) mice display increased plasma cholesterol and increased atherosclerotic plaque burden which may be driven by unchecked elastase activity.

**A functional dissection of angiopoietin-like 3**

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Dyslipidemia is a risk factor for metabolic diseases, emphasizing the importance of regulating lipoprotein metabolism. Levels of lipoproteins are regulated, in part, by extracellular lipases including lipoprotein lipase (LPL) and endothelial lipase (EL). Angiopoietin-like-3 (ANGPTL3) regulates both EL and LPL activity, inhibiting EL on its own and LPL when ANGPTL3 forms a complex with angiopoietin-like-8 (ANGPTL8). ANGPTL3 and ANGPTL3-8 complexes are both considered targets for the treatment of dyslipidemia. Understanding the residues of ANGPTL3 that contribute to each of its functions could enable the development of more effective therapies. The goal of our study was to identify residues in ANGPTL3 that contribute to complex formation, EL inhibition, and LPL inhibition. We generated a panel of mutations that spanned the N-terminal domain of ANGPTL3, and the ability of each mutant to inhibit EL and LPL was assessed using lipase activity assays. The oligomerization state of wild-type and mutant ANGPTL3 and wild-type and mutant ANGPTL3-ANGPTL8 complexes was determined using mass photometry and blue native gel electrophoresis. We found that mutations in the SE1 domain and surrounding regions disrupted the ability of ANGPTL3 to inhibit EL or LPL. Several leucine residues throughout the N-terminal region were also essential for lipase inhibition. Our oligomeric studies found that wild-type ANGPTL3 primarily forms a homotrimer and that trimer formation is necessary for the inhibition of EL. Mutations that disrupted trimer formation, which included the leucine mutations we identified in our inhibition studies, had no capacity to inhibit EL. Blue native electrophoresis with ANGPTL3-ANGPTL8 strongly suggests that these complexes form as trimers in a two ANGPTL3 to one ANGPTL8 ratio. Our results also show that some residues of ANGPTL3 are critical for inhibiting both EL and LPL, but some are specifically important only for EL or LPL inhibition. Identification of these residues allows us to dissect distinct pathways by which ANGPTL3 regulates lipoprotein metabolism.

# MSIonization: a machine learning tool for ionization mode prediction of small molecules

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## ABSTRACT

Ionization mode selection is a crucial step in mass spectrometry (MS), particularly when analyzing chemically diverse small molecules in high-throughput workflows, such as metabolomics, drug development, and environmental monitoring. Yet, selecting the appropriate ionization mode, whether a molecule ionizes efficiently in positive or negative mode, remains challenging. It often relies on trial-and-error, which is time-consuming and resource-intensive, representing a major barrier in large-scale MS analyses. To address this challenge, we invented MSIonization, a first machine learning (ML)-driven tool to predict optimal ionization mode (positive or negative) for small molecules, including lipids. It performs binary classification using molecular structure (SMILES) as input to deliver rapid, scalable predictions with probability scores and applicability domain assessments, enhancing sensitivity, specificity, and reproducibility. When we applied to the NPAtlas database of 36,454 molecules, MSIonization achieved a processing rate of 8.03 molecules per second, highlighting its suitability for high-throughput applications. While currently limited to non-volatile, polar to mid-polar compounds within the 50-800 Da mass range, MSIonization can significantly improve the efficiency, reproducibility, and sensitivity of MS workflows. It offers a user-friendly, offline ML-driven solution that streamlines MS workflows and reduces trial-and-error. In the future, it holds significant potential to integrate with AI-driven MS pipelines to predict the ionization mode of small molecules and/or large molecules to support research across a wide range of scientific disciplines. MSIonization is publicly available, and a tutorial for installing and running the MSIonization package can be downloaded at https://www.moleculardetective.org/Links.html.

investigating ANGPTL Interactions with AlphaFold3

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The angiopoietin-like protein (ANGPTL) family consists of 8 members which are structurally similar to angiopoietins. ANGPTL3, ANGPTL4, and ANGPTL8 play important roles in regulating lipoprotein metabolism by inhibiting lipoprotein lipase (LPL) and endothelial lipase (EL). While the atomic structures of ANGPTL proteins have yet to be solved, ANGPTL3 and ANGPTL4 have been shown to form homotrimers, and ANGPTL3 and ANGPTL8 have been shown to form a 2:1 heterotrimer. We have found that formation of these oligomeric structures is essential for inhibiting lipases. Is has been suggested that ANGPTL4 might inhibit other lipases, including hepatic lipase, pancreatic lipase, and lysosomal acid lipase, but these interactions have not been strongly validated. Moreover, other, less studied, ANGPTL family members such as ANGPTL5 and ANGPTL6 have been implied to be associated with triglyceride metabolism in humans. However, their interactions with lipases and with other ANGPTL proteins have not been characterized. Using AlphaFold3 as a prediction tool to explore these interactions could highlight probable interacting partners within the ANGPTL family and predict an interaction with target lipases. To investigate this idea, I developed a Python and PyMOL-based pipeline using AlphaFold3 to predict ANGPTL complex formation as well as identify interactions between predicted ANGPTL trimers and target lipases. Using a method that calculates the interacting residues from multiple predicted models, I show that AlphaFold3 can successfully predict the interactions between experimentally validated ANGPTL oligomers and the lipases they interact with, including the interaction of ANGPTL4 homotrimers and ANGPTL3/ANGPTL8 heterotrimers with LPL. I also show that AlphaFold3 consistently predicts that ANGPTL4 and ANGPTL5 can form a heterotrimer that interacts within the catalytic pocket of EL akin to the interaction ANGPTL3 homotrimers are known to have with EL.

**The cobalamin trafficking protein Mmadhc is a modifier of Pparg-driven adipocyte differentiation**

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Adipogenesis, or the differentiation of preadipocytes into adipocytes, is a coordinated process driven by the transcription factor peroxisome proliferator-activated receptor gamma (Pparg or PPARγ). The regulation of adipogenesis is of particular importance to the understanding of the pathophysiology of obesity, a global health concern that is a primary risk factor for conditions like type 2 diabetes and cardiovascular disease. Several factors contribute to adipogenesis, including genetics. Using the key role of PPARγ in adipogenesis, our lab developed a unique computational genetics pipeline to identify genetic modifiers of PPARγ associated with metabolic traits in a genetically diverse mouse population. With this pipeline, we identified a potential genetic interaction between PPARγ and Mmadhc (Metabolism of Cobalamin Associated D; CblD) that is associated with fat mass gain in mice. CblD is a cytosolic and mitochondrial protein involved in metabolism of vitamin $B_{12}$ (cobalamin) and its conversion into the two biologically active forms of cobalamin: adenosylcobalamin and methylcobalamin. We show that knockdown of CblD in human and mouse preadipocytes blocks adipocyte differentiation and significantly suppresses PPARγ mRNA and protein expression as well as transcription of PPARγ target genes. However, at the start of differentiation, CblD knockdown and control preadipocytes have similar expression of PPARγ. This indicates that CblD knockdown inhibits the amplification of PPARγ early in the differentiation process. In contrast, overexpression of CblD in preadipocytes significantly increases adipocyte differentiation, PPARγ expression, and transcriptional activity of PPARγ. Structure-function studies of the CblD protein show that mitochondrial targeting of CblD mediates its modifier effect on PPARγ, as overexpression of CblD without the mitochondrial targeting sequence fails to increase differentiation or PPARγ expression. Moreover, conditioned media from control adipocytes partially restores the impaired differentiation and suppressed PPARγ expression in CblD knockdown preadipocytes. This suggests that a CblD-dependent factor is secreted early in differentiation that plays an important role in the amplification of PPARγ and adipogenesis. Collectively, our knockdown and overexpression results establish that CblD is a critical regulator of PPARγ-driven adipogenesis, and the structure-function and conditioned media data provide insight into potential mechanisms of this interaction. This novel interaction between CblD and PPARγ may contribute to the pathogenesis of obesity and type 2 diabetes.

## Deletion of Carnitine Palmitoyltransferase 1a From Adipocytes Leads to Subcutaneous Adiposity and Insulin Resistance in Female Mice

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**BACKGROUND:** Carnitine palmitoyltransferase 1 (CPT1) is the rate-limiting enzyme in mitochondrial fatty acid oxidation (FAO). Our laboratory and others have shown that CPT1a is the most abundant CPT1 isoform in white adipose tissue (WAT) in mice and humans, prompting an investigation into its role in adipocyte biology.

**METHODS:** Subcutaneous WAT was collected from lean and obese females with body mass indexes (BMIs) ranging from 19-42. Human adipose derived stem cells (hADSCs) and murine 3T3L1 fibroblasts were used to study adipocyte differentiation *in-vitro*. The genome editing tool CRISPR-Cas9N was used to delete CPT1a in 3T3L1 fibroblasts. Seahorse and stable isotope-resolved metabolomics were employed to measure glycolysis and FAO in WT and CPT1a KO cultured adipocytes. Insulin responses, cellular triglycerides, and proliferation assays were also utilized in this cell culture system. For *in-vivo* studies, eight-week old male and female AKO (*Cpt1a*$^{\Delta Adipo}$) and littermate controls (*Cpt1a*$^{F/F}$) were placed on a high-fat diet (HFD; 60% kcal fat) for 16 weeks. Glucose and insulin tolerance tests were completed after 11 and 13 weeks on diet, respectively. Mice were necropsied after a 16 hour fast, and tissues and serum were collected for insulin and C-peptide analysis, bulk RNA sequencing, and protein expression by immunoblotting.

**RESULTS:** Mitochondrial fatty acid oxidation proteins CPT1a, SCAD, and HADHA were all significantly (>50%) lower in subcutaneous WAT from obese individuals (BMIs 35-42). Further, CPT1a RNA and protein levels decreased by ~75% throughout hADSC and 3T3L1 adipogenesis. Deletion of CPT1a in 3T3L1 fibroblasts led to a ~40% reduction in $^{13}$C-oleate derived enrichment into TCA cycle intermediates (citrate, fumarate), but increased extracellular acidification rates indicative of a shift from mitochondrial FAO to non-mitochondrial metabolism. CPT1a KO fibroblasts exhibited 2.5x greater proliferation rates and increased adipocyte differentiation, which was accompanied by a ~50% increase in triglycerides and a 4-5 fold increase in adipogenic markers (*Cd36*, *Cidec*). In complete growth media, CPT1a KO adipocytes had 3-10x greater Akt and Erk signaling; yet, exogenous insulin treatment led to insulin resistance in CPT1a KO adipocytes.

Complementary in-vivo studies showed that female CPT1a AKO mice had higher body weight and subcutaneous adiposity in response to HFD, as compared to littermate controls. Further, female AKO mice displayed 2-fold higher fasting insulin and insulin to C-peptide ratios, which coincided with glucose intolerance and insulin resistance in these mice. Bulk RNA-sequencing and subsequent enrichment analyses revealed insulin signaling as the most upregulated pathway in subcutaneous adipose tissue of female AKO mice. No changes were observed in male mice across all parameters tested.

**CONCLUSIONS:** Deletion of CPT1a in adipose tissue promotes sex-specific responses in adiposity and insulin resistance. Future research will determine mechanisms by which substrates (fatty acids) and products (acylcarnitines) of CPT1a impact insulin signaling in adipocytes.

# Visceral Adipose Pcpe2 Attenuates Healthy Adipogenesis Through TGFb-Signaling

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The prevalence of obesity in the United States is a persistent and growing concern, contributing to increased rates of cardiovascular disease (CVD) and cancer. Maintaining a healthy cardiometabolic state is multifactorial but mainly involves the remodeling and expansion of visceral adipose tissue (VAT) following excessive caloric intake. The healthiest way to store incoming fat is through the differentiation of precursor cells (PC's) which become new adipocytes, a process called adipogenesis. Our studies show that **pro-collagen endopeptidase enhancer protein 2 (Pcpe2)**, an extracellular matrix glycoprotein, expressed in PCs and 3T3-L1 fibroblasts attenuates healthy adipose expansion. Mice specifically lacking adipose tissue Pcpe2 expression ($Adip^{+}Pcpe2^{KO}$), are resistant to diet-induced obesity body weight gain (25% reduction, $p<0.001$), VAT mass gain (29% reduction, $p<0.001$), as well as plasma glucose levels (30% reduction, $p<0.01$), compared to control mice. **Therefore, we hypothesize that Pcpe2 attenuates healthy adipose expansion by facilitating the signaling of transforming growth factor-beta (TGFβ) in PCs, thereby, reducing transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ) levels, and turning off adipogenesis.**

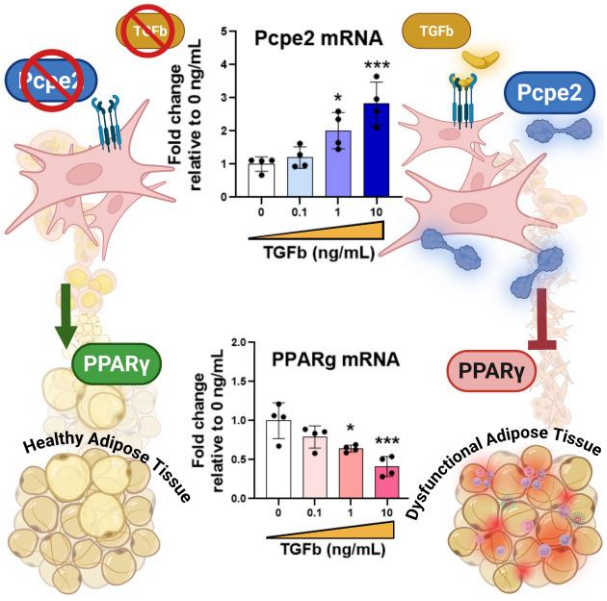


**Figure 1. Healthy Adipogenesis is Attenuated by Pcpe2 Expression.** Dose-dependent treatment of 3T3-L1 cells with TGFb1 shows increased Pcpe2 mRNA expression **(top)** and a reciprocal decrease in PPARg mRNA expression **(bottom).** Since PPARg acts as a master regulator of healthy adipogenesis these data suggest that Pcpe2 acts in response to TGFb1 signaling to redirect PCs away from adipogenesis and towards a fibroblast morphology, resulting in lipid storage inefficiency and consequent inflammation.

To test this, we treated 3T3-L1 cells with increasing doses of TGFβ for 4hrs and measured the mRNA abundance of selected genes using RT-qPCR. **Figure 1 top** shows cellular Pcpe2 mRNA levels were *increased* 1.4-fold when treated with 1 ng/mL of TGFβ, $p<0.05$, and 2.7-fold with 10 ng/mL, $p<0.0001$ compared to no-treatment controls. In a reciprocal fashion, shown in **Figure 1 bottom**, increasing TGFβ treatment *reduced* PPARγ mRNA abundance ~1.6-fold, $p<0.05$, and 3.3-fold, $p<0.001$, respectively. Since PPARγ is a master regulator of adipogenesis, a decrease in its expression will significantly alter PC differentiation into mature adipocytes, thus blocking healthy expansion.

Furthermore, **Figure 2** shows when 3T3-L1 cell Pcpe2 expression was silenced using siRNA, we observed PPARγ levels were elevated compared to nonsense siRNA controls under TGFβ treatment conditions. This data suggests TGFβ-suppressed adipogenesis acts in concert with Pcpe2 to shunt differentiation. In conclusion, our data strongly implies that Pcpe2 contributes to TGFβ signaling and cellular stemness in PCs and thus plays a pivotal role in adipose expansion and remodeling.

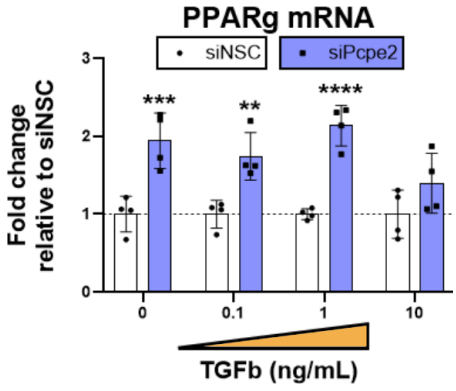


**Figure 2. Pcpe2 knockdown weakens TGFb's role in downregulating PPARγ.** siRNA silencing of Pcpe2 was shown to partially recover PPARg mRNA levels despite TGFb treatment. This data infers Pcpe2 may function to assist in initiating the TGFb signaling cascade extracellularly to fully shunt activation of PPARg and consequent adipogenesis.

## Increased HuR expression in adipose tissue impairs fatty acid oxidation and promotes lipotoxic stress and loss of white adipose tissue

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Expression of the RNA binding protein HuR in adipose tissue has been shown to decrease with obesity and age. Recent work from our lab has established a functional role for the RNA binding protein Human antigen R (HuR) in adipose tissue, with distinct HuR-dependent phenotypes in brown and white adipose tissue. We previously demonstrated that adipocyte-specific deletion of HuR (Adipo-HuR$^{-/-}$) impairs brown adipose tissue (BAT)-mediated thermogenesis through dysregulation of calcium cycling. Additionally, Adipo-HuR$^{-/-}$ results in the development of a spontaneous cardiac hypertrophy and fibrosis, which appears to be dependent on loss of HuR in subcutaneous white adipose tissue (scWAT).

To further elucidate the role of HuR in adipose tissue function, we generated mice with an adipocyte-specific overexpression of HuR (Adipo-HuR$^{OE}$) as a gain-of-function model. Despite no changes in total food intake, activity, or body weight, Adipo-HuR$^{OE}$ mice show a significant decrease in WAT mass, and fail to gain WAT mass even under high fat diet feeding. Indirect calorimetry via CLAMS reveals an increase in respiratory exchange ratio coupled with reduced energy expenditure, consistent with impaired fatty acid oxidation in Adipo-HuR$^{OE}$ mice. Lipidomic profiling revealed an increase in ceramides, DAGs, FAs, and PC/PE lipid species in Adipo-HuR$^{OE}$ mice, suggestive of a lipotoxic phenotype.

Taken together, these results identify HuR as a bidirectional regulator of adipose tissue homeostasis. While HuR deficiency promotes cardiac pathology via WAT dysfunction, HuR overexpression drives WAT wasting through an imbalance between lipogenesis, lipolysis, and fatty acid oxidation, culminating in lipotoxic stress and loss of adipose mass.

**Liver-specific Deletion of Carnitine Palmitoyltransferase 1a Promotes Tumorigenesis in a Mouse Model of Obesity-driven Hepatocellular Carcinoma**

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**Background:** Metabolic dysfunction-associated steatotic liver disease (MASLD) is the fastest-growing etiology of hepatocellular carcinoma (HCC). The primary goal of this project is to determine the contribution of carnitine palmitoyltransferase 1a (CPT1a)-mediated fatty acid oxidation (FAO) in MASLD-driven HCC.

**Methods:** Paired tumor (n=8) and adjacent non-tumor tissue (n=8) were collected from patients with suspected MASLD-driven HCC at the University of Kentucky Markey Cancer Center. Hematoxylin and eosin (H&E) staining was used for pathological determination of tumor and adjacent nontumor tissue. Lipids were extracted via methyl-tert-butyl ether and subjected to lipidomics by the West Coast Metabolomics Center. Bulk RNA-sequencing was employed to assess gene expression changes across paired samples. For murine studies, four-to-five-day old $CPT1a^{F/F}$ and liver-specific CPT1a KO (LKO) pups were treated with 7,12-dimethylbenz[a]anthracene and fed GAN diet (40% kcal fat; Research Diets) until 34 weeks of age. Mice were necropsied after a 24-hour fast. Livers were excised, and total tumor number was calculated.

**Results:** H&E staining showed significant lipid vacuole accumulation in human HCC tumors relative to nontumor tissue. Lipidomic analysis revealed significant increases in long-chain nonesterified monounsaturated fatty acids (MUFAs; C16:1, C18:1, C20:1) and MUFA-enriched phospholipids (PC30:1, PC32:1, PE32:1, and PC36:1) in HCC. On the contrary, both MUFA-(C14:1, C18:1) and PUFA-enriched acylcarnitines (C18:2, C18:3) were collectively reduced in human tumors. Consistent with this lipid profile, fatty acid oxidation genes (*CPT1A*, *CPT2*, *ACADL*, *ACADM*, *ACADS, HADHA*) were significantly reduced in tumor versus nontumor tissue. In mice, CPT1a deletion increased liver to body weight ratios by 50% ($P$=0.0003) and increased overall tumor number by ~3.4X (*3.7 vs. 12.4 average nodules per mouse;* $P$=0.0055). H&E analysis suggests that tumors in mice replicate the histopathology of human HCC.

**Conclusions:** These results suggest human HCC tumors exhibit a reduced capacity to undergo mitochondrial β-oxidation resulting in accumulation of free- and esterified-MUFAs with a concomitant reduction in MUFA-carnitines. Complimentary mouse studies show that CPT1a deletion in hepatocytes promotes HCC in male mice. Future studies are underway to identify mechanisms governing these differences. These findings identify mitochondrial FAO as a potential therapeutic target for MASLD-HCC prevention and treatment.

**Title**

Lipid dysregulation in ER+ breast cancer induced by endocrine therapy sensitizes drug-tolerant persister cells to ferroptosis.

**Authors**

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**Abstract**

Recurrence due to treatment resistance in estrogen receptor alpha (ER)-positive breast cancer occurs in ~30% of patients. Endocrine therapy is effective but leaves behind drug-tolerant persister cancer cells (DTPs) with high oxidative stress and reliance upon lipid metabolism. Suppression of ER signaling with endocrine therapy derepresses the enzyme lysophosphatidylcholine acyltransferase 3 (LPCAT3), which promotes incorporation of polyunsaturated fatty acids (PUFAs) into membranes. The lipidome of DTPs showed increased incorporation of PUFAs that are prone to peroxidation by reactive oxygen species (ROS), eventually leading to cell death through ferroptosis. DTPs evaded ferroptosis through upregulation of the antioxidant enzyme glutathione peroxidase 4 (GPX4). Treatment with the GPX4 inhibitor RSL3 enhanced the effects of endocrine therapy by promoting ferroptosis in DTPs. Our findings make a case for development of therapies to target metabolic vulnerabilities due to lipid dysregulation for treatment of ER+ breast cancer.

## Metaorganismal Choline Metabolism Impacts the Development of Obesity-Driven Hepatocellular Carcinoma (HCC)

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Obese individuals have higher risk of developing liver cancer compared to those with a healthy body weight. This elevated risk is primarily attributed to hepatic fat accumulation and chronic inflammation, that can progressively lead to hepatocellular carcinoma (HCC). In addition to well-known metabolic drivers, recent studies have highlighted the role of microbial metabolites as emerging risk factors in various cancers, including HCC. One such metabolite, trimethylamine (TMA), which is produced by the gut microbiota from dietary nutrients rich in choline. Once absorbed, TMA is converted in the liver into trimethylamine N-oxide (TMAO), a compound that has been implicated in the development of cardiovascular diseases and liver cancer. Although microbial metabolites are known to act as ligands for host receptors and play a significant role in modulating host physiology and disease, targeting these metabolites to inhibit disease progression remains underexplored. In this study, we hypothesized that elevated circulating levels of TMAO contribute to the progression of obesity-driven HCC. To test this, we manipulated circulating TMAO levels through dietary interventions in a mouse model of obesity-driven HCC. C57bl/6J mice were treated with a chemical carcinogen (DMBA) and subsequently subjected to a high-fat diet supplemented either with choline substrate, with TMAO itself, or with a pharmacological inhibitor that blocks the gut bacterial conversion of choline to TMA. This experimental design allowed us to comprehensively assess how altering the levels of this gut microbe-associated metabolite affects HCC development. Our preliminary findings indicate that inhibiting TMAO production attenuates liver tumor progression, suggesting a potential therapeutic angle for targeting microbiota-derived metabolites in obesity-associated liver cancer.

Overexpression of SAA in the liver impairs hepatic lipid metabolism and promotes atherosclerosis in apolipoprotein E-deficient mice lacking SAA1.1, SAA2.1, and SAA3

Ailing Ji, Luke Meredith, Hui Yu, Andrea C Trumbauer, Victoria P Noffsinger, Nancy R Webb, Lisa R Tannock, Preetha Shridas

Objectives: Chronic elevation of acute-phase serum amyloid A (SAA) is a hallmark of persistent inflammation and is linked to increased cardiovascular risk in humans. While SAA deficiency reduces atherosclerosis in mouse models, the direct effects of hepatic SAA expression remain unclear. To investigate this, we utilized a doxycycline-inducible, liver-specific transgenic system to examine how SAA1.1 expression impacts lipid metabolism and atherosclerosis in apolipoprotein E-knockout (apoE KO) mice lacking endogenous SAA1.1, SAA2.1, and SAA3 (TKE mice).

Approach and Results: Twelve-week-old male and female apoE KO and TKE mice were studied, including TKE cohorts carrying either a liver-specific, doxycycline-inducible SAA1.1 transgene (TgL-TKE) or a liver-specific reverse tetracycline transactivator without the SAA gene (TetL-TKE). Mice were maintained on standard chow and treated with doxycycline (1 mg/mL in drinking water) for 8 weeks. Plasma lipid levels were measured at weeks 3, 5, and 8. At study endpoint, liver histology, lipid profiling, and RNA sequencing were performed. In TgL-TKE mice, plasma total cholesterol (TC), triglycerides (TG), and SAA levels increased markedly compared to controls. FPLC analysis revealed a redistribution of SAA from its typical HDL association to VLDL and LDL fractions. Hepatic TC was significantly elevated, while TG levels and lipid droplet abundance were reduced in TgL-TKE livers. Histological examination revealed extensive hepatic fibrosis and potential amyloid deposition. RNA sequencing showed significant alterations in genes related to lipid metabolism, and western blotting revealed increased expression of ApoA1 and ApoB in both plasma and liver tissues. These changes were accompanied by a marked increase in atherosclerotic lesion area in both male and female TgL-TKE mice relative to TetL-TKE and apoE KO controls.

Conclusions: Liver-specific overexpression of SAA1.1 profoundly disrupts hepatic lipid homeostasis, promotes hepatic fibrosis with potential amyloid deposition, and accelerates atherosclerosis.

# Hepatic Expression of Human Cholesteryl Ester Transfer Protein Promotes Steatosis Progression in Male Mice Through an Androgen-Dependent Mechanism

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**Background**
Metabolic associated fatty liver disease (MAFLD) encompasses a spectrum from simple steatosis to steatohepatitis and hepatocellular carcinoma. There are notable sex differences in pathophysiology and associated dyslipidemia that influence cardiovascular risk with females generally lower risk. Understanding the molecular basis of these sex differences may reveal novel therapeutic targets. Cholesteryl ester transfer protein (CETP), a lipid transfer protein expressed in human liver and adipose tissue, facilitates the exchange of triglycerides and cholesteryl esters among lipoproteins. However, the role of CETP in mediating sex-specific susceptibility to MAFLD remains poorly defined.

**Methods**
To dissect the sex-specific role of CETP in hepatic lipid metabolism, we utilized two complementary mouse models: (1) transgenic mice expressing human CETP under its native regulatory elements (huCETP), and (2) wild-type (C57BL/6) mice administered AAV8-TBG-GFP or AAV8-TBG-CETP (hAAV-CETP) to generate liver-enriched CETP expression. Male and female mice were fed a 60% high-fat diet (HFD) for 15 weeks. Metabolic phenotyping included glucose, insulin, and fat tolerance tests performed after 8 weeks of diet. Mice were sac collected serum and tissue followed by analysis of serum lipids, liver histology, mRNA expression, and protein levels.

**Results**
Hepatic expression of CETP exerted divergent effects on MAFLD progression in male versus female mice. While HFD increased adiposity across sexes independent of CETP, male hAAV-CETP mice exhibited increased liver weight, worsened glucose tolerance, and marked hepatic steatosis compared to GFP controls. Conversely, CETP expression improved glucose tolerance in female mice. Histological analysis revealed greater lipid accumulation in CETP-expressing male livers, with upregulation of genes involved in lipid synthesis and gluconeogenesis, while these pathways were downregulated in CETP-expressing females. In vitro, testosterone impaired insulin signaling, supporting androgen-mediated modulation. Fibrogenic gene expression was selectively increased in CETP-expressing male livers. RNA sequencing following orchiectomy and testosterone replacement in huCETP mice identified ~1500 androgen-dependent, CETP-regulated transcripts.

**Conclusion**
CETP drives sexually dimorphic progression of MAFLD, exacerbating steatosis and metabolic dysfunction in males through androgen-dependent mechanisms. These findings position CETP as a potential therapeutic target for MAFLD, particularly in men, and highlight the value of sex-specific strategies in metabolic disease treatment.

**Key words:** MAFLD, Cholesteryl ester transfer protein, lipid metabolism, testosterone.

## Acute Ethanol Exposure Improves Cholestatic Liver Injury in the Humanized *Cyp2c70*$^{-/-}$ Mouse Model: Serendipity Uncovers Novel Pathways Promoting Fibrosis Resolution

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Bile acids (BAs) play important roles in intestinal fat absorption and initiate signaling processes that maintain lipid homeostasis throughout the body. Therefore, BA-focused therapeutic strategies have been leveraged for several diseases including diverse liver diseases. Unfortunately, differences in BA metabolism between mice and humans hamper translation of preclinical outcomes. *Cyp2c70* knockout (*Cyp2c70*$^{-/-}$) mice represent a “humanized” mouse model lacking the liver enzyme responsible for synthesis of 6-hydroxylated muricholate BA species that predominate in rodents. *Cyp2c70*$^{-/-}$ mice instead possess a more hydrophobic “human-like” bile acid composition enriched in lithocholic acid (LCA) and deoxycholic acid (DCA). *Cyp2c70*$^{-/-}$ mice are prone to female-dominant progressive hepatobiliary injury and portal fibrosis at an early age due to excessive levels of LCA and DCA in the liver. Here, we hypothesized that *Cyp2c70*$^{-/-}$ mice would have accelerated liver injury in response to alcohol consumption, given excessive alcohol use is well known to promote liver injury through parallel mechanisms. However, to our surprise after only 10 days of ethanol exposure, the cholestatic liver injury known to occur in *Cyp2c70*$^{-/-}$ mice was markedly improved when compared to *Cyp2c70*$^{-/-}$ mice not consuming ethanol. Acute ethanol exposure unexpectedly reduced circulating markers of liver injury (ALT/AST) and improved histological and gene expression markers of fibrosis, liver inflammation, and ductular reaction normally seen in *Cyp2c70*$^{-/-}$ mice. Here, we have uncovered diverse mechanisms underlying this ethanol-induced resolution of fibrosis including reorganization of the gut microbiome, bile acid pool, and hepatic gene/protein expression that collectively results in striking fibrosis resolution. It has long been appreciated that excessive accumulation of human-relevant BAs (LCA and DCA) can promote hepatic inflammation and fibrosis. The general consensus is that once bridging fibrosis occurs there is little hope for recovery of liver function. However, here we have serendipitously discovered a novel interaction between ethanol consumption and BA metabolism that has important implications in the development of drugs that could potentially reverse advanced liver fibrosis.

**Title:** Cpt1a-Dependent Transcriptional Responses to Lipid-Sensing Transcription Factors in Mouse Primary Hepatocytes

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## Introduction

Carnitine Palmitoyltransferease 1a (CPT1a) is a key regulatory enzyme that mediates the entry of fatty acids into mitochondria for oxidation. Rare and common variants of CPT1a are associated with plasma levels of apolipoprotein B-containing lipoproteins in humans. However, the underlying molecular mechanism for these associations is poorly understood. We recently published that genes in the PPAR pathway were differentially expressed in wildtype and Cpt1a deficient mouse liver. We subsequently confirmed that responses to PPARα and LXRα/β agonists differed in control and Cpt1a knockdown H2.35 hepatocytes. We sought to confirm these differences in mouse primary hepatocytes and extend our findings to the cholesterol-sensing transcription factor Srebp2.

## Methods

Primary hepatocytes were isolated from wildtype (WT) mice. A dose response curve was conducted to optimize transcriptional responses to PPARα and LXRα/β agonists. Following the dose response curve, primary hepatocytes were isolated from both WT and Cpt1a knockout ($Cpt1a^{LKO}$) mice. The cells were seeded on collagen-coated 6-well plates. After 3 hours, the cells were treated overnight with either a control, PPARα (GW7647, 250nm), or LXRα/β (GW3965, 500nm) treatment. RNA was isolated from the cells and the expression of PPARα, LXRα/β and SREBP2 target genes analyzed by rtPCR. Additional cells were imaged by immunofluorescence microscopy for the abundance and subcellular localization of ApoB.

## Results

$Cpt1a^{LKO}$ cells imaged by immunofluorescence microscopy showed significantly greater signal for ApoB within cells, as compared to the WT cells and appeared in a reticular pattern. Irrespective of treatment, CPT1a was reduced by 70% in hepatocytes isolated from $Cpt1a^{LKO}$ mice. In response to PPARα agonist, Fgf21 increased in control but not $Cpt1a^{LKO}$ hepatocytes. Baseline Hmgcr was greater in $Cpt1a^{LKO}$ hepatocytes, but unaffected by PPARα agonist. In response to LXRα/β agonist, Fgf21 increased two-fold, an effect blunted by the absence of Cpt1a. This pattern of gene expression was also observed for LXRα/β target genes Srebp1c and Abca1. In mice lacking hepatic Cpt1a, nuclear Srebp2 was increased as was the abundance of LDL receptor protein in whole cell lysates. These results indicate that the absence of Cpt1a alters cellular responses mediated by lipid-sensing transcription factors and may provide mechanistic insight underlying the relationship between Cpt1a variants that contribute to differences in plasma lipids in patients.

Title: Liver-specific ABCG5 ABCG8-deficiency contributes to Metabolic Dysfunction Associated Steatotic Liver Disease (MASLD) phenotypes in Mice

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**Background:** The ABCG5 ABCG8 sterol transporter is the body's primary defense against the accumulation of dietary sterols. Loss of function mutations in ABCG5 or ABCG8 result in Sitosterolemia, a rare disorder that presents as familial hypercholesterolemia but is distinguished by recessive genetics and the accumulation of phytosterols in plasma and tissues. We previously published that whole-body G5G8 deficiency contributes to hepatic steatosis in mice maintained on high fat diets. More recent literature establishes that cholesterol contributes to MASLD phenotypes in animal models. Therefore, we tested the hypothesis that disruptions in biliary cholesterol secretion would contribute to MASLD phenotypes in mice maintained on a diet that promotes hepatic injury.

**Methods:** Control and liver-specific ABCG5 ABCG8-deficient ($G5G8^{LKO}$) mice were maintained on the NASH-GAN diet (40 kCal% (Mostly Palm Oil, fat, 20 kcal% fructose, 2% cholesterol) for 16 weeks to induce liver injury. Body weight was monitored weekly. A glucose tolerance test was conducted at 15 weeks. Before necropsy, body composition was determined by MRI. Plasma and tissues were collected in the fasted state. Tissues were analyzed for differences in lipid composition, protein expression, and transcriptional landscape.

**Results & Conclusions:** Differences in body weight and composition were not appreciated at the termination of the study. Liver weight was elevated along with hepatic lipids and liver enzymes in $G5G8^{LKO}$ mice relative to Controls. Fasting glucose was unaffected, but insulin levels were elevated, suggesting insulin resistance. C-peptide levels were modestly reduced, consistent with delayed insulin clearance. Glucose levels did not differ at two hours following glucose challenge between genotypes, but reached greater peak levels in both male and female $G5G8^{LKO}$ mice. Endothelial lipase was modestly reduced, while lipoprotein lipase levels were elevated in $G5G8^{LKO}$ mice, suggesting that ApoB-containing lipoproteins emanating from the liver may be hydrolyzed upon secretion and contribute to hepatic steatosis and liver injury. These data suggest that biliary cholesterol secretion protects hepatocytes from injury in mice challenged with the NASH-GAN dietary model of MASLD.

**Dietary β-carotene reduces Dysfunction-Associated Fatty Liver Disease (MAFLD) in mouse models for reversible hyperlipidemia**

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Introduction: Atherosclerosis is the main underlying cause of cardiovascular disease, accounting for millions of deaths worldwide. Our recent findings indicate that β-carotene, the main source of vitamin A, accelerates atherosclerotic lesion resolution in mice.
Hypothesis: Based on these findings, we hypothesize that β-carotene supplementation during atherosclerosis resolution mitigates liver steatosis and inflammation, two characteristic features of Metabolic Dysfunction-Associated Fatty Liver Disease (MAFLD).
Methods: We performed a secondary analysis on liver samples in two experimental models for atherosclerosis resolution. In both models, we fed our mice a vitamin A-deficient Western diet (41% fat + 0.3% cholesterol) for either 12 or 16 weeks. We harvested a subset of mice (Baseline), while the remaining animals underwent resolution by normalizing hyperlipidemia on either a diet with (β-carotene group) or without (Control group) 50 mg/kg of β-carotene.
Results: Normalization of plasma lipids resulted in a reduction of fat and macrophage contents in the liver in comparison to Baseline groups. This reduction was greater in the β-carotene group. The effects of β-carotene were abrogated in mice lacking the enzyme responsible for the cleavage of β-carotene to vitamin A, implicating retinoic acid signaling on the protective effects of β-carotene on MAFLD. We did not observe changes in adipose tissue lipid or macrophage contents. These data suggest that dietary β-carotene could serve as an adjuvant to plasma lipid normalization not only in atherosclerosis resolution, but also in the mitigation of MAFLD.

**16α-hydroxylation of bile acid enhances fatty acid oxidation and decreases lipid accumulation in the hepatocytes through PPARα activation.**

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Metabolic dysfunction-associated steatotic liver disease (MASLD) is characterized by triglyceride (TGs) accumulation in the liver, which can progress into non-alcoholic steatohepatitis and hepatocellular carcinoma. Bile acids (BAs) are expected to be therapeutic targets against MASLD. BAs are synthesized from cholesterol in the liver and act as signaling molecules to regulate energy, lipid, and glucose homeostasis by activating BA receptors, Takeda G protein-coupled receptor 5, and transcription factor farnesoid X receptor (FXR). Activation of FXR has a preventive effect against MASLD by suppressing fatty acid uptake and lipogenesis in the liver. In addition to this, BAs regulate bioactive lipid, oleoyl ethanolamide (OEA) synthesis via an enzymatic reaction. OEA is an endogenous ligand for peroxisome proliferator-activated receptor alpha (PPARα), which enhances fatty acid oxidation (FAO) and suppresses intrahepatic lipid accumulation. Preclinical studies have shown that OEA-PPARα signaling has a preventive effect against MASLD.

Previously, we reported that 3α, 12α and 16α hydroxylated BA, pythocholic acid (PCA), strongly enhances OEA synthesis. Our study showed that PCA treatment increased hepatic PPARα and its target gene expression in mice. Thus, we hypothesized that BAs, especially PCA, regulate hepatic lipid metabolism through PPARα activation. To test this, we evaluated the effect of PCA on mitochondrial activation and TG accumulation in the hepatocytes.

FAO was evaluated using the Seahorse XF Palmitate Oxidation Stress Test Kit (Agilent). PCA treatment significantly increased fatty acid-induced mitochondrial oxygen consumption rate (OCR) and decreased TG levels in WT primary mouse hepatocytes. However, this effect was not observed in PPARα knock-out (KO) primary mouse hepatocytes, suggesting that PPARα is required for PCA-induced FAO and suppression of TG in the hepatocytes. We evaluated the effect of PCA in PPARα activation using a reporter assay. For this, PPARα reporter assay plasmids were transfected into HepG2 cells. PCA treatment significantly increased PPARα activation.

Our findings indicated that BAs induce 1) PPARα activation, 2) enhanced FAO and 3) a preventive effect of lipid accumulation in the hepatocytes. BAs will be potential rategets for MASLD treatment.

**A novel mechanism of bile acids protecting against atherosclerotic cardiovascular diseases**

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**Background and rationale:** Cardiovascular disease (CVD) is a leading cause of death worldwide, and atherosclerosis, or the accumulation of apolipoprotein B (apoB)-containing lipoproteins, such as low-density lipoprotein (LDL), remains a major contributor to CVD pathogenesis. Interestingly, a recent study demonstrated that patients affected by coronary artery disease exhibited decreased levels of bile acids in serum when compared to patients without (Chong Nguyen et al., 2021, *Scientific Reports*). Moreover, beyond its well-accepted role in fat emulsification, bile acids have been reported to interact with tissue-type plasminogen activator (tPA), an essential serine protease involved in the physiological dissolution of blood clots, by blocking tPA from binding to its inhibitor, plasminogen activator inhibitor-1 (PAI-1) (Boonstra et al., 2012, *Annals of Surgery*). Our lab recently ascribed a novel function for free tPA within hepatocytes, revealing its direct interaction with apoB in the endoplasmic reticulum (ER), inhibiting apoB lipidation, and thus, the assembly of very-low density lipoprotein (VLDL) (Dai et al., 2023, *Science*). Upon lipid-loading the cells, PAI-1 sequestered tPA away from apoB, leading to enhanced apoB lipidation and VLDL production (Dai et al., 2023, *Science*). As bile acids are also synthesized in the ER of hepatocytes and found to interact with tPA, this raises an important question of whether bile acids play a protective role against CVDs by increasing the availability of free tPA and thereby reducing VLDL production from hepatocytes.

**Hypothesis:** Bile acids within hepatocytes increase the availability of free tPA to limit VLDL production.

**Methods and Results:** To investigate this, we knocked down cholesterol 7α-hydroxylase (CYP7A1), the rate limiting enzyme for bile acid synthesis, in freshly isolated human primary hepatocytes received through the Human Hepatocyte Isolation Distribution (HHID) at the University of Pittsburgh. Successful knockdown of CYP7A1 was observed at both the mRNA level (79% reduction) and at the protein level (30% reduction) when compared to hepatocytes treated with scramble control siRNA. Further, by knocking down CYP7A1 in hepatocytes, we observed reduced free tPA levels; this was accompanied by a concomitant increase of apoB secretion into the cell culture media when comparing CYP7A1 knockdown hepatocytes to their respective controls. Moreover, we performed an enzyme-linked immunosorbent assay (ELISA) to assess whether bile acids, such as taurocholic acid (TCA), had the ability to bind to tPA and interfere with complex formation with PAI-1. With increasing concentrations of TCA, free tPA that was not bound to PAI-1 was increased. In fact, bile acids competed off PAI-1 binding to tPA with concentrations as low as 2.5 μM. Altogether, our findings suggest that bile acids have the capacity to increase free tPA within hepatocytes, which may then reduce the assembly of apoB-containing lipoproteins.

**Conclusion:** These results implicate a role for bile acids in increasing the availability of free tPA within hepatocytes, and thus, reducing apoB secretion. Collectively, our data suggests that bile acids are involved in regulating VLDL production and atherosclerosis at large.

# Investigating the relationship between the Gut Microbe-TMA-FMO3 Pathway and Steroid Hormone Synthesis in the Hypothalamic-Pituitary-Adrenal Axis

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The gut microbial metabolite trimethylamine-N-oxide (TMAO) has been found to alter cholesterol metabolism in the liver, intestines, and macrophages, impairing reverse cholesterol transport and increasing risk for atherosclerosis. Despite growing knowledge of TMAO's alteration of cholesterol metabolism in the gut and liver, no published studies to date have investigated whether TMAO also disrupts cholesterol metabolism in the adrenal glands, which are responsible for synthesizing cholesterol-derived steroid hormones to regulate glucose and lipid metabolism in both basal and stress conditions. Pilot studies in our lab found that female C57BL6/J mice treated with TMAO-lowering drug fluoromethylcholine exhibited altered expression of various cholesterol-related genes in the adrenal cortex, including the genes encoding the rate-limiting enzymes in steroid hormone (*Cyp11a1*) and corticosterone (*Cyp11b1*) synthesis. These results prompted us to investigate whether TMAO could impact the body's stress response through the hypothalamic-pituitary-adrenal (HPA) axis. Both sexes of wild-type or Fmo3-Tg C57BL6/J mice were subjected to 3-hour restraint stress and compared to their non-stressed controls, where flavin-containing monooxygenase 3 (FMO3) is the main hepatic enzyme responsible for converting gut microbial metabolite trimethylamine (TMA) to TMAO. We found that the stress-induced corticosterone response was significantly blunted in female transgenic mice. Moreover, several of the genes encoding enzymes in steroidogenesis (*Cyp11a1*, *Cyp11b1*, and *Cyp11b2*) were significantly upregulated in female Fmo3-Tg mice. While male mice did not display alterations in these same genes, male Fmo3-Tg mice displayed a strong stress-induced response by upregulating *Hsd3b2*. While our studies indicate that altered TMAO metabolism can alter the activity of the HPA axis in a sexually dimorphic manner, it is not yet clear if this is due to microbial metabolism, TMAO activity, or host FMO3 activity. Future studies in our lab aim to further investigate this relationship and evaluate if it can provoke pathological outcomes in the form of inappropriate HPA signaling.

# 16α-hydroxylated bile acid, pythocholic acid, mediates bioactive lipid and PPARα signaling to regulate food intake

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Obesity can cause serious disease problems such as diabetes and cardiovascular disease. Especially, cardiovascular disease is a leading cause of death in the U.S. Only 5% body weight reduction can lower the risk of these metabolic diseases in obese patients. Currently, only five drugs are approved as anti-obesity drugs by the FDA. However, some adverse effects of these drugs have been reported. Therefore, establishing novel and alternative approaches for obesity and metabolic disease treatment has been expected. Thus, we aim to propose a novel approach to treat obesity. Bile acids (BAs) are potential therapeutic targets for obesity because BAs regulate food intake, energy expenditure, and glucose homeostasis. The previous study showed that the hydroxyl group position of BA plays an important role in its function. We hypothesized that 16α-hydroxylated bile acid, pythocholic acid (PCA), enhances the hypophagic effect due to its unique structure. PCA was isolated from pythons, and their effect on mammals was evaluated. PCA treatment significantly reduced food intake independent of gastric emptying and lipid absorption while increasing jejunal bioactive lipid, oleoyl ethanolamide (OEA), in mice. 7 days of treatment with tauro-conjugated-PCA showed a trend to decrease body weight and significantly improved glucose homeostasis in mice. Next, we evaluated the mechanism of PCA in reduced food intake. The mechanism of OEA-induced satiety is suggested to be mediated by PPARα activation. Thus, we administered PCA (20 mg/kg) by oral gavage in wild-type (WT) and PPARα knock-out (KO) mice. PCA treatment decreased food intake in WT. However, this effect of PCA on food intake was canceled in KO mice. Furthermore, reporter assay demonstrated that PCA treatment significantly increased PPARα activity. These results indicate that PCA decreases food intake via OEA-PPARα signaling. Overall, our study elucidates the role of PCA in food intake and the signaling pathway involved in hypophagia, suggesting PCA to be a novel therapeutic target for obesity and related metabolic disorders.

Monocyte recruitment and fate specification within atherosclerotic lesions

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Atherosclerosis in the arteries is manifested by the accumulation of lipids and immune cells in the arterial intima. Blood monocytes are recruited to the atherosclerotic lesion, differentiate into macrophages, and serve to engulf accumulating lipids. However, they are prone to becoming foam cells, a lipid-saturated and dysfunctional phenotype that serves as a primary driver of plaque progression and eventual rupture, the root cause of heart attack and stroke. While single cell RNA sequencing has expanded our understanding of macrophage heterogeneity within plaques, there have been limited analyses exploring regulators of monocyte lineage commitment in disease. Pseudotime trajectory analysis of scRNAseq gene expression data of plaque immune cells predicted monocytes undergo a mutually exclusive binary fate decision by becoming either inflammatory or lipid loaded, foamy macrophages. An *in vivo* reporter model able to track monocyte recruitment into atherosclerotic plaques showed small, rounded morphology consistent with newly recruited monocytes shortly after labeling. However, over the course of days, labeled cells appeared lipid-loaded with bloated morphology. Additionally, IL-1beta expressing cells were no longer present at this later time point, consistent with data supporting that inflammatory cells are short lived in atherosclerotic plaques whereas foamy cells persist for longer. However, it remains unclear whether recruited monocytes self-renew in spatially restricted regions of atherosclerotic plaques, which may be associated with specific fate decisions. To test this, we established an inducible multicolor reporter model ($CCR2^{CreERT2}$ $Rosa26^{lsl\text{-}Rainbow}$) to analyze clonally expanding monocyte clusters within plaques at early and late stages of atherosclerosis. Ongoing work in our lab will further address cellular crosstalk modulating macrophage fate commitment and continued monocyte recruitment. Understanding the dynamics of monocyte differentiation and interaction within the plaque will illuminate novel mechanisms of atherosclerosis progression and potential therapeutic targets.

## Multi-Omics Analysis of Postprandial Mesenteric Lymph Reveals Role for SAA in Systemic Postprandial Inflammation

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**Background:** Postprandial triglyceride (TAG) levels are an independent predictor of cardiovascular risk in humans. Chylomicrons (CMs) are the primary carrier of dietary TAG and they travel to the circulation via intestinal lymph. The protein components of CM's can impact their metabolism in the circulation, however, the composition of CM's is difficult to study because these particles are rapidly metabolized upon entry to the circulation.

**Objectives:** 1) Determine the protein and lipid composition of mesenteric lymph during fasting and after a lipid bolus, and 2) identify proteins in lymph that may impact the metabolism of newly secreted chylomicrons prior to entry into the circulation.

**Methods and Results:** A conscious lymph fistula procedure was used to collect mesenteric lymph from mice before and after infusion of a mixed lipid bolus into the duodenum. Wild type mice were compared to *Dennd5b*$^{-/-}$ mice (n=5-8 mice/group), a model of impaired chylomicron secretion. This study revealed that, compared to wild type mice, *Dennd5b*$^{-/-}$ mice exhibit significantly reduced TAG content in the lymph (-94%, $p<0.001$). Electron micrographs revealed that the lymph of wild type mice had both greater number and larger sized lipid particles compared to *Dennd5b*$^{-/-}$ mice. In addition, lipidomics analysis of fasting and postprandial lymph showed significant increases in triglycerides and free fatty acids in wildtype, but not *Dennd5b*$^{-/-}$ mice. Shotgun proteomics revealed significant changes in the lymph proteome of wild type mice after lipid bolus (14 proteins increased and 18 decreased; $p<0.01$). Key protein changes were confirmed by western blotting. One notable finding was an increase in lymph Saa1 after the lipid bolus (+200%, $p<0.001$). Size-exclusion chromatography was used to determine the distribution of Saa1 across lipoprotein fractions in lymph and demonstrated that Saa1 was associated primarily with fractions that contain chylomicrons and to a lesser extent with HDL-containing fractions. To understand the functional role of Saa association with lipoproteins under postprandial conditions intralipid gavage studies were performed in wild type and Saa$^{-/-}$ mice and plasma triglycerides and plasma IL6 levels were measured over 6 hours. While Saa$^{-/-}$ mice had no significant difference in postprandial plasma triglyceride concentrations compared to wild type mice, Saa$^{-/-}$ mice have significantly higher postprandial plasma IL6 concentrations compared to wild type mice.

**Conclusions:** We identified several proteins in intestinal lymph that respond to dietary lipid ingestion. In wildtype mice, lipid feeding increases lymphatic Saa1, which appears to be predominantly associated with chylomicrons. This response was not observed in *Dennd5b*$^{-/-}$ mice, indicating that it is dependent on successful CM secretion from intestinal epithelium. Our studies reveal that Saa contributes to attenuation of postprandial inflammation, but may not significantly impact chylomicron lipid metabolism in the lymph or circulation.

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**Lipid modification of cell-free small RNA drives innate immune training through TLR2**

Clark Massick, Dani Michell, Eldaah Pualraj, Jeffrey Franklin, Robert Coffey, **Kasey C. Vickers**

Mature macrophages have stories to tell. Innate immune cells, e.g., myeloid cells, display immunological memory of past insults through epigenetic changes. Pathogen-associated (PAMP) and damage-associated molecular patterns (DAMP) reset cellular gene expression programs through DNA methylation changes within regulatory elements of inflammatory cytokines and receptors. Whether to increase efficiency of responsiveness or to provide tolerance of repeated stimuli, trained innate immune cells often mount altered responses to disparate stimuli and provide cross-protection to infections and sterile inflammation. A major driver of systemic inflammation and DAMP activity is conferred by cell-free RNA (cf-RNA); however, the links between cf-RNA, DNA methylation, and innate immune training were unknown. Cf-RNA are transported in plasma and biofluids by various carriers, including extracellular vesicles and lipoproteins; however, a large majority of RNA in plasma and interstitial fluid are likely bound to small, multi-protein complexes termed Non-Vesicle Extracellular Particles (NVEP). Recently, we discovered a NVEP sub-species, termed supermeres. Based on native gel electrophoresis and RNA-to-peptide cross-linking mass spectrometry studies, supermeres were found to harbor the RNA-binding metabolic enzyme - lactate dehydrogenase (LDHA/B). Plasma levels of LDH are historical markers of tissue damage and ischemia. The contribution of LDH-sRNA supermeres to innate immune responses were not previously known; however, these particles appear to be strong drivers of trained immunity. RNA-bound LDH+ particles were found to significantly decrease *DNMT3A* expression and activity in primary macrophages, a key DNA methyltransferase in trained immunity. LDH+ supermeres were also shown to reduce 5-methylcytidine (5-mC) DNA levels in macrophages, as quantified by global ELISA and LC-MS/MS. Conversely, LDH+ particles were found to significantly increase *TET1* expression, a ten-eleven translocation demethylase enzyme responsible removing 5-mC DNA adducts. LDH+ supermeres were be strong drivers of training in monocytes/macrophages, as mouse monocytes stimulated with LDH+ supermeres showed dramatically altered responses to repeated or disparate stimuli after long washout periods. Moreover, these changes were associated with suppression of DNMT3A and hypo-methylation of DNMT3A-target genes in trained macrophages. These epigenetic changes may be linked to LDH activation of toll-like receptor 2 (TLR2) signaling, as both small molecule inhibitors of TLR2 (c29) and *Tlr2*-deficienct models showed reduced supermere activation of primary macrophages. LDH+ supermeres were also found activate TLR2 in a NF-kB transactivation screen. Most interestingly, LDH+ supermeres were found to transport endogenously acylated-cf-sRNAs, which may serve as ligands for TLR2. Results from these studies support that LDH+ supermeres likely confer innate memory, potentially through lipid-modified cf-sRNA-induced TLR2-mediated DNA hypomethylation. Identifying the extracellular signals, protein carriers, and receptors of immunological memory are critical to understanding this phenomenon and designing better therapeutic approaches to prevent and treat infections and chronic inflammatory diseases.

## The Metabolite 3-Phenyllactic Acid Can Modulate Alternative Aactivation of Macrophages: Implications in Systemic and Metabolic Inflammation

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Macrophage polarization during an immune response is a crucial phenomenon that dictates the body's ability to respond to pathogens. Often dysregulation in macrophage skewing to either the proinflammatory M1 or anti-inflammatory M2 phenotype can exacerbate systemic inflammation, which can be observed in several cardiometabolic diseases. Interleukin 4 (IL-4) signaling through the IL-4 receptor $\alpha$ on macrophages leads to their alternative activation towards a M2 phenotype, which has a protective effect against cardiac dysfunction and metabolic inflammation. Although both bacterial and viral patterns can elicit a proinflammatory response in macrophages, their metabolites might have other immunomodulatory abilities as well. Therefore, we designed an experiment to identify metabolites originating from metaorganismal metabolism that can elicit an immunomodulatory response. We screened ~200 metabolites to determine their effects on alternative activation of macrophages. 3-Phenyllactic acid (3PLA) was one such hits that showed significant upregulation of IL-4 mediated macrophage activation markers. It is interesting to note that 3PLA can be synthesized by both the host (L-3PLA) and gut bacterial (D-3PLA) metabolism. To further understand its role in *in vivo* immune modulation, male, 10 weeks old C57BL/6J mice were supplemented with 3PLA in diet for 2 weeks, subcutaneously challenged with IL-4 on the 15$^{th}$ day and necropsied 6 h post injection. LC-MS/MS analysis showed that 3PLA was systemically elevated in the mice supplemented with the same metabolite in diet. An analysis of circulating levels of plasma cytokines revealed that 3PLA was able to significantly reduce several pro-inflammatory cytokines, like IL-1β, IFN-γ, IL-12p70, TNF-α and KC/GRO, suggesting a strong systemic anti-inflammatory effect of the metabolite. In addition, 3PLA supplementation did not alter endogenous IL-4 levels, however, when injected with IL-4, these mice had significantly lower levels of plasma IL-4 – suggesting a possible effect of 3PLA in IL-4 turnover, which might affect immune homeostasis. Taken together, these data strongly suggest that 3PLA might be an important immunomodulatory metabolite that can regulate macrophage function. Mechanistic understanding of how this metabolite affects immunometabolism in macrophage activation can help develop 3PLA as a potential therapeutically important metabolite, especially in context of cardiometabolic diseases.

**Keywords:** Macrophage polarization, 3-phenyllactic acid, metabolite, immunomodulation, cardiometabolic diseases

**Title:** Suboptimal vitamin A status promotes sterile inflammation in rodents and is associated with increased inflammatory markers in humans

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**Abstract**

Vitamin A deficiency, defined as plasma retinol levels < 0.7 μM, is a global health issue particularly in developing countries. Vitamin A deficiency not only impairs normal growth in children but also disrupts the adequate development of the immune system and is associated with increased inflammation. Even though vitamin A deficiency is considered rare in the US, vitamin A insufficiency (plasma retinol < 1.05 μM) is relatively common in children, pregnant women, and people with specific genetic variants. We hypothesize that vitamin A insufficiency is a previously uncharacterized risk factor for the development of cardiometabolic diseases by sustaining low-grade inflammation. Exploration of National Health and Nutrition Examination Survey datasets reveal a significant association between inflammatory markers and vitamin A insufficiency in humans. Vitamin A-deficient diet in developing rats stunted growth and increased monocytosis and neutrophilia. To assess the effects of chronic vitamin A insufficiency in the context of cardiometabolic disease, we optimized a method to sustain vitamin A insufficiency in hyperlipidemic mice fed Western Diet. Vitamin A insufficiency resulted in increased systemic and tissue inflammation, including atherosclerosis, without alterations in plasma lipid levels. The combination of vitamin A deficiency with hyperlipidemia resulted in the proliferation of myeloid-biased hematopoietic progenitors in the bone marrow. Our findings highlight the complex interplay between vitamin A, inflammation, and hyperlipidemia in the context of cardiovascular health.

**Therapeutic Trem2 Blockade Attenuates Atherosclerosis Progression and Alters Local Plaque Transcriptome and Macrophage Metabolome**

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Atherosclerosis is driven by LDL cholesterol deposition in the aortic intima, forming a plaque. Monocytes infiltrate atherosclerotic lesions and differentiate into macrophages, including lipid-loaded foamy macrophages that promote plaque progression and are defined by expression of the lipid sensor Trem2. Our lab implicated Trem2 as a key factor modulating atherosclerosis in vivo. We found that Trem2 mediated foam cell formation, and deletion of Trem2 in myeloid cells resulted in increased foam cell death, reduced macrophage proliferation, and reduced plaque size. Further, we show that administration of a Trem2 blocking antibody also results in attenuated plaque progression. We performed single cell RNA sequencing on aortas from atherosclerotic mice with or without myeloid-specific Trem2 deletion. scRNAseq analysis aligned with our previous work, revealing that Trem2-deleted aortas had a fewer foamy macrophages than controls, as well as impaired cholesterol homeostasis and oxidative phosphorlyation. Lastly, metabolomics analysis of Trem2-sufficient and -deficient macrophages *in vitro* revealed reduced cholesterol ester formation in Trem2-deficient cells, suggesting a defect in lipid storage. In all, this study highlights potential mechanisms of Trem2 signaling contributing to macrophage function in plaque.

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Title: NAPE-PLD role in the regulation of macrophage function and atherosclerosis

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Efferocytosis, the phagocytic clearance of apoptotic cells, is an important component of the resolution of inflammation and impaired efferocytosis contributes to the expansion of necrotic cores within atherosclerotic plaques, making these plaques vulnerable to rupture, which can in turn leads to myocardial infarction and death. A better understanding of the factors driving this impaired efferocytosis could lead to therapeutic strategies to rescue this function. Human coronary arteries with vulnerable atherosclerotic plaques have significantly reduced expression of N-acyl-phosphatidylethanolamine hydrolyzing phospholipase D (NAPE-PLD, as do the aortas of Apoe-/- mice fed a Western diet. NAPE-PLD catalyzes three known activities: 1) Hydrolysis of N-acyl-phosphatidylethanolamines (NAPEs) to generate bioactive N-acyl-ethanolamines, including palmitoylethanolamide (PEA), oleoylethanolamide (OEA), and anandamide (AEA); 2) Hydrolysis of N-aldehyde modified phosphatidyethanolamines (NALPEs) such as N-isolevuglandin-PE (N-IsoLG-PE) and N-hydroxynonenal-PE (N-HNE-PE) to inactivate these pro-inflammatory phospholipids; and 3) the transport of pyridoxal-5'O-phosphate and potentially other related compounds across organelle membranes. Previous studies showed that treating atherosclerotic mice with OEA or PEA inhibits atherosclerosis and that PEA treatment of bone marrow-derived macrophages (BMDM) improved their efferocytosis capacity, leading us to hypothesize that NAPE-PLD critically regulates macrophage phenotype and function. We found that BMDM from *Napepld*$^{-/-}$ (KO) mice or BMDM from wild-type (WT) mice had reduced efferocytosis capacity compared to vehicle treated WT BMDM, while WT BMDM treated with NAPE-PLD activators had increased efferocytosis capacity. Furthermore, treatment of WT BMDM with N-IsoLG-PE reduced efferocytosis, while treatment with NAPE-PLD product OEA enhanced efferocytosis. Together, these findings suggest that NAPE-PLD regulates efferocytosis through both its synthesis OEA and PEA and by its ability to clear pro-inflammatory NALPEs such as N-IsoLG-PE. To understand how loss of NAPE-PLD altered macrophage function, we treated BMDM with the NAPE-PLD inhibitor LEI-401 or with OEA or both and then performed RNA-Seq. LEI-401 markedly increased expression of inflammatory genes (Adgre1, Cd14, Ccr-1,-3,-5; Ccl-2, -5,-7, -8, -9, -12; Cxcl-2, -3, 2, Saa3, Mmp-9,-13) and oxidized LDL scavenger receptors (Msr1, Cd36, and Marco) and co-treatment with OEA only partially blocked the effects of LEI-401. Treatment of RAW264.7 mouse macrophage cells with LEI-401 or NAPE-PLD activator altered their oxLDL uptake and efflux of cholesterol to HDL. KEGG pathway analysis also identified changes in genes related to DNA replication, mismatch repair, and cell cycle, which may also play a role in reduced efferocytosis, but future studies are needed to confirm their functional alteration and contribution.

Ldlr-/- mice that received bone marrow transplants from Napepld-/- mice had worsened atherosclerosis than those that received transplants from Napepld+/+ littermates.

# Identification of Oxidized Diacyl-PUFA phosphatidylcholines as inducers of ferroptosis in macrophages

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Ferroptosis is a regulated form of cell death characterized by iron-dependent lipid peroxidation, glutathione (GSH) depletion, and inactivation of glutathione peroxidase 4 (GPX4). It plays a critical role in the pathogenesis of various diseases, including cancer, neurodegeneration, and cardiovascular disorders. Recent studies have identified diacyl polyunsaturated fatty acid phospholipids (PUFA2s) as key initiators of ferroptosis via mitochondrial reactive oxygen species (ROS) production and subsequent lipid peroxidation in the endoplasmic reticulum. However, the role of PUFA2s inducing ferroptosis in macrophages remains largely unexplored. Here, we use Diarachidonoyl-sn-glycero-3-phosphocholine (DAPC) as a PUFA2s.

We tested the hypothesis that oxidized DAPC (OxDAPC) is essential for initiating ferroptosis in macrophages. Treatment of RAW 264.7 macrophages with OxDAPC (80 μM) for 24 hours led to 50% cell death as measured by LDH release (CytoTox 96® Assay) and ATP-based luminescence (CellTiter-Glo®). To assess ferroptosis, macrophages were pre-treated with ferroptosis inhibitors ebselen (5 μM), ferrostatin-1 (1 μM), liproxstatin-1 (100 nM), or Trolox (50 μM) for 1 hour. Surprisingly, none of the ferroptosis inhibitors rescued OxDAPC -treated cells, suggesting that oxidized DAPC may bypass the early lipid peroxidation steps targeted by these inhibitors. This implies that once lipid peroxidation is established, radical scavengers are no longer effective. Furthermore, apoptosis (zVAD-fmk), necroptosis (Necrostatin-1), pyroptosis (TAK242) inhibitors also did not rescue cells death. RT-qPCR analysis revealed upregulation of ferroptosis- and NRF2-associated genes (Acsl4, Srxn1, Txnrd1, Alox5, Keap1, Got1) in response to OxDAPC treatment, with reversal observed upon co-treatment with ferrostatin-1. To further investigate phenotype-specific sensitivity, macrophages were polarized into M1 (LPS, 1 μg/mL), M2 (IL-4, 10 ng/mL), or Mox (OxPAPC, 50 μg/mL) subtypes prior to OxDAPC exposure. M2 macrophages were more susceptible to OxDAPC induced ferroptosis whereas M1 macrophages displayed increased resistance.

Ongoing studies involve LC–MS/MS-based structural characterization and quantification of oxidized DAPC species as potential ferroptosis biomarkers in cells, tissues, and plasma. These findings enhance our understanding of lipid oxidation-driven ferroptosis and underscore the importance of macrophage phenotypic polarization in determining susceptibility to ferroptosis.

**Exploring the mechanisms of non-enzymatic carotenoid disposal**

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**Abstract:**

Introduction: Carotenoids are lipophilic compounds with antioxidant properties. Their structural differences result in varying degrees of polarity, which can influence their absorption and bioavailability. Humans store intact carotenoids predominantly in the liver and adipose tissue, where these compounds can be progressively cleaved to apocarotenoids by the two carotenoid-cleaving enzymes: β-carotene oxygenase 1 (BCO1) and BCO2. As wild-type mice have high activity of these enzymes, they do not accumulate intact carotenoids in tissues; therefore, we use mice lacking BCO1 and BCO2 to study carotenoid homeostasis. Using these "humanized" mouse models, we recently identified a novel fecal carotenoid disposal pathway for β-carotene. Building on these findings, we now aim to investigate the mechanism(s) regulating carotenoid homeostasis.

Hypothesis: Mobilization and elimination routes of carotenoids depend on their polarity.

Methods: *Bco1*$^{-/-}$ and *Bco2*$^{-/-}$ mice were fed diets containing the same concentration of different carotenoids with increasing polarities: β-carotene (no polar groups), β-cryptoxanthin (one hydroxyl group), or lutein (two hydroxyl groups) for four weeks. By this time, mouse tissue carotenoid levels become comparable to those found in humans. At the end of the 4-week supplementation period, a set of mice was sacrificed to serve as the baseline group. The remaining mice were switched to a carotenoid-free diet for two weeks to assess the depletion of tissue carotenoids. Carotenoid levels in tissues and plasma were measured by high-performance liquid chromatography (HPLC).

Results and conclusion: Our results showed a similar decline in carotenoid levels across all washout groups, regardless of carotenoid polarity. This decline was observed in plasma and liver but not in adipose tissue. Our findings suggest that polarity does not play a major role in carotenoid mobilization and fecal disposal but highlight tissue-specific differences in carotenoid retention. Further studies are needed to clarify the mechanism(s) behind carotenoid mobilization from storage tissues.

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