Discovery of low molecular weight novel serum biomarkers for Preeclampsia and Alzheimer's disease

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Project I : Low molecular weight serum biomarkers of preeclampsia

PREECLAMPSIA

- ***Pregnancy related disorder***
  - a major cause of maternal and fetal mortality and morbidity
  - affects nearly 8 % pregnancies worldwide
  - cases reported yearly ~ 8.5 million
  - maternal deaths per year worldwide ~ 50-75,000

- ***Major symptoms include-***
  - Hypertension >140/90
  - Proteinuria > 0.3g/24h
  - Edema
  - Can progress to eclampsia/convulsions

Walker. J.J, Lancet; Pre-eclampsia. 2000, 356, 1260-1265

NEED FOR DIAGNOSIS

- Diagnosis through symptoms is possible mainly after 20 weeks

- No developed prevention and treatment therapies available

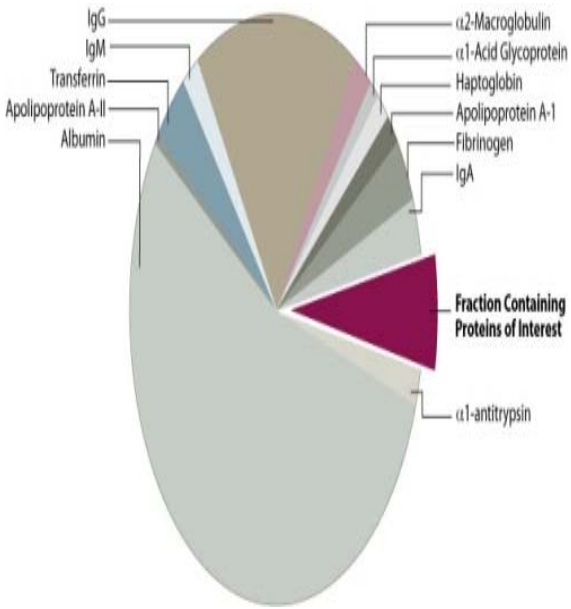
  Delivery of the baby is the only available cure

- Early diagnosis for confirmation and risk stratification of PE

BIOMARKERS

WHY PEPTIDES NOT PROTEINS ?

- ***Low molecular weight proteins and peptide***
- Produced by specific diseased related enzymatic cleavage
- informative
- ***Large proteins***
- Mainly albumins, globulins, transferrin, lipoproteins
- House keeping proteins
- Ten orders of magnitude higher in abundance than low M.W. peptides.
- Signal suppression (removal leads to appearance of ~5,000-30,000 molecular species)
- Column fouling



LAB APPROACH

Sera from 24 cases and 24 controls collected at 12-14 weeks of pregnancy

Protein depletion

↓

cLC/MS (QTOF) separation

↓

Mass spectral data

↓

Spectral overlays for quantitative differences

RESULTS

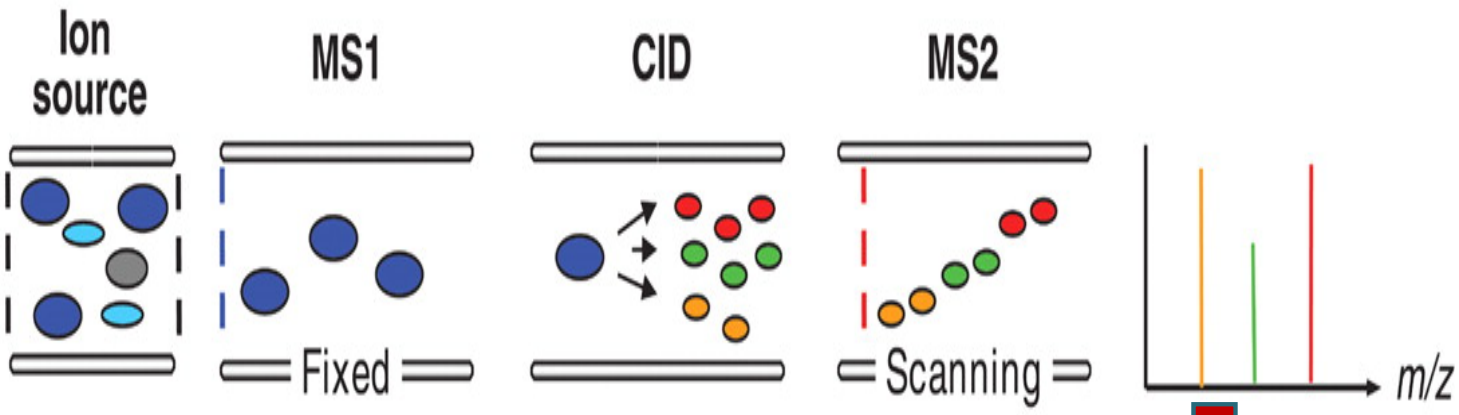
39 statistically significant (p<0.05) markers detected using Student's t-test

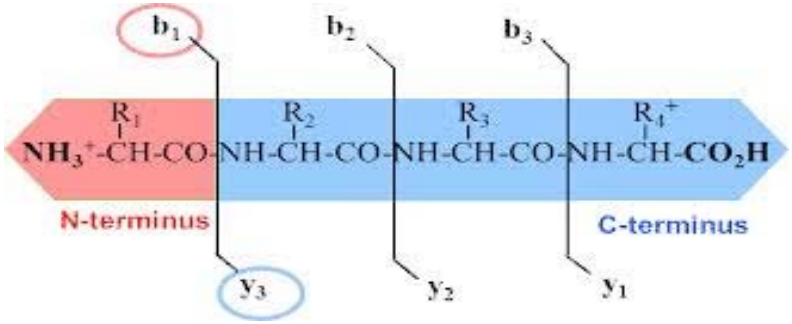
30- ↓ PE cases

9- ↑ PE cases

Anand, Swati, et al. "Serum biomarkers predictive of pre-eclampsia."*Biomarkers in medicine* 9.6 (2015): 563-575.

TANDEM MS





MASCOT

http://www.sciencemag.org/content/312/5771/212/F1.large.jpg

6 PEPTIDES SEQUENCED USING CID

S. no.	m/z	Higher in cases/or controls	Sequence	Parent Protein
1	1238.5	controls	QGVND---	Beta fibrinogen
2	942.5	controls	HWESA---	Complement C3
3	676.6	cases	QLGLP---	ITIH 4
4	1070.8	cases	NWHSA---	ITIH 4

Anand, Swati, et al. "Serum biomarkers predictive of pre-eclampsia."*Biomarkers in medicine* 9.6 (2015): 563-575.

19 LIPID BIOMARKERS

GLYCEROPHOSPHOCHOLINES

PC, SM, LPC

m/z	Higher in	Subclasses
594.3	cases	Oxidized glycerophosphocholines
508.3	controls	1Z-alkenylglycerophosphocholines
756.5	controls	Diacylglycerophosphocholines

Anand, Swati, et al. "Serum biomarkers predictive of pre-eclampsia."*Biomarkers in medicine* 9.6 (2015): 563-575.

AUC'S FOR INDIVIDUAL MARKERS

m/z	AUC
739	0.8
639	0.79
1026	0.76
553	0.75
508	0.74
634	0.7
1074	0.7

Anand, Swati, et al. "Serum biomarkers predictive of pre-eclampsia."*Biomarkers in medicine* 9.6 (2015): 563-575.

MULTIPLE MARKER PANELS WITH AUC>0.9

m/z for panels	AUC
619, 739, 649, 1111, 756, 723	1
568, 734, 718, 719, 593, 1026, 503	0.99
621, 639, 734, 649, 503, 756, 634, 1069	0.98

Anand, Swati, et al. "Serum biomarkers predictive of pre-eclampsia."*Biomarkers in medicine* 9.6 (2015): 563-575.

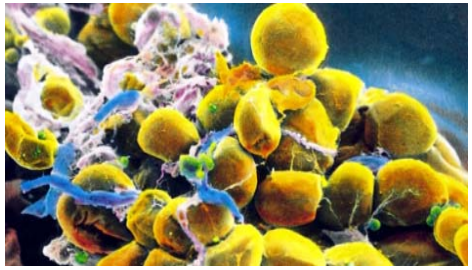
CONCLUSIONS

- Discovery of 39 novel statistically significant biomarkers
- MS2 studies on 39 markers
- Sequencing of 6 peptide biomarkers
- Characterization of lipid biomarkers
  - 3 PC, 1 LPC, 1 oxidized PC, 6 PC dimers, 5 SM
  - 3 unidentified peptides
  - 14 uncharacterized likely lipids
- Production of multi marker model AUC >0.98

Project II. Discovery and confirmation of novel serum lipidomic biomarkers for Preeclampsia

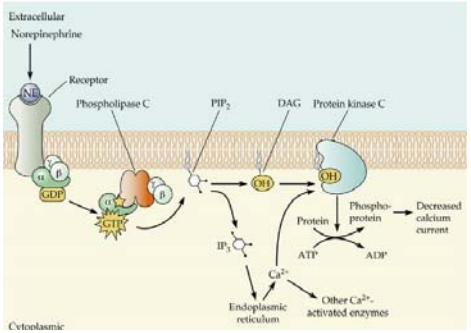
WHY LIPIDS?

- Energy Metabolism/Reserves

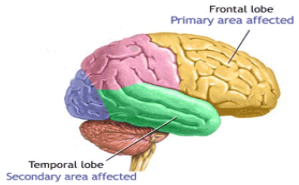


- Membranes

- Cellular Regulation
  (signaling messengers)

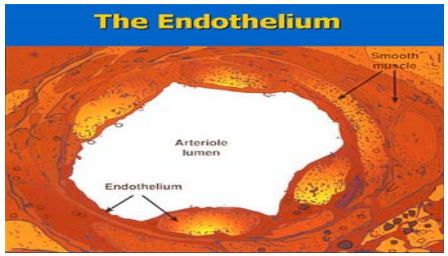


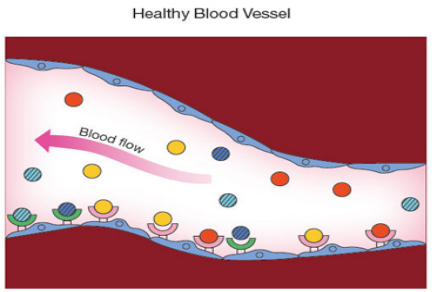
- Dysfunctioning of lipid signaling -central role in many diseases

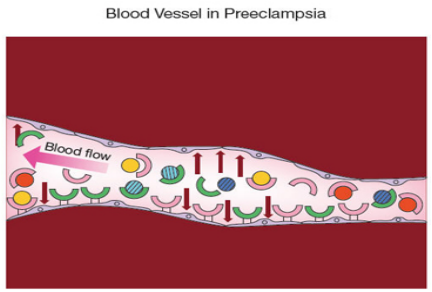


LIPIDS IN PREECLAMPSIA

Dyslipidemia → Endothelial dysfunction → Preeclampsia







Evidence of Imbalanced of PGI2 and TXA2

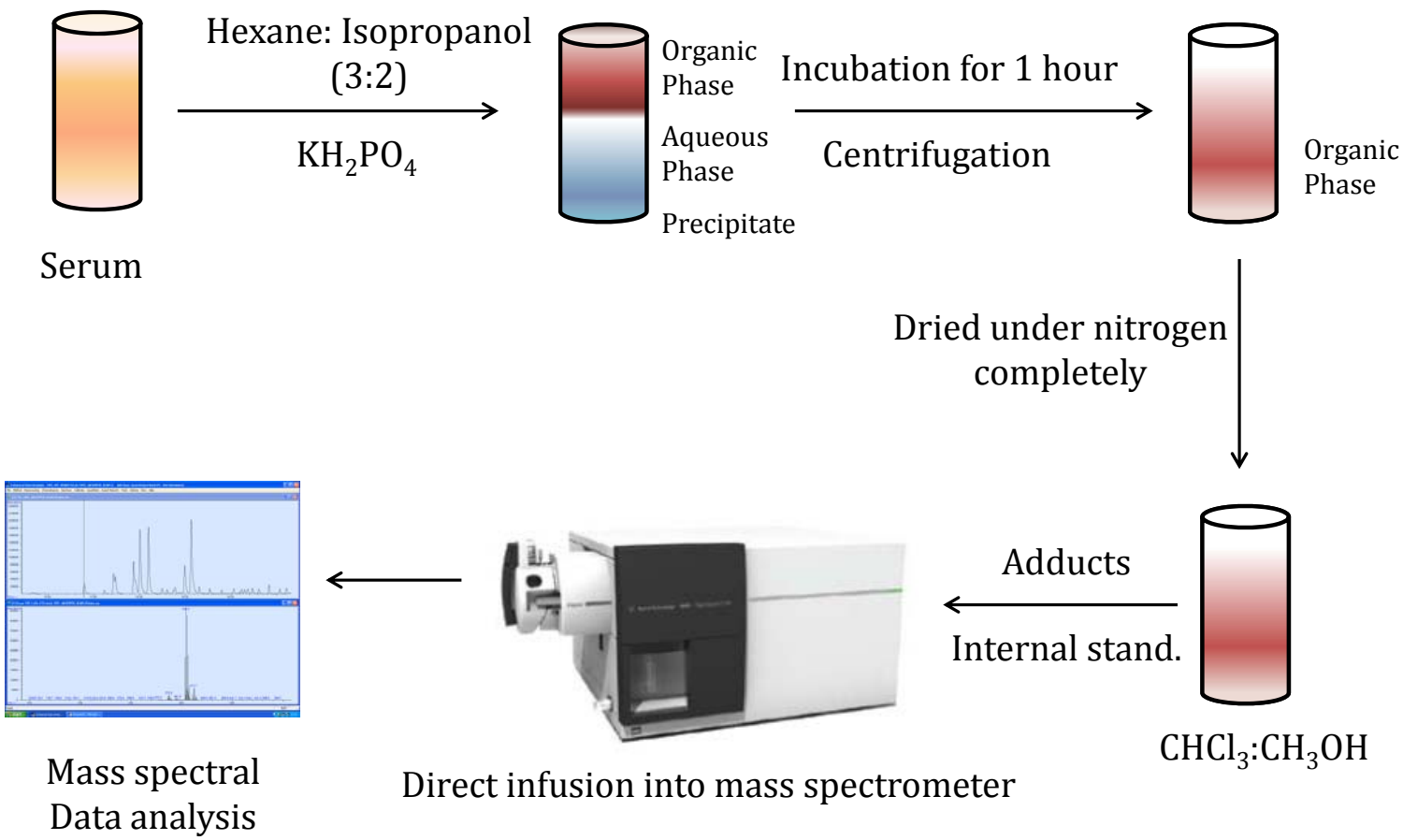
Evidence of Reactive oxygen species

Evidence of Apoptosis

http://www.medscape.org/viewarticle/557238

http://www.nichd.nih.gov/news/releases/Pages/080311-levine.aspx

LAB APPROACH
DISCOVERY SET – 27 CONTROL AND 29 CASES (12-14 WKS)



RESULTS

45 statistically significant lipid markers detected using Student's t-test

19- ↓ PE cases

26- ↑ PE cases

Anand, Swati, et al. "Detection and confirmation of serum lipid biomarkers for preeclampsia using direct infusion mass spectrometry." *Journal of lipid research* 57.4 (2016): 687-696.

VALIDATION OF POTENTIAL BIOMARKERS IN A SECOND CONFIRMATORY SET

43 CONTROLS and 37 CASES

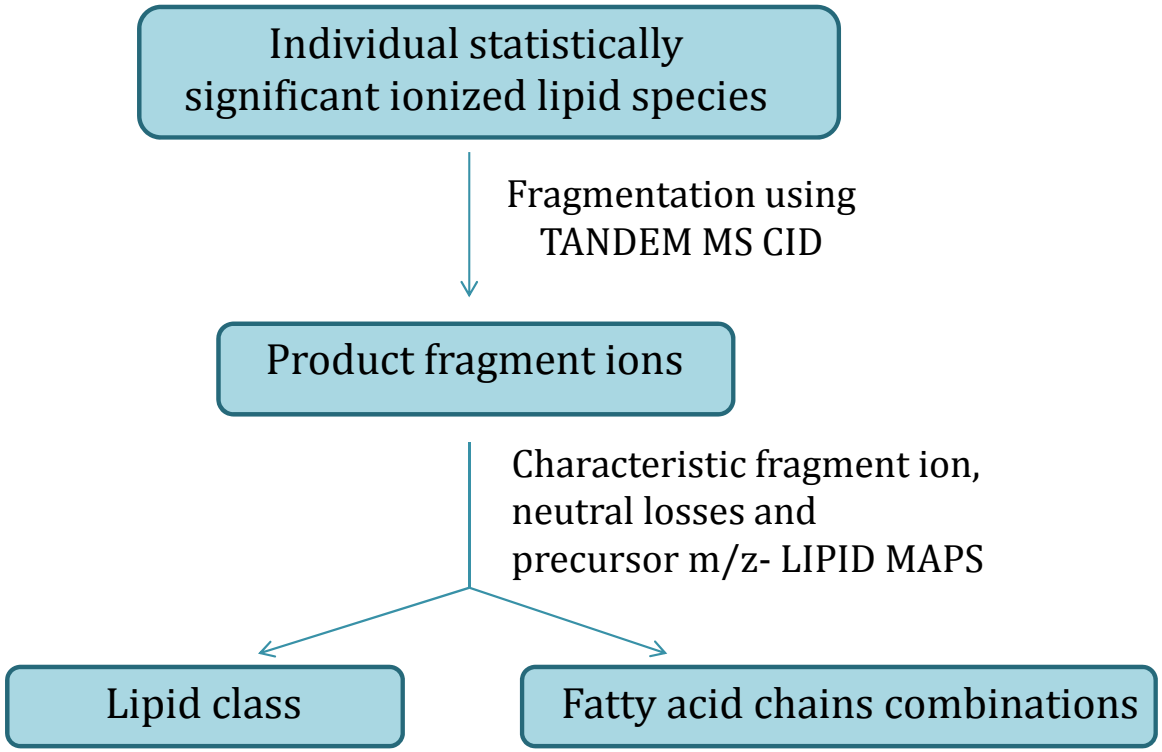
23/45 – validated

13 ↓ PE cases

10 ↑ PE cases

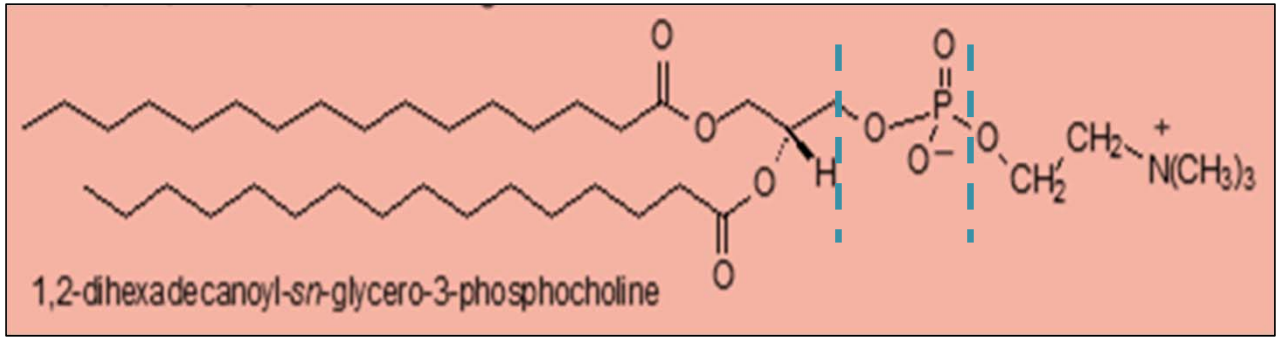
Anand, Swati, et al. "Detection and confirmation of serum lipid biomarkers for preeclampsia using direct infusion mass spectrometry." *Journal of lipid research* 57.4 (2016): 687-696.

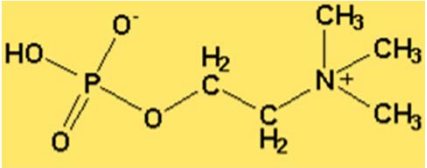
IDENTIFICATION OF THE POTENTIAL CANDIDATE BIOMARKERS



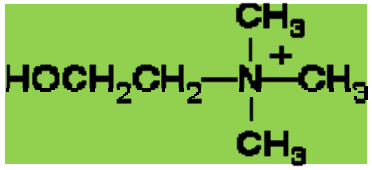
INTERPRETATION OF FRAGMENTATION SPECTRA

Phosphatidylcholine





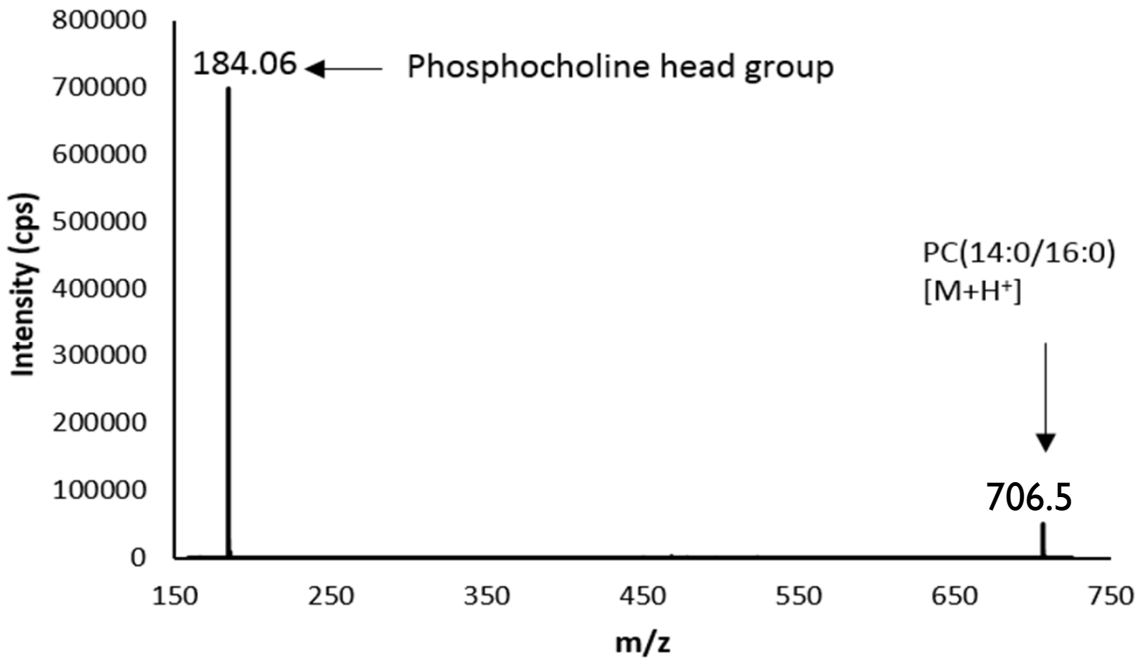
m/z 184 Phosphocholine



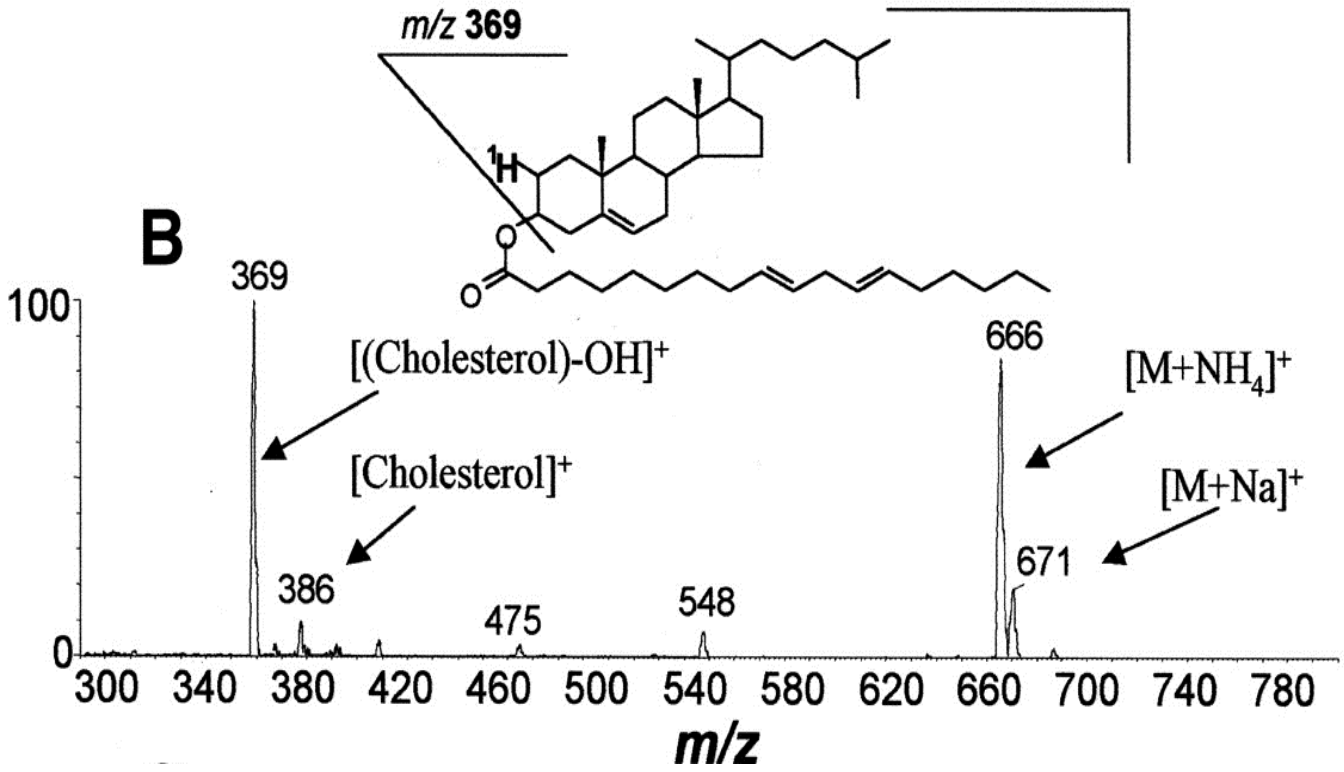
m/z 104 Choline

http://lipidlibrary.aocs.org/lipids/pc/index.htm

FRAGMENTATION SPECTRUM OF A PC

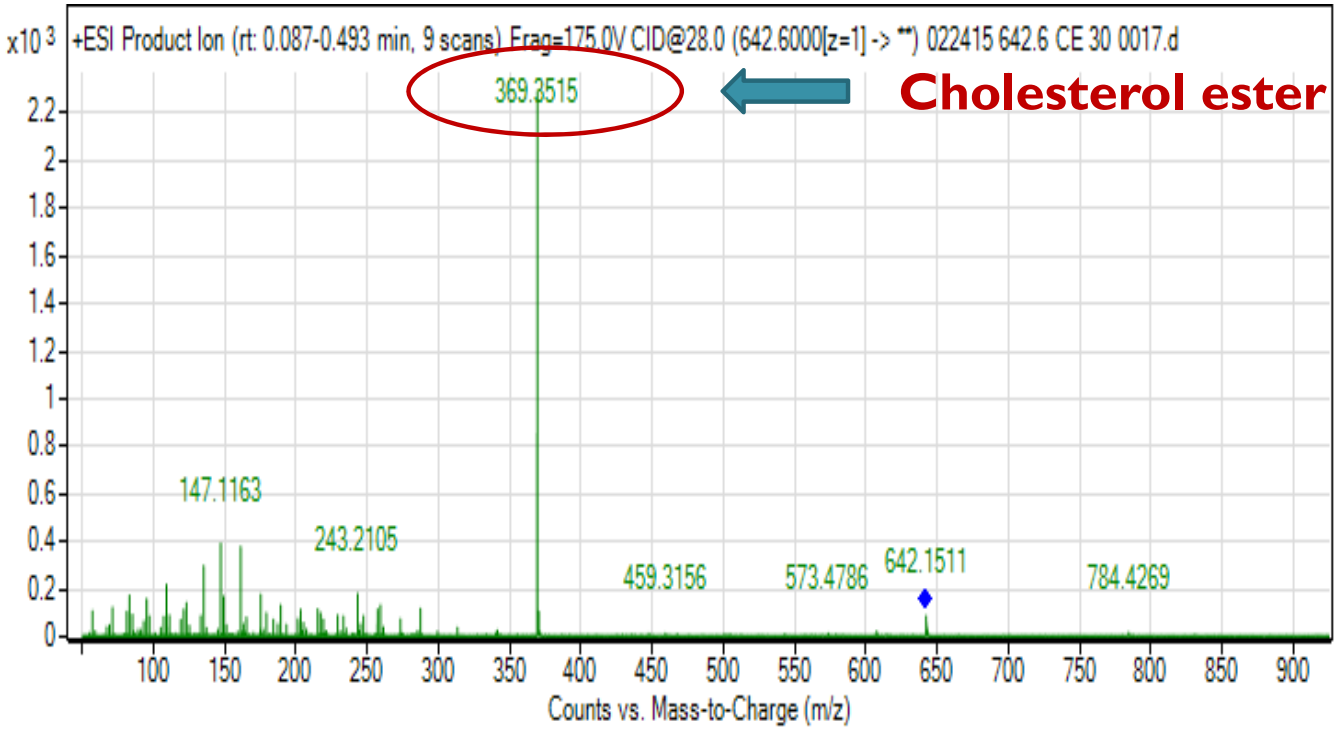


FRAGMENTATION SPECTRUM OF CHOLESTEROL ESTERS



Lima E S et al. J. Lipid Res. 2003;44:1660-1666

FRAGMENTATION SPECTRUM OF CHOLESTEROL ESTERS



IDENTIFIED LIPID CLASSES

m/z	Lipid classes
263, 301	Fatty alcohols/aldehydes
383, 445	Sterols
645, 714	Cholesterol esters
734, 760, 784, 788, 796, 798, 810, 836, 895	PC, SM
798, 895	Oxidized PC and SM
956, 958	Triacylglycerols
916, 920, 954	Oxidized triacyglycerols

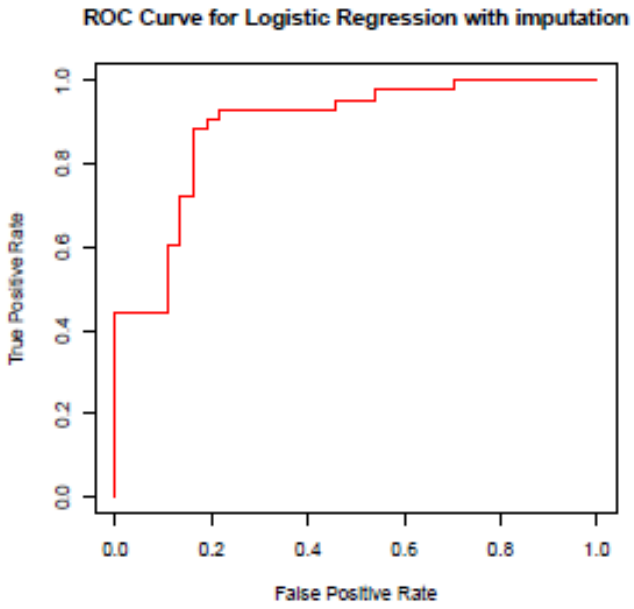
Anand, Swati, et al. "Detection and confirmation of serum lipid biomarkers for preeclampsia using direct infusion mass spectrometry." *Journal of lipid research* 57.4 (2016): 687-696.

MULTIMARKER PANELS

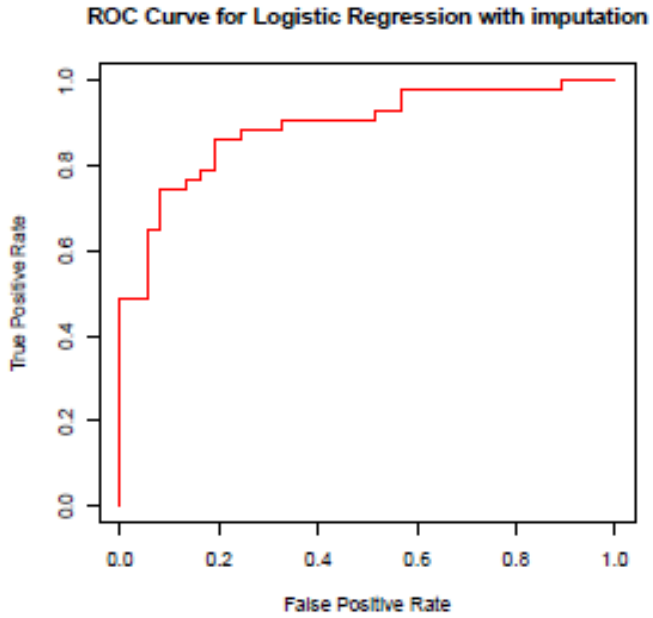
AUC	Potential biomarker combinations
0.89	263, 383, 462, 645, 784, 916
0.89	383, 462, 784, 796, 798, 920
0.88	263, 383, 462, 645, 784, 920
0.86	263, 383, 462, 645
0.83	263, 383, 462
0.88	263, 383, 462, 645, 836

Anand, Swati, et al. "Detection and confirmation of serum lipid biomarkers for preeclampsia using direct infusion mass spectrometry." *Journal of lipid research* 57.4 (2016): 687-696.

ROC CURVES



AUC -0.89



AUC -0.89

Anand, Swati, et al. "Detection and confirmation of serum lipid biomarkers for preeclampsia using direct infusion mass spectrometry." *Journal of lipid research* 57.4 (2016): 687-696.

CONCLUSIONS

- Discovery of 45 statistically significant biomarkers
- Confirmation of 23 lipid marker
- MS2 studies on 23 markers
- Characterization of 21 lipid biomarkers
- Production of multi marker model ROC curves with AUC > 0.89

SIGNIFICANCE

- Determine specific lipid compositional changes in response to a PE
- Early diagnosis of PE
- Severity of the disease
- Development of treatment therapies
- Roles of individual molecular species of lipids in PE

PROJECT III. Discovery and confirmation of novel serum lipidomic biomarkers for Alzheimer's disease

ALZHEIMER'S

- Leading cause of dementia in U.S
- Fourth leading cause of death after heart disease, cancer and stroke in U.S
- Progressive loss of cognitive functions- Memory, language, motor control, spatial ability, executive function and behavior and poor judgement
- Current diagnosis

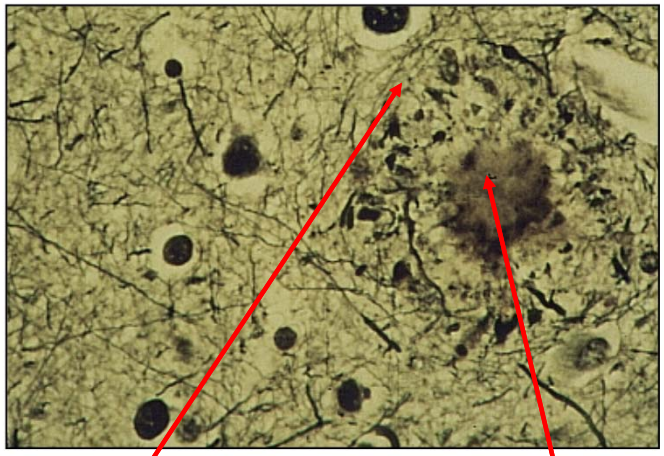
➢ PROBABLE → Dementia; Progressive memory and cognitive deterioration; Ruling out other diseases

➢ DEFINITE → Neuroimaging, lab tests; Brain autopsy

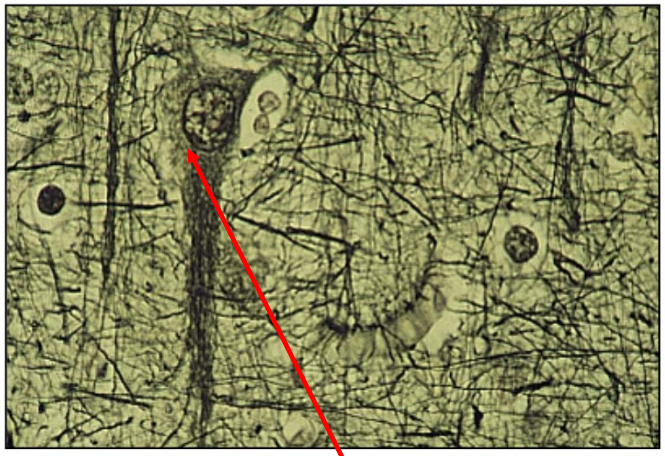
STAGES OF AD

Impairment: **CDR:**	**None** **0**	**Questionable** **0.5**	**Mild** **1**	**Moderate** **2**	**Severe** **3**
Memory	No memory loss or slight inconsistent forgetfulness	Consistent slight forgetfulness; partial recollection of events' "benign" forgetfulness	Moderate memory loss; more marked for recent events; defect interferes with everyday activities	Severe memory loss; only highly learned material retained; new material rapidly lost	Severe memory loss; only fragments remain
Orientation	Fully	Fully	Moderate	Severe	Oriented to

Plaques and Neurofibrillary Tangles

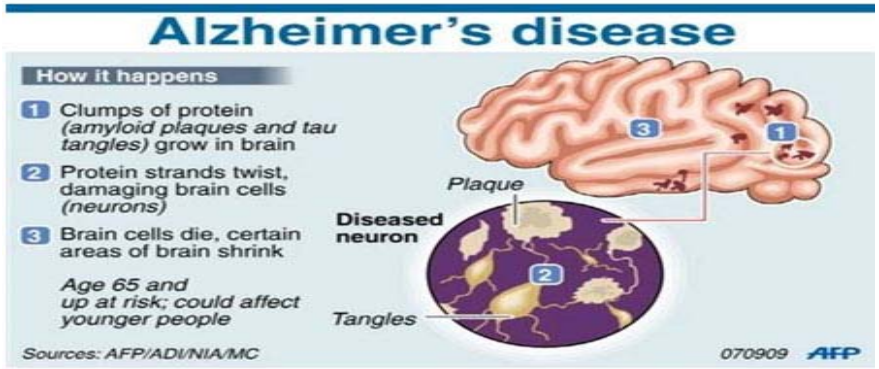


Plaque surrounding amyloid deposit



Neurons filled with neurofibrillary tangles

NEED FOR DIAGNOSIS



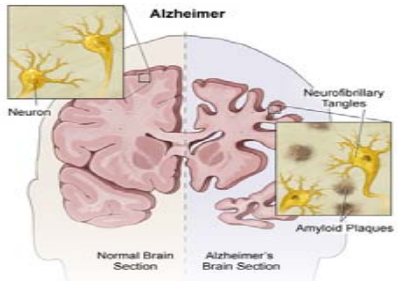
- Neurodegeneration estimated to start 20 to 30 years before the onset of first clinical symptoms
- Treatment therapies might be most effective before pathological changes spread throughout the brain
- Early diagnosis with reliable biomarkers is essential

LIPIDS IN ALZHEIMER'S DISEASE

- e4 allele of apolipoprotein E

↓

Susceptibility to AD



- Evidence of Oxidative stress
- Reports of accumulation of oxidized lipids like isoprostanes in AD brain
- Evidence of neurodegeneration leading to cell lysis

↓

Abnormal cell membrane lipid profile

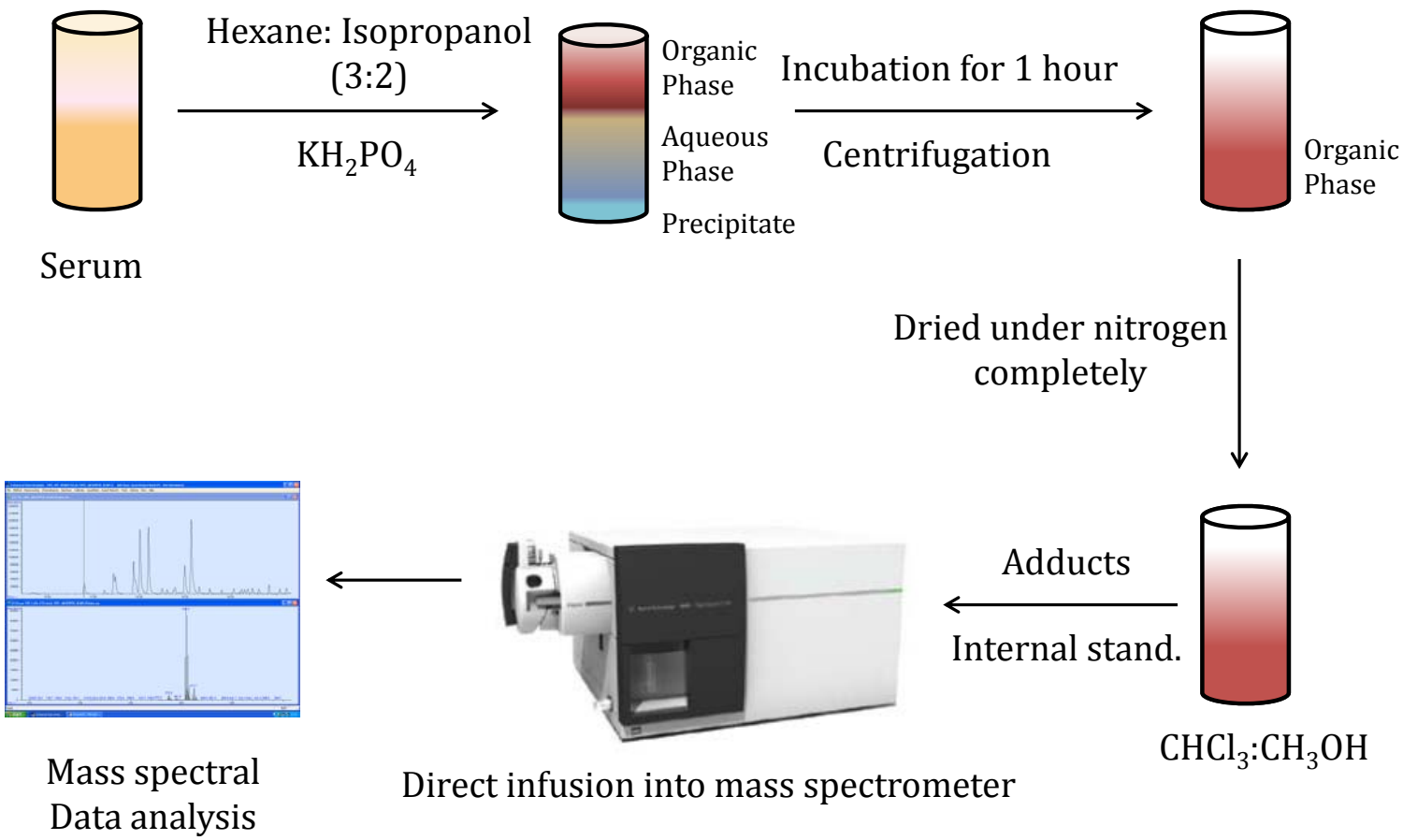
- Evidence of alteration of Plasmalogens, Ceramides and other glycerophospholipids in AD

SAMPLE SIZE

- Discovery set
- Controls (CDR-0): 32
- Cases:29
- a) Mild Cognitive impairment or very mild (CDR-0.5):10
- b)Mild (CDR-1):10
- c)Moderate (CDR-2):9

- Confirmation set
- Controls (CDR-0): 30
- Cases:27
- a) Mild Cognitive impairment or very mild (CDR-0.5):9
- b)Mild (CDR-1):9
- c)Moderate (CDR-2):9

LAB APPROACH



RESULTS

Discovery set -87 markers

Validation set- 35/87 markers

9 ↓ AD Cases

26 ↑ AD Cases

SOME OF THE IDENTIFIED LIPID CLASSES

No. of identified species	Lipid classes
2	Phosphatidylethanolamines
1	Cholesterol esters
1	Phosphatidylcholines
2	Sphingomyelins
1	Oxidized PC
2	Lysophosphatidylcholines
13	Triacylglycerols
3	Others
1	Sterols
1	Oxidized triacylglycerols

MULTIMARKER PANELS FOR ALL STAGE AD VS. CONTROLS

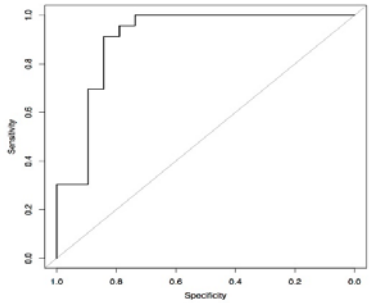
AUC	Potential marker combinations
0.90	496.3, 714.6, 778.5, 864.6, 488.3, 602.3
0.90	522.3, 778.5, 864.6, 488.3, 610.5
0.88	864.6, 714.6, 620.4, 778.5
0.87	778.5, 864.6, 610.5, 488.3

MULTIMARKER PANELS FOR ALL STAGE AD VS. CONTROLS

CDR	AUC	Potential marker combinations
All stage combined	0.90	229.1, 514.4, 602.4, 610.5, 620.4, 630.5, 778.5, 799.7, 824.6
0.5	0.94	229.1, 430.4, 514.4, 602.4, 610.5, 620.4, 778.5, 850.8, 864.6
1	0.91	430.4, 514.4, 602.4, 620.4, 630.5, 714.6, 766.6, 778.5, 824.6
2	0.93	229.1, 514.4, 620.4, 630.5, 778.5, 842.7

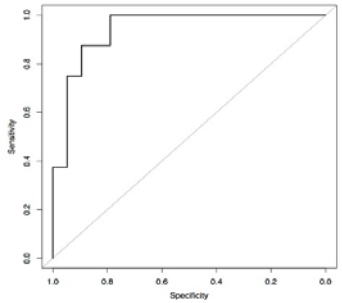
ROC curves for all stage AD, CDR 0.5, 1 and 2

All stage AD vs. Controls



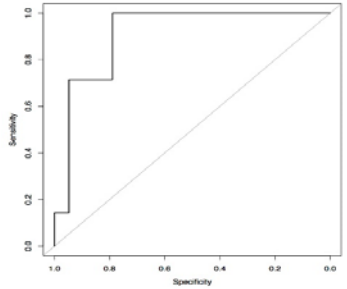
sensitivity~91% specificity of ~84%

CDR 0.5 vs. Controls



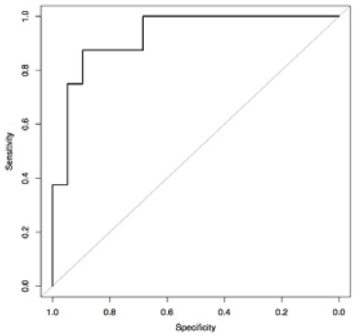
sensitivity ~100% specificity ~79%.

CDR 1 vs. Controls



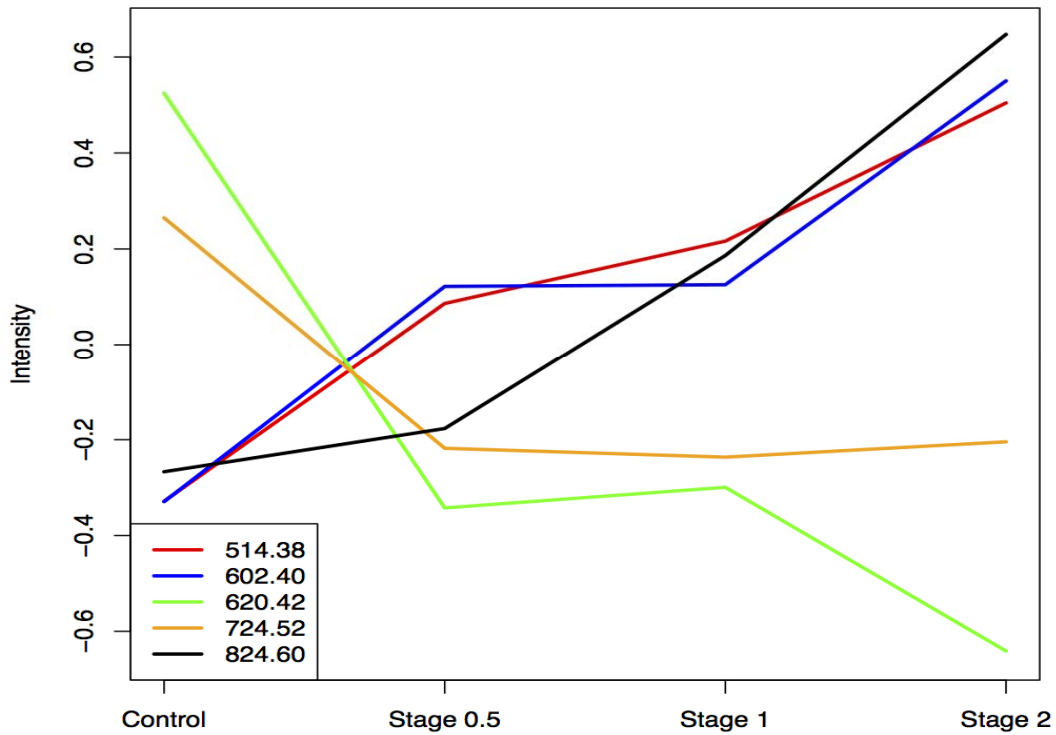
sensitivity ~100% specificity ~79%.

CDR 2 vs. Controls



sensitivity of ~88% at a specificity of ~90%

Correlations of the markers with disease stage



SIGNIFICANCE

- Determine specific lipid compositional changes in response to a dementia
- Early diagnosis of AD
- Stage of the disease (CDR 0.5,1 and 2)
- Mechanism of the AD
- Development of treatment therapies
- Roles of individual molecular species of lipids in AD

ACKNOWLEDGEMENTS

My Mentor

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Undergrads

Sydney Young

Diana Garcia

Others

Justin

Our Collaborator

Dr. Thulin, Dr Kauwe,

Dr. Tolley, Dr. Esplin, Dr. Johnson

Brigham Young University

My Co-Workers

Tanielle, Dipti, Komal, Ying

Dept. engineer

Bruce Jackson

QUESTIONS

Thank you

MARKERS FOR EARLY DIAGNOSIS

- ***Serum***

  - preferred specimen of choice
  - minimally invasive
  - blood in continuous contact with diseased regions

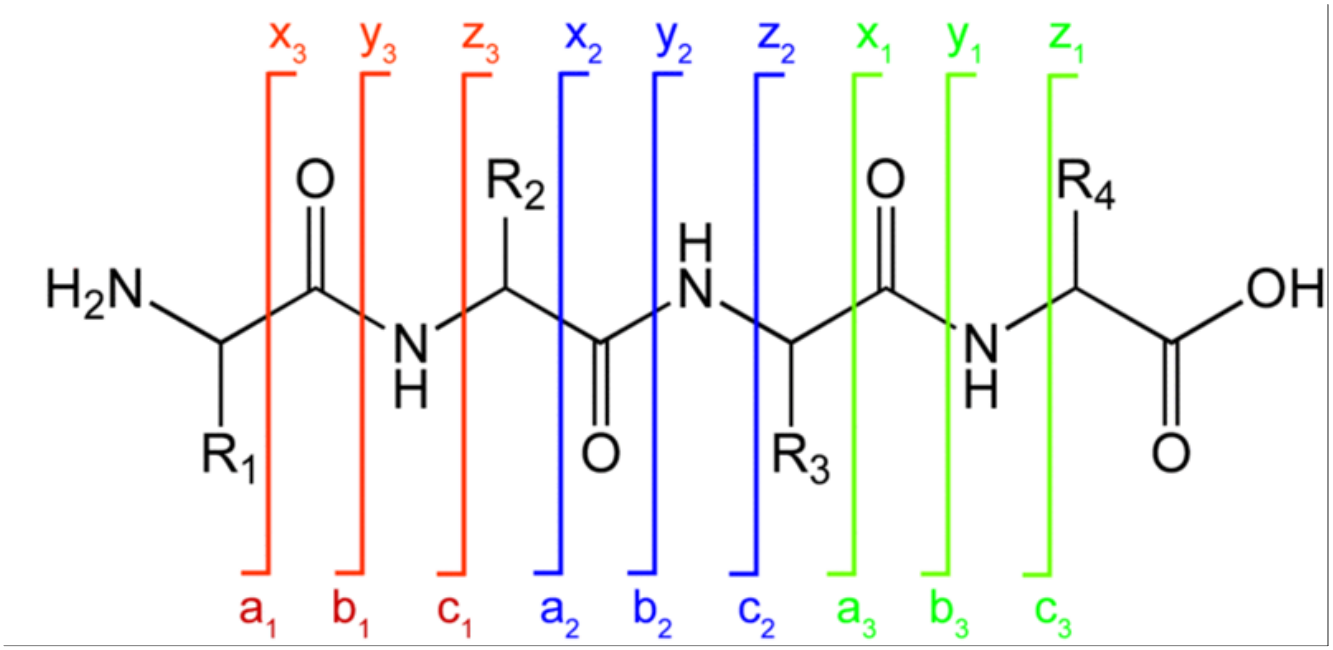
- ***Currently proposed biomarkers***

  - *Angiogenic factors*
  - *P-selectin*
  - *Plasma protein 13*
  - *Pentraxin 3*

Low detection sensitivity and specificity

Can't diagnose at early stage

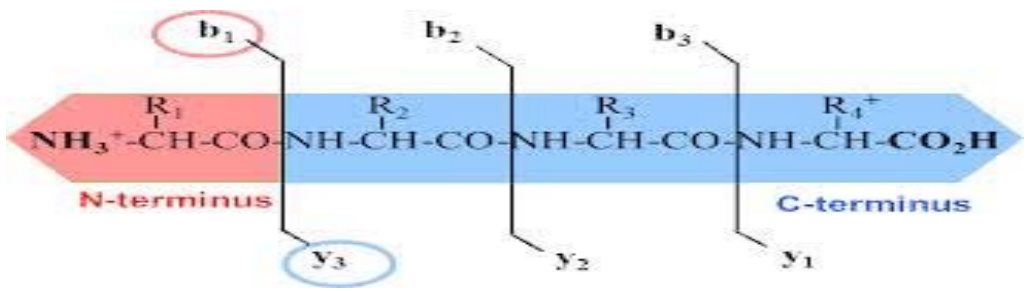
Types of ions produced by fragmentation



http://www.mbc.manchester.ac.uk

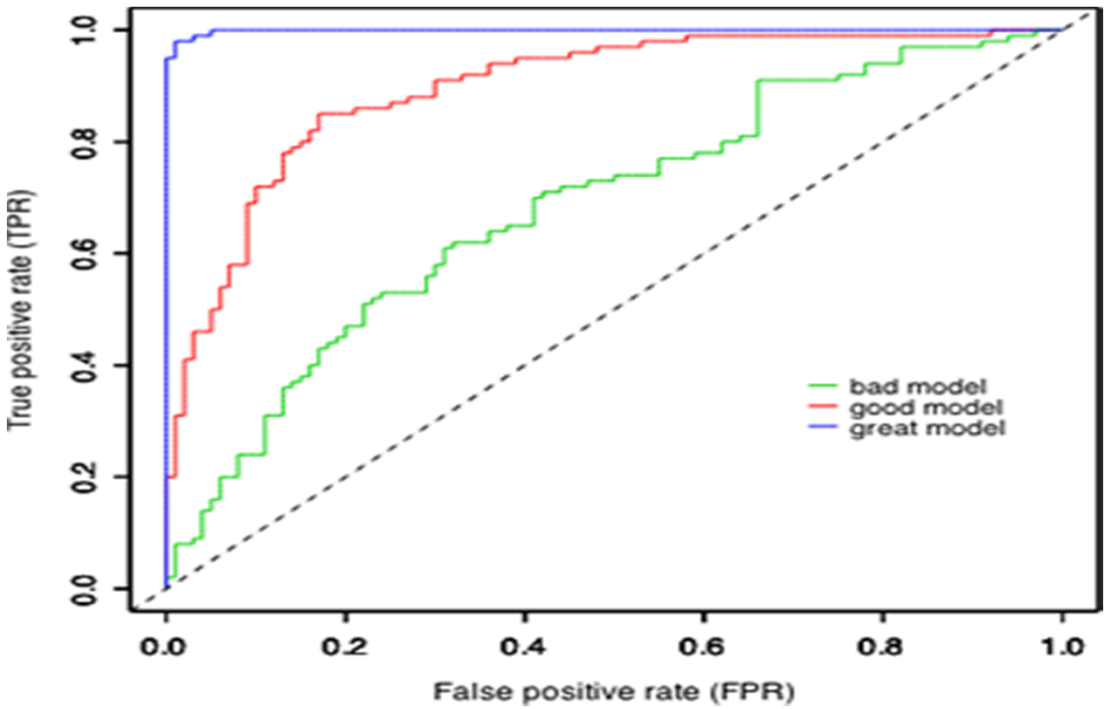
COLLISION INDUCED DISSOCIATION

- Involves collision of peptide ions with neutral collision gas molecules
- Kinetic energy is converted to internal energy of the ions
- Ergodic process
- Dissociates peptide bonds



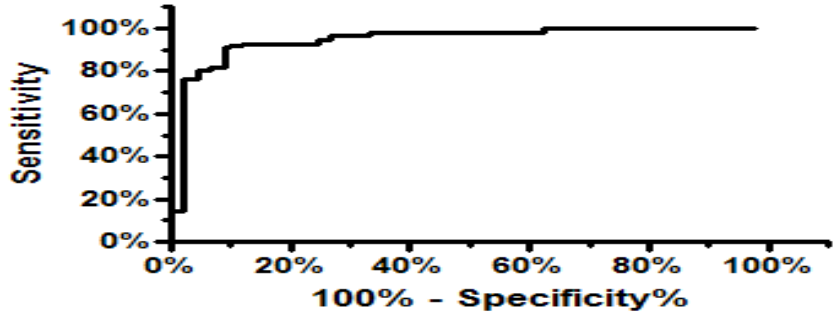
http://www.molgen.mpg.de/101151/Proteomics

COMPARISON OF ROC CURVES



http://gim.unmc.edu/dxtests/roc3.htm

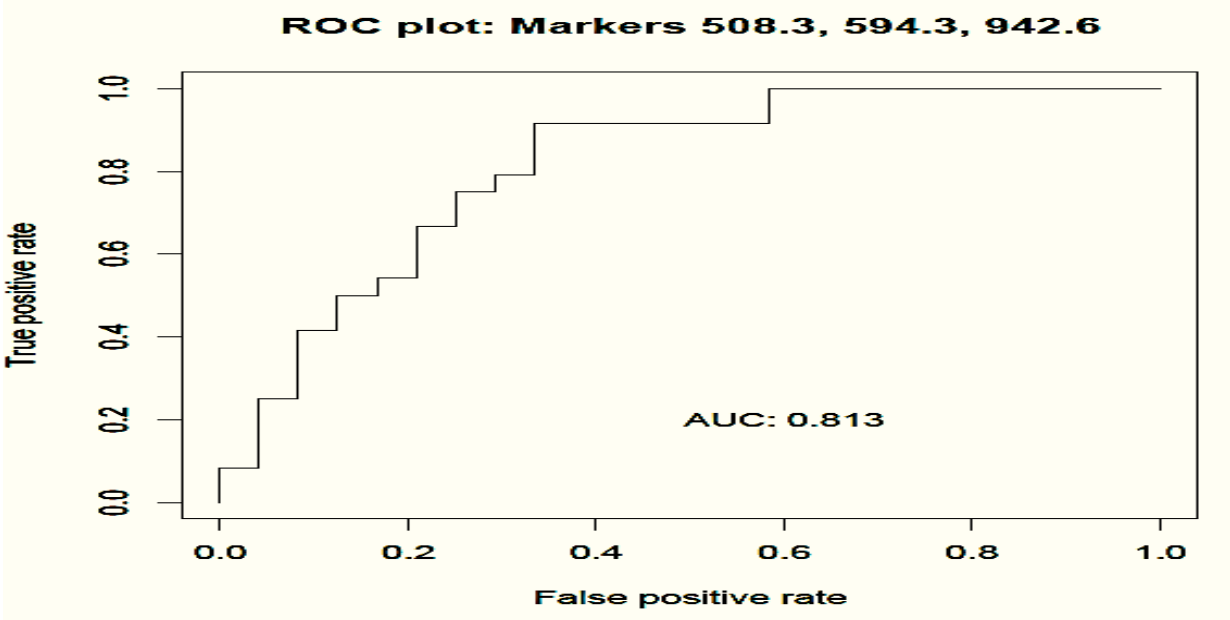
RECEIVER OPERATOR CURVE ANALYSIS



- Evaluating the performance of a diagnostic test
- Curve for sensitivity vs. 1- specificity
- 100% sensitivity and 100% specificity – Perfect Classification
- Area under the curve(AUC) – ability to distinguish between cases and control
- AUC -1 Perfectly accurate

http://www.graphpad.com/guides/prism/6/statistics/stat_howto_roc.htm

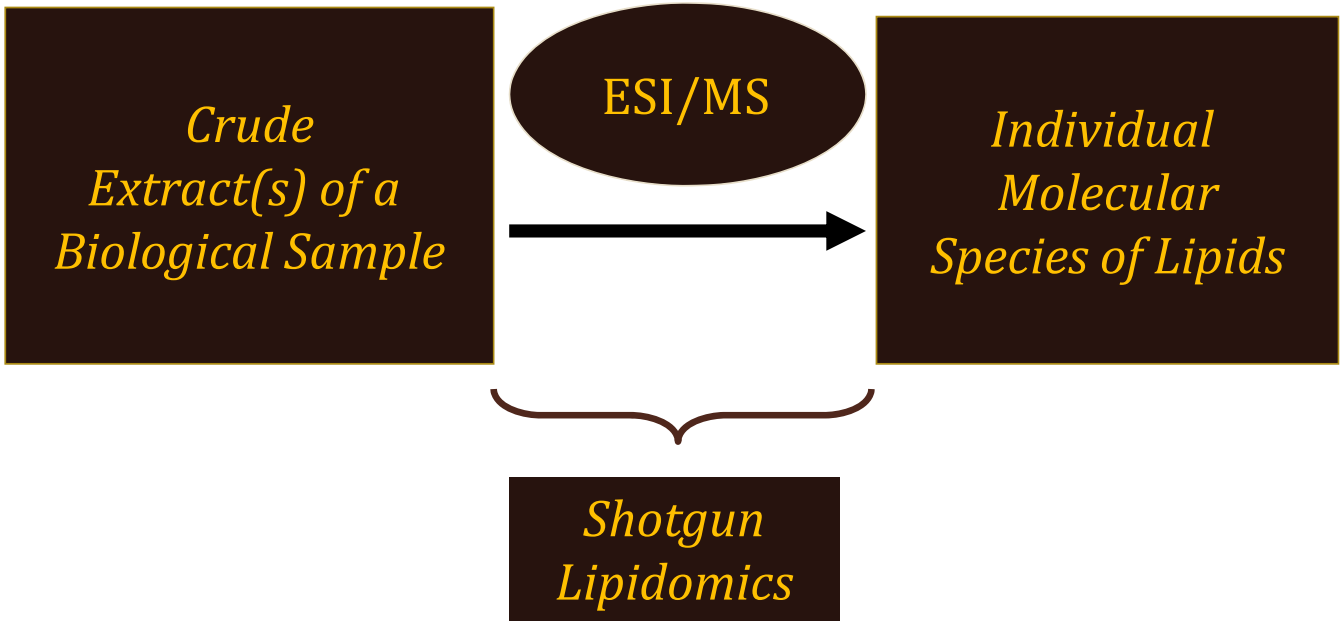
MULTI MARKER MODELING



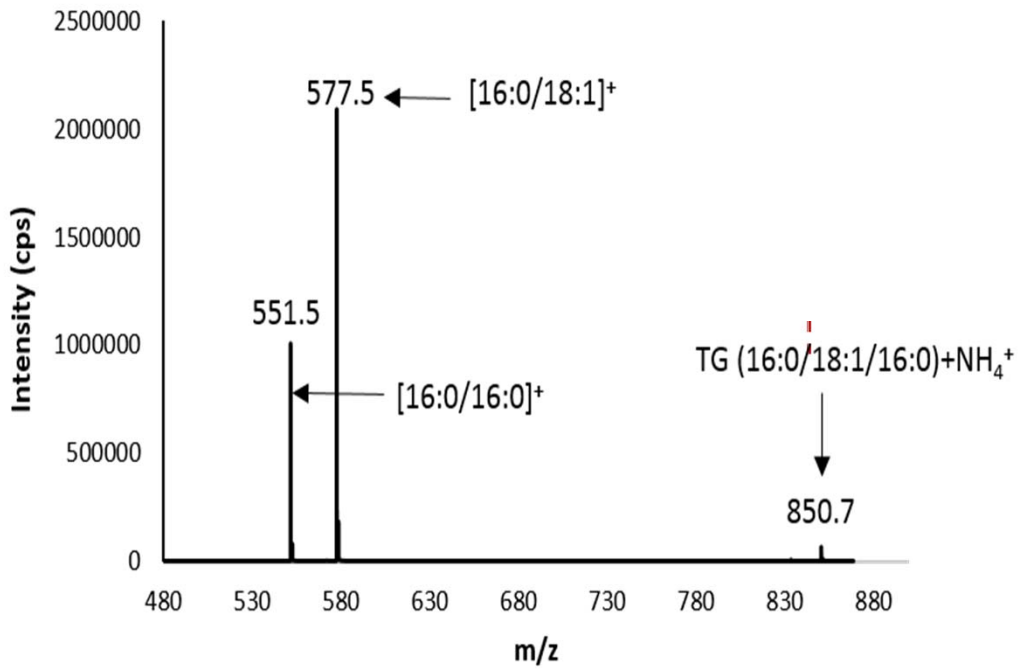
ROC plot for a combination of potential markers having m/z 508, 594 and 942

WHAT IS SHOTGUN LIPIDOMICS?

Developed by Han and Gross in 2003



FRAGMENTATION SPECTRUM OF A TG STANDARD



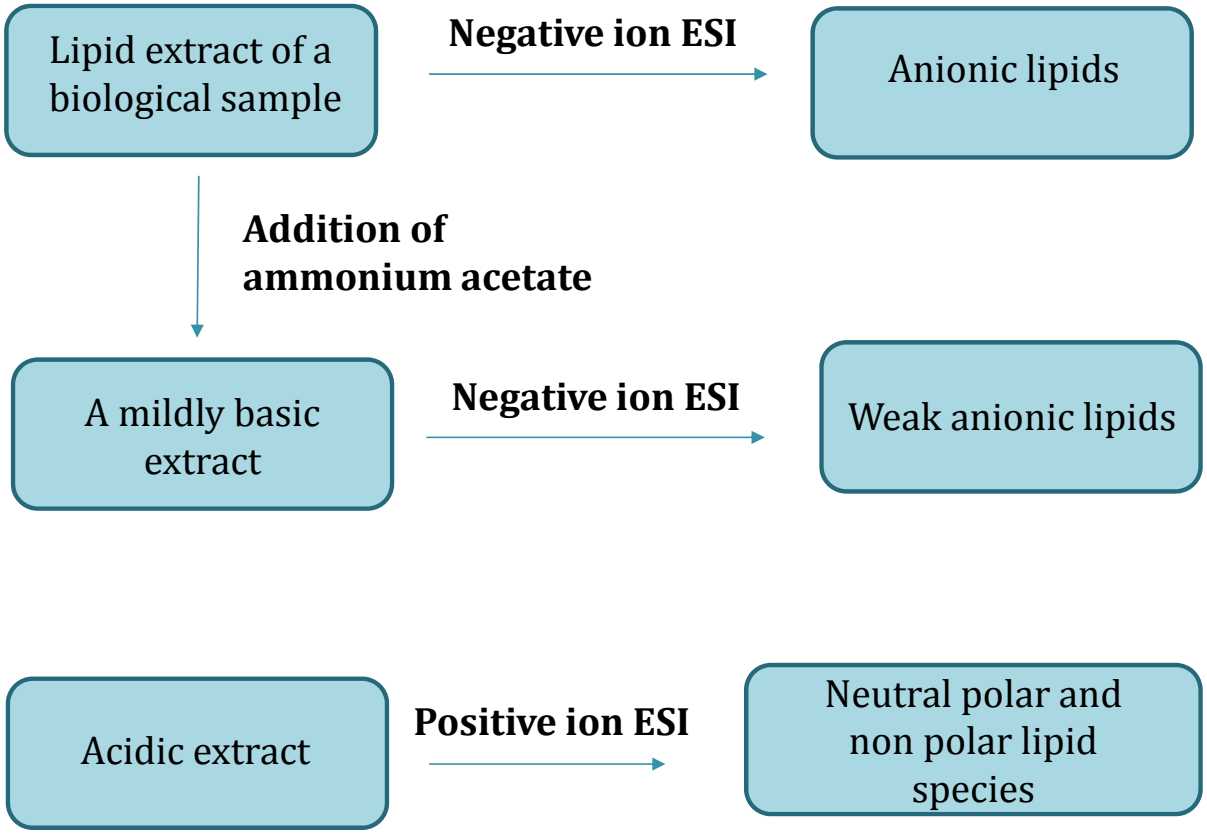
Classification of lipids based on charges retained

Group	Electrical Propensity	Lipid Classes
Anionic lipids	Carry net negative charge(s) at physiological pH	Cardiolipin, acylCoA, sulfatide, PtdIns (PtdInsP, $PtdInsP_2$, $PtdInsP_3$), PtdGro, PtdSer, PtdH, etc.
Weak anionic lipids	Carry a net negative charge at alkaline pH	PE, lysoPE, ceramide, NEFA, eicosanoids, etc.
Neutral polar lipids	Neutral at alkaline pH	PC, lysoPC, SM, glycolipid, TAG, etc.
Special lipids	Vary	Acylcarnitine, sterols, etc.

The ionization efficiency of an analyte greatly depends on the electrical propensity of an individual analyte in its own microenvironment to lose or gain a charge

Source: Gross and Han,, 2004

Lipidome analysis



Source: Gross and Han, methods in Enzymology, 2007

- Leading cause of dementia in Europe, India, Australia, Africa- AD
- Stages and symptoms

CDR 0.5- frequent memory loss, problems expressing and understanding language, reminders for daily activities.

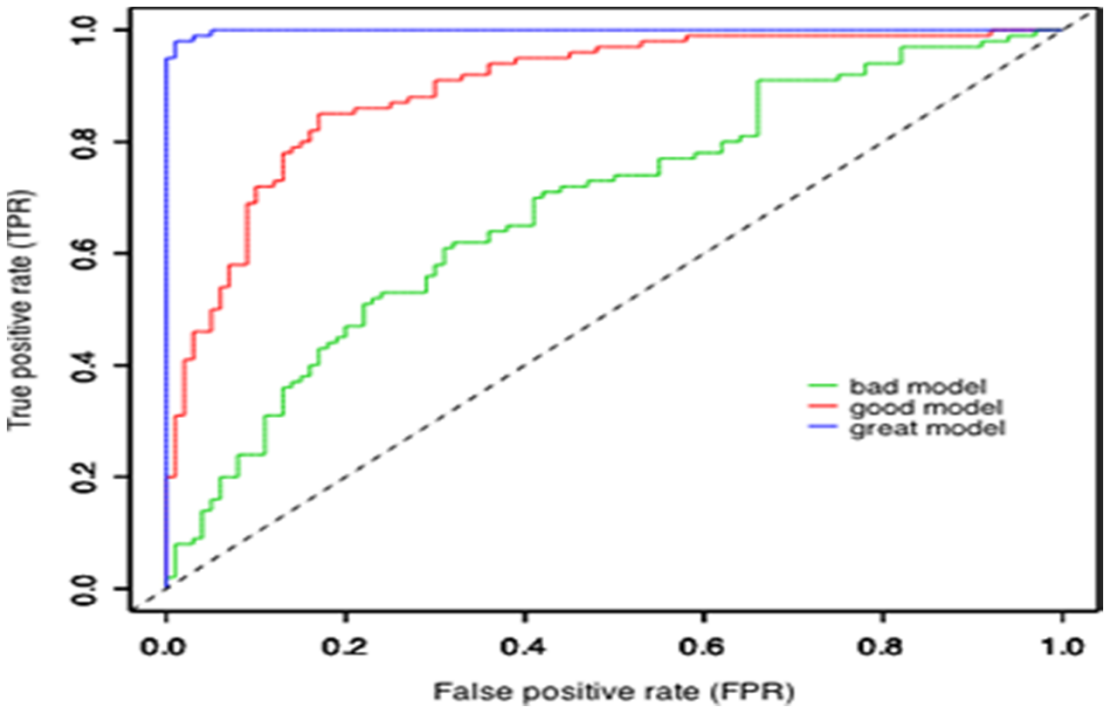
CDR 1- persistent memory loss, inability to recognize friends and family, assistance with daily living activities

CDR 2- loss of ability to remember and communicate, possible falls and likely immobility, always need full support and care

Leading causes of death

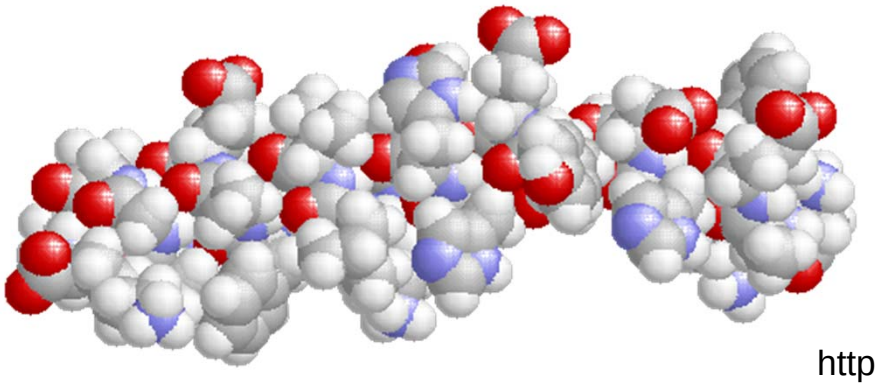
US	Europe	Australia	India	Africa
Heart disease	Heart disease	Heart disease	Heart disease	HIV/AIDS
Cancer	Cancer	AD and dementia	Lung disorders	Lower res tract
Stroke	Stroke	stroke	Stroke	Diarrhea
AD	AD	Lung cancer	Diarrhea	Malaria
accident	suicide	Other lung disorders	Lower resp (pneumonia, influenza)	stroke
			TB	

COMPARISON OF ROC CURVES



http://gim.unmc.edu/dxtests/roc3.htm

Amyloid Beta-42- forms plaque



http://molbio.info.nih.gov

- 42 amino acid A/b peptide
- Produced from APP by **b**/**g** secretase
- Will self-associate to form toxic fibrils
- Insoluble and resistant to proteolysis once aggregated

Tau Protein- forms neurofibrillary tangles

- Normally dephosphorylated by phosphatases
- Binds and stabilizes microtubules normally
- Abnormal tau in AD is phosphorylated
- Fails to bind when phosphorylated
- Paired helical filaments form → tangles
- Axonal transport blocked → degeneration