The Use of Tissue and Serum 'Omics' Methods To Characterize Disease

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ABSTRACT

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Preeclampsia (PE) is a multisystem disorder that contributes to maternal and fetal mortality and morbidity worldwide. It is characterized by de-novo hypertension and proteinuria or other maternal organ damage after 20 weeks of gestation. Evidence suggested that endogenous digitalis-like factor (EDLF) contributes to the pathogenesis of PE, and that the potential source of EDLF is the placenta. EDLF can inhibit the sodium pump (SP) specifically and may lead to hypertension, it has also been associated with hypoxia, oxidative stress and other abnormalitites present in PE.

We studied whether normal human placenta responded to SP inhibition casued by EDLF with a change in abundance of lipids in the placental cytosol, and whether there was a characteristic set of lipid changes that could serve as a signature for EDLF exposure if there were such changes. Placenta tissues from 20 normal pregnancies were incubated for 48 hr in the presence and absence of ouabain, a widely studied EDLF, followed by tissue homogenization, lipid extraction, and the study of lipids using a mass spectrometery (MS) based lipidomics approach. 1207 lipidomic markers were surveyed by paired Student t-test, among which 26 markers had significantly different abundances between cases and control at the FDR=0.05 level. A set of 8 lipidomic markers were selected by a statistical model built with a sparse partial least squares discriminant analysis method (sPLS-DA) and a bootstrap procedure. All eight markers were then chemically characterized and partially identified using tandem MS. These markers might be used to identify placentas that have been previously exposed to EDLF in return.

Endogenous peptides and small proteins might contribute to the pathophysiology of various diseases. Therefore, we investigated the potential peptidomic profile of placenta tissues in response to EDLF exposure as well. Placenta tissues from 20 normal pregnancies were incubated for 25 hr with and without the addition of ouabain, followed by homogenization, protein depletion, and the study of the peptides by a LC-MS based peptidomics approach. 275 peptidomic markers were evaluated by Student t-test. A set of 8 markers was chosen using a logistic regression model build with the Akaike information criterion (AIC). However, no peptidomics markers or set of markers showed specific, statististically significantly different changes in abundances between cases and controls after applying a false discovery rate (FDR) correction or using more conservative methods to overcome over-fitting. Using an optimal sPLS-DA, cross-validation studies and logistic regression models, we also found that the addition of any peptidomic marker to the previously selected lipidomic profile was unlikely to help identify placentas that had been exposed to EDLF.

Alzheimer's disease (AD) is the most common form of dementia and the number of AD cases worldwide is currently estimated to be 36 million. The exact pathogenesis of AD remains

elusive and available therapeutic strategies can only delay its progession temporarily. Several hypotheses have been proposed regarding the pathophysiology of AD and the beta-amyloid (Aβ) hypothesis is considered the core mechanism. However, the majority of studies concerning AD, or AD biomarkers specifically, have ignored a potentially important variable that is gender, despite reported gender differences in the risk of developing AD, the risk factors, clinical symptoms and CSF biomarkers of the disease, among many other aspects.

We analyzed data obtained from a previous study of diagnostic serum lipid biomarkers for AD with the consideration of potential gender difference. Firstly, we studied the interaction between gender and disease stage using analysis of variance (ANOVA) and analysis of covariance (ANCOVA). Lipid markers that showed statistically significant interaction were selected after applying a FDR correction. Secondly, using a lasso logistic regression model with binary classification (control vs. all AD stages), we identified gender-specific markers and found different coefficient estimates for different genders as well. Lastly, we build a new ordinal model with the addition of a gender-specific marker using a Bayesian lasso probit ordinal regression model. The predictive performance of the new model was found to be statistically significantly better than the previous model which was built without the consideration of gender.

In conclusion, we successfully discovered, chemically characterized lipidomic markers indicative of EDLF exposure in placenta and detected gender-specific lipid markers for AD.

Keywords: Preeclampsia, Alzheimer's disease, lipidomics, peptidomics, endogenous digitalis-like factor, placenta, biomarkers, gender, serum, diagnosis

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1D PAGE	One-dimensional polyacrylamide gel electrophoresis
2-D	2-dimensional
2D-DIGE	Two-dimensional differential gel electrophoresis
3-D	3-dimensional
α	Alpha
A2M	Alpha-2-macrogloblin
AA-KIR	Killer cell immunoglobulin-like receptor AA genotype
Aβ	Amyloid beta
ACN	Acetonitrile
ACOG	American College of Obstetrics and Gynecology
AD	Alzheimer's disease
ADAM12	A disintegrin and metalloprotease 12
ADL	Activities of daily living
ADMA	Dimethylarginine
ADRC	Alzheimer's Disease Research Center
AIC	Akaike information criterion
ALK5	Activin receptor-like kinase 5
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
APOE	apolipoprotein E
APP	Amyloid precursor protein
APPI	Atmospheric pressure photoionization
AT1-AAs	AGT II type 1 receptor autoantibodies
AT1-R	Angiotensin type I receptor
ATPase	Adenosine triphosphatase
AUC	Area under curve
β	Beta
BACE1	β-site amyloid precursor protein-cleaving enzyme 1
BDNF	Brain derived neurotrophic factor
BIC	Bayesian information criterion
BUME	Butanol and methanol
Ca	Calcium
CDR	Clinical dementia rating
CE	Capillary electrophoresis
CHF	Congestive heart failure
CI	Chemical ionization
CI	credible intervals
CID	Collision-induced dissociation
cLC	Capillary liquid chromatography
cm	centimeter
CNS	Central nervous system

CSDD	Cornell scale for depression in dementia
CSF	Cerebrospinal fluid
CT	Computed tomography
CTS	Cardiotonic steroids
Cys	Cysteine
DBM	Disease biomarkers
DBP	Diastolic blood pressure
DESI	Desorption electrospray ionization
DIF	Digoxin Immune Fab
DLF	Digitalis-like factor
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DTI	Diffusion-tensor imaging
EC	Endothelial cells
EDLF	Endogenous digitalis-like factor
EEG	Electroencephalography
eFAD	Early onset familial Alzheimer's disease
e.g.	exempli gratia
EGFR	Epidermal growth factor receptor
EI	Electron ionization
EIC	Extracted ion chromatogram
ELIZA	Enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ERLIC	Electrostatic repulsion hydrophilic interaction chromatography
ESI	Electrospray ionization
ESRD	End-stage renal disease
EST	Expressed sequence tag
ET-1	Endothelin-1
Etc	Et cetera
ETD	Electron transfer dissociation
FA	formic acid
FAB	Fast atom bombardment
FDA	Food and Drug Administration
FDR	False discovery rate
FTICR	Fourier transform ion cyclotron resonance
g	gram
GC	Gas chromatography
GFR	Glomerular filtration rate
HDL	High density lipoprotein
HELLP	Hemolysis, elevated liver enzymes, and low platelet count
HILIC	Hydrophilic interaction LC
HLA-C2	Human leukocyte antigen-C2
HMDB	Human metabolome database
^{1}H-MRS	^{1}H-MR spectroscopy
HPLC	High-performance liquid chromatography

HPTLC	High-performance TLC
hr	hour
HRT	Hormone replacement therapy
HVLT	Hopkins verbal learning test
ICAT	Isotope-coded affinity tags
IDE	Insulin-degrading enzyme
i.e.	id est
IL-6	Interleukin-6
IMS	Imaging mass spectrometry
IMS	Ion-mobility spectrometry
INF-γ	Interferon gamma
IRB	Institutional Review Board
ISSHP	International Society for the Study of Hypertension in Pregnancy
iTRAQ	Isobaric tag for relative and absolute quantification
IVH	Intraventricular hemorrhage
K	Potassium
KDR	Kinase domain receptor
LB	Lower bound
LDL	Low-density lipoproteins
LIPID MAPS	Lipid metabolite and pathway strategy
LIT-MS	Linear ion trap
LLE	Liquid-liquid extraction
LMSD	LIPID MAPS Structure Database
LPPE	Late postpartum eclampsia
MALDI	Matrix-assisted laser desorption/ionization
MAP	Microtubule-associated protein
MBG	Marinobufagenin
MBT	Marinobufotoxin
MCI	Mild cognitive impairment
MDMS-SL	Multi-dimensional mass spectrometry-base shotgun lipidomics
mg	Milligram
min	minute
miRNA	microRNA
mL	milliliter
mm	millimeter
MMCD	Madison Metabolomics Consortium Database
mmHg	Millimeter of mercury
MMSE	Mini-mental state examination
MOA	Mechanism of action
MR	Magnetic resonance
MRI	Magnetic resonance imaging
MRM	Multiple-reaction monitoring
MS	Mass spectrometry
MS/MS or MS^2	Tandem mass spectrometry
MSE	Mean standard error
MTBE	Methyl-tert-butyl ether

MudPIT	Multi-dimensional protein identification technology
MW	molecular weight
m/z	mass-to-charge ratio
Na	Sodium
NEC	Necrotizing enterocolitis
NFT	Neurofibrillary tangles
NK cells	Natural killer cells
NLS	Neutral loss scanning
nM	nanomolar
NMDA	N-methyl D-aspartate
NMR	Nuclear magnetic resonance
NPLC	Normal-phase LC
PBS	Phosphate-buffered saline
PC	Phosphatidylcholines
PC	Glycerophosphocholines
PE	Preeclampsia
PE	Phosphatidylethanolamines
PET	Positron emission tomography
PG	Phosphatidylglycerol
PGRN	Progranulin
pI	Iso-electric point
PI	Phosphatidylinositol
PIS	Precursor ion scanning
PlGF	Placental growth factor
P-MCI	Progressive MCI
PS	glycerophosphoserine
PSEN1	Presenilin-1
P-tau	Phosphorylated tau protein
PTM	Posttranslational modifications
QIT-MS	3D ion trap
QqLIT	Quadrupole-linear ion trap
QqQ	Triple quadrupole
QqTOF	Quadrupole time-of-flight mass spectrometer
γ	gamma
RBMT	Rivermead behavioral memory test
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RPF	Renal plasma flow
rpm	revolutions per minute
SBP	Systolic blood pressure
SCX	Strong cation exchange
SEC	Size-exclusion chromatography
SELDI	Surface-enhanced laser desorption/ionization
sEng	Soluble endoglin
SFC	Supercritical fluid chromatography
sFlt-1	Soluble fms-like tyrosine kinase-1

SILAC	Stable isotope labeling with amino acids in cell culture
SIM	Selected ion monitoring
SM	sphingolipids
SM	sphingomyelin
S-MCI	Stable MCI
SNP	Single-nucleotide polymorphism
SP	Sodium pump
SPECT	Single photon emission computed tomography
sPLS-DA	Sparse partial least squares discriminant analysis
SRM	Selected reaction monitoring
STOX1	Storkhead box 1
TβRII	TGF-beta receptor type II
TC	Total cholesterol
TCB	Telocinobufagin
TGF-β	Transforming growth factor-β
TGF-β1	Transforming growth factor beta-1
TGF-βII	Transforming growth factor, beta receptor II
TIC	Total ion current, total ion chromatogram
TLC	Thin-layer chromatography
TMAB	4-trimethylammoniumbutyryl
TMT	Tandem mass tags
TNF-α	Tumor necrosis factor-alpha
TSH	Thyroid stimulating hormone
T-tau	total-tau
TTR	transthyretin
UB	Upper bound
UBQLN1	Ubiquilin-1
UHPLC	Ultrahigh performance LC
μL	microliter
VCap	Capillary voltage
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoproteins
vs.	versus
VSM	Vascular smooth muscle
v/v	Volume/volume

Chapter 1 Introduction

My research has sought to use mass spectrometer-based methods to probe the molecular composition of biological specimens seeking to find, initially, specific biochemical signatures for a disease or signatures in response to factors thought to mediate disease. Then there has been additional research to identify the relevant factors and the biological or physiological pathways involved in the transition from a normal state of health to one of disease. In this dissertation I will focus on two diseases: preeclampsia and Alzheimer's disease and ask focused questions about molecular changes typifying and potentially mediating them.

1.1 Preeclampsia (PE)

1.1.1 History

Around 400 BC, Hippocrates stated in the Coan Prognosis that headache accompanied by heaviness and convulsions during pregnancy was considered bad[1]. This marks the earliest recognition of the medical problem of eclampsia[2], one of the most serious complications of pregnancy. According to McMillen, it was Francois Mauriceau who first systematically described eclampsia[3]. Boissier de Sauvages (1739) successfully distinguished eclampsia from epilepsy[4], despite the fact that eclampsia had been mistaken for epilepsy for centuries. In 1797, severe swelling, medically termed edema, in women with eclampsia was recognized by Demanet[4]. Pierre Rayer (1840) first discovered proteinuria in eclamptic women[5], followed by John Lever's finding in 1843 that the preeclampsia-associated proteinuria was specific to the disorder[6]. In the same year, Robert Johns brought to light the connection between an increased

risk of convulsions and the following symptoms: headache, loss of vision, severe abdominal pain and edema[7]. In 1897, eclamptic hypertension was finally documented by Vaquez and Nobecourt[4]. Since then, the concept of preeclampsia as a unique disease or set of closely related diseases unique to pregnancy has been widely recognized and accepted.

1.1.2 Epidemiology

Preeclampsia (PE) is a multisystem disorder that complicates 2-5% of all pregnancies in the United States, Canada and Western Europe[8] and 3-10% of all births around the world[9]. It is a major source of maternal and fetal mortality and morbidity worldwide[9]. PE can be life-threatening, especially in less economically developed countries, due to the lack of adequate antenatal and intrapartum care[12].

PE is currently defined and characterized by hypertension and proteinuria after 20 weeks gestation. Every year, approximately 50,000 women die from PE globally[10]. Eclampsia, the most dramatic form of PE, can account for up to 10 percent of all maternal mortality[10]. Complications associated with PE include stroke, renal or liver failure, cardiac dysfunction or arrest, pulmonary edema, eclampsia, the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count), and placental abruption[9, 14, 18]. PE is also a potential risk factor of cardiovascular diseases and related mortality later in life[9, 13, 19]. Risk factors for PE (Table 1.1) encompass nulliparity, vascular/endothelial/renal disease, preexisting hypertension, diabetes mellitus, obesity, ≥10 years birth interval, having donated a kidney, high altitude, molar pregnancy, etc[13-17].

PE is associated with fetal and neonatal mortality and morbidity as well. It contributes to about 12 to 25% of fetal growth restriction and small for gestational age fetuses. and 15 to 20% of all preterm births additionionally[9]. Stillbirth, neonatal death and associated complications of

Table 1.1 List of maternal risk factors for preeclampsia. (Reprinted with permission from Mol, B. W.; Roberts, C. T.; Thangaratinam, S.; Magee, L. A.; de Groot, C. J.; Hofmeyr, G. J., Pre-eclampsia. *Lancet* 2016, *387* (10022), 999-1011.)

(i) Previous preeclampsia (PE)
(ii) Previous early onset PE and preterm delivery at <34 weeks' gestation
(iii) PE in more than one prior pregnancy
(iv) Chronic kidney disease
(v) Autoimmune disease such as systemic lupus erythematosis or antiphospholipid syndrome
(vi) Heritable thrombophilias
(vii) Type 1 or type 2 diabetes
(viii) Chronic hypertension
(ix) First pregnancy
(x) Pregnancy interval of more than 10 years
(xi) New partner
(xii) Reproductive technologies
(xiii) Family history of PE (mother or sister)
(xiv) Excessive weight gain in pregnancy
(xv) Infection during pregnancy
(xvi) Gestational trophoblastic disease
(xvii) Multiple pregnancies
(xviii) Age 40 years or older
(xix) Ethnicity: Nordic, Black, South Asian, or Pacific Island
(xx) Body mass index of 35 kg/m^2 or more at first visit
(xxi) Booking systolic blood pressure >130 mmHg or diastolic blood pressure >80 mmHg
(xxii) Increased prepregnancy triglycerides
(xxiii) Family history of early onset cardiovascular disease
(xxiv) Lower socioeconomic status
(xxv) Cocaine and methamphetamine use
(xxvi) Nonsmoking

prematurity from early delivery are some of the other examples of PE related fetal complications[9, 18]. Children born to preeclamptic mothers have been reported to be more susceptible to coronary heart disease, stroke, and metabolic syndrome in adult life[13].

1.1.3 Diagnosis

PE is characterized by new onset hypertension (systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg, on two occasions at least 6 hours apart) and proteinuria (excretion of ≥300 mg protein in a 24 hour urine collection or a protein/creatinine ratio ≥0.3 or a urine dipstick reading of 1+) after 20 weeks of gestation[9]. In the absence of proteinuria, the diagnostic criteria for PE are de-novo hypertension with new onset of any of the following symptoms: pulmonary edema, thrombocytopenia, renal insufficiency, impaired liver function, cerebral or visual distrubances[20].

Features of severe PE include SBP ≥160 mmHg or DBP ≥110 mmHg (on two occasions, at least 6 hours apart), proteinuria ≥5g in a 24 hour collection, CNS disturbances, pulmonary edema, generalized edema, **h**emolysis, **e**levated **l**iver function tests, **l**ow **p**latelet count, i.e. thrombocytopenia (collectively the HELLP syndrome), epigastric/right upper quadrant pain, progressive renal insufficiency, placental abruption, fetal growth restriction or oligohydramnios[9,17,20].

Apart from the diagnostic criteria and risk factors for PE listed above, several other methods have been utilized to possibly predict this complex disorder of pregnancy. For example, Doppler ultrasonography, a noninvasive method, can be used to assess uteroplacental circulation, owing to the fact that inadequate placental perfusion is associated with the development or presence of PE[15]. Moreover, a number of maternal biochemical markers have been proposed to

potentially predict PE (Table 1.2) with modest predictive value, although the clinical application of most of these biomarkers needs to be further evaluated in prospective, unselected, interventional studies[18]. Evidence also suggests that better predictability can be achieved by combining biochemical markers with biophysical measurements (e.g. Doppler ultrasound evaluation)[17]. However, the predictive value of serum uric acid has been somewhat controversial[21-23]. Urinary calcium excretion appears to have no predictive value and provocative tests are unreliable[24]. One aspect of note regarding the prediction of PE involves false positive and false negative results. It is more desirable to apply tests that are highly sensitive in the diagnosis of PE, because a false positive prediction may lead to increased monitoring and screening which are rarely harmful. A false negative prediction, in contrast, could prevent or delay necessary treatment and lead to the development of PE, eclampsia, or other relevant complications[17].

1.1.4 Pathophysiology

PE, a multisystem disorder of pregnancy, remains "a disease of theories" due to its complex and incompletely understood pathophysiology. Despite the fact that the exact cause and earliest events of PE are still elusive, much understanding of its pathogenesis has been gained over the years.

It is generally believed that the placenta is the most important organ in the pathogenesis of PE. This can be demonstrated by the following evidence: 1. PE can be resolved quickly by removing the placenta in most cases; 2. Patients with a molar pregnancy have an increased risk in developing PE even though a fetus is absent; 3. In the case of postpartum eclampsia (LPPE), which has been linked to retained placental fragments, uterine curettage can lead to rapid

Table 1.2 List of potential maternal biochemical markers for predicting preeclampsia. (Reprinted with permission from Mol, B. W.; Roberts, C. T.; Thangaratinam, S.; Magee, L. A.; de Groot, C. J.; Hofmeyr, G. J., Pre-eclampsia. *Lancet* 2016, *387* (10022), 999-1011.)

A disintegrin and metalloprotease 12 (ADAM12)	L-Arginine
Activin-A	L-Homoarginine
Adiponectin	Leptin
Adrenomedullin	Magnesium
Alpha fetoprotein	Matrix metalloproteinase-9
Alpha-1-microglobulin	Microalbuminuria
Ang-2 angiopoietin-2	Microtransferrinuria
Antiphospholipid antibodies	N-Acetyl-β-glucosaminidase
Antithrombin III	Neurokinin B
Atrial natriuretic peptide	Neuropeptide Y
Beta2-microglobulin	Neutrophil gelatinase-associated lipocalin
C-reactive protein	P-Selectin
Calcium	Pentraxin 3
Cellular adhesion molecules	**Placenta growth factor**
Circulating trophoblast	Placental protein 13
Corticotropin release hormone	Plasminogen activator inhibitor-2
Cytokines	Platelet activation
Dimethylarginine (ADMA)	Platelet count
Endothelin	**Pregnancy associated plasma protein-A**
Estriol	Prostacyclin
Ferritin	Relaxin
Fetal DNA	Resistin
Fetal RNA	Serum lipids
Free fetal hemoglobin	Soluble endoglin
Fibronectin	Soluble fms-like tyrosine kinase
Genetic markers	Thromboxane
Haptoglobin	Thyroid function
Hematocrit	Total proteins
Homocysteine	Transferrin
Human chorionic gonadotropin	Tumor necrosis factor receptor-1
Human placental growth hormone	Uric acid
Inhibin A	Urinary calcium to creatinine ratio
Insulin-like growth factor	Urinary kallikrein
Insulin-like growth factor binding protein	Vascular endothelial growth factor
Insulin resistance	Visfatin
Isoprostanes	Vitamin D

recovery; and lastly, 4. in women with an extrauterine pregnancy complicated by PE, clinical symptoms remain even after delivering the fetus, but the symptoms resolve after subsequent delivery of the placenta[22, 121, 123].

It is thought by many that the principal cause of PE is abnormal placentation[10, 13, 22, 121], which is characterized by impaired endovascular trophoblast invasion mainly and defective interstitial trophoblast invasion of the decidualized endometrium and myometrium secondarily[126]. In this proposed model of PE during endovascular invasion, cytotrophoblasts, derived from the embryo, fail to adopt an endothelial cell-surface adhesion phenotype, and consequently fail to adequately invade and remodel the uterine spiral arteries of the maternal decidua and myometrium[121], resulting in the continued presence of low-flow, high-resistance vessels. These vessels cannot adequately supply maternal blood to the villous space and the fetoplacental unit, thus leading to underperfusion and hypoxia of the placenta and potentially the fetus[13, 125].

The imbalance between proangiogenic factors and antiangiogenic factors has been identified as a central characteristic of PE[22]. It was suggested[22, 121] that the placental hypoxia resulting from abnormal placentation leads to the release of antiangiogenic factors, which in turn result in generalized damage to maternal endothelium in the kidney, liver, and brain, etc. Though it has also been suggested that placental hypoxia could be a result of PE development instead of its cause[121]. Proangiogenic factors include vascular endothelial growth factor (VEFG), placental growth factor (PlGF), transforming growth factor-β (TGF-β) and surface endoglin. VEGF can bind to Flt-1 and kinase domain receptor (KDR), both of which are receptors on vascular endothelial cells. It has been suggested that VEGF is crucial in placental and embryonic vasculogenesis and angiogenesis, vascular homeostasis, as well as nitric oxide-dependent

vasodilation. PlGF binds to Flt-1 only and could amplify VEGF effects by displacing VEGF from Flt so that it can bind to the more active KDR receptor. TGF-β could bind to the type I TGF-β receptor ALK5 and type II receptor TGF-βII that are important in angiogenesis, vascular homeostasis and regulation of VEGF expression. Endoglin is a co-receptor for TGF-β and is necessary in endothelial cell proliferation and migration[13, 22]. In preeclamptic women, the levels of antiangiogenic factors including soluble Flt-1 (sFlt-1) and soluble endoglin (sEng), are both higher than those in women having normal pregnancies. sFlt-1 is a potent inhibitor of VEGF and PlGF, whereas sEng is a TGF-β1 inhibitor (Figure 1.1). The binding of sFlt-1 to VEGF and PlGF may lead to reduced levels of free VEGF and free PlGF. Therefore, the levels of free PlGF, sFlt-1/PlGF, and the combination of sEng and sFlt-1/PlGF have all been suggested as markers of PE in clinical setting[22]. It has also been reported that aberrant regulation of angiogenic factors could contribute to poor cytotrophoblast invasion. As early as about 11 weeks of gestation, the expression of sFlt-1 could be upregulated, leading to inadequate cytotrophoblast invasion. Similarly, excessive production of TGF-β or endoglin during early gestation has been hypothesized to cause shallow cytotrophoblast invasion and subsequent PE[121]. However, there are serious questions about these factors causing or mediating PE. For example, many women with PE have 'normal' levels of both the anti-angiogenic and pro-angiogenic factors, especially women with milder or later PE[198]. Furthermore, a large, prospective, blinded trial of anti-oxidant therapy to prevent or reduce PE showed no reduction in PE in the treatment group, however, levels of sFlt-1 and PlGF both normalized in the PE women[199]. Moreover, a recent large study found that the sFlt-1/PlGF ratio, long touted as a strong predictive marker for PE, failed to predict women with PE. A very high ratio provided a sensitivity of 34%. There was almost complete overlap of the values of the ratio for women with later with PE versus women of

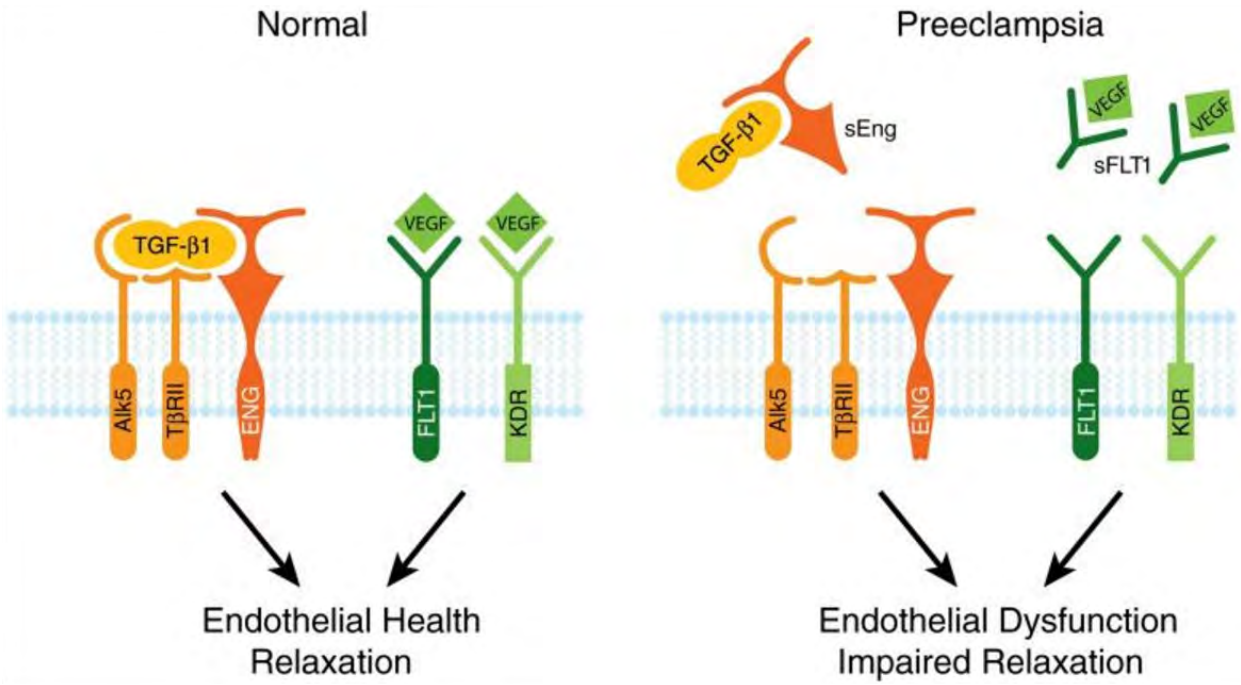

Figure 1.1 Imbalance between proangiogenic factors and antiangiogenic factors that contributes to the pathogenesis of PE. (Reprinted with permission from powe, C. E.; Levine, R. J.; Karumanchi, S. S., Preeclampsia, a disease of the maternal endothelium: the role of antiangiogenic factors and implications for later cardiovascular disease. *Circulation* 2011, *123* (24), 2856-69.)

comparable gestational age having uncomplicated pregnancies although there was a weak but statistically significant difference between the groups[243].

Oxidative stress has also been associated with PE. It was reported that preeclamptic placentas produced greater amount of superoxide than normal placentas. Evidence from an animal model of placental ischemia-induced hypertension in pregnancy[128] has also suggested an association between placental hypoxia and reactive oxygen species (ROS) production in the maternal liver. Moreover, oxidation of protein and lipoprotein particles, elevated levels of lipid peroxidation isoprostanes, and lower levels of antioxidants have all been detected in women with PE[22, 121], although contradictory results are present as well[244, 245]. In addition, it was suggested that shedding of placental debris, which possibly results from placental hypoxia, could lead to increased oxidative stress, vascular endothelial damage, inflammation, and possibly elevated circulating levels of sFlt-1 in preeclamptic women[125].

The immune system has been suggested to contribute to PE as well. Evidence has demonstrated the importance of an intact immune system in the development of PE[121]. It was suggested that altered immune adaptation to fetal antigens[24] might be responsible for shallow cytotrophoblast invasion. Statistically significant elevations in dendritic cells and macrophage infiltration have been reported in preeclamptic placentas, together with increased levels of chemokines[121]. At the fetal-maternal interface, natural killer (NK) cells and altered regulation of the complement system have both been associated with abnormal placentation[22]. It was indicated that the combination of a HLA-C2 genotype, expressed by invading cytotrophoblasts, and an AA-KIR genotype, the receptor on uterine natural killer (NK) cells was linked to PE[13]. In addition, increased levels of inflammatory cytokines[127] and AGT II type 1 receptor autoantibodies (AT1-AAs) have both been detected in women with PE. AT1-AA can bind to and

activate the angiotensin type I receptor (AT1-R) through the TNF-α-dependent pathway, inducing the production of anti-angiogenic factors sEng and sFlt-1[130]. The binding of AT1-AA to AT1-R could also induce vasoconstriction in the absence of TNF-α signaling, or mediate hypertension by increasing ROS production[131]. It has also been reported that AT1-AA could lead to elevated TNF-α/IL-6 signaling, resulting in increased production of endothelin-1 (ET-1)[122], the elevated levels of which have been suggested as a contributing factor to PE. In addition, elevated expression of TNF-α and AT1-AAs has both been correlated to placental hypoxia in the setting of PE. It was reported that TNF-α could lead to increased production of sEng and sFlt-1[129], and that TNF-α, together with interleukin 6 (IL-6), could lead to a number of observations mentioned above by producing AT1-AA[124].

Genetics has been suggested to play a role in the pathogenesis of PE. People who have a maternal or paternal family history of PE are more likely to develop the disease or father a preeclamptic pregnancy[14, 22]. Aberrant expression of or the Y153H mutant of transcription factor storkhead box 1 (STOX1) has been associated with abnormal placentation in PE, although inconsistent findings exist[263]. It was also noted that STOX1 is not a common cause of PE. In addition, PE had been linked to impaired DNA methylation, defective Notch signaling pathway (which is essential in vasculogenesis), polymorphisms of genes that control oxidative stress, angiotensinogen, endothelial nitric oxide synthase (eNOS) to name a few, and multiple PE implicated loci, for example, 2p25, 9p13, 10q22, etc[125, 127].

Last but not least, endogenous digitalis-like factor (EDLF) has been associated with PE and proposed as a factor that may mediate the hypertension of PE. This is supported by the evidence that the levels of EDLF in sera and placental homogenates from patients with PE are significantly higher than those in women having a normal pregnancy[71, 72], that EDLF can cause

vasoconstriction and increased blood pressure and that Digibind, the Fab fragment of a polyclonal anti-digoxin antibody that can inactivate EDLF, has reduced blood pressure or the use of anti-hypertensives in women with EDLF-positive PE and led to improvement of other neonatal and maternal complications leading to better outcomes[117]. For a detailed explanation regarding the association between EDLF and PE, please refer to the second part of the Chapter 1.

1.1.5 Treatment, management and prevention

The only definitive treatment for PE is the delivery of the placenta and the fetus[9, 14, 17], the criteria of which are based on gestational age at the time of diagnosis and the severity of the disease[13]. The potential benefit of fetal growth in utero should be carefully balanced with the maternal and fetal risk of complications of PE[27]. Digibind, the Fab fragment of the anti-digoxin antibody raised in sheep, has been used to treat women with PE in multiple clinical trials and improved maternal and neonatal outcomes were observed without any adverse effects[76, 77, 104].

Prior to delivery, calcium supplementation has been recommended, especially for women with low calcium diets, since calcium supplementation during pregnancy is associated with significantly reduced risk of PE[26]. Anti-hypertensive drugs are typically used only with more severely elevated blood pressures whereas magnesium sulfate is often used in the US to treat women with PE to prevent eclamptic seizures[12-14, 17]. Additionally, corticosteroids are administered if possible to the mother for 48 hours before delivery, if the woman is not at severe risk and can be stabilized, to allow for the acceleration of fetal lung maturation[18, 24]. Hemodynamic, neurological and laboratory monitoring are typically done for women with severe PE following delivery, since rarely, complications of PE can occur postpartum[13]. In such cases

long-term monitoring for cardiovascular disease is also recommended.

1.2 Endogenous digitalis like factors (EDLF)

1.2.1 Digitalis-like factors (DLF) or Endogenous digitalis-like factors

Digitalis-like factors (DLF), which are also referred to as ouabain-like factor, digoxin-like factor, cardiotonic steroids (CTS), or sometimes cardiac glycosides, are a family of compounds that currently consist of cardenolides and bufadienolides[46]. Examples of plant-derived cardenolides are ouabain, digoxin, and digitalis, and examples of animal-originated bufadienolides include marinobufagenin (MBG), telocinobufagin (TCB) and proscillaridin A[30] to name a few (Figure 1.2). DLFs have a cyclopentaphenanthrene nucleus with l AB cis, BC trans, and CD cis fused ring system, a C-14 hydroxyl group or a C14-C15 epoxide[78], and a β-unsaturated (5-membered in cardenolides, 6-membered in bufadienolides) lactone ring[30].

Digitalis and related compounds have been and still are used to treat congestive heart failure (CHF)[30]. By specifically inhibiting the Na^+/K^+ -ATPase or sodium pump, they increase the intracellular Na^+ concentration, resulting in cell membrane potential depolarization with activation of voltage-dependent Ca^{2+} channels or the Na^+/Ca^{2+} exchanger. Intracellular Ca^{2+} levels thus increase in heart muscle leading to enhanced myocardial contractility in CHF patients. The highly conserved nature of the binding site for plant-derived DLFs such as digitalis on the Na^+/K^+-ATPase raises questions as to the existence of one or more endogenous digitalis-like factors[68]. Indeed, in 1961 de Wardener et al.[89] suggested the existence of 'the third factor' based on the demonstration that natriuresis induced by saline infusion can still be maintained even if both renal perfusion pressure and glomerular filtration rate remain normal. Since then,

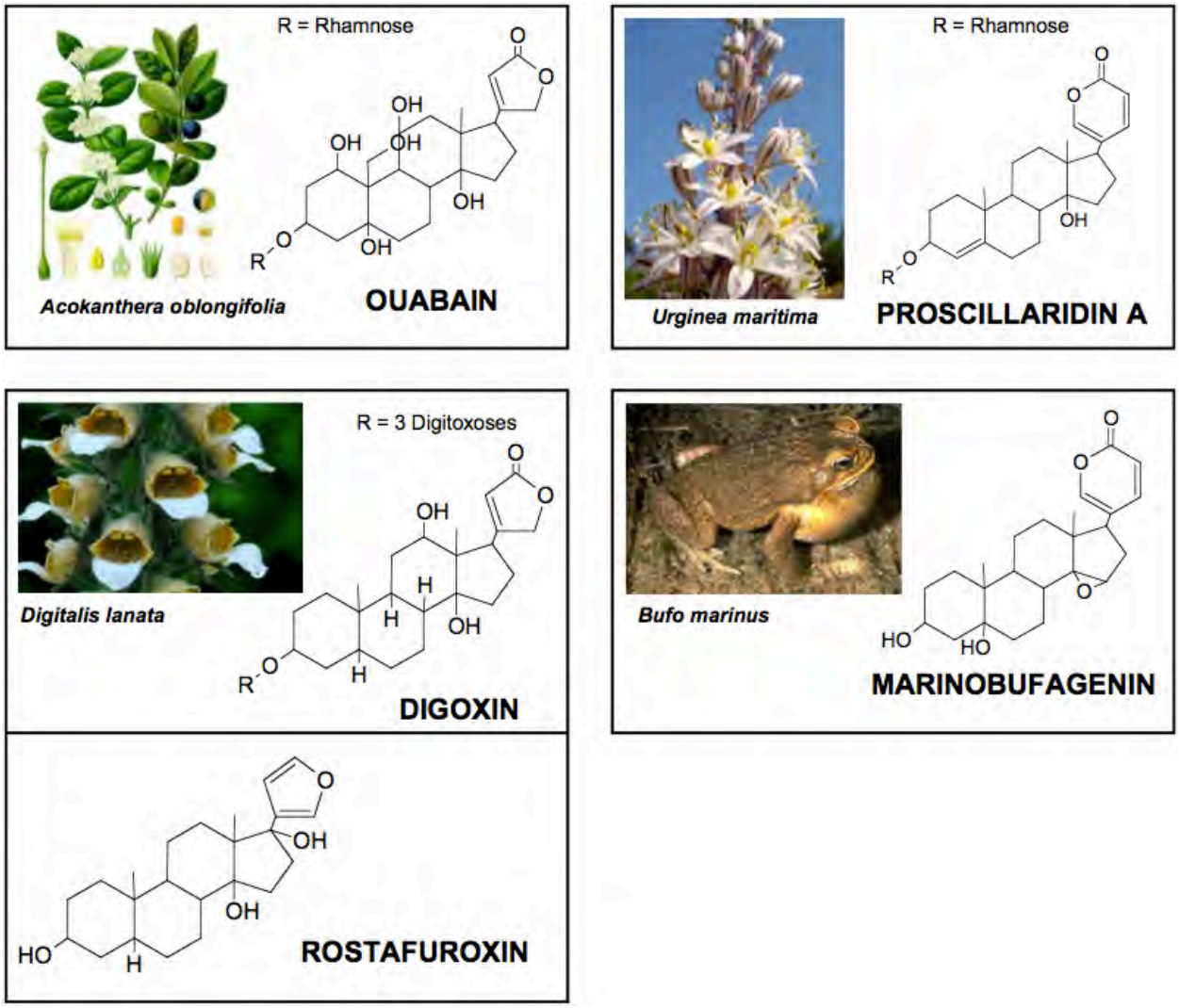

Figure 1.2 Chemical structures of digitalis-like factors (DLF): cardenolides and bufadienolides. (Reprinted with permission from Bagrov, A. Y.; Shapiro, J. I.; Fedorova, O. V., Endogenous cardiotonic steroids: physiology, pharmacology, and novel therapeutic targets. *Pharmacological reviews* 2009, *61* (1), 9-38.)

'the third factor', now termed EDLF, has been shown to respond to volume expansion and is capable of inhibiting the activity of the Na^+/K^+-ATPase, has remained a research topic of great interest.

Elevated concentrations of a circulating EDLF have been associated with a number of diseases that are marked by salt or volume overload, such as renal disease, hepatic failure, several forms of hypertension in both the human and in animal models, and in PE[91]. It is thought that increased levels of EDLF detected in various pathological conditions are one of the body's attempts to advance sodium and volume excretion in the kidney by reducing renal sodium reabsorption[35, 91]. Additionally, Graves et al. detailed four different responses to increased levels of EDLF in the cardiovascular system, all of which can result in blood pressure elevation and facilitate salt and water excretion. He suggested that elevated concentrations of an EDLF in clinical settings, consequently, may lead to chronic hypertension[91] either directly by causing vascular smooth muscle (VSM) contraction or by sensitizing the VSM to other vasoconstrictors.

Over the years, EDLF has been "detected and identified" in a number of human tissues and fluids under various clinical settings. Below is a summary of these findings:

An EDLF indistinguishable from ouabain has been identified in human plasma using NMR and mass spectrometry[48]. Evidence suggested that the endogenous ouabain is produced by the adrenal cortex and/or hypothalamus[51, 55]. This is supported by the observation that the circulating level of EDLF is reduced following adrenalectomy[49]; and in patients with adrenal tumors, increased circulating level of EDLF is associated with elevation of blood pressure, both of which were normalized following adenoma removal[50]. Elevated levels of an endogenous ouabain-like factor have also been detected in the plasma of preeclamptic women[65]. It was suggested that the placenta could be the source of EDLF in women with PE. Animal models also

indicated the association of ouabain with hypertention and cardiac hypertrophy[25, 56]. It is thought that inhibition of the SP caused by increased levels of endogenous ouabain in vascular smooth muscle is one of the three mechanisms that lead to vasoconstriction in hypertension[25]. At the same time, however, there are contradictory results concerning even the existence of endogenous ouabain[69. 70].

An EDLF indistinguishable from digoxin had also been isolated from human urine[57], although Qazzaz et al. suggested that the EDLF was deglycosylated or non-glycosylated analogs of digoxin instead of digoxin itself, and the former was identified in bovine adrenal glands[58].

Research has provided evidence for the existence of endogenous bufadienolides in mammals as well. An endogenous bufalin-like factor has been detected in human placenta[73], human bile and plasma by multiple research groups[59, 60]. EDLFs that appear to be derivatives of bufalin were identified in the lens of several mammals using mass spectroscopy[61]. An EDLF that can cross-react with an antibody to proscillaridin A[62] was reported in hypertensive human plasma in 1996. Additionally, an EDLF identical to marinobufagenin (MBG) at increased concentrations was also found in human urine and plasma and in patients with PE, hypertension, end-stage renal disease, acute myocardial infarction and CHF, and in one study identified by NMR, immunoassay and mass spectrometry[63-66]. Takahisa et al. suggested that marinobufotoxin (MBT), purified from cultured adrenocortical cells, is a novel EDLF associated with an animal model of hypertension. Lastly, elevated levels of telecinobufagin (TCB)[67] were detected by NMR and tandem mass spectrometry in uremic plasma in 2005.

The above findings concerning the identity of EDLF, though, should be interpreted with caution, due to the non-specificity of the assays for EDLF, which include the ATPase-based assay and radioimmunoassay[91], etc. For detailed suggestions and explanations regarding the

criteria for EDLF (Table 1.3), please refer to relevant review articles in the literature[91, 92].

1.2.2 EDLF and the sodium pump

The Na^+/K^+ ATPase or sodium pump (SP) was discovered by Skou[31] in 1957, it uses the energy derived from the hydrolysis of ATP to maintain the low intracellular Na^+/K^+ ratio, which is achieved by transporting three sodium ions out of the cell and two potassium ions into the cell against existing concentration gradients of both[32]. This also generates a cell membrane potential. The sodium pump is a highly conserved heterotrimeric enzyme/ion pump in eukaryote cells. It consists of an α subunit, a β subunit, and a FXYD (the γ subunit)[28, 29] (Figure 1.3). The α subunit has ten transmembrane segments with a molecular mass of 110 kDa. It is the catalytic subunit of the SP and it has the binding sites for Na^+, K^+, ATP and extracellularly EDLF[28]. The binding site for ATP is on the intracellular region of TM4-TM5 loop. The binding sites for EDLF include the TM1-TM2, TM5-TM6, TM7-TM8 loops and a few amino acids on the transmembrane segments of M4, M6, and M10[25], among which the extracellular section of the TM1-TM2 loop is the most important part. The β subunit is a single transmembrane protein that has a molecular mass of 36 kDa[30], which is important in assisting plasma membrane localization and activation of the α subunit. The FXYD subunit has a molecular weight of 7.3 kDa and regulates SP activity in a tissue-specific and isoform-specific manner[30].

There are four isoforms of the α subunit (α1, α2, α3, and α4), three isoforms of the β subunit (β1, β2, and β3) and at least seven isoforms of FXYD[30] documented so far. The α1 isoform is expressed ubiquitously but is the sole isoform expressed in the kidney. The α2 isoform is expressed in heart, vascular smooth muscle, brain, skeletal muscle, bone, cartilage, lung and adipocytes[37-41]. The α3 isoform is expressed in neural tissues, heart and ovaries[41-43]. The α4

Table 1.3 Proposed criteria for DLF. (Reprinted with permission from Hollenberg, N. K.; Graves, S. W., Koch's postulates and the digitalis-like factor. *Hypertension research: official journal of the Japanese society of hypertension* 1995, *18* (1), 1-6.)

(1) Action at physiological concentration
(2) Relevant physiological and clinical correlates
(3) Appropriate biochemical activity
(4) Appropriate bioactivity
(5) Surgical, pharmacologic, or immunological reversal
(6) Biosynthesis

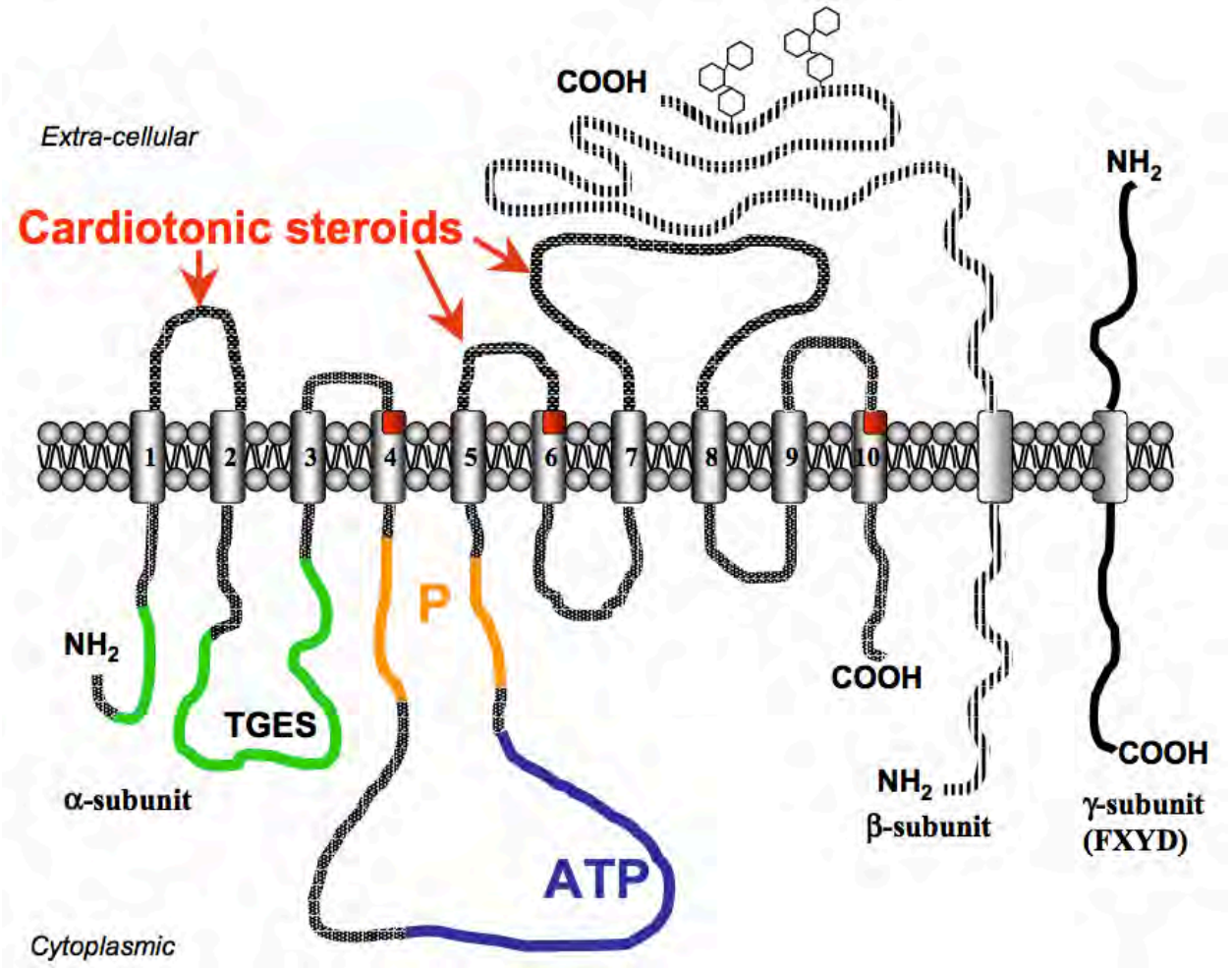

Figure 1.3 Structure of the sodium pump and its binding sites for EDLF. (Reprinted with permission from Bagrov, A. Y.; Shapiro, J. I.; Fedorova, O. V., Endogenous cardiotonic steroids: physiology, pharmacology, and novel therapeutic targets. *Pharmacological reviews* 2009, *61* (1), 9-38.)

isoform is expressed in sperm[44]. The β isoforms are expressed in a tissue-specific fashion as well. The β1 isoform is ubiquitously expressed. Both β2 and β3 isoforms can be found in the brain, erythrocytes and cartilage, additionally, the β2 isoform is expressed in cardiac tissues and the β3 isoform can also be found in lung tissues[25]. The variable combinations of αβ complexes, the discrepancies in the amino acid sequence among different species and several other mechanisms underlie the diverse functions of the SP and its differential sensitivities to different EDLFs[25, 36]. For example, the α1 isoform, which can be found in the brain tissue and the kidney[25], is less sensitive to ouabain, whereas the α3 isoform, which can also be found in the brain, has high sensitivity toward ouabain[28].

The SP has many cellular functions: for instance, the electrochemical membrane potential necessary for neuron excitability is created by the SP[33]. The low intracellular sodium concentration essential for the normal function of smooth muscle and cardiac myocytes is maintained by the SP[45]. The Na^+ gradient generated by the SP can be coupled to the Na^+/Ca^{2+} - exchanger[34]. Last but not least, it is also important for sodium reabsorption from the glomerular filtrate in the distal tubule of the kidney[35].

The effects of EDLF on the Na^+/K^+ ATPase under physiological conditions are examined here. It had been reported that elevated circulating and/or urinary levels of EDLF are associated with acute saline infusion, volume expansion, acute fluid ingestion and salt ingestion[91, 93-95]. Bagrov et al. proposed that there are two pathways that "work in parallel and synergistically" regarding the effects of EDLF on the SP. One is the ionic pathway, in which the inhibition of SP by EDLF coupled with the Na^+/Ca^{2+} exchanger leads to elevated intracellular calcium concentrations, which can act as a second messenger for a number of cell functions[31]. The other is the signaling pathway in caveolae that includes the activation of the protein Scr upon the

binding of EDLF to the SP, the subsequent transactivation of the protein EGFR, and a cascade of events that follow which involves multiple signaling proteins, and ultimately, leads to a variety of genomic and non-genomic effects[25].

The effect of EDLF on the SP has not been extensively studied in women with uncomplicated pregnancy[97]. Jie et al. indicated that increased concentrations of EDLF were detected in women with normotensive pregnancy using radioimmunoassay[71]. However, in a different study, Hopate-Sitake et al. reported that the reduction of SP activity in sera from women with uncomplicated pregnancy compared with those from non-pregnant women was not a result of the presence of an EDLF[72]. Since increasing amount of sera from women with normal pregnancy did not lead to enhanced SP inhibition, combined with the fact that Digibind, an antibody that can cross-react with and bind EDLF, failed to reverse the SP inhibition displayed in sera from women with normal pregnancies. Some other studies described the presence of an immunoreactive factor with high concentrations in sera of pregnant women, with or without measurement of modest inhibition of the SP in the same sera[97]. It had also been reported that concentrations of the factors that can cross-react with anti-digoxin antibodies displayed substantial increase during the course of normotensive pregnancy. However, other studies suggest that the immunoreactive EDLF is largely protein bond and is not filterable without heat treatment[246]. It is essential, though, for any researcher to recognize and consider the criteria for EDLF[91, 92] when it comes to the detection and identification of such factors. And it should be noted that in some cells sodium can be regulated by multiple transport systems, the most important of which is the Na^+/K^+-ATPase[97]. Despite the suggestion that (small elevation in concentrations of) a SP inhibitor may be present in the circulation of women with uncomplicated pregnancy[98, 99], most studies favor an increase in SP activity in women with normal pregnancy. It

had been proposed that even if elevated levels of a SP inhibitor are exhibited in women with normal pregnancy, the elevation in the number and activity of the SP would have more than adequately offset their existence[97].

1.2.3 EDLF in various clinical settings

1.2.3.1 EDLF in PE

It has been suggested that EDLF plays a role in the pathogenesis of PE[78]. This is supported by the evidence that significantly higher levels of EDLF have been detected in the placental homogenates and sera from preeclamptic women compared with women having a normal pregnancy, using assays that measure the degree of SP inhibition and/or apparent concentration of the substance that can cross-react with Digibind[71, 72, 102]. Furthermore, increased levels of an EDLF were reported in cord blood from neonates born to women with PE compared with those in cord blood from infants born to women with normotensive pregnancies[86]. The natriuretic factor EDLF was suggested to be released in an attempt to correct renal salt retention or to improve fetal-placental perfusion in the setting of PE. This however, unfortunately leads to hypertension. The addition of Digibind, the anti-digoxin Fab fragment from sheep sera that can inactivate EDLF, resulted in 43% and 35% reduction in SP inhibition in the placental homogenates and sera from women with PE respectively[72]. Digibind also led to reduced blood pressure in PE and pregnancy-induced hypertension associated with significantly elevated levels of EDLF[65, 76, 77]. Similarly, anti-EDLF antibodies effectively decreased blood pressure in an animal model of PE as well[100, 101]. It was also found that Digibind could mitigate the inflammatory responses induced by TNF-α and protect endothelial cells in PE[113].

Additionally, it has been suggested that the placenta is the potential source of EDLF in the setting of PE, based on the evidence that EDLF concentration in placental homogenates from preeclamptic women was reported to be (much) higher than that in sera from women with PE[71], and EDLF continued to be present in fetal blood after birth for a few days, and that EDLF quickly disappears from maternal blood postpartum[104]. In the attempt to study the synthesis and regulation of EDLF, it was demonstrated that an EDLF, capable of cross-reacting with Digibind and causing SP inhibition, could be produced and released from cultured human placenta explants into the surrounding media[71]. Furthermore, EDLF release could be increased by adding 17-OH-progesterone or decreased by adding ketoconazole, a known steroid synthesis inhibitor, to the cultured placental tissue. Moreover, EDLF produced from human placenta has been associated with a number of abnormalities present in PE for example, hypoxia, oxidative stress and proinflammatory cytokines. Statistically significant higher levels, sometimes a doubling of EDLF, were found to be produced and released from placenta explant culture in the presence of hypoxia, hydrogen peroxide and TNF α, respectively[71].

In other experiments Digibind was also able to reverse the vasoconstrictive effect of an EDLF in perfused preeclamptic placenta[105]. Not only did the expression of SP increase in the preeclamptic placenta, but these SPs now displayed an increased sensitivity to an ouabain-like factor inhibition[106]. Apart from the placenta[53, 71, 72], EDLF had also been detected in cord serum[87], fetal and neonatal plasma[88], and amniotic fluid[120]. Substantially higher levels of EDLF were detected in amniotic fluid in women with hypertensive complication of pregnancy in comparison with women with normal pregnancy, using both a radioimmunoassay and a functional assay that measures SP inhibition[83].

It has been proposed that the EDLF detected in the plasma of preeclamptic women[100] is

very similar to ouabain. Using an 'ouabain-specific' antibody assay, elevated levels of EDLF capable of SP inhibition have been reported in patients with PE[65]. Evidence also suggests that the EDLF present in human placenta could be in the category of bufadienolides[73]. MBG was proposed to be one such candidate, because elevated levels of MBG and SP inhibition were reported in the plasma and placenta of women with mild and severe PE. The administration of anti-MBG antibodies effectively neutralized the SP inhibition present in PE[65, 103, 109]. Similarly, increased levels of MBG and heightened sensitivity to MBG have also been reported in different animal models of PE. The addition of anti-MBG antibody significantly reduced blood pressure in both models[107, 108]. Additionally, MBG was suggested to play a role in the dysfunction of cytotrophoblasts and the subsequent abnormal placentation in the pathogenesis of PE[110].

However, other studies have reported the absence of immunoreactive factors or factors that are capable of inhibiting the SP in the setting of PE[111, 112, 117]. In the secondary analysis of the DEEP trial, women with PE were classified as being EDLF negative or EDLF positive[117]. This was based on an earlier report that demonstrated higher levels of urinary EDLF could be detected in 82% of preeclamptic women compared with women with normotensive pregnancy[119], a percentage comparable to the 78% reported in the DEEP clinical trial. In patients who were EDLF positive, multiple findings were reported. For example, it was found that Digibind treatment significantly lowered EDLF levels in preeclamptic women[76, 77, 104]. Digibind also preserved renal function by preventing GFR reduction in women with severe PE[117, 118]. The association between EDLF and pulmonary edema was also suggested for the first time, which was supported by the observation that women who were administered the Digibind Fab fragment had a substantially reduced rate of maternal pulmonary edema compared with those who were administered placebo[117]. Lastly, it was reported that the rate of intraventricular hemorrhage

(IVH) in neonates was substantially reduced in EDLF positive women treated with Digibind. This Fab fragment may also then neutralize an endogenous ouabain-like factor in cord blood[80] that is capable of inhibiting the SP. Digibind treatment therefore might bring about improved neonatal outcomes in babies born to preeclamptic women[117].

1.2.3.2 EDLF in hypertension

Higher levels of SP inhibitor have been reported in many studies of essential hypertension and several types of secondary hypertension including hypertension associated with chronic kidney disease[30]. In hypertensive individuals with increased levels of EDLF, three mechanisms have been proposed regarding the hypertensinogenic effects of EDLF[25].

In an animal model designed to study the pathogenesis of salt-sensitive hypertension, higher concentrations of free EDLF have been detected in cataract-prone Dahl salt-sensitive rats[84], a subgroup of Dahl salt-sensitive rats[85], compared with non-cataract prone Dahl salt-sensitive rats and control Dahl salt-resistant rats. Digibind, a polyclonal antibody Fab used to treat digoxin poisoning, effectively neutralized the EDLF found in several animal models of hypertension[74, 75]. Digibind also led to decreased blood pressure in DOCA-salt rat model, 5/6 reduced renal mass model[75, 115] and animal models of hypertension with increased EDLF concentrations. No such effect was observed in the controls of the 5/6 reduced renal mass model. EDLF that cross-reacts with anti-ouabain antibody has been detected in the hypothalamus and pituitary gland in several rat models of hypertension as well[114]. The administration of Digibind successfully prevented blood pressure elevation associated with brain EDLF[68]. In dogs, natriuresis was found to be blocked by Digibind during saline infusion[116].

1.2.3.3 EDLF in renal disease

It was suggested that a circulating EDLF plays a role in the pathogenesis of renal failure and the uremic syndrome according to research done on experimental rat models and human subjects[50, 90]. In rodents, elevated circulating levels of EDLF in plasma have been reported in clinical and experimental renal failure[25]. Similarly, significantly increased levels of TCB and/or MBG were also detected in plasma of patients with end-stage renal disease (ESRD)[67] and animal models of chronic kidney failure[66]. These animal models developed symptoms comparable to human uremic cardiomyopathy as well. Immunization against MBG in rats was able to attenuate the features that characterized the uremic cardiomyopathy[82].

Elevated levels of a circulating EDLF have also been reported in patients with acute and chronic kidney failure using assays that measure the degree of SP inhibition and immunometric apparent EDLF concentration[91]. In several animal models of defective kidney function that include renal artery clipped primates, renal artery wrapped rats and more, increased circulating levels of EDLF were shown to occur to some extent before or along with the clinical manifestation of hypertension. Unclipping or unwrapping, on the other hand, effectively led to hypertension reversal and EDLF reduction[91]. It was also found that Digibind could inactivate an ouabain-like factor and reverse EDLF inhibition of the SP found in human peritoneal dialysate[79].

Apart from the association of EDLF with PE, hypertension and renal failure, EDLF had also been linked to myocardial ischemia/infarction, cardiorenal syndrome[52], uremic cardiomyopathy[82], diabetes, polycystic kidney disease[27], necrotizing enterocolitis (NEC)[87], acromegaly, hepatic failure, severe liver disease, obstructive sleep apnea and behavioral stress. The list goes on[25].

In my research described hereafter I attempt to use mass spectrometer-based profiling of tissue components to identify a pattern of change in those tissues that have been exposed to EDLF. To this end, we sought to find whether normal human placenta responded to SP inhibition with a change in the abundance of biomolecules in the cytosol and if there were changes whether there was a characteristic set or pattern of responses. To do this, we studied the changes in lipidomic and peptidomic profiles of placenta tissues in the presence and absence of ouabain, a known SP inhibitor and a proposed EDLF, using mass spectrometry based 'omics' methods. The findings, in return, could potentially advance our understanding of the pathogenesis of preecalmpsia and a great number of other diseases that had been associated with EDLF.

1.3 Lipidomics

1.3.1 Introduction

Lipids are biomolecules that are soluble in non-polar organic solvents but insoluble in water. A better definition of lipids is as follows: hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion based condensation of thioesters and/or by carbocation based condensations of isoprene units[132]. Lipids can be divided into eight major categories: 1) fatty acids, 2) glycerolipids, 3) glycerophospholipids, 4) sphingolipids, 5) sterol lipids, 6) prenol lipids, 7) saccharolipids, and 8) polyketides (Figure 1.4), with further divisions into classes, subclasses, and sometimes fourth-level classes. The functions of these highly diverse lipids include membrane-formation, membrane anchoring, energy storage, hormones, cell signaling, protein modification, regulation, etc. Abnormal lipid metabolism has been associated with a number of human diseases such as diabetes, cancer, atherosclerosis, liver

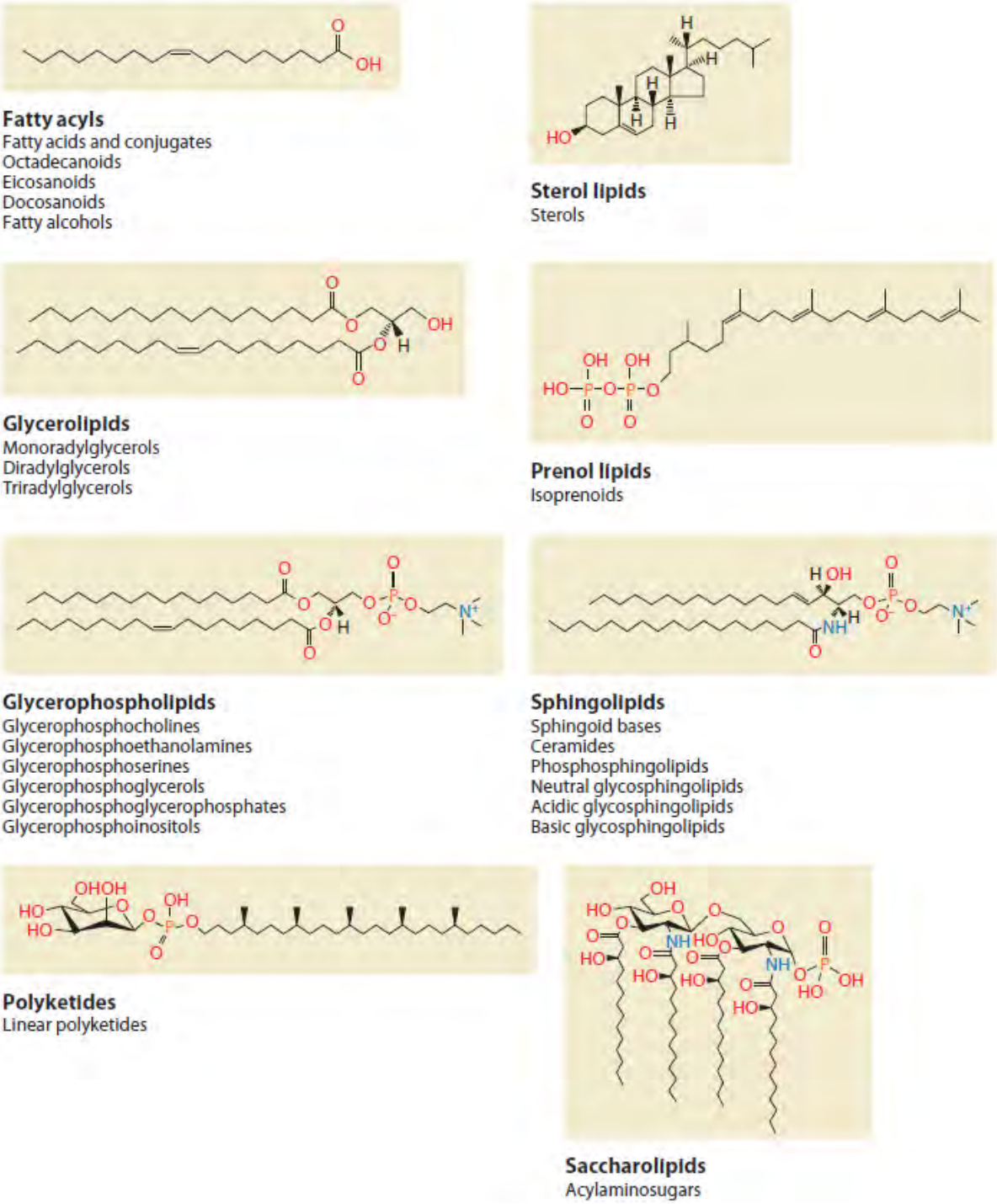

Figure 1.4 Eight lipid categories and examples. (Reprinted with permission from Brugger, B., Lipidomics: analysis of the lipid composition of cells and subcellular organelles by electrospray ionization mass spectrometry. *Annual review of biochemistry* 2014, *83*, 79-98.)

steatosis, chronic inflammation, PE, and many neurological disorders including Alzheimer's disease[135, 136].

The lipidome refers to the complete collection of chemically distinct lipids within a cell, tissue, organ or a biological system. It has been estimated that there are as many as tens of thousands to millions of lipids in a cellular lipidome[137]. Lipidomics, as a branch of metabolomics, is defined as the full characterization of lipid molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation. It has become more and more important in our understanding of many diseases and physiological processes[138]. Lipidomic studies[137] typically involve the identification and quantification of individual lipid species of interest, the study of the interaction between lipid and protein, other lipids or metabolites, etc. There are a variety of analytical methods utilized in lipidomics research, including: gas chromatography (GC), high-performance liquid chromatography (HPLC), mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, enzyme-linked immunosorbent assays (ELIZA) to name a few (Table 1.4). However, many of these focus on a single targeted lipid or group of lipids and are therefore limited in the number of lipids surveyed. We will provide brief introductions to several methods routinely used in lipid analysis below.

1.3.2 Lipid extraction

Extraction of lipids from complex biological fluids, membranes, cell or tissue homogenates is generally the first step in a lipidomics analysis. By eliminating proteins, saccharides, and minerals[138] that potentially interfere with lipid analysis, better results can be achieved.

Experimental approach	Lipid classes covered	Advantages	Disadvantages
Chromatography			
Thin-layer chromatography (TLC)	Solvent systems established for most lipid classes	Very established technique; technically relatively easy; does not require sophisticated instrumentation; spot chromatograms allow for rapid screening of mutant extract libraries.	Low resolution and sensitivity limits many lipidomic applications; detection of lipid by iodine vapour and (class-specific) dyes and radioactivity.
High-performance liquid chromatography (HPLC)	Many lipids, including sterols, GP, TG, DG, FA and lipid headgroup derivatives.	Well established with worked out reverse- and normal-phase conditions available; ease of automation; very quantitative.	Detection by refractive index or mass detector (lipids, as defined here, in general do not absorb visible and UV light effectively); medium sensitivities in general.
Gas chromatography (GC)	Non-polar compounds such as TG; derivatized FA and sterols.	Very widely used for determination of fatty-acid composition, detection generally by MS.	Requires volatile compounds or derivatization of polar lipids.
Mass spectrometry			
ESI	Polar compounds such as GP (more apolar compounds can be analysed by APCI)	Direct detection by *m/z*; high sensitivity and resolution; direct profiling of complex lipid mixtures; ease of automation; compatible with upfront LC separation.	Supression of ionization, in particular in the case of crude extracts and when low-abundance species are to analysed; absolute quantification requires considerable efforts (for example, class and mass dependent internal standards).
MALDI	Many lipids including complex glycolipids.	Direct detection by *m/z*; buffer and salt contaminants generally well tolerated; can be combined with prior TLC separation.	Supression of ionization, in particular in the case of crude extracts and when low-abundance species are to analysed; matrix backgrounds.
NMR			
^{31}P	Phospholipids.	Direct measurement; non-destructive; quantitative.	Line broadening of lipids in aqueous solutions; low sensitivity.
^{1}H	All lipids.	Direct measurement; non-destructive; powerful technique for structural analysis of purified compounds.	low sensitivity, spectra dominated by very abundant lipids (cholesterol, PC).
Biochemistry			
Assays using immobilized lipids (for example, monolayer adsorption, langmiur blodget films, immobilized lipid biosensors, lipid blots/beads)	Many lipids both in pure form or mixtures.	Sensitive approaches which allow determination of interaction of ligands with lipids.	Functional immobilization of lipids difficult; technically tedious; automation and throughput generally limited.
Assays using lipid in solution/suspension (for example, optical, calorimetric, radiometric approaches using liposomes and micelles)	Many lipids both in pure form or mixtures.	Solution conditions; binding studies; often quantitative; enzyme assays, some which of high throughput.	Often experimentally challenging; optimization of conditions can require significant effort.
Lipid antibodies	Very few.	Cell biological studies (for example, subcellular localization) possible.	Specificity of antibodies.
Reactive lipids (for example, photoactivatable)	Few.	Identification of lipid-binding proteins.	Specificity; probes limited.

DG, diacylglycerol; ESI, electrospray ionization; FA, fatty acid; GP, glycerophospholipids; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; TG, triacylglycerol.

Table 1.4 Analytical methods in lipidomics. (Reprinted with permission from Wenk, M. R., The emerging field of lipidomics. *Nature reviews. Drug discovery* 2005, *4* (7), 594-610.)

Lipid extraction usually involves a phase separation between immiscible solvents in which the lipids partition into the hydrophobic phase. The most commonly used method involves the addition of chloroform and methanol (2:1, v/v), developed by Folch et al[140]. This method was later modified by Bligh and Dyer[141] through the addition of water or acetic acid to maximize extraction of both polar and nonpolar lipids. However, these protocols may need to be further modified[137] in order to extract low-abundance lipids, labile lipid metabolites, etc. Another liquid-liquid extraction (LLE) method involves using hexane and isopropanol (3:2, v/v) to extract apolar lipids[142]. Other LLE methods include a methyl-tert-butyl ether (MTBE)-based method, a butanol and methanol (BUME)-based method, a metal complex "Phos-tag"-based method, etc[134, 138, 139].

Because of the diverse chemical properties of different lipids, no single method can extract lipids of all classes with optimal recovery. In addition, a pre-clearing step before extraction has been suggested to remove interfering agents such as high salts, detergents and density gradient media. Lastly, it has become a standard practice recently to add internal lipid standards prior to lipid extraction to account for variability in quantitative lipid analysis[133]. This of course assumes that you know the particular lipid you wish to analyze.

1.3.3 Lipid separation

Due to its excellent separation efficiency, chromatography plays an important role in the analysis of complex lipid extracts. A variety of chromatographic approaches[138] can be coupled with MS in lipid analysis, for instance, thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), hydrophilic interaction LC (HILIC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE), etc. No single

method can completely resolve a complex extract into individual lipid species. We will briefly review the first three chromatographic methods below.

TLC is a simple, inexpensive method that can analyze lipids with acceptable resolution in some applications. With the advent of high-performance TLC (HPTLC), 2-D TLC, and micro-TLC combined with certain other techniques, TLC resolution has been increased substantially. Various combinations of a stationary phase and mobile phase allow for separation of almost all lipid classes[248, 249]. However, it is challenging to identify lipid subclasses utilizing TLC. In lipidomic studies, TLC can be coupled with ESI-MS, MALDI-MS, or DESI-MS[250]. In addition, TLC-blot-MALDI-MS has also been used in lipid analysis[251].

GC is frequently used in lipid analysis, and it has exceptional capabilities for isomer separation and highly sensitive quantitation. The resolution and sensitivity of GC can be further improved if the analysis is focused on only one specific class of lipids. However, derivatization is essential since most lipids are nonvolatile. Multidimensional GC is preferred for the analysis of complex lipid extracts. However, GC-based lipidomic methods have their own limitations. Structural information of lipids may be lost or modified substantially following derivatization. At the high temperatures needed for GC, certain lipids are prone to modification or degradation[138].

The coupling of high performance liquid chromatography (HPLC) with ESI-MS is the most commonly used method for lipid analysis in biological samples. There are primarily two strategies in lipidomic analysis: targeted analysis that focuses on sensitive detection of pre-specified known lipids, and non-targeted analysis that focuses on simultaneous detection of as many lipid species as possible. It has been recommended that two-dimensional LC (2D LC) be utilized in non-targeted analysis, in which normal-phase LC (NPLC) separates lipid classes based on the polarity of head groups, and reversed-phase LC (RPLC) separates lipids of one

class preferably or different classes[137] based on the hydrophobicity of fatty acyl chains. Virtually all lipids can be separated by HPLC, a separation method with high resolving power, high sensitivity and good reproducibility. Much higher resolution can be achieved by utilizing an ultrahigh performance LC (UHPLC) column, which is frequently used in global analysis of complex lipid extracts. With reduced flow rates and higher detection sensitivity, both nano-LC and capillary LC can be used to effectively analyze trace amounts of lipid samples[135, 138, 139].

1.3.4 Ionization technologies

Analytes need to be ionized by an ion source first to allow a mass spectrometer to measure the mass of analytes that have an electrical charge. A number of ionization technologies have been developed over the years.

Electron ionization (EI) is usually used to analyze gases and volatile organic molecules separated by gas chromatography. Derivatization is necessary for the analysis of non-volatile molecules using EI. Chemical ionization (CI), unlike EI that involves high energy collisions, can generate an intact molecular ion species through low energy collisions with a reagent gas. However, the application of EI or CI in lipidomics is limited due to their low sensitivity and inconvenient derivatization steps.

Fast atom bombardment (FAB) can be used to identify non-volatile lipids, but it is challenging to quantitate lipids using FAB considering its incompatibility with chromatography, high chemical backgrounds, and the complexity of lipid extracts[134, 139].

Electrospray ionization (ESI) is the most widely used method to generate positively or negatively charged lipid molecular ions with minimal in-source fragmentation. It is one of the softest ionization techniques and is capable of producing multiply charged ions at atmospheric

pressure. Due to its compatibility with the liquid phase, ESI can be readily used to profile lipids from blood, cells, biofluids, biological tissues, bacteria, virus and fungi with very high sensitivity[138, 139]. ESI is considered a better choice for lipids because organic solvents can enhance ionization and increase lipid solubility at the same time[137]. In addition, chemicals like formic acid, lithium ion or ammonium acetate are routinely added to lipid extracts in order to enhance ionization or the formation of charged adducts. Intra-source separation can also be utilized to separate individual lipid classes based on variable electrical propensities of different analytes (Figure 1.5) in ESI-MS.

Atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) are two other approaches that ionize compounds at atmospheric pressure. Both of them are more suitable for the analysis of nonpolar lipids[138], although they have differential sensitivities for polar and nonpolar classes. In addition, APCI and APPI are less prone to ion suppression and salt buffer effects[139].

Matrix-assisted laser desorption ionization (MALDI) is a laser-based soft ionization approach that can be used to analyze both large proteins and lipids from biological samples. The choice of the matrix is essential in optimizing MALDI-MS. HPLC or TLC usually can be combined with MALDI-MS for the analysis of both polar and nonpolar lipids but this requires spotting and drying each separation fraction on a MALDI plate prior to MS[139]. Ion suppression, a major drawback shared with ESI-MS, can also be overcome by coupling TLC with MALDI-MS when it comes to the analysis of complex mixtures[135]. MALDI-MS analysis is rapid, convenient, and has high sensitivity compared to other ionization technologies. However, substantial off-line purification and preparation, poor reproducibility and an inability to accurately quantitate are its major limitations[137, 139].

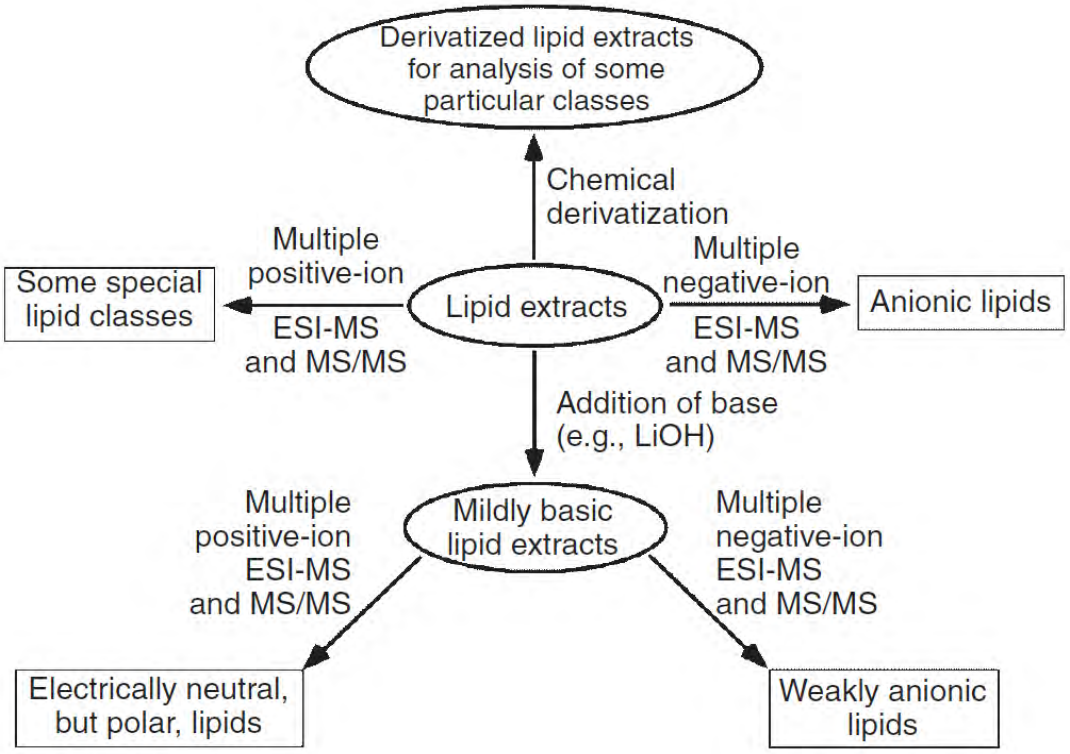

Figure 1.5 Experimental strategies for global analyses of cellular lipidomes directly from crude extracts of biological samples. (Reprinted with permission from Han, X. L. *Lipidomics: Comprehensive Mass Spectrometry of Lipids*, 1st ed.; Wiley: New Jersey, 2016.)

1.3.5 Mass analysis

Soft ionization methods lead to minimal in-source fragmentation, which means the identification and quantitation of lipids rely heavily on tandem MS analysis. There are four common MS/MS techniques (Figure 1.6) in lipidomic analysis: 1) product ion scanning, 2) precursor ion scanning (PIS), 3) neutral loss scanning (NLS), and 4) selected reaction monitoring (SRM) or multiple-reaction monitoring (MRM). In product ion scanning mode, the first mass analyzer selects a precursor ion of defined mass-to-charge ratio (m/z), which undergoes collision-induced dissociation (CID) in a collision cell, the resulting product ions are then scanned in the second mass analyzer. Product ion scanning can be used to obtain structural information of selected precursor ions. However, MS alone cannot separate isomers with the same molecular weight, chiral chromatography would need to be used prior to mass analysis in that case[134]. In the PIS mode, the first mass analyzer operates in scanning mode, whereas the second mass analyzer monitors a fragment ion of defined m/z. PIS is usually used to detect all precursor ions that generate a particular product ion after CID, e.g. you could monitor the presence of species containing a particular head group, such as choline. In NLS, both mass analyzers scan but with a particular offset of the m/z of a selected neutral loss fragment. Analytes that lose a common neutral fragment after CID are detected in the NLS mode. In the SRM mode, both the first and the second mass analyzers monitor selected precursor and product ion, respectively. This mode is termed MRM when any of the two mass analyzers or both monitor multiple ions, usually specific and characteristic set of product ions. SRM is commonly used to monitor a defined CID reaction. Both NLS and PIS have been widely used to filter classes of compounds from complex lipid mixtures in shotgun lipidomics, whereas SRM has been frequently utilized in LC-MS-based lipidomics for determination of a compound of interest in a

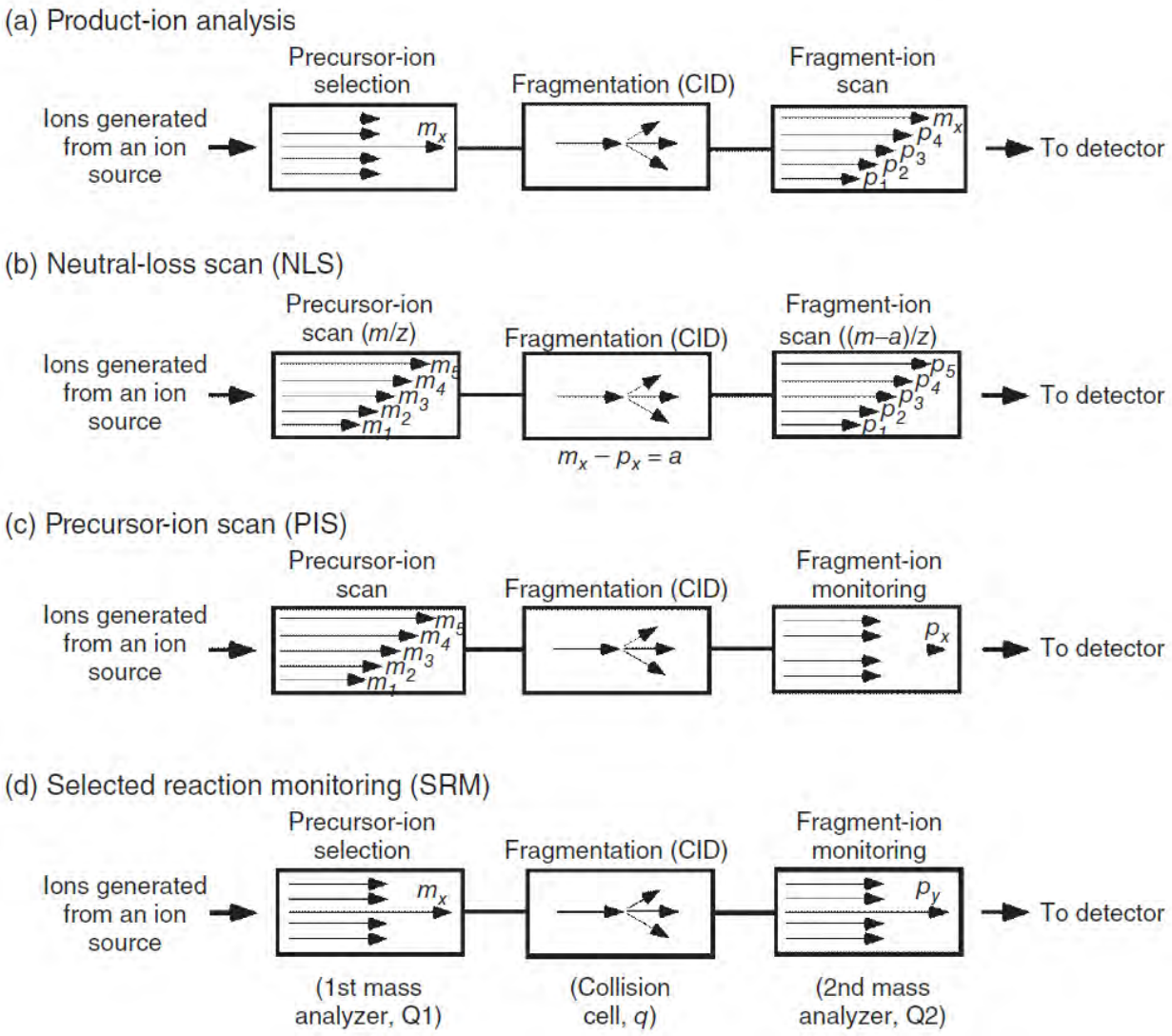

Figure 1.6 Different modes of data acquisition for tandem mass spectrometry. (Reprinted with permission from Han, X. L. *Lipidomics: comprehensive mass spectrometry of lipids*, 1st ed.; Wiley: New Jersey, 2016.)

mixture with high sensitivity and selectivity, and quantitative analysis of lipids with the addition of relevant internal standards[135, 137].

A variety of hybrid mass spectrometers can be employed to perform MS/MS experiments in lipidomic analysis. The triple quadrupole (QqQ) mass spectrometer enables lipid identification and quantitation through different MS/MS modes with high selectivity and broad linear dynamic range. However, this is a targeted approach requiring knowledge of the lipid to be measured. The hybrid quadrupole time-of-flight mass spectrometer (QqTOF) or qualdrupole-Orbitrap allows for product ion analysis of complex samples with high sensitivity, mass accuracy and mass resolution. The tandem quadrupole-linear ion trap (QqLIT) allows for NLS, PIS, and MS^n analysis. Linear quadrupole ion trap and three-dimensional (3D) ion trap are also commonly used in lipidomics. Their merits include good sensitivity, high-throughput ability, and capability of multi-dimensional MS analysis. In addition, the Orbitrap analyzers have improved in ability to where they rival Fourier transform ion cyclotron resonance (FTICR) analyzers for high resolving power, ability to distinguish most isobaric lipid species and reduced chemical noise in shotgun lipidomics studies[133, 137, 139].

1.3.6 MS-based lipidomics

1.3.6.1 Shotgun lipidomics

Shotgun lipidomics is a direct infusion MS-based lipidomics approach. The constant concentration of the lipid sample has many advantages. It allows a number of instrumental variables to be ramped as part of the same infusion. It also allows for almost unlimited time to carry out different MS/MS scanning modes and MS^n analysis. In addition, lipid species of one

class can often be easily visualized and quantified in shotgun lipidomics. Devices commonly utilized in this lipidomic approach include a syringe pump or a tightly-sealed high quality glass syringe to deliver the specimen. Chip-based devices have been recommended for their superior capabilities.

There are three major methods in shotgun lipidomics. In tandem MS-based shotgun lipidomics, NLS or PIS has been used to profile lipid species of a specific class of interest with high mass spectral S/N ratio. However, non-specificity may be present in MS/MS scanning; the fatty acyl substituents of lipids cannot be identified using this approach. In high mass accuracy-based shotgun lipidomics, product ion scanning can be performed using QqTOF or qualdrupole-Orbitrap mass spectrometers to identify and quantify individual lipid species in a non-targeted manner. PIS and/or NLS can also be extracted from product-ion mass spectra, an approach termed multi-PIS high mass accuracy shotgun lipidomics. In addition, high-resolution MS has the advantage of identifying novel lipid species, which cannot be covered by using a QqQ mass spectrometer[133]. The third approach is MDMS-based shotgun lipidomics. The interrelationships among the four different MS/MS modes have laid the foundation for MDMS-SL. With this approach, differential hydrophobicity, stability, electrical properties of different lipid classes or subclasses can be exploited as part of lipid analysis through sample preparation (multiplexed extraction) and intrasource fragmentation. Moreover, NLS or PIS of the head group can be used to identify a particular lipid class, and fatty acyl side chains can be utilized to identify individual lipid species of one particular class[137, 139].

1.3.6.2 LC-MS-based lipidomics

Another category of MS-based lipidomics is LC-MS-based lipidomics, in which the

concentration of the lipid sample is always changing. LC-MS-based lipidomic approaches aim to combine the excellent separation capabilities of LC[138] with the highly sensitive detection power of MS. In NPLC-MS-based lipidomics, lipid extracts are separated into different lipid classes based on the polarity of head groups. MS detection is achieved through selected ion monitoring (SIM), in which the ion(s) of interest can be extracted from total ion chromatogram after the chromatographic separation. The short elution time of individual lipid species renders MRM and data-dependent analysis unsuitable. In RPLC-MS-based lipidomics, in most cases lipids of one particular class can be separated based on the hydrophobicity of fatty acyl chains through MRM, which is preferred for detection of one specific ion since it can be done in the short elution time of the lipid species. It has also been reported that certain classes of lipids could be analyzed by performing both PIS and product ion scanning on the hybrid QqQ mass spectrometer coupled with RP-HPLC[143]. In addition, UHPLC has shown its potential to replace 2D-HPLC (NP-HPLC followed by RP-HPLC) because of its improved chromatographic resolution in several global lipid studies, in which SIM is the current preferred method. Other possible LC-MS-based lipidomic approaches include HILIC-MS-based approach, silver-ion LC-MS-based approach, and chiral chromatography-MS-based approach[137], etc.

Mass spectrometry is often applied in qualitative and quantitative lipidomic analysis owing to its high sensitivity and specificity[253, 255]. A multitude of MS/MS (tandem mass spectrometry) strategies, in particular, are often combined with various bioinformatic analysis tools and databases to aid data processing, i.e. lipid identification and quantitation[253, 255]. However, unlike proteomics, in which the analysis of the fragmentation spectra of proteins and peptides is straightforward due to the fact that they present highly conserved fragmentation patterns when fragmented by MS/MS, the analysis of lipidomics MS/MS spectra is challenging

because of the high degree of molecular heterogeneity of lipids and their more diverse and complicated fragment mass spectra[253-255]. Lipids do not have single-favored ionization polarity as proteins and peptides do, and frequently structural information of lipids cannot be deduced without specific experimental setups, additionally the MS/MS spectra of lipids can be tremendously diverse depending on various factors, for instance, the choice of mass spectrometer, the collision energy used, ion mode and the choice of adduct ions, etc[255]. Therefore, it is often impossible to deduce structural information of lipids by simply submitting actual MS/MS data to lipid databases like we do in the identification of proteins and peptides. Still, various lipidomic data-processing software and search tools have been developed despite the lack of standardization of data processing[253], improvement of data interpretation[252] and the fact that databases for lipids are far from complete[254]. Examples include the lipid metabolite and pathways strategy (LIPID MAPS), the human metabolome database (HMDB), XCMS2 (METLIN database), MassBank, Lipid View, LipidXplorer, m/zMine2, to name a few[252, 253, 255]. The spectral databases of lipids have limited use due to the presence of isobaric precursors and the fact that only lipids that have been included in the databases can possibly be identified, if the databases are not extended by end-users[255]. Lipidomics is still at its early stage before these issues can be solved[252].

1.4 Proteomics and Peptidomics

1.4.1 Proteomics

The proteome is defined as all the proteins expressed in a specific biological system at a certain time[144]. Therefore, the large-scale analysis of the 'entire' protein complement of a cell,

tissue, or organism at a given time under defined conditions is termed proteomics[145, 146]. Mass spectrometry (MS) is the most comprehensive and popular tool in the analysis of complex protein samples[147, 148]. There are two broad proteomic approaches: the bottom-up approach and the top-down approach. Top-down proteomics uses the masses and fragments of intact proteins for characterization whereas the bottom-up approach, the most popular method, uses the masses and fragments of peptides for protein identification after proteins are enzymatically or chemically digested into peptides prior to MS analysis[147, 151, 152]. The applications of MS-based proteomics[148, 149, 151] include protein identification in biochemical or large-scale proteomic studies, the detection and identification of posttranslational modifications (PTMs), the study of protein-protein interactions, the characterization of macromolecules such as recombinant proteins in the biotechnology field, and the identification of biological markers or novel therapeutic targets, etc. The strategies for MS-based proteomics involve protein purification/sample preparation, sample separation, ionization, instrumentation, data acquisition, protein identification, the analysis of PTMs, and quantitative MS[147, 149], each of which will be briefly summarized below.

A variety of separation techniques or combination of separation techniques can be used for sample preparation and separation, depending on the sample complexity, the specific proteomic approach to be used, and different research goals. Electrophoretic gel-based proteomics strategies include one-dimensional polyacrylamide gel electrophoresis (1D PAGE), which is a low-resolution method for protein separation, and two-dimensional (2D) PAGE, that can be utilized in the separation of complex protein mixtures in the 10-200 kDa range[162] based on iso-electric point (pI) followed by molecular mass[149]. However, gel-based approaches are labor intensive. Other drawbacks of these techniques include lack of reproducibility, insensitivity

with limited dynamic range, and the inability to detect highly acidic or basic proteins[149, 150]. Gel free proteomics strategies encompass RP-HPLC, affinity chromatography, ion-exchange chromatography, and multi-dimensional protein identification technology (MudPIT) coupled to MS. In these approaches, complex protein mixtures are usually digested into peptides prior to sample preparation. RPLC separates peptide mixtures based on their hydrophobicity. With high resolution, reproducibility, efficiency and compatibility with ESI, RP-HPLC has been suggested to be used as a single phase or the last dimension of MudPIT[147]. Affinity chromatography is more frequently used to enrich proteins and peptides with PTMs, examples of such studies include phosphoproteomics and glycoproteomics[147]. Multi-dimensional separation is applicable to samples with high complexity. In MudPIT, the coupling of strong cation exchange (SCX) chromatography to RP-HPLC leads to sample separation with unbiased high sensitivity, high resolution and high capacity[147, 150]. An alternative to MudPIT is the RP-RP approach, in which the first dimension is high pH HPLC, and the second dimension is low pH HPLC[161]. Other separation strategies include HILIC, electrostatic repulsion hydrophilic interaction chromatography (ERLIC), and capillary electrophoresis (CE)[161].

Both proteins and peptides are polar, nonvolatile, and thermally unstable biomolecules that are necessarily ionized by soft ionization techniques like ESI and MALDI. Most of the ions generated by MALDI are singly charged species, which makes MALDI better suited for the analysis of high-molecular-weight proteins with pulsed MS instruments in top-down proteomics[147]. However, MALDI suffers from low reproducibility and strong dependence on prior sample preparation and purification. MALDI is more frequently utilized in the analysis of relatively simple peptide mixtures[148] since proteins can be fragmented to some extent during MALDI, resulting in low sensitivity[149]. ESI is routinely used in either direct infusion mode or in

combination with HPLC. It has been suggested that ESI-LC-MS is the preferred method for the analysis of complex peptide mixtures[148]. Large ions generated by ESI from proteins and peptides are predominantly multiply charged, therefore, they are within the m/z range of typical quadrupole sector containing mass spectrometers[149]. The lowered flow rates of micro-ESI and nano-ESI can lead to sample separation with improved sensitivity[147].

The mass analyzers most frequently used in proteomics include 3D ion trap (QIT-MS), linear ion trap (LIT-MS), QqQ-MS, LTQ-Orbitrap MS, LTQ-FTICR MS, QqTOF-MS, and IT-TOF-MS. The applications and features of the mass analyzers mentioned above are elegantly summarized in Table 5. Among the various mass spectrometers, QqTOF-MS, with the merits of high sensitivity and high mass resolution, most often can unequivocally determine the charge state and provide very high specificity in database searching[149]. Despite its higher sensitivity and mass accuracy, FTICR is not widely used in biological mass spectrometry, due to cost and the very high Tesla permanent magnet. TOF, Q, and trapping mass spectrometers such as ion trap, Orbitrap, and FTICR can all be coupled to both ESI and MALDI. However, MALDI is routinely coupled with TOF-MS whereas ESI is typically coupled with Q and trapping MS[148, 149].

Protein identification can in many cases be achieved through either database searching or de novo sequencing. The sequence databases that can be searched by MS data include non-redundant protein databases, expressed sequence tag (EST) databases, and genomic databases[149]. Database searching can be further divided into two different approaches. One approach is peptide mass fingerprinting, in which a protein is enzymatically cut into peptides first, then the set of experimentally observed peptide masses are compared with in silico generated peptide masses in the database. Peptide mass fingerprinting is usually utilized for the identification of a single protein, or sometimes very simple protein mixtures[149]. The success rate for identification of

Table 1.5 Comparison of characteristics and applications of different mass spectrometers. (Reprinted with permission from Yates, J. R.; Ruse, C. I.; Nakorchevsky, A., Proteomics by mass spectrometry: approaches, advances, and applications. *Annual review of biomedical engineering* 2009, *11*, 49-79.)

Instrument	Applications	Resolution	Mass accuracy	Sensitivity	Dynamic range	Scan rate
LIT (LTQ)	Bottom-up protein identification in high-complexity, high-throughput analysis, LC-MS^n capabilities	2000	100 ppm	Femtomole	1e4	Fast
TQ (TSQ)	Bottom-up peptide and protein quantification; medium complexity samples, peptide and protein quantification (SRM, MRM, precursor, product, neutral fragment monitoring)	2000	100 ppm	Attomole	1e6	Moderate
LTQ-Orbitrap	Protein identification, quantification, PTM identification	100,000	2 ppm	Femtomole	1e4	Moderate
LTQ-FTICR, Q-FTICR	Protein identification, quantification, PTM identification, top-down protein identification	500,000	<2 ppm	Femtomole	1e4	Slow, slow
Q-TOF, IT-TOF	Bottom-up, top-down protein identification, PTM identification	10,000	2–5 ppm	Attomole	1e6	Moderate, fast
Q-LIT	Bottom-up peptide and protein quantification; medium complexity samples, peptide and protein quantification (SRM, MRM, precursor, product, neutral fragment monitoring)	2,000	100 ppm	Attomole	1e6	Moderate, fast

proteins from organisms with completely sequenced genome has been estimated[149] to be 50-90%. A more widely used approach for protein identification involves peptide sequencing by tandem MS. In the peptide sequence tag method, an accurate tag is first picked out, then this short sequence ladder, together with enzyme specificity, peptide mass, and fragment ion masses can all be used in a sequence homology search for protein identification[149, 152]. However, nowadays sequence tag searches are more frequently used in the 'error tolerant' mode when a peptide has an unknown modification or amino acid sequence variation[152]. The other method involves database searching with uninterpreted MS/MS data directly. The experimental MS/MS spectra of individual peptides from proteins of interest are matched against theoretical spectra generated in the database in a continuous, real time way. This approach can be highly automated and potentially can use MS/MS spectra of marginal quality for protein identification[152], especially in small data sets. However, this method does require manual inspection of matched sequences in most cases[149]. The search parameters for this method have been discussed in greater detail in the tutorial written by John S. Cottrell[152]. The database searching algorithms for sequence identification using MS/MS spectra include Mascot, Sequest, X!Tandem, and PEAKS. If the appropriate database entries for the protein of interest do not exist or if the abundance of protein is low or if the number of matching fragments is too few, then de novo sequencing has to be used. The software for de novo sequencing of MS/MS data includes PEAKS, DeNovoX, and PepSeq. In addition, SPIDER can be used for homology search, and either SIMS or Ascore's algorithm can be utilized in PTM analysis[161].

The fields of quantitative proteomics include relative quantitative proteomics and absolute quantitative proteomics. Relative quantification in MS-based proteomics can be achieved through either stable-isotope labeling methods or label-free methods[147]. Isotope labels

can be attached to proteins enzymatically (during or after digestion), metabolically[153, 154] (through in vivo isotope labeling of organisms or SILAC, which stands for stable isotope labeling by amino acids in cell culture), or chemically[155, 156] (using ICAT, which stands for isotope-coded affinity tags, or iTRAQ, which is the abbreviation for isobaric tags for relative and absolute quantification, or TMTs, which stands for tandem mass tags)[147, 161]. The label-free method utilizes either peptide peak intensity/area or spectral counting for relative quantification of proteins in the sample[147, 161]. In addition, evidence has shown that spectral counting is strongly correlated to isotope labeling methods for quantitative proteomic analysis via MudPIT[147, 157]. Absolute abundance of proteins, on the other hand, can be obtained with SILAC, artificial QCAT proteins, or synthetic sequence identify, isotope labeled internal standard peptides[147, 158-160]. All of the above quantitative methods are non-gel based. However, protein quantification can also be achieved possibly though gel-based methods, for example, by comparing the intensities of protein stains on the gel in 2D PAGE or 2D-DIGE (two-dimensional differential gel electrophoresis) with much better quantitative capability and reliability. But due to the inherent limitations of 2D gel techniques, including sensitivity, reproducibility and poor separation resolution, gel-based protein quantification is less preferred than non-gel based protein quantification[161].

1.4.2 Peptidomics

The definition of the peptidome is all the endogenous peptides that are present in a biological system at a certain time. It forms the low molecular weight proteome and its distribution within an organism is usually dynamic[145, 170, 195]. Peptidomics is defined as the comprehensive qualitative and/or quantitative analysis of all peptides in cells, tissues, organs,

fluids, or an organism[162-164]. Peptidomics is considered a subset of proteomics[165] and many of the MS approaches are similar. The applications of peptidomics range from the study of dietary protein digestion in food science to the discovery and identification of disease biomarkers (DBM) or novel signaling molecules like neuropeptides and peptide hormones using chemical modification and LC-MS/MS[163, 164, 177]. It has been shown that endogenous peptides are crucial to almost all physiological processes. They can also be seen as a measure of health status[163, 196]. In addition, peptides are thought to play roles in pathologic processes resulting from abnormal regulation of protease activity[195]. Despite the similarities between peptidomics and proteomics, intrinsic differences in conceptual and analytical techniques exist (Figure 1.7 and Table 1.6).

Enzymatic digestion of peptide mixtures is not necessary in peptidomics, although skipping this step does not make peptidomic analysis any easier, especially for larger peptides[164, 166]. Peptidomic analysis usually starts with peptide enrichment to overcome the presence of proteins, lipids, salts and carbohydrates[163] that interfere with downstream analysis of peptides. Peptides can be separated from proteins using size exclusion chromatography or ultrafiltration[167, 168]. Proteins can also be precipitated with the addition of organic solvents or acid[163, 169] to a biological sample. It has been suggested that hot acid should be avoided because it can lead to peptide degradation[166]. The removal of proteins can improve the resolution and loading capacity of chromatographic separations of peptides afterwards[165]. In addition, protease activity can be controlled by protein denaturation or with the addition of protease inhibitors[163], thus post-sample collection proteolysis can be avoided and subsequently peptides of interest can be detected by LCMS with higher signals. However, for peptide samples like urine that are relatively stable, protease inhibition is not required[178]. It has been suggested that multiple peptide fractionation strategies can be employed due to the diversity of peptides chemically or physically, for instance,

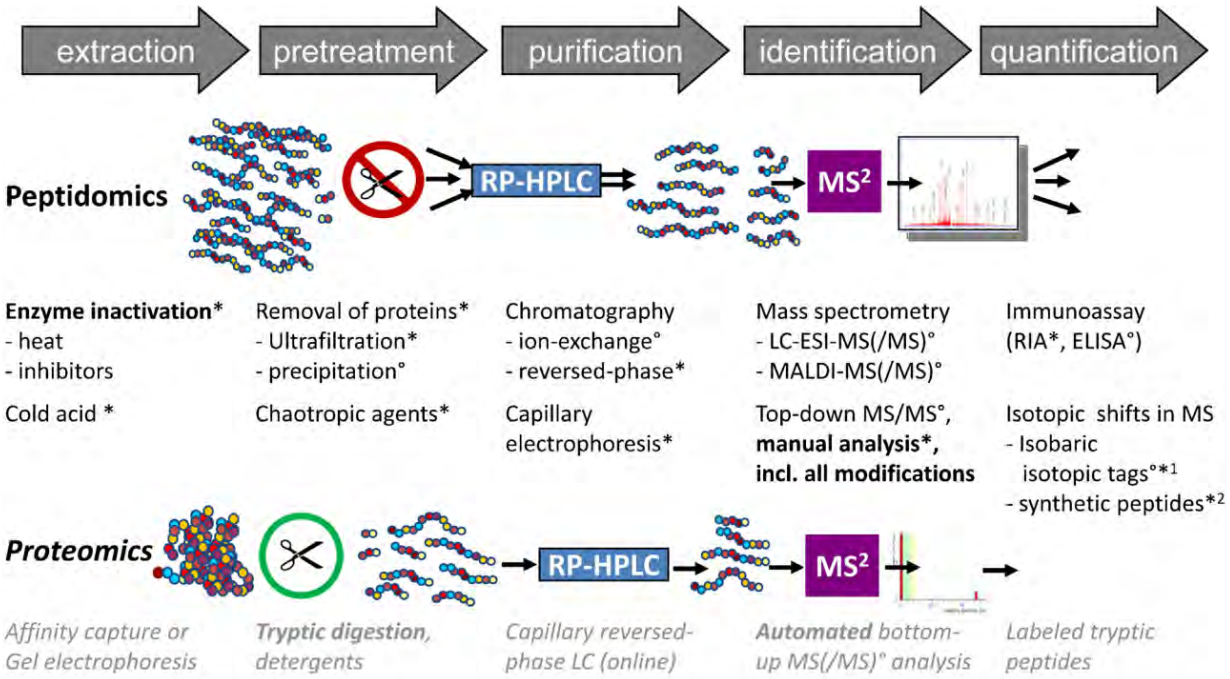

Figure 1.7 Comparison of analytical methods between proteomics and peptidomics. (This image is used under a Creative Commons Attribution 3.0 Unported license. Reprinted with permission from Schrader, M.; Knappe, P. S.; Fricker, L. D., Historical perspective of peptidomics. *EuPA Open Proteomics* 2014, *3*, 171-182.)

Table 1.6 Advantages and disadvantages of proteomics and peptidomics. (Reprinted with permission from Di Meo, A.; Pasic, M. D.; Yousef, G. M., Proteomics and peptidomics: moving toward precision medicine in urological malignancies. *Oncotarget* 2016, *7* (32), 52460-52474.)

Proteomics	**Advantages** • Allows an in-depth analysis of dynamic protein expression, PTMs, cellular and sub-cellular protein distribution, and protein-protein interaction • Allows the detection of protein isoforms • Accurately reflects actual cellular processes • Can be utilized for functional analysis • Allows the discovery of protein sequence similarity
	Limitations • Lack of validation studies • Technologies (mass spectrometry) require specialized staff • Susceptible to biological variability • Reduced detection of low abundant proteins due to the presence of high abundant proteins (masking effect) • Digested protein fragments may match to several proteins • Digestion-induced modifications may result in a failure to identify specific interactions • Requires sophisticated bioinformatic algorithms for accurate analysis
Peptidomics	**Advantages** • Provides insight regarding proteolytic activity • Allows for the study of the disease microenvironment • Requires no chemical or enzymatic digestion for sample processing
	Limitations • Lack of validation studies • Technologies (mass spectrometry) require specialized staff • Susceptible to biological variability • Low molecular weight (LMW) peptides are low abundant • LMW peptides may associate with highly abundant proteins, reducing their detection • Data analysis is challenging due to the absence of chemical or enzymatic digestion • Endogenous peptides may match to several proteins • Requires sophisticated bioinformatic algorithms for accurate analysis

size, charge state, and PTMs[163].

Bioactive peptides usually exist in very low concentrations in biological samples, therefore it is important that peptides are purified from complex biological matrices, which can be achieved by RP-HPLC and/or ion exchange HPLC[166] with high resolution. The advantages of the popular column chromatography in peptidomic analysis include the absence of peptide loss, increased number of peptide identifications[194], the high peptide recovery rate due to the fact that the folding behavior of peptides are reversible in most cases, and its applicability to peptide mixtures of various dynamic range[165]. Affinity chromatography has been reported useful for peptide purification and selection[194]. In addition, capillary electrophoresis has also been successfully applied to peptidomic analysis[176]. However, multi-dimensional chromatography in peptidomics is not recommended unless more than 1,000 peptides need to be analyzed from a single source. It has also been reported that 2D PAGE cannot provide sufficient separation and detection of peptides from complex samples[165].

The combination of HPLC and MS has been considered a standard in peptide research since the total peptide content can be sensitively detected in complex samples by MS[165, 166]. It has been suggested that ESI is the preferred ionization method for tryptic or natural peptides, although overlapping signals may result from ESI of larger peptides because of multiple charge states[165]. Another advantage of ESI is that increased number of product ions resulting from fragmentation of multiply charged peptides can be detected[194]. Alternatively, MALDI is considered the ionization method of choice for larger peptides if the peptide is known and can be targeted. In this case, LC needs to be connected offline to MS, but the generation of singly charged species leads to MS analysis with higher resolving power and higher sensitivity[165, 173, 174]. MALDI-MS also has the advantage of tolerance against salts and other interfering

compounds when detecting intact peptides from complex mixtures[166]. In addition, surface-enhanced laser desorption/ionization (SELDI) has also been utilized in peptidomic analysis[175].

The fragmentation methods for peptide identification include low energy CID, electron transfer dissociation (ETD), high-energy CID[183, 194]. QTOF, Orbitrap, QIT, and micro-TOF are some of the most frequently used mass analyzers[163, 194]. Once MS/MS spectra are obtained, either database searching (if the peptide target is known) or de novo sequencing can be applied to identify peptides of interest. One aspect of note regarding peptide identification is that manual verification of peptide assignment is indispensible[193]. Examples of de novo sequencing programs are UStags, PepNovo, Sherenga, etc[184-186]. Database searching, however, can be challenging for peptidomics because of unspecified cleavage enzymes, incomplete protein databases, and deficient scoring scheme, incomplete b- and y-series fragments, etc[197]. It has been suggested that a "no enzyme" setting should be used for the comparison between experimental MS/MS spectra with in silico generated spectra from peptide spectral databases or protein libraries[163], and that the search programs used should take into account possible posttranslational modifications[194]. Examples of database searching programs include Mascot, MS-Fit, and OMSSA, etc[187-189]. In addition, programs that make use of both database searching and de novo sequencing have been reported for successful peptide identification[163, 190]. It has also been suggested that using multiple approaches for peptide identification can lead to higher rates of true positives and improved coverage[194].

Quantitative peptidomics consists of labeled quantification and label-free quantification. The isobaric tags that can be used to label peptides include iTRAQ and TMT[166], both of which allow more accurate quantification of peptides with improved throughput[179, 180]. Quantitative peptidomic analysis can also be achieved by isotopic labeling of endogenous peptides through

SILAC, ICAT, and 4-trimethylammoniumbutyryl (TMAB) tags[163, 166], among which TMAB tags have been considered ideal because of its many advantages including capability for multivariate analysis[181]. However, due to the fact that free Cys residues are absent in most endogenous peptides, the application of ICAT in quantitative peptidomics is limited[166, 181]. For isotopic labeling like TMAB, MS/MS is not required for quantification[181], however, an increased number of ion signals can lead to more complex spectra[163]. Label-free quantification methods include the spectral counting approach and the peptide signal intensity approach. Though label-free methods are thought to be less quantitative, neither complex sample preparation nor a chemical reaction step is required, and they are applicable to most, if not all, analytical platforms[163]. In addition, MRM has been coupled with stable isotope dilution for absolute peptide quantification with high sensitivity and specificity, although identification of the peptide of interest is required prior to quantitative analysis[182].

Following identification and quantification of peptides of interest, additional programs, for example, Pepstr[191] and SwePep[192] can be utilized to predict the 3D structure and biological functions of peptides, respectively. It may also be interesting to identify the PTMs within peptides of interest, to find out the parent proteins and predict relevant proteolytic enzymes based on available peptidomic data if the peptides of interest are not produced in situ[163, 195]. If peptidomic analysis of tissues with spatial resolution is desired, MALDI-imaging mass spectrometry (IMS) may be used for such purpose[194].

1.5 Gender-specific biomarkers for Alzheimer's disease (AD)

1.5.1 Alzheimer's Disease

Alzheimer's disease was first defined by Alois Alzheimer, a Bavarian psychiatrist, in Munich in 1906. He reported a woman named Auguste D., who had shown progressive memory impairment, loss of cognitive functions, delusions, and psychosocial incompetence, all of which still characterize this disease today[202]. Alzheimer's disease (AD) is the most prevalent form of late-life mental affliction in humans. It is "a disorder of the most complex of physiological systems, the human cerebral cortex"[202], and it attacks "the structures that harbor the very essence of who we are"[200]. The number of AD cases worldwide is currently estimated to be 36 million, and this number will approximately triple by 2050[200]. Currently, the exact pathogenesis of AD remains elusive, the definitive diagnosis of AD is only made post-mortem, and available therapeutic strategies can only delay AD progression temporarily[221]. Alzheimer's disease brings enormous psychological and financial burdens on patients, caregivers, communities and society. As life expectancy increases with improved medical care, Alzheimer's disease has been predicted to be the greatest public health crisis in the 21st century[201].

Alzheimer's disease is the most common form of dementia[222]. There are generally three stages in dementia: early stage (first year or two), middle stage (2nd to 4th or 5th year), and late stage (5th year and later)[222]. Correspondingly, the clinical dementia rating (CDR) scale can be used to classify dementia based on disease severity into mild (CDR 1), moderate (CDR 2), and severe (CDR 3). In addition, mild cognitive impairment (MCI) or very mild AD denotes the transitional state between normal aging and mild AD, and it has a CDR of 0.5[229]. The symptoms of AD vary with regard to different stages of the disease, yet they typically include: progressive episodic memory impairment, language disturbance, deficits of visuospatial perception and associative visual agnosia, executive dysfunction and attention shifting problems, ideomotor apraxia, ideational apraxia, and constructional apraxia, apathy, disinhibition, agitation, social

isolation and withdrawal, delusions, hallucinations, sleep disturbances, cognitive anosognosia and many other behavioral symptoms[201].

The clinical diagnosis of AD is complicated. It involves initial clinical interview, physical examination and routine laboratory tests, often to rule out other causes of symptoms[222]. Neuropsychological assessment can be done through a multitude of standard tests. For example, the Mini-Mental State Examination (MMSE) can provide information on the severity of cognitive dysfunction, the Activities of Daily Living (ADL) scale can be used to assess functional status, AD-associated depression may be detected by utilizing the specifically designed Cornell Scale for Depression in Dementia (CSDD) with high reliability and sensitivity, The Hopkins Verbal Learning Test (HVLT) can be used for detection and measurement of memory impairment. Executive functioning can be assessed through the Rivermead Behavioral Memory Test (RBMT), the list goes on and on[201]. In addition, a variety of neuroimaging, potential biochemical and possibly genomic biomarkers of AD have been proposed and evaluated in greater detail for their potential diagnostic value[223-242]. Currently, none are considered diagnostic and none are used in routine clinical practice. Again, the definitive diagnosis of AD can only be achieved by examining the brain tissue for senile plaques and NFT on autopsy or rarely, brain biopsy during life[222, 227].

There are two forms of Alzheimer's disease: the familial form and the sporadic form (non-familial). Conceivably, as many as 5% of all AD cases are familial or genetic (although several reports suggest that the actual prevalence to be closer to 1%) which are caused by mutations in one of three genes: the gene encoding for the amyloid precursor protein (APP), and the genes encoding for presenilin-1 and presenilin-2 (PSEN1 and PSEN2)[201]. Presenilin is important for proper cleavage of APP by γ-secretase. The missense mutations in PSEN1 have

been suggested to be causative of the most aggressive and earliest from of AD[202]. The mutations in the three genes above can result in altered production of different amyloid beta (Aβ) isoforms and therefore increased amounts of the more neurotoxic Aβ42 are produced leading to the formation of senile plaques eventually. Early onset (<65 years) familial Alzheimer's disease (eFAD) is inherited in an autosomal-dominant fashion. Currently, 18 mutations in APP and 152 mutations in PSEN1 and PSEN2 genes associated with Alzheimer's disease have been identified[203]. Sporadic forms of the Alzheimer's disease are much more common, multifactorial and have complex pathophysiology[201]. It has been reported that the phenotype and clinical manifestations of familial cases are highly similar or often indistinguishable from those of the sporadic AD, except that familial AD cases often have an early onset (before age 65), and that several distinctive features have been reported in multiple family members having sporadic forms of AD[202].

A number of risk factors have been identified for AD. Examples of genes that predispose individuals to sporadic AD are the apolipoprotein E (APOE) gene ε4 allele (which has been linked to late-onset familial AD with semi-dominant inheritance), several variants of the insulin-degrading enzyme (IDE) gene, ubiquilin-1 (UBQLN1), SORL1 genetic variants to name a few[200, 201]. Genetic variations in the APP promoter sequences that lead to increased APP gene expression have also been reported as a potential risk factor for late-onset AD[217]. In addition, evidence has also suggested the association between AD risk and dysregulated epigenetic mechanisms representing abnormal DNA methylation and histone modifications[218]. Aging has been reported as the greatest known non-genetic risk factor for late onset Alzheimer's disease (age>65 years)[200]. Individuals beyond age 85 have an ~50% chance of developing AD[222]. Other possible environmental risk factors associated with late-onset AD include small white matter

infarcts, traumatic brain injury, female gender, estrogen replacement therapy[221], sleep apnea, a history of depression, hyperlipidemia, hypertension, homocysteinemia, diabetes mellitus, obesity, smoking, low education levels and low mental ability[204-207], although some of these risk factors are disputed[219].

Several hypotheses have been proposed in the attempt to explain the pathogenesis of AD, including the beta-amyloid (Aβ) hypothesis, cholinergic hypothesis, tau hypothesis, and inflammation hypothesis[208, 210]. The amyloid cascade hypothesis is considered by many as the core mechanism in the pathophysiology of AD, although it alone cannot adequately account for this complex multifactorial disease. Based on the amyloid cascade model, the aberrant accumulation of Aβ in the brain is thought to be a very early event in the development of AD that ultimately leads to neurodegeneration[211]. APP is a transmembrane protein widely expressed in tissues, particularly in synapses of neurons. Its normal biological function involves modulating synapse structure and function[212]. When cleaved by α-secretase, the APP releases α-sAPP and α-CTF (Figure 1.8). Because α-secretase cleaves within the Aβ domain of the APP, this prevents the release of Aβ polypeptide. Following cleavage of α-CTF by γ-secretase, a harmless fragment p3 is produced. However, cleavage of the APP by β-secretase results in the release of β-sAPP and β-CTF. As shown in Figure 1.8, the Aβ domain is still attached to β-CTF and remains intact. The subsequent cleavage of β-CTF by γ-secretase leads to the release of Aβ polypeptide [208]. Both pathways take place in normal brain[201]. In Alzheimer's disease, however, increased β-secretase activity and decreased α-secretase have been reported[213]. The exact mechanism of Aβ accumulation remains elusive, though multiple hypothesized mechanisms have been proposed[201, 211]. Accumulating Aβ peptides can aggregate into oligomers, which can transition into large fibrils and eventually form senile plaques[208]. Aβ oligomers have been suggested to induce

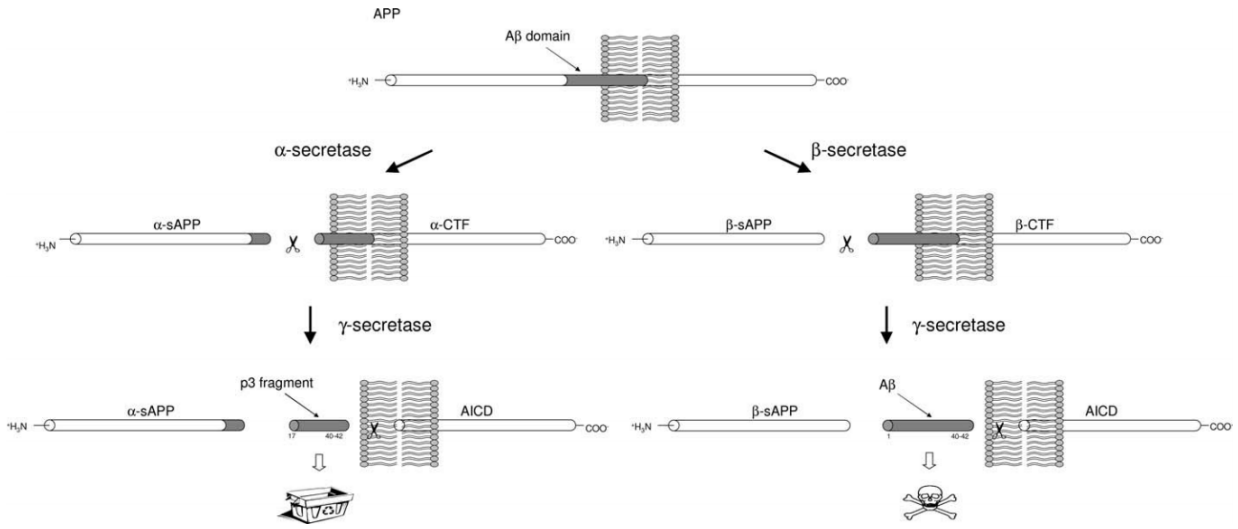

Figure 1.8 Secretase cleavage of amyloid precursor protein (APP) in normal and disease states. (Reprinted with permission from Minati, L.; Edginton, T.; Bruzzone, M. G.; Giaccone, G., Current concepts in Alzheimer's disease: a multidisciplinary review. *American journal of Alzheimer's disease and other dementias* 2009, *24* (2), 95-121.)

oxidative stress and tau hyperphosphorylation, which can lead to synapse and mitochondria damage. They may also contribute to vascular and neuronal degeneration[210, 214]. Aβ plaques have been reported to cause microglial activation, which can lead to release of pro-inflammatory cytokines and neuritic degeneration[208, 215]. However, the exact mechanism of Aβ neurotoxicity remains unknown. Senile plaques and neurofibrillary tangles (NFT) have long been considered the two main pathological hallmarks of AD. NFT are aggregates of abnormally phosphorylated protein tau, which is a highly soluble microtubule-associated protein (MAP)[220]. Evidence suggests that Aβ accumulation may lead to oxidative stress and impaired neuronal metabolic and ionic homeostasis, which can result in hyperphosphorylation of tau protein through an imbalance between protein kinases and phosphatases, although NFT have also been reported to occur independently of senile plaques[202]. The formation of NFT then lead to widespread axonal loss, synaptic dysfunction, neuronal death in the cerebral cortex and hippocampus, and progressive neurotransmitter deficits, manifesting eventually as Alzheimer's disease[201, 202].

Currently only five drugs are approved by the US Food and Drug Administration (FDA) to treat the clinical symptoms of AD. The drug mechanism of action (MOA) involves preventing the breakdown of the neurotransmitter acetylcholine, the inadequate amount of which has been associated with AD[208]. Among the five drugs, memantine (Namenda), a N-methyl D-aspartate (NMDA) receptor antagonist, may be the only drug that can be used to treat patients in the later stages of the disease, namely moderate to severe AD; rivastigmine (Exelon), galantamine (Reminyl) and donepezil (Aricept), all of which are inhibitors of cholinesterase[201], have been reported to be most effective for early stages of AD[208, 209]. However, it has also been reported that only half of the patients who undergo treatment respond to the drugs, and among these patients the progression of symptoms is only modestly and temporarily delayed[228]. Once the

treatment is terminated, rapid cognitive decline to placebo levels has been observed[201]. Recently, a drug shown to bind and remove Aβ was tested in early AD. Despite the drug being effective against Aβ, it failed to slow disease progression or limit severity. This draws into question whether Aβ is the cause of AD or simply a later contributing factor. Clearly, measuring Aβ may be the wrong target for an AD biomarker[264].

1.5.2 Biomarkers for AD

Several neuroimaging techniques have been employed to facilitate clinical diagnosis of AD. For instance, both computed tomography (CT) and magnetic resonance imaging (MRI) can be used to exclude secondary causes of dementia, the combination of magnetic resonance (MR) volumetry and ^{1}H-MR spectroscopy (^{1}H-MRS) has been reported to be able to diagnose AD with 90% accuracy in the late stages of AD[223]. Diffusion-tensor imaging (DTI) may be used to predict the progression from MCI to AD, although the application of structural and functional MRI is limited due to complex data postprocessing and other reasons[224]. More expensive radiologic and radioimaging techniques have also been used in the diagnosis of AD. Both single photon emission computed tomography (SPECT) and positron emission tomography (PET) may be valuable for differential diagnosis and prediction of the progression from MCI to AD, though SPECT is much more frequently used in clinical practice[225, 226]. Electroencephalography (EEG) may also be used for the purpose of differential diagnosis, although its clinical application is somewhat limited due to the lack of advanced signal analysis methods[201]. Though it can increase diagnosis specificity by 25%, genomic biomarkers such as APOE ε4 genotyping also leads to decreased sensitivity, and therefore genotyping has very limited application in predicting AD in the clinical setting[201, 242].

A variety of biochemical biomarkers have been explored for their potential application in the diagnosis of Alzheimer's disease. Three cerebrospinal fluid (CSF) biomarkers, for example, Aβ42, total-tau (T-tau) and phosphorylated tau protein (P-tau) have been consistently implemented in the differential diagnosis of AD[233]. It was suggested that only the combination of these three biomarkers above could lead to over 95% sensitivity and over 85% specificity in the diagnosis of sporadic AD[237]. However, the application of either Aβ42 or total-tau in differential diagnosis of AD is limited since their changes in concentration are associated with a variety of dementias or disorders[201]. The elevated concentration of CSF P-tau seems to be linked to AD more specifically compared with the other two biomarkers[236]. The following ratios, for example, Aβ42:40, T-tau:Aβ42, Aβ42:P-tau have also been suggested to be used in the clinical diagnosis of preclinical AD or MCI[239]. In addition, YKL-40 protein, carnosinase I and chromogranin A were reported to lead to increased diagnostic accuracy in combination with Aβ42 and phosphorylated tau [231]. Aβ oligomers, β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) and secreted APP isoforms are among other candidate CSF biomarkers[238, 239]. Although CSF biomarkers appear to aid the clinical diagnosis of AD, their application is still limited due to the high cost, increased patient risk, and patient discomfort[201]. Better diagnostic accuracy of AD or predictive accuracy of MCI has been reported, however, when combining neuroimaging and CSF biomarkers[239].

Compared to CSF biomarkers, blood-based biomarkers seem more practical because it is less invasive to obtain specimens. It costs less to obtain blood and it can be repeatedly evaluated in a clinical setting[230, 242]. The potential application of plasma proteomic biomarkers in AD diagnosis had been investigated by several different research groups. For instance, a panel of 18 plasma protein biomarkers that had been externally validated was reported in one prospective

longitudinal study. These biomarkers were capable of differentiating subjects with AD from normal control subjects with high sensitivity and specificity in that study[231]. In another study, 18 signaling proteins in plasma were reported that could distinguish individuals with AD from healthy control subjects with almost 90% accuracy and identify subjects with MCI that developed to AD with 91% accuracy[232]. A panel of a mere three markers: cortisol, oxidized low-density lipoprotein and von Willebrand factor was reported to discriminate AD individuals from healthy controls with more than 80% accuracy[241]. It was shown previously that plasma Aβ has poor diagnostic power since studies showed that changes in Aβ concentrations in AD have been inconsistent. However, now it has been suggested that changes in the levels of plasma Aβ42 or Aβ42:Aβ40 ratio may be applicable to AD diagnosis[240]. Using a metabolomic and lipidomic approach, researchers of a five-year study reported a panel of ten plasma phospholipids that can detect preclinical AD with higher than 90% accuracy, although numbers were very small and external validation is still needed[230]. Additionally, circulating microRNAs (miRNAs) have been suggested as potential biomarkers for early AD diagnosis as well[235]. Despite many reports of potential markers for AD, blood-based biomarkers still have their drawbacks due to the fact that follow-up studies either have failed to find diagnostic usefulness or such studies have yet to be done[238]. Additionally, the application of blood biomarkers is somewhat challenging owing to the fact that the concentration of blood biomarkers for AD is usually low on account of the blood-brain barrier and their dilution in the plasma volume[239].

Since the concentration of CSF and blood biomarkers may be reduced by homeostatic mechanisms, urinary biomarkers of AD were investigated in a variety of animal models, such as Tg2576 transgenic AD mice[256] and TgCRND8 transgenic AD mice[257]. A number of biomarkers, for example, 3-hydroxykynurenine and 2-oxoglutarate have been reported to be effective

metabolic biomarkers for AD prior to clinical onset of dementia and late-stage AD, respectively[234].

Lastly, while Aβ40, Aβ42 and clusterin are considered candidate biomarkers for AD for their potential roles in the neurodegenerative pathology of AD, a variety of other biomarkers that may have potential association with vascular risk and inflammation states of AD have been listed and investigated in detail in one review article[242], examples of such markers include total cholesterol, sphingolipids, oxysterols, cobalamin, insulin, leptin, and thyroid stimulating hormone (TSH), etc[242].

The majority of studies on AD, and biomarkers for AD in particular, have ignored a potentially important variable: gender. It had been suggested that women are more likely to develop AD in their lifetime than men[13]. One study reported that the remaining lifetime risk of developing AD is 6.3% in a 65-year-old man, whereas 12% in a woman of the same age[5]. Various gender differences in the symptoms of the disease have also been found. For example, female AD patients showed substantially worse performance on a variety of neuropsychological tasks than their male counterparts[258]. In addition, men outperformed women in language, semantic abilities, visuospatial abilities and episodic memory among AD patients, even after adjusting for age, education or disease severity[259]. It has been reported that there are gender differences in certain CSF biomarkers. One study found that women have 1%-1.5% faster annual rates of brain atrophy than men, and the atrophy rates are associated with CSF changes of Aβ and tau[260, 261]. Furthermore, gender differences in APOE ε4 (best know genetic risk factor), brain physiology and various socialcultural aspects have been investigated and reported as well[261, 262]. Therefore, it is more than reasonable to take into consideration the effect of gender in studies of AD biomarker discovery.

In summary, the cause of AD is unknown. While many have focused on Aβ pathology, recent studies draw into question its central causative role. Currently, CSF ligand markers are sensitive to Aβ, but this approach is too expensive and slow to use for screening and more importantly it may be the wrong diagnostic target. There is therefore a critical need for clinically useful AD biomarkers, especially markers of early disease. Importantly, given the evidence for AD being different for women and men, markers that can diagnose early AD in a gender specific way would greatly facilitate other drug studies and may provide insight into the disease itself.

1.6 References

1. Chadwick, J.; Mann, W. N. The medical works of Hippocrates, 1st ed.; Blackwell Scientific Publications: England, 1950. (Original work published 5th century B.C.

2. Bell, M. J., A historical overview of preeclampsia-eclampsia. *Journal of obstetric, gynecologic, and neonatal nursing : JOGNN* **2010,** *39* (5), 510-8.

3. Kiple, K. *The Cambridge historical dictionary of disease*, 1st ed.; Cambridge University Press: New York, 2003.

4. Chesley, L.C. *Hypertensive disorders in pregnancy*. Appleton-Century-Crofts: New York, 1978

5. Rayer, P.F. *Traite des maladies des reins et des alterations de la secretion urinaire*. Bailliere: Paris, 1837-41.

6. Lever, J.W. Cases of puerperal convulsions with remarks. *Guys Hosp Rep* **1843**, *2*, 495-517.

7. Johns, R. Observations of puerperal convulsions. *Dublin Journal of Medical Science* **1843**, *24* (1), 101–115.

8. Villar, J.; Say, L.; Gulmezoglu, A.M.; Meraldi, M.; Lindheimer, M.D.; Betran, A.P. Piaggio G. Eclampsia and pre-eclampsia: a health problem for 2000 years. *Pre-eclampsia* **2003**. pp. 189–207.

9. Jeyabalan, A., Epidemiology of preeclampsia: impact of obesity. *Nutrition reviews* **2013,** *71 Suppl 1*, S18-25.

10. Uzan, J.; Carbonnel, M.; Piconne, O.; Asmar, R.; Ayoubi, J. M., Pre-eclampsia: pathophysiology, diagnosis, and management. Vascular health and risk management 2011, 7, 467-74.

11. Grill, S.; Rusterholz, C.; Zanetti-Dallenbach, R.; Tercanli, S.; Holzgreve, W.; Hahn, S.; Lapaire, O., Potential markers of preeclampsia--a review. *Reproductive biology and endocrinology : RB&E* **2009,** *7*, 70.

12. Poon, L. C.; Nicolaides, K. H., Early prediction of preeclampsia. *Obstetrics and gynecology international* **2014,** *2014*, 297397.

13. Noris, M.; Perico, N.; Remuzzi, G., Mechanisms of disease: Pre-eclampsia. *Nature clinical practice. Nephrology* **2005,** *1* (2), 98-114; quiz 120.

14. Turner, J. A., Diagnosis and management of pre-eclampsia: an update. *International journal of women's health* **2010,** *2*, 327-37.

15. Mol, B. W.; Roberts, C. T.; Thangaratinam, S.; Magee, L. A.; de Groot, C. J.; Hofmeyr, G. J., Pre-eclampsia. *Lancet* **2016,** *387* (10022), 999-1011.

16. Ahmed, R.; Dunford, J.; Mehran, R.; Robson, S.; Kunadian, V., Pre-eclampsia and future cardiovascular risk among women: a review. *Journal of the American College of Cardiology* **2014,** *63* (18), 1815-22.

17. American College of Obstetricians and Gynecologists. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet Gynecol* **2013**, *122*(5).

18. Cnossen, J. S.; de Ruyter-Hanhijarvi, H.; van der Post, J. A.; Mol, B. W.; Khan, K. S.; ter Riet, G., Accuracy of serum uric acid determination in predicting pre-eclampsia: a systematic review. *Acta obstetricia et gynecologica Scandinavica* **2006,** *85* (5), 519-25.

19. Thangaratinam, S.; Ismail, K.; Sharp, S.; Coomarasamy, A.; Khan, K. Accuracy of serum uric acid in predicting complications of pre-eclampsia: a systematic review. *BJOG* **2006**, *113*, 369-378.

20. Koopmans, C. M.; van Pampus, M. G.; Groen, H.; Aarnoudse, J. G.; van den Berg, P. P.; Mol, B. W., Accuracy of serum uric acid as a predictive test for maternal complications in pre-eclampsia: bivariate meta-analysis and decision analysis. *European journal of obstetrics, gynecology, and reproductive biology* **2009,** *146* (1), 8-14.

21. Mustafa, R.; Ahmed, S.; Gupta, A.; Venuto, R. C., A comprehensive review of hypertension in pregnancy. Journal of Pregnancy 2012, 2012, Article ID 105918, 19

pages.

22. Powe, C. E.; Levine, R. J.; Karumanchi, S. A., Preeclampsia, a disease of the maternal endothelium: the role of antiangiogenic factors and implications for later cardiovascular disease. Circulation 2011, 123 (24), 2856-69.

23. Hofmeyr, G. J.; Atallah, A. N.; Duley, L., Calcium supplementation during pregnancy for preventing hypertensive disorders and related problems. *The Cochrane database of systematic reviews* **2006,** (3), CD001059.

24. Pennington, K. A.; Schlitt, J. M.; Jackson, D. L.; Schulz, L. C.; Schust, D. J., Preeclampsia: multiple approaches for a multifactorial disease. *Disease models & mechanisms* **2012,** *5* (1), 9-18.

25. Bagrov, A. Y.; Shapiro, J. I.; Fedorova, O. V., Endogenous cardiotonic steroids: physiology, pharmacology, and novel therapeutic targets. *Pharmacological reviews* **2009,** *61* (1), 9-38.

26. Blanco, G.; Mercer, R. W., Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *The American journal of physiology* **1998,** *275* (5 Pt 2), F633-50.

27. Khundmiri, S. J., Advances in understanding the role of cardiac glycosides in control of sodium transport in renal tubules. *The Journal of endocrinology* **2014,** *222* (1), R11-24.

28. Skou, J. C., The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochimica et biophysica acta* **1957,** *23* (2), 394-401.

29. Skou, J. C.; Esmann, M., The Na,K-ATPase. *Journal of bioenergetics and biomembranes* **1992,** *24* (3), 249-61.

30. Lingrel, J. B., Na,K-ATPase: isoform structure, function, and expression. *Journal of bioenergetics and biomembranes* **1992,** *24* (3), 263-70.

31. Blaustein, M. P., Physiological effects of endogenous ouabain: control of intracellular Ca2+ stores and cell responsiveness. *The American journal of physiology* **1993,** *264* (6 Pt 1), C1367-87.

32. Jorgensen, P. L.; Skou, J. C., Preparation of highly active (Na+ + K+)-ATPase from the outer medulla of rabbit kidney. *Biochemical and biophysical research communications* **1969,** *37* (1), 39-46.

33. Adams, R. J.; Schwartz, A.; Grupp, G.; Grupp, I.; Lee, S. W.; Wallick, E. T.;

Powell, T.; Twist, V. W.; Gathiram, P., High-affinity ouabain binding site and low-dose positive inotropic effect in rat myocardium. *Nature* **1982,** *296* (5853), 167-9.

34. Mobasheri, A.; Avila, J.; Cozar-Castellano, I.; Brownleader, M. D.; Trevan, M.; Francis, M. J.; Lamb, J. F.; Martin-Vasallo, P., Na+, K+-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions. *Bioscience reports* **2000,** *20* (2), 51-91.

35. Blanco, G., Na,K-ATPase subunit heterogeneity as a mechanism for tissue-specific ion regulation. *Seminars in nephrology* **2005,** *25* (5), 292-303.

36. Bystriansky, J. S.; Kaplan, J. H., Sodium pump localization in epithelia. *Journal of bioenergetics and biomembranes* **2007,** *39* (5-6), 373-8.

37. Donnet, C.; Sweadner, K. J., The mechanism of Na-K interaction on Na,K-ATPase. *Annals of the New York Academy of Sciences* **2003,** *986*, 249-51.

38. Lingrel, J. B., The physiological significance of the cardiotonic steroid/ouabain-binding site of the Na,K-ATPase. *Annual review of physiology* **2010,** *72*, 395-412.

39. Kaplan, J. H., Biochemistry of Na,K-ATPase. *Annual review of biochemistry* **2002,** *71*, 511-35.

40. Blanco, G.; Mercer, R. W., Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *The American journal of physiology* **1998,** *275* (5 Pt 2), F633-50.

41. Shamraj, O. I.; Lingrel, J. B., A putative fourth Na+,K(+)-ATPase alpha-subunit gene is expressed in testis. *Proceedings of the National Academy of Sciences of the United States of America* **1994,** *91* (26), 12952-6.

42. Blanco, G.; Mercer, R. W., Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *The American journal of physiology* **1998,** *275* (5 Pt 2), F633-50.

43. Yoshika, M.; Komiyama, Y.; Konishi, M.; Akizawa, T.; Kobayashi, T.; Date, M.; Kobatake, S.; Masuda, M.; Masaki, H.; Takahashi, H., Novel digitalis-like factor, marinobufotoxin, isolated from cultured Y-1 cells, and its hypertensive effect in rats. *Hypertension* **2007,** *49* (1), 209-14.

44. Nicholls, M. G.; Lewis, L. K.; Yandle, T. G.; Lord, G.; McKinnon, W.; Hilton, P. J., Ouabain, a circulating hormone secreted by the adrenals, is pivotal in cardiovascular disease. Fact or fantasy? *Journal of hypertension* **2009,** *27* (1), 3-8.

45. Boulanger, B. R.; Lilly, M. P.; Hamlyn, J. M.; Laredo, J.; Shurtleff, D.; Gann, D. S., Ouabain is secreted by the adrenal gland in awake dogs. *The American journal of physiology* **1993,** *264* (3 Pt 1), E413-9.

46. Masugi, F.; Ogihara, T.; Hasegawa, T.; Sakaguchi, K.; Kumahara, Y., Normalization of high plasma level of ouabain-like immunoreactivity in primary aldosteronism after removal of adenoma. *Journal of human hypertension* **1988,** *2* (1), 17-20.

47. Doris, P. A.; Kilgore, M. W.; Durham, D.; Alberts, D.; Stocco, D. M., An endogenous digitalis-factor derived from the adrenal gland: studies of adrenocortical tumor cells. *Endocrinology* **1989,** *125* (5), 2580-6.

48. Hamlyn, J. M.; Blaustein, M. P.; Bova, S.; DuCharme, D. W.; Harris, D. W.; Mandel, F.; Mathews, W. R.; Ludens, J. H., Identification and characterization of a ouabain-like compound from human plasma. *Proceedings of the National Academy of Sciences of the United States of America* **1991,** *88* (14), 6259-63.

49. Mohmand, B.; Malhotra, D. K.; Shapiro, J. I., Uremic cardiomyopathy: role of circulating digitalis like substances. *Frontiers in bioscience : a journal and virtual library* **2005,** *10*, 2036-44.

50. Bricker, N. S.; Bourgoignie, J.; Weber, H.; Schmidt, R. W.; Slatopolsky, E., Pathogenesis of the uremic state: a new perspective. *Advances in nephrology from the Necker Hospital* **1972,** *2*, 263-76.

51. Hamlyn, J. M.; Ringel, R.; Schaeffer, J.; Levinson, P. D.; Hamilton, B. P.; Kowarski, A. A.; Blaustein, M. P., A circulating inhibitor of (Na+ + K+)ATPase associated with essential hypertension. *Nature* **1982,** *300* (5893), 650-2.

52. Bagrov, A. Y.; Dmitrieva, R. I.; Fedorova, O. V.; Kazakov, G. P.; Roukoyatkina, N. I.; Shpen, V. M., Endogenous marinobufagenin-like immunoreactive substance. A possible endogenous Na, K-ATPase inhibitor with vasoconstrictor activity. *American journal of hypertension* **1996,** *9* (10 Pt 1), 982-90.

53. Fedorova, O. V.; Tapilskaya, N. I.; Bzhelyansky, A. M.; Frolova, E. V.; Nikitina, E. R.; Reznik, V. A.; Kashkin, V. A.; Bagrov, A. Y., Interaction of Digibind with endogenous cardiotonic steroids from preeclamptic placentae. *Journal of hypertension* **2010,** *28* (2), 361-6.

54. Nguyen, A. N.; Wallace, D. P.; Blanco, G., Ouabain binds with high affinity to the Na,K-ATPase in human polycystic kidney cells and induces extracellular signal-regulated kinase activation and cell proliferation. *Journal of the American Society of Nephrology : JASN* **2007,** *18* (1), 46-57.

55. Komiyama, Y.; Nishimura, N.; Munakata, M.; Mori, T.; Okuda, K.; Nishino, N.; Hirose, S.; Kosaka, C.; Masuda, M.; Takahashi, H., Identification of endogenous ouabain in culture supernatant of PC12 cells. *Journal of hypertension* **2001,** *19* (2), 229-36.

56. Rossoni, L. V.; Xavier, F. E.; Moreira, C. M.; Falcochio, D.; Amanso, A. M.; Tanoue, C. U.; Carvalho, C. R.; Vassallo, D. V., Ouabain-induced hypertension enhances left ventricular contractility in rats. *Life sciences* **2006,** *79* (16), 1537-45.

57. Goto, A.; Ishiguro, T.; Yamada, K.; Ishii, M.; Yoshioka, M.; Eguchi, C.; Shimora, M.; Sugimoto, T., Isolation of a urinary digitalis-like factor indistinguishable from digoxin. *Biochemical and biophysical research communications* **1990,** *173* (3), 1093-101.

58. Qazzaz, H. M.; Goudy, S. L.; Valdes, R., Jr., Deglycosylated products of endogenous digoxin-like immunoreactive factor in mammalian tissue. *The Journal of biological chemistry* **1996,** *271* (15), 8731-7.

59. Kieval, R. S.; Butler, V. P., Jr.; Derguini, F.; Bruening, R. C.; Rosen, M. R., Cellular electrophysiologic effects of vertebrate digitalis-like substances. *Journal of the American College of Cardiology* **1988,** *11* (3), 637-43.

60. Numazawa, S.; Honma, Y.; Yamamoto, T.; Yoshida, T.; Kuroiwa, Y., A cardiotonic steroid bufalin-like factor in human plasma induces leukemia cell differentiation. *Leukemia research* **1995,** *19* (12), 945-53.

61. Lichtstein, D.; Gati, I.; Samuelov, S.; Berson, D.; Rozenman, Y.; Landau, L.; Deutsch, J., Identification of digitalis-like compounds in human cataractous lenses. *European journal of biochemistry* **1993,** *216* (1), 261-8.

62. Sich, B.; Kirch, U.; Tepel, M.; Zidek, W.; Schoner, W., Pulse pressure correlates in humans with a proscillaridin A immunoreactive compound. *Hypertension* **1996,** *27* (5), 1073-8.

63. Bagrov, A. Y.; Fedorova, O. V.; Dmitrieva, R. I.; Howald, W. N.; Hunter, A. P.; Kuznetsova, E. A.; Shpen, V. M., Characterization of a urinary bufodienolide Na+,K+-ATPase inhibitor in patients after acute myocardial infarction. *Hypertension* **1998,** *31* (5), 1097-103.

64. Bagrov, A. Y.; Fedorova, O. V.; Austin-Lane, J. L.; Dmitrieva, R. I.; Anderson, D. E., Endogenous marinobufagenin-like immunoreactive factor and Na+, K+ ATPase inhibition during voluntary hypoventilation. *Hypertension* **1995,** *26* (5), 781-8.

65. Lopatin, D. A.; Ailamazian, E. K.; Dmitrieva, R. I.; Shpen, V. M.; Fedorova, O. V.; Doris, P. A.; Bagrov, A. Y., Circulating bufodienolide and cardenolide sodium pump

inhibitors in preeclampsia. *Journal of hypertension* **1999,** *17* (8), 1179-87.

66. Gonick, H. C.; Ding, Y.; Vaziri, N. D.; Bagrov, A. Y.; Fedorova, O. V., Simultaneous measurement of marinobufagenin, ouabain, and hypertension-associated protein in various disease states. *Clinical and experimental hypertension* **1998,** *20* (5-6), 617-27.

67. Komiyama, Y.; Dong, X. H.; Nishimura, N.; Masaki, H.; Yoshika, M.; Masuda, M.; Takahashi, H., A novel endogenous digitalis, telocinobufagin, exhibits elevated plasma levels in patients with terminal renal failure. *Clinical biochemistry* **2005,** *38* (1), 36-45.

68. Buckalew, V. M., Endogenous digitalis-like factors: an overview of the history. *Frontiers in endocrinology* **2015,** *6*, 49.

69. Baecher, S.; Kroiss, M.; Fassnacht, M.; Vogeser, M., No endogenous ouabain is detectable in human plasma by ultra-sensitive UPLC-MS/MS. *Clinica chimica acta; international journal of clinical chemistry* **2014,** *431*, 87-92.

70. Lewis, L. K.; Yandle, T. G.; Hilton, P. J.; Jensen, B. P.; Begg, E. J.; Nicholls, M. G., Endogenous ouabain is not ouabain. *Hypertension* **2014,** *64* (4), 680-3.

71. Ma, J.; Esplin, M. S.; Adair, C. D.; Mason, L. A.; Graves, S. W., Increasing evidence for and regulation of a human placental endogenous digitalis-like factor. *Reproductive sciences* **2012,** *19* (4), 437-48.

72. Hopoate-Sitake, M. L.; Adair, C. D.; Mason, L. A.; Torres, C.; Kipikasa, J.; Graves, S. W., Digibind reverses inhibition of cellular rb+ uptake caused by endogenous sodium pump inhibitors present in serum and placenta of women with preeclampsia. *Reproductive sciences* **2011,** *18* (2), 190-9.

73. Hilton, P. J.; White, R. W.; Lord, G. A.; Garner, G. V.; Gordon, D. B.; Hilton, M. J.; Forni, L. G.; McKinnon, W.; Ismail, F. M.; Keenan, M.; Jones, K.; Morden, W. E., An inhibitor of the sodium pump obtained from human placenta. *Lancet* **1996,** *348* (9023), 303-5.

74. Li, M.; Wen, C.; Whitworth, J. A., Hemodynamic effects of the Fab fragment of digoxin antibody (digibind) in corticotropin (ACTH)-induced hypertension. *American journal of hypertension* **1997,** *10* (3), 332-6.

75. Kaide, J.; Ura, N.; Torii, T.; Nakagawa, M.; Takada, T.; Shimamoto, K., Effects of digoxin-specific antibody Fab fragment (Digibind) on blood pressure and renal water-sodium metabolism in 5/6 reduced renal mass hypertensive rats. *American journal of hypertension* **1999,** *12* (6), 611-9.

76. Goodlin, R. C., Antidigoxin antibodies in eclampsia. *The New England journal of medicine* **1988,** *318* (8), 518-9.

77. Adair, C. D.; Buckalew, V.; Taylor, K.; Ernest, J. M.; Frye, A. H.; Evans, C.; Veille, J. C., Elevated endoxin-like factor complicating a multifetal second trimester pregnancy: treatment with digoxin-binding immunoglobulin. *American journal of nephrology* **1996,** *16* (6), 529-31.

78. Pullen, M. A.; Brooks, D. P.; Edwards, R. M., Characterization of the neutralizing activity of digoxin-specific Fab toward ouabain-like steroids. *The Journal of pharmacology and experimental therapeutics* **2004,** *310* (1), 319-25.

79. Tao, Q. F.; Soszynski, P. A.; Hollenberg, N. K.; Graves, S. W., Specificity of the volume-sensitive sodium pump inhibitor isolated from human peritoneal dialysate in chronic renal failure. *Kidney international* **1996,** *49* (2), 420-9.

80. Balzan, S.; Montali, U.; Biver, P.; Ghione, S., Digoxin-binding antibodies reverse the effect of endogenous digitalis-like compounds on Na,K-ATPase in erythrocytes. *Journal of hypertension. Supplement : official journal of the International Society of Hypertension* **1991,** *9* (6), S304-5.

81. Yoshika, M.; Komiyama, Y.; Konishi, M.; Akizawa, T.; Kobayashi, T.; Date, M.; Kobatake, S.; Masuda, M.; Masaki, H.; Takahashi, H., Novel digitalis-like factor, marinobufotoxin, isolated from cultured Y-1 cells, and its hypertensive effect in rats. *Hypertension* **2007,** *49* (1), 209-14.

82. Kennedy, D. J.; Vetteth, S.; Periyasamy, S. M.; Kanj, M.; Fedorova, L.; Khouri, S.; Kahaleh, M. B.; Xie, Z.; Malhotra, D.; Kolodkin, N. I.; Lakatta, E. G.; Fedorova, O. V.; Bagrov, A. Y.; Shapiro, J. I., Central role for the cardiotonic steroid marinobufagenin in the pathogenesis of experimental uremic cardiomyopathy. *Hypertension* **2006,** *47* (3), 488-95.

83. Graves, S. W.; Williams, G. H., An endogenous ouabain-like factor associated with hypertensive pregnant women. *The Journal of clinical endocrinology and metabolism* **1984,** *59* (6), 1070-4.

84. Estape, E. S.; Torres-Negron, I.; Gonzalez, L.; Martinez-Maldonado, M., A New Animal Model to Study Endogenous Cardiotonic Steroids and the Progression of Cardiovascular Events in Salt-Sensitive Hypertension. *International archives of translational medicine* **2015,** *1* (1).

85. Dahl, L. K.; Knudsen, K. D.; Iwai, J., Humoral transmission of hypertension: evidence from parabiosis. *Circulation research* **1969,** *24* (5), Suppl:21-33.

86. Beyers, A. D.; Odendaal, H. J.; Spruyt, L. L.; Parkin, D. P., The possible role of endogenous digitalis-like substance in the causation of pre-eclampsia. *South African*

medical journal = Suid-Afrikaanse tydskrif vir geneeskunde **1984,** *65* (22), 883-5.

87. Graves, S. W.; Esplin, M. S.; McGee, P.; Rouse, D. J.; Leveno, K. J.; Mercer, B. M.; Iams, J. D.; Wapner, R. J.; Sorokin, Y.; Thorp, J. M.; Ramin, S. M.; Malone, F. D.; O'Sullivan, M. J.; Peaceman, A. M.; Hankins, G. D. V.; Dudley, D. J.; Caritis, S. N.; Eunice Kennedy Shriver National Institute of Child, H.; Human Development Maternal-Fetal Medicine Units, N., Association of cord blood digitalis-like factor and necrotizing enterocolitis. *American journal of obstetrics and gynecology* **2014,** *210* (4), 328 e1-328 e5.

88. Valdes, R., Jr.; Graves, S. W.; Brown, B. A.; Landt, M., Endogenous substance in newborn infants causing false positive digoxin measurements. *The Journal of pediatrics* **1983,** *102* (6), 947-50.

89. De Wardener, H. E.; Mills, I. H.; Clapham, W. F.; Hayter, C. J., Studies on the efferent mechanism of the sodium diuresis which follows the administration of intravenous saline in the dog. *Clinical science* **1961,** *21*, 249-58.

90. Bricker, N. S.; Klahr, S.; Purkerson, M.; Schultze, R. G.; Avioli, L. V.; Birge, S. J., In vitro assay for a humoral substance present during volume expansion and uraemia. *Nature* **1968,** *219* (5158), 1058-9.

91. Graves, S. W.; Williams, G. H., Endogenous digitalis-like natriuretic factors. *Annual review of medicine* **1987,** *38*, 433-44.

92. Hollenberg, N. K.; Graves, S. W., Koch's postulates and the digitalis-like factor. *Hypertension research : official journal of the Japanese Society of Hypertension* **1995,** *18* (1), 1-6.

93. Espiner, E. A.; Richards, A. M.; Yandle, T. G.; Nicholls, M. G., Natriuretic hormones. *Endocrinology and metabolism clinics of North America* **1995,** *24* (3), 481-509.

94. Valdes, R. Jr; Siegfried, B., Digoxin immunoreactivity in urine of persons not taking digoxin. *Clin Chem* **1983**, *29*, 1166. Abstract.

95. Gault, M. H.; Vasdev, S. C.; Longerich, L. L.; Fernandez, P.; Prabhakaran, V.; Dawe, M.; Maillet, C., Plasma digitalis-like factor(s) increase with salt loading. *The New England journal of medicine* **1983,** *309* (23), 1459.

96. Wang, Y.; Lewis, D. F.; Adair, C. D.; Gu, Y.; Mason, L.; Kipikasa, J. H., Digibind attenuates cytokine TNFalpha-induced endothelial inflammatory response: potential benefit role of digibind in preeclampsia. *Journal of perinatology : official journal of the California Perinatal Association* **2009,** *29* (3), 195-200.

97. Graves, S. W., Sodium regulation, sodium pump function and sodium pump inhibitors in uncomplicated pregnancy and preeclampsia. *Frontiers in bioscience : a journal and virtual library* **2007,** *12*, 2438-46.

98. Gallery, E. D.; Brown, M. A., Volume homeostasis in normal and hypertensive human pregnancy. *Bailliere's clinical obstetrics and gynaecology* **1987,** *1* (4), 835-51.

99. Gilson, G. J.; Graves, S. W.; Qualls, C. R.; Curet, L. B., Digoxin-like immunoreactive substance and sodium-potassium-adenosine triphosphatase inhibition in normal pregnancy: a longitudinal study. *Obstetrics and gynecology* **1997,** *89* (5 Pt 1), 743-6.

100. Agunanne, E.; Horvat, D.; Uddin, M. N.; Puschett, J., The treatment of preeclampsia in a rat model employing Digibind. *American journal of perinatology* **2010,** *27* (4), 299-305.

101. Horvat, D.; Severson, J.; Uddin, M. N.; Mitchell, B.; Puschett, J. B., Resibufogenin prevents the manifestations of preeclampsia in an animal model of the syndrome. *Hypertension in pregnancy* **2010,** *29* (1), 1-9.

102. Graves, S. W.; Valdes, R., Jr.; Brown, B. A.; Knight, A. B.; Craig, H. R., Endogenous digoxin-immunoreactive substance in human pregnancies. *The Journal of clinical endocrinology and metabolism* **1984,** *58* (4), 748-51.

103. Averina, I. V.; Tapilskaya, N. I.; Reznik, V. A.; Frolova, E. V.; Fedorova, O. V.; Lakatta, E. G.; Bagrov, A. Y., Endogenous Na/K-ATPase inhibitors in patients with preeclampsia. *Cellular and molecular biology* **2006,** *52* (8), 19-23.

104. Adair, C. D.; Luper, A.; Rose, J. C.; Russell, G.; Veille, J. C.; Buckalew, V. M., The hemodynamic effects of intravenous digoxin-binding fab immunoglobulin in severe preeclampsia: a double-blind, randomized, clinical trial. *Journal of perinatology : official journal of the California Perinatal Association* **2009,** *29* (4), 284-9.

105. Di Grande, A.; Boura, A. L.; Read, M. A.; Malatino, L. S.; Walters, W. A., Release of a substance from the human placenta having digoxin-like immunoreactivity. *Clinical and experimental pharmacology & physiology* **1993,** *20* (9), 603-7.

106. Amler, E.; Cester, N.; Salvolini, E.; Staffolani, R.; Burkhard, M.; Mazzanti, L.; Kotyk, A.; Romanini, C., Human hypertensive placenta contains an increased amount of Na,K-ATPase with higher affinity for cardiac glycosides. *Cell biology international* **1994,** *18* (7), 723-7.

107. Fedorova, O. V.; Kolodkin, N. I.; Agalakova, N. I.; Namikas, A. R.; Bzhelyansky, A.; St-Louis, J.; Lakatta, E. G.; Bagrov, A. Y., Antibody to marinobufagenin lowers blood

pressure in pregnant rats on a high NaCl intake. *Journal of hypertension* **2005,** *23* (4), 835-42.

108. Vu, H. V.; Ianosi-Irimie, M. R.; Pridjian, C. A.; Whitbred, J. M.; Durst, J. M.; Bagrov, A. Y.; Fedorova, O. V.; Pridjian, G.; Puschett, J. B., Involvement of marinobufagenin in a rat model of human preeclampsia. *American journal of nephrology* **2005,** *25* (5), 520-8.

109. Fedorova, O. V.; Simbirtsev, A. S.; Kolodkin, N. I.; Kotov, A. Y.; Agalakova, N. I.; Kashkin, V. A.; Tapilskaya, N. I.; Bzhelyansky, A.; Reznik, V. A.; Frolova, E. V.; Nikitina, E. R.; Budny, G. V.; Longo, D. L.; Lakatta, E. G.; Bagrov, A. Y., Monoclonal antibody to an endogenous bufadienolide, marinobufagenin, reverses preeclampsia-induced Na/K-ATPase inhibition and lowers blood pressure in NaCl-sensitive hypertension. *Journal of hypertension* **2008,** *26* (12), 2414-25.

110. Uddin, M. N.; Horvat, D.; Glaser, S. S.; Danchuk, S.; Mitchell, B. M.; Sullivan, D. E.; Morris, C. A.; Puschett, J. B., Marinobufagenin inhibits proliferation and migration of cytotrophoblast and CHO cells. *Placenta* **2008,** *29* (3), 266-73.

111. Seely, E. W.; Williams, G. H.; Graves, S. W., Markers of sodium and volume homeostasis in pregnancy-induced hypertension. *The Journal of clinical endocrinology and metabolism* **1992,** *74* (1), 150-6.

112. Didden, M. A.; Vollebergh, J. H.; Hoekstra, M. P.; Schellekens, A.; de Jong, P. A., Erythrocytic ouabain-sensitive sodium efflux rate constant in pregnancy. *European journal of obstetrics, gynecology, and reproductive biology* **1993,** *48* (3), 177-81.

113. Wang, Y.; Lewis, D. F.; Adair, C. D.; Gu, Y.; Mason, L.; Kipikasa, J. H., Digibind attenuates cytokine TNFalpha-induced endothelial inflammatory response: potential benefit role of digibind in preeclampsia. *Journal of perinatology : official journal of the California Perinatal Association* **2009,** *29* (3), 195-200.

114. Ferrandi, M.; Manunta, P.; Balzan, S.; Hamlyn, J. M.; Bianchi, G.; Ferrari, P., Ouabain-like factor quantification in mammalian tissues and plasma: comparison of two independent assays. *Hypertension* **1997,** *30* (4), 886-96.

115. Krep, H.; Price, D. A.; Soszynski, P.; Tao, Q. F.; Graves, S. W.; Hollenberg, N. K., Volume sensitive hypertension and the digoxin-like factor. Reversal by a Fab directed against digoxin in DOCA-salt hypertensive rats. *American journal of hypertension* **1995,** *8* (9), 921-7.

116. Bagrov, A. Y.; Fedorova, O. V.; Dmitrieva, R. I.; French, A. W.; Anderson, D. E., Plasma marinobufagenin-like and ouabain-like immunoreactivity during saline volume expansion in anesthetized dogs. *Cardiovascular research* **1996,** *31* (2), 296-305.

117. Lam, G. K.; Hopoate-Sitake, M.; Adair, C. D.; Buckalew, V. M.; Johnson, D. D.; Lewis, D. F.; Robinson, C. J.; Saade, G. R.; Graves, S. W., Digoxin antibody fragment, antigen binding (Fab), treatment of preeclampsia in women with endogenous digitalis-like factor: a secondary analysis of the DEEP Trial. *American journal of obstetrics and gynecology* **2013,** *209* (2), 119 e1-6.

118. Adair, C. D.; Haupert, G. T., Jr.; Koh, H. P.; Wang, Y.; Veille, J. C.; Buckalew, V., Erythrocyte sodium/potassium ATPase activity in severe preeclampsia. *Journal of perinatology : official journal of the California Perinatal Association* **2009,** *29* (4), 280-3.

119. Agunanne, E.; Horvat, D.; Harrison, R.; Uddin, M. N.; Jones, R.; Kuehl, T. J.; Ghanem, D. A.; Berghman, L. R.; Lai, X.; Li, J.; Romo, D.; Puschett, J. B., Marinobufagenin levels in preeclamptic patients: a preliminary report. American journal of perinatology 2011, 28 (7), 509-14.

120. Valdes, R., Jr.; Graves, S. W.; Brown, B. A.; Landt, M., Endogenous substance in newborn infants causing false positive digoxin measurements. *The Journal of pediatrics* **1983,** *102* (6), 947-50.

121. Young, B. C.; Levine, R. J.; Karumanchi, S. A., Pathogenesis of preeclampsia. *Annual review of pathology* **2010,** *5*, 173-92.

122. Zhou, C. C.; Irani, R. A.; Dai, Y.; Blackwell, S. C.; Hicks, M. J.; Ramin, S. M.; Kellems, R. E.; Xia, Y., Autoantibody-mediated IL-6-dependent endothelin-1 elevation underlies pathogenesis in a mouse model of preeclampsia. *Journal of immunology* **2011,** *186* (10), 6024-34.

123. Matsuo, K.; Kooshesh, S.; Dinc, M.; Sun, C. C.; Kimura, T.; Baschat, A. A., Late postpartum eclampsia: report of two cases managed by uterine curettage and review of the literature. *American journal of perinatology* **2007,** *24* (4), 257-66.

124. LaMarca, B.; Speed, J.; Fournier, L.; Babcock, S. A.; Berry, H.; Cockrell, K.; Granger, J. P., Hypertension in response to chronic reductions in uterine perfusion in pregnant rats: effect of tumor necrosis factor-alpha blockade. *Hypertension* **2008,** *52* (6), 1161-7.

125. Palei, A. C.; Spradley, F. T.; Warrington, J. P.; George, E. M.; Granger, J. P., Pathophysiology of hypertension in pre-eclampsia: a lesson in integrative physiology. *Acta physiologica* **2013,** *208* (3), 224-33.

126. Lyall, F.; Bulmer, J. N.; Duffie, E.; Cousins, F.; Theriault, A.; Robson, S. C., Human trophoblast invasion and spiral artery transformation: the role of PECAM-1 in

normal pregnancy, preeclampsia, and fetal growth restriction. *The American journal of pathology* **2001,** *158* (5), 1713-21.

127. Craici, I. M.; Wagner, S. J.; Weissgerber, T. L.; Grande, J. P.; Garovic, V. D., Advances in the pathophysiology of pre-eclampsia and related podocyte injury. *Kidney international* **2014,** *86* (2), 275-85.

128. Gilbert, J. S.; Ryan, M. J.; LaMarca, B. B.; Sedeek, M.; Murphy, S. R.; Granger, J. P., Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction. *American journal of physiology. Heart and circulatory physiology* **2008,** *294* (2), H541-50.

129. Laresgoiti-Servitje, E.; Gomez-Lopez, N., The pathophysiology of preeclampsia involves altered levels of angiogenic factors promoted by hypoxia and autoantibody-mediated mechanisms. *Biology of reproduction* **2012,** *87* (2), 36.

130. Zhou, C. C.; Ahmad, S.; Mi, T.; Abbasi, S.; Xia, L.; Day, M. C.; Ramin, S. M.; Ahmed, A.; Kellems, R. E.; Xia, Y., Autoantibody from women with preeclampsia induces soluble Fms-like tyrosine kinase-1 production via angiotensin type 1 receptor and calcineurin/nuclear factor of activated T-cells signaling. *Hypertension* **2008,** *51* (4), 1010-9.

131. LaMarca, B.; Wallace, K.; Herse, F.; Wallukat, G.; Martin, J. N., Jr.; Weimer, A.; Dechend, R., Hypertension in response to placental ischemia during pregnancy: role of B lymphocytes. *Hypertension* **2011,** *57* (4), 865-71.

132. Yetukuri, L.; Katajamaa, M.; Medina-Gomez, G.; Seppanen-Laakso, T.; Vidal-Puig, A.; Oresic, M., Bioinformatics strategies for lipidomics analysis: characterization of obesity related hepatic steatosis. *BMC systems biology* **2007,** *1*, 12.

133. Brugger, B., Lipidomics: analysis of the lipid composition of cells and subcellular organelles by electrospray ionization mass spectrometry. *Annual review of biochemistry* **2014,** *83*, 79-98.

134. Watson, A. D., Lipidomics: a global approach to lipid analysis in biological system. *J Lipid Res* **2006**, *14*, 2101-2111.

135. Wenk, M. R., The emerging field of lipidomics. *Nature reviews. Drug discovery* **2005,** *4* (7), 594-610.

136. Kofeler, H. C.; Fauland, A.; Rechberger, G. N.; Trotzmuller, M., Mass spectrometry based lipidomics: an overview of technological platforms. *Metabolites* **2012,** *2* (1), 19-38

137. Han, X. L. Lipidomics: Comprehensive Mass Spectrometry of Lipids, 1st ed.; Wiley: New Jersey, 2016.

138. Li, M.; Yang, L.; Bai, Y.; Liu, H., Analytical methods in lipidomics and their applications. *Analytical chemistry* **2014,** *86* (1), 161-75.

139. Li, L.; Han, J.; Wang, Z.; Liu, J.; Wei, J.; Xiong, S.; Zhao, Z., Mass spectrometry methodology in lipid analysis. *International journal of molecular sciences* **2014,** *15* (6), 10492-507.

140. Folch, J.; Lees, M.; Sloane Stanley, G. H., A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry* **1957,** *226* (1), 497-509.

141. Bligh, E. G.; Dyer, W. J., A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* **1959,** *37* (8), 911-7.

142. Hara, A.; Radin, N. S., Lipid extraction of tissues with a low-toxicity solvent. *Analytical biochemistry* **1978,** *90* (1), 420-6.

143. Khaselev, N.; Murphy, R. C., Structural characterization of oxidized phospholipid products derived from arachidonate-containing plasmenyl glycerophosphocholine. *Journal of lipid research* **2000,** *41* (4), 564-72.

144. Ibanez, C.; Simo, C.; Garcia-Canas, V.; Cifuentes, A.; Castro-Puyana, M., Metabolomics, peptidomics and proteomics applications of capillary electrophoresis-mass spectrometry in Foodomics: a review. *Analytica chimica acta* **2013,** *802*, 1-13.

145. Soloviev, M., Andren, P., Shaw, C. *Peptidomics: Methods and Applications*, 1st ed.; Wiley: New Jersey, 2007.

146. Chandrasekhar, K.; Dileep, A.; Lebonah, D. E.; Kumari, J. P., A short review on proteomics and its applications. *International letters of natural sciences* **2014**, *12*(1), 77-84.

147. Yates, J. R.; Ruse, C. I.; Nakorchevsky, A., Proteomics by mass spectrometry: approaches, advances, and applications. *Annual review of biomedical engineering* **2009,** *11*, 49-79.

148. Aebersold, R.; Mann, M., Mass spectrometry-based proteomics. *Nature* **2003,** *422* (6928), 198-207.

149. Mann, M.; Hendrickson, R. C.; Pandey, A., Analysis of proteins and proteomes by mass spectrometry. *Annual review of biochemistry* **2001,** *70*, 437-73.

150. Delahunty, C.; Yates, J. R., 3rd, Protein identification using 2D-LC-MS/MS. *Methods* **2005,** *35* (3), 248-55.

151. Di Meo, A.; Pasic, M. D.; Yousef, G. M., Proteomics and peptidomics: moving toward precision medicine in urological malignancies. *Oncotarget* **2016,** *7* (32), 52460-52474.

152. Cottrell, J. S., Protein identification using MS/MS data. *Journal of proteomics* **2011,** *74* (10), 1842-51.

153. Ong, S. E.; Mann, M., Mass spectrometry-based proteomics turns quantitative. *Nature chemical biology* **2005,** *1* (5), 252-62.

154. Wu, C. C.; MacCoss, M. J.; Howell, K. E.; Matthews, D. E.; Yates, J. R., 3rd, Metabolic labeling of mammalian organisms with stable isotopes for quantitative proteomic analysis. *Analytical chemistry* **2004,** *76* (17), 4951-9.

155. Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R., Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature biotechnology* **1999,** *17* (10), 994-9.

156. Ross, P. L.; Huang, Y. N.; Marchese, J. N.; Williamson, B.; Parker, K.; Hattan, S.; Khainovski, N.; Pillai, S.; Dey, S.; Daniels, S.; Purkayastha, S.; Juhasz, P.; Martin, S.; Bartlet-Jones, M.; He, F.; Jacobson, A.; Pappin, D. J., Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Molecular & cellular proteomics : MCP* **2004,** *3* (12), 1154-69.

157. Zybailov, B.; Coleman, M. K.; Florens, L.; Washburn, M. P., Correlation of relative abundance ratios derived from peptide ion chromatograms and spectrum counting for quantitative proteomic analysis using stable isotope labeling. *Analytical chemistry* **2005,** *77* (19), 6218-24.

158. Gerber, S. A.; Rush, J.; Stemman, O.; Kirschner, M. W.; Gygi, S. P., Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proceedings of the National Academy of Sciences of the United States of America* **2003,** *100* (12), 6940-5.

159. Beynon, R. J.; Doherty, M. K.; Pratt, J. M.; Gaskell, S. J., Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. *Nature methods* **2005,** *2* (8), 587-9.

160. Hanke, S.; Besir, H.; Oesterhelt, D.; Mann, M., Absolute SILAC for accurate

quantitation of proteins in complex mixtures down to the attomole level. *Journal of proteome research* **2008,** *7* (3), 1118-30.

161. Cunningham, R.; Ma, D.; Li, L., Mass Spectrometry-based Proteomics and Peptidomics for Systems Biology and Biomarker Discovery. *Frontiers in biology* **2012,** *7* (4), 313-335.

162. Bini, L.; Heid, H.; Liberatori, S.; Geier, G.; Pallini, V.; Zwilling, R., Two-dimensional gel electrophoresis of Caenorhabditis elegans homogenates and identification of protein spots by microsequencing. *Electrophoresis* **1997,** *18* (3-4), 557-62.

163. Dallas, D. C.; Guerrero, A.; Parker, E. A.; Robinson, R. C.; Gan, J.; German, J. B.; Barile, D.; Lebrilla, C. B., Current peptidomics: applications, purification, identification, quantification, and functional analysis. *Proteomics* **2015,** *15* (5-6), 1026-38.

164. Fricker, L. D.; Lim, J.; Pan, H.; Che, F. Y., Peptidomics: identification and quantification of endogenous peptides in neuroendocrine tissues. *Mass spectrometry reviews* **2006,** *25* (2), 327-44.

165. Schulz-Knappe, P.; Zucht, H. D.; Heine, G.; Jurgens, M.; Hess, R.; Schrader, M., Peptidomics: the comprehensive analysis of peptides in complex biological mixtures. *Combinatorial chemistry & high throughput screening* **2001,** *4* (2), 207-17.

166. Schrader, M.; Knappe, P. S.; Fricker, L. D., Historical perspective of peptidomics. *EuPA Open Proteomics* **2014**, *3*, 171-182.

167. Hu, L.; Li, X.; Jiang, X.; Zhou, H.; Jiang, X.; Kong, L.; Ye, M.; Zou, H., Comprehensive peptidome analysis of mouse livers by size exclusion chromatography prefractionation and nanoLC-MS/MS identification. *Journal of proteome research* **2007,** *6* (2), 801-8.

168. Sigdel, T. K.; Ling, X. B.; Lau, K. H.; Li, L.; Schilling, J.; Sarwal, M. M., Urinary peptidomic analysis identifies potential biomarkers for acute rejection of renal transplantation. *Clinical proteomics* **2009**, *5*(2), 103-113.

169. Manes, N. P.; Gustin, J. K.; Rue, J.; Mottaz, H. M.; Purvine, S. O.; Norbeck, A. D.; Monroe, M. E.; Zimmer, J. S.; Metz, T. O.; Adkins, J. N.; Smith, R. D.; Heffron, F., Targeted protein degradation by Salmonella under phagosome-mimicking culture conditions investigated using comparative peptidomics. *Molecular & cellular proteomics : MCP* **2007,** *6* (4), 717-27.

170. Schrader, M.; Selle, H., The process chain for peptidomic biomarker discovery.

Disease markers **2006,** *22* (1-2), 27-37.

171. Svensson, M.; Skold, K.; Svenningsson, P.; Andren, P. E., Peptidomics-based discovery of novel neuropeptides. *Journal of proteome research* **2003,** *2* (2), 213-9.

172. Che, F. Y.; Lim, J.; Pan, H.; Biswas, R.; Fricker, L. D., Quantitative neuropeptidomics of microwave-irradiated mouse brain and pituitary. *Molecular & cellular proteomics : MCP* **2005,** *4* (9), 1391-405.

173. Uttenweiler-Joseph, S.; Moniatte, M.; Lagueux, M.; Van Dorsselaer, A.; Hoffmann, J. A.; Bulet, P., Differential display of peptides induced during the immune response of Drosophila: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study. *Proceedings of the National Academy of Sciences of the United States of America* **1998,** *95* (19), 11342-7.

174. Jurgens, M.; Schrader, M.; Raida, M.; Forssmann W.; Knappe, P. S., Multi-dimensional mapping of human blood peptides by mass spectrometry. *Journal of biomolecular techniques* **1998**, *9*(4), 24-30.

175. Cadieux, P. A.; Beiko, D. T.; Watterson, J. D.; Burton, J. P.; Howard, J. C.; Knudsen, B. E.; Gan, B. S.; McCormick, J. K.; Chambers, A. F.; Denstedt, J. D.; Reid, G., Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS): a new proteomic urinary test for patients with urolithiasis. *Journal of clinical laboratory analysis* **2004,** *18* (3), 170-5.

176. Herrero, M.; Ibanez, E.; Cifuentes, A., Capillary electrophoresis-electrospray-mass spectrometry in peptide analysis and peptidomics. *Electrophoresis* **2008,** *29* (10), 2148-60.

177. Sillard, R.; Jornvall, H.; Carlquist, M.; Mutt, V., Chemical assay for cyst(e)ine-rich peptides detects a novel intestinal peptide ZF-1, homologous to a single zinc-finger motif. *European journal of biochemistry* **1993,** *211* (1-2), 377-80.

178. Kolch, W.; Neususs, C.; Pelzing, M.; Mischak, H., Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. *Mass spectrometry reviews* **2005,** *24* (6), 959-77.

179. Ross, P. L.; Huang, Y. N.; Marchese, J. N.; Williamson, B.; Parker, K.; Hattan, S.; Khainovski, N.; Pillai, S.; Dey, S.; Daniels, S.; Purkayastha, S.; Juhasz, P.; Martin, S.; Bartlet-Jones, M.; He, F.; Jacobson, A.; Pappin, D. J., Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Molecular & cellular proteomics : MCP* **2004,** *3* (12), 1154-69.

180. Xiang, F.; Ye, H.; Chen, R.; Fu, Q.; Li, L., N,N-dimethyl leucines as novel isobaric

tandem mass tags for quantitative proteomics and peptidomics. *Analytical chemistry* **2010,** *82* (7), 2817-25.

181. Morano, C.; Zhang, X.; Fricker, L. D., Multiple isotopic labels for quantitative mass spectrometry. *Analytical chemistry* **2008,** *80* (23), 9298-309.

182. Wen, Q.; Liu, L. Y.; Yang, T.; Alev, C.; Wu, S.; Stevenson, D. K.; Sheng, G.; Butte, A. J.; Ling, X. B., Peptidomic Identification of Serum Peptides Diagnosing Preeclampsia. *PloS one* **2013,** *8* (6), e65571.

183. Shen, Y.; Tolic, N.; Xie, F.; Zhao, R.; Purvine, S. O.; Schepmoes, A. A.; Moore, R. J.; Anderson, G. A.; Smith, R. D., Effectiveness of CID, HCD, and ETD with FT MS/MS for degradomic-peptidomic analysis: comparison of peptide identification methods. *Journal of proteome research* **2011,** *10* (9), 3929-43.

184. Shen, Y.; Tolic, N.; Hixson, K. K.; Purvine, S. O.; Pasa-Tolic, L.; Qian, W. J.; Adkins, J. N.; Moore, R. J.; Smith, R. D., Proteome-wide identification of proteins and their modifications with decreased ambiguities and improved false discovery rates using unique sequence tags. *Analytical chemistry* **2008,** *80* (6), 1871-82.

185. Frank, A.; Pevzner, P., PepNovo: de novo peptide sequencing via probabilistic network modeling. *Analytical chemistry* **2005,** *77* (4), 964-73.

186. Dancik, V.; Addona, T. A.; Clauser, K. R.; Vath, J. E.; Pevzner, P. A., De novo peptide sequencing via tandem mass spectrometry. *Journal of computational biology : a journal of computational molecular cell biology* **1999,** *6* (3-4), 327-42.

187. Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S., Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999,** *20* (18), 3551-67.

188. Jimenez, C. R.; Huang, L.; Qiu, Y.; Burlingame, A. L., Searching sequence databases over the internet: protein identification using MS-Fit. *Current protocols in protein science* **2001,** *Chapter 16*, Unit 16 5.

189. Geer, L. Y.; Markey, S. P.; Kowalak, J. A.; Wagner, L.; Xu, M.; Maynard, D. M.; Yang, X.; Shi, W.; Bryant, S. H., Open mass spectrometry search algorithm. *Journal of proteome research* **2004,** *3* (5), 958-64.

190. Searle, B. C.; Dasari, S.; Wilmarth, P. A.; Turner, M.; Reddy, A. P.; David, L. L.; Nagalla, S. R., Identification of protein modifications using MS/MS de novo sequencing and the OpenSea alignment algorithm. *Journal of proteome research* **2005,** *4* (2), 546-54.

191. Kaur, H.; Garg, A.; Raghava, G. P., PEPstr: a de novo method for tertiary structure prediction of small bioactive peptides. *Protein and peptide letters* **2007,** *14* (7), 626-31.

192. Falth, M.; Skold, K.; Norrman, M.; Svensson, M.; Fenyo, D.; Andren, P. E., SwePep, a database designed for endogenous peptides and mass spectrometry. *Molecular & cellular proteomics : MCP* **2006,** *5* (6), 998-1005.

193. Falth, M.; Skold, K.; Svensson, M.; Nilsson, A.; Fenyo, D.; Andren, P. E., Neuropeptidomics strategies for specific and sensitive identification of endogenous peptides. *Molecular & cellular proteomics : MCP* **2007,** *6* (7), 1188-97.

194. Boonen, K.; Landuyt, B.; Baggerman, G.; Husson, S. J.; Huybrechts, J.; Schoofs, L., Peptidomics: the integrated approach of MS, hyphenated techniques and bioinformatics for neuropeptide analysis. *Journal of separation science* **2008,** *31* (3), 427-45.

195. Bauca, J. M.; Martinez-Morillo, E.; Diamandis, E. P., Peptidomics of urine and other biofluids for cancer diagnostics. *Clinical chemistry* **2014,** *60* (8), 1052-61.

196. Schulz-Knappe, P.; Schrader, M.; Zucht, H. D., The peptidomics concept. *Combinatorial chemistry & high throughput screening* **2005,** *8* (8), 697-704.

197. Menschaert, G.; Vandekerckhove, T. T.; Baggerman, G.; Schoofs, L.; Luyten, W.; Van Criekinge, W., Peptidomics coming of age: a review of contributions from a bioinformatics angle. *Journal of proteome research* **2010,** *9* (5), 2051-61.

198. Zeisler, H.; Llurba, E.; Chantraine, F.; Vatish, M.; Staff, A. C.; Sennstrom, M.; Olovsson, M.; Brennecke, S. P.; Stepan, H.; Allegranza, D.; Dilba, P.; Schoedl, M.; Hund, M.; Verlohren, S., Predictive Value of the sFlt-1:PlGF Ratio in Women with Suspected Preeclampsia. *The New England journal of medicine* **2016,** *374* (1), 13-22.

199. Poston, L.; Igosheva, N.; Mistry, H. D.; Seed, P. T.; Shennan, A. H.; Rana, S.; Karumanchi, S. A.; Chappell, L. C., Role of oxidative stress and antioxidant supplementation in pregnancy disorders. *The American journal of clinical nutrition* **2011,** *94* (6 Suppl), 1980S-1985S.

200. Huang, Y.; Mucke, L., Alzheimer mechanisms and therapeutic strategies. *Cell* **2012,** *148* (6), 1204-22.

201. Minati, L.; Edginton, T.; Bruzzone, M. G.; Giaccone, G., Current concepts in Alzheimer's disease: a multidisciplinary review. *American journal of Alzheimer's disease and other dementias* **2009,** *24* (2), 95-121.

202. Selkoe, D. J., Alzheimer's disease: genes, proteins, and therapy. *Physiological reviews* **2001,** *81* (2), 741-66.

203. Papassotiropoulos, A.; Fountoulakis, M.; Dunckley, T.; Stephan, D. A.; Reiman, E. M., Genetics, transcriptomics, and proteomics of Alzheimer's disease. *The Journal of clinical psychiatry* **2006,** *67* (4), 652-70.

204. Longstreth, W. T., Jr.; Dulberg, C.; Manolio, T. A.; Lewis, M. R.; Beauchamp, N. J., Jr.; O'Leary, D.; Carr, J.; Furberg, C. D., Incidence, manifestations, and predictors of brain infarcts defined by serial cranial magnetic resonance imaging in the elderly: the Cardiovascular Health Study. *Stroke* **2002,** *33* (10), 2376-82.

205. Van Den Heuvel, C.; Thornton, E.; Vink, R., Traumatic brain injury and Alzheimer's disease: a review. *Progress in brain research* **2007,** *161*, 303-16.

206. Geerlings, M. I.; den Heijer, T.; Koudstaal, P. J.; Hofman, A.; Breteler, M. M., History of depression, depressive symptoms, and medial temporal lobe atrophy and the risk of Alzheimer disease. *Neurology* **2008,** *70* (15), 1258-64.

207. Koepsell, T. D.; Kurland, B. F.; Harel, O.; Johnson, E. A.; Zhou, X. H.; Kukull, W. A., Education, cognitive function, and severity of neuropathology in Alzheimer disease. *Neurology* **2008,** *70* (19 Pt 2), 1732-9.

208. Kumar, A.; Singh, A.; Ekavali, A review on Alzheimer's disease pathophysiology and its management: an update. *Pharmacological reports : PR* **2015,** *67* (2), 195-203.

209. Auld, D. S.; Kornecook, T. J.; Bastianetto, S.; Quirion, R., Alzheimer's disease and the basal forebrain cholinergic system: relations to beta-amyloid peptides, cognition, and treatment strategies. *Progress in neurobiology* **2002,** *68* (3), 209-45.

210. Kurz, A.; Perneczky, R., Novel insights for the treatment of Alzheimer's disease. *Progress in neuro-psychopharmacology & biological psychiatry* **2011,** *35* (2), 373-9.

211. Hardy, J.; Selkoe, D. J., The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **2002,** *297* (5580), 353-6.

212. Priller, C.; Bauer, T.; Mitteregger, G.; Krebs, B.; Kretzschmar, H. A.; Herms, J., Synapse formation and function is modulated by the amyloid precursor protein. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2006,** *26* (27), 7212-21.

213. Stockley, J. H.; O'Neill, C., The proteins BACE1 and BACE2 and beta-secretase activity in normal and Alzheimer's disease brain. *Biochemical Society transactions* **2007,** *35* (Pt 3), 574-6.

214. Roth, A. D.; Ramirez, G.; Alarcon, R.; Von Bernhardi, R., Oligodendrocytes damage in Alzheimer's disease: beta amyloid toxicity and inflammation. *Biological research* **2005,** *38* (4), 381-7.

215. Meyer-Luehmann, M.; Spires-Jones, T. L.; Prada, C.; Garcia-Alloza, M.; de Calignon, A.; Rozkalne, A.; Koenigsknecht-Talboo, J.; Holtzman, D. M.; Bacskai, B. J.; Hyman, B. T., Rapid appearance and local toxicity of amyloid-beta plaques in a mouse model of Alzheimer's disease. *Nature* **2008,** *451* (7179), 720-4.

216. Brion, J. P., The role of neurofibrillary tangles in Alzheimer disease. *Acta neurologica Belgica* **1998,** *98* (2), 165-74.

217. Brouwers, N.; Sleegers, K.; Engelborghs, S.; Bogaerts, V.; Serneels, S.; Kamali, K.; Corsmit, E.; De Leenheir, E.; Martin, J. J.; De Deyn, P. P.; Van Broeckhoven, C.; Theuns, J., Genetic risk and transcriptional variability of amyloid precursor protein in Alzheimer's disease. *Brain : a journal of neurology* **2006,** *129* (Pt 11), 2984-91.

218. Chouliaras, L.; Rutten, B. P.; Kenis, G.; Peerbooms, O.; Visser, P. J.; Verhey, F.; van Os, J.; Steinbusch, H. W.; van den Hove, D. L., Epigenetic regulation in the pathophysiology of Alzheimer's disease. *Progress in neurobiology* **2010,** *90* (4), 498-510.

219. Daviglus, M. L.; Bell, C. C.; Berrettini, W.; Bowen, P. E.; Connolly, E. S., Jr.; Cox, N. J.; Dunbar-Jacob, J. M.; Granieri, E. C.; Hunt, G.; McGarry, K.; Patel, D.; Potosky, A. L.; Sanders-Bush, E.; Silberberg, D.; Trevisan, M., NIH state-of-the-science conference statement: Preventing Alzheimer's disease and cognitive decline. *NIH consensus and state-of-the-science statements* **2010,** *27* (4), 1-30.

220. Grundke-Iqbal, I.; Iqbal, K.; Tung, Y. C.; Quinlan, M.; Wisniewski, H. M.; Binder, L. I., Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences of the United States of America* **1986,** *83* (13), 4913-7.

221. Defina, P. A.; Moser, R. S.; Glenn, M.; Lichtenstein, J. D.; Fellus, J., Alzheimer's disease clinical and research update for health care practitioners. *Journal of aging research* **2013,** *2013*, 207178.

222. Duthey, B., Update on 2004 background paper, BP 6.11 Alzheimer disease and other dementias. *A Public Health Approach to Innovation* **2013**, pp. 1-74.

223. Minati, L.; Grisoli, M.; Bruzzone, M. G., MR spectroscopy, functional MRI, and diffusion-tensor imaging in the aging brain: a conceptual review. *Journal of geriatric psychiatry and neurology* **2007,** *20* (1), 3-21.

224. Dubois, B.; Feldman, H. H.; Jacova, C.; Dekosky, S. T.; Barberger-Gateau, P.; Cummings, J.; Delacourte, A.; Galasko, D.; Gauthier, S.; Jicha, G.; Meguro, K.; O'Brien, J.; Pasquier, F.; Robert, P.; Rossor, M.; Salloway, S.; Stern, Y.; Visser, P. J.; Scheltens, P., Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria. *The Lancet. Neurology* **2007,** *6* (8), 734-46.

225. Matsuda, H., Role of neuroimaging in Alzheimer's disease, with emphasis on brain perfusion SPECT. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **2007,** *48* (8), 1289-300.

226. Chetelat, G.; Desgranges, B.; de la Sayette, V.; Viader, F.; Eustache, F.; Baron, J. C., Mild cognitive impairment: Can FDG-PET predict who is to rapidly convert to Alzheimer's disease? *Neurology* **2003,** *60* (8), 1374-7.

227. Warren, J. D.; Schott, J. M.; Fox, N. C.; Thom, M.; Revesz, T.; Holton, J. L.; Scaravilli, F.; Thomas, D. G.; Plant, G. T.; Rudge, P.; Rossor, M. N., Brain biopsy in dementia. *Brain : a journal of neurology* **2005,** *128* (Pt 9), 2016-25.

228. Kurz, A.; Perneczky, R., Novel insights for the treatment of Alzheimer's disease. *Progress in neuro-psychopharmacology & biological psychiatry* **2011,** *35* (2), 373-9.

229. Williams, M. M.; Storandt, M.; Roe, C. M.; Morris, J. C., Progression of Alzheimer's disease as measured by Clinical Dementia Rating Sum of Boxes scores. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2013,** *9* (1 Suppl), S39-44.

230. Mapstone, M.; Cheema, A. K.; Fiandaca, M. S.; Zhong, X. G.; Mhyre, T. R.; MacArthur, L. H.; Hall, W. J.; Fisher, S. G.; Peterson, D. R.; Haley, J. M.; Nazar, M. D.; Rich, S. A.; Berlau, D. J.; Peltz, C. B.; Tan, M. T.; Kawas, C. H.; Federoff, H. J., Plasma phospholipids identify antecedent memory impairment in older adults. *Nature medicine* **2014**, *20*, 415-418.

231. Doecke, J. D.; Laws, S. M.; Faux, N. G.; Wilson, W.; Burnham, S. C.; Lam, C. P.; Mondal, A.; Bedo, J.; Bush, A. I.; Brown, B.; De Ruyck, K.; Ellis, K. A.; Fowler, C.; Gupta, V. B.; Head, R.; Macaulay, S. L.; Pertile, K.; Rowe, C. C.; Rembach, A.; Rodrigues, M.; Rumble, R.; Szoeke, C.; Taddei, K.; Taddei, T.; Trounson, B.; Ames, D.; Masters, C. L.; Martins, R. N.; Alzheimer's Disease Neuroimaging, I.; Australian Imaging, B.; Lifestyle Research, G., Blood-based protein biomarkers for diagnosis of Alzheimer disease. *Archives of neurology* **2012,** *69* (10), 1318-25.

232. Ray, S.; Britschgi, M.; Herbert, C.; Takeda-Uchimura, Y.; Boxer, A.; Blennow, K.; Friedman, L. F.; Galasko, D. R.; Jutel, M.; Karydas, A.; Kaye, J. A.; Leszek, J.; Miller, B. L.; Minthon, L.; Quinn, J. F.; Rabinovici, G. D.; Robinson, W. H.; Sabbagh, M. N.; So, Y. T.; Sparks, D. L.; Tabaton, M.; Tinklenberg, J.; Yesavage, J. A.; Tibshirani, R.; Wyss-Coray, T., Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. *Nature medicine* **2007,** *13* (11), 1359-62.

233. Tsai, A.; Malek-Ahmadi, M.; Kahlon, V.; Sabbagh, M. N., Differences in Cerebrospinal Fluid Biomarkers between Clinically Diagnosed Idiopathic Normal Pressure Hydrocephalus and Alzheimer's Disease. *Journal of Alzheimer's disease & Parkinsonism* **2014,** *4* (4).

234. An, M.; Gao, Y., Urinary Biomarkers of Brain Diseases. *Genomics, proteomics & bioinformatics* **2015,** *13* (6), 345-54.

235. Geekiyanage, H.; Jicha, G. A.; Nelson, P. T.; Chan, C., Blood serum miRNA: non-invasive biomarkers for Alzheimer's disease. *Experimental neurology* **2012,** *235* (2), 491-6.

236. Steinerman, J. R.; Honig, L. S., Laboratory biomarkers in Alzheimer's disease. *Current neurology and neuroscience reports* **2007,** *7* (5), 381-7.

237. Humpel, C., Identifying and validating biomarkers for Alzheimer's disease. *Trends in biotechnology* **2011,** *29* (1), 26-32.

238. Hampel, H.; Frank, R.; Broich, K.; Teipel, S. J.; Katz, R. G.; Hardy, J.; Herholz, K.; Bokde, A. L.; Jessen, F.; Hoessler, Y. C.; Sanhai, W. R.; Zetterberg, H.; Woodcock, J.; Blennow, K., Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives. *Nature reviews. Drug discovery* **2010,** *9* (7), 560-74.

239. Rosen, C.; Hansson, O.; Blennow, K.; Zetterberg, H., Fluid biomarkers in Alzheimer's disease - current concepts. *Molecular neurodegeneration* **2013,** *8*, 20.

240. Chintamaneni, M.; Bhaskar, M., Biomarkers in Alzheimer's disease: a review. *ISRN pharmacology* **2012,** *2012*, 984786.

241. Snyder, H. M.; Carrillo, M. C.; Grodstein, F.; Henriksen, K.; Jeromin, A.; Lovestone, S.; Mielke, M. M.; O'Bryant, S.; Sarasa, M.; Sjogren, M.; Soares, H.; Teeling, J.; Trushina, E.; Ward, M.; West, T.; Bain, L. J.; Shineman, D. W.; Weiner, M.; Fillit, H. M., Developing novel blood-based biomarkers for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2014,** *10* (1), 109-14.

242. Weinstein, G.; Seshadri, S., Circulating biomarkers that predict incident dementia. *Alzheimer's research & therapy* **2014,** *6* (1), 6.

243. Zeisler, H.; Llurba, E.; Chantraine, F.; Vatish, M.; Staff, A. C.; Sennstrom, M.; Olovsson, M.; Brennecke, S. P.; Stepan, H.; Allegranza, D.; Dilba, P.; Schoedl, M.; Hund, M.; Verlohren, S., Predictive Value of the sFlt-1:PlGF Ratio in Women with Suspected Preeclampsia. *The New England journal of medicine* **2016,** *374* (1), 13-22.

244. Regan, C. L.; Levine, R. J.; Baird, D. D.; Ewell, M. G.; Martz, K. L.; Sibai, B. M.; Rokach, J.; Lawson, J. A.; Fitzgerald, G. A., No evidence for lipid peroxidation in severe preeclampsia. *American journal of obstetrics and gynecology* **2001,** *185* (3), 572-8.

245. Spinnato, J. A., 2nd; Freire, S.; Pinto, E. S. J. L.; Cunha Rudge, M. V.; Martins-Costa, S.; Koch, M. A.; Goco, N.; Santos Cde, B.; Cecatti, J. G.; Costa, R.; Ramos, J. G.; Moss, N.; Sibai, B. M., Antioxidant therapy to prevent preeclampsia: a randomized controlled trial. *Obstetrics and gynecology* **2007,** *110* (6), 1311-8.

246. Valdes, R., Jr.; Graves, S. W., Protein binding of endogenous digoxin-immunoactive factors in human serum and its variation with clinical condition. *The Journal of clinical endocrinology and metabolism* **1985,** *60* (6), 1135-43.

247. Roher, A. E.; Maarouf, C. L.; Kokjohn, T. A.; Belden, C.; Serrano, G.; Sabbagh, M. S.; Beach, T. G., Chemical and neuropathological analyses of an Alzheimer's disease patient treated with solanezumab. *American journal of neurodegenerative disease* **2016,** *5* (4), 158-170.

248. Fuchs, B.; Suss, R.; Teuber, K.; Eibisch, M.; Schiller, J., Lipid analysis by thin-layer chromatography--a review of the current state. *Journal of chromatography. A* **2011,** *1218* (19), 2754-74.

249. Christie, W. W.; Dobson, G., Thin-layer chromatography-revisited. *Lipid technology* **1999**, *11*, 64-66.

250. Paglia, G.; Ifa, D. R.; Wu, C.; Corso, G.; Cooks, R. G., Desorption electrospray ionization mass spectrometry analysis of lipids after two-dimensional high-performance thin-layer chromatography partial separation. *Analytical chemistry* **2010,** *82* (5), 1744-50.

251. Goto-Inoue, N.; Hayasaka, T.; Taki, T.; Gonzalez, T. V.; Setou, M., A new lipidomics approach by thin-layer chromatography-blot-matrix-assisted laser desorption/ionization imaging mass spectrometry for analyzing detailed patterns of phospholipid molecular species. *Journal of chromatography. A* **2009,** *1216* (42), 7096-101.

252. Li, L.; Han, J.; Wang, Z.; Liu, J.; Wei, J.; Xiong, S.; Zhao, Z., Mass spectrometry methodology in lipid analysis. *International journal of molecular sciences* **2014,** *15* (6), 10492-507.

253. Kofeler, H. C.; Fauland, A.; Rechberger, G. N.; Trotzmuller, M., Mass spectrometry based lipidomics: an overview of technological platforms. *Metabolites* **2012,** *2* (1), 19-38.

254. Smith, R.; Mathis, A. D.; Ventura, D.; Prince, J. T., Proteomics, lipidomics, metabolomics: a mass spectrometry tutorial from a computer scientist's point of view. *BMC bioinformatics* **2014,** *15 Suppl 7*, S9.

255. Hartler, J.; Tharakan, R.; Kofeler, H. C.; Graham, D. R.; Thallinger, G. G., Bioinformatics tools and challenges in structural analysis of lipidomics MS/MS data. *Briefings in bioinformatics* **2013,** *14* (3), 375-90.

256. Fukuhara, K.; Ohno, A.; Ota, Y.; Senoo, Y.; Maekawa, K.; Okuda, H.; Kurihara, M.; Okuno, A.; Niida, S.; Saito, Y.; Takikawa, O., NMR-based metabolomics of urine in a mouse model of Alzheimer's disease: identification of oxidative stress biomarkers. *Journal of clinical biochemistry and nutrition* **2013,** *52* (2), 133-8.

257. Peng, J.; Guo, K.; Xia, J.; Zhou, J.; Yang, J.; Westaway, D.; Wishart, D. S.; Li, L., Development of isotope labeling liquid chromatography mass spectrometry for mouse urine metabolomics: quantitative metabolomic study of transgenic mice related to Alzheimer's disease. *Journal of proteome research* **2014,** *13* (10), 4457-69.

258. Koran, M. E. I.; Wagener, M.; Hohman, T. J.; Alzheimer's Neuroimaging, I., Sex differences in the association between AD biomarkers and cognitive decline. *Brain imaging and behavior* **2017,** *11* (1), 205-213.

259. Luders, E.; Gaser, C.; Narr, K. L.; Toga, A. W., Why sex matters: brain size independent differences in gray matter distributions between men and women. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2009,** *29* (45), 14265-70.

260. Hua, X.; Hibar, D. P.; Lee, S.; Toga, A. W.; Jack, C. R., Jr.; Weiner, M. W.; Thompson, P. M.; Alzheimer's Disease Neuroimaging, I., Sex and age differences in atrophic rates: an ADNI study with n=1368 MRI scans. *Neurobiology of aging* **2010,** *31* (8), 1463-80.

261. Tapiola, T.; Alafuzoff, I.; Herukka, S. K.; Parkkinen, L.; Hartikainen, P.; Soininen, H.; Pirttila, T., Cerebrospinal fluid {beta}-amyloid 42 and tau proteins as biomarkers of Alzheimer-type pathologic changes in the brain. *Archives of neurology* **2009,** *66* (3), 382-9.

262. Apostolova, L. G.; Dinov, I. D.; Dutton, R. A.; Hayashi, K. M.; Toga, A. W.; Cummings, J. L.; Thompson, P. M., 3D comparison of hippocampal atrophy in amnestic

mild cognitive impairment and Alzheimer's disease. *Brain : a journal of neurology* **2006,** *129* (Pt 11), 2867-73.

263. George, E. M.; Bidwell, G. L., STOX1: a new player in preeclampsia? *Hypertension* **2013,** *61* (3), 561-3.

264. Herrup, K, The case for rejecting the amyloid cascade hypothesis. *Nature Neuroscience* **2015**, *18*, 794-799.

Chapter 2 Lipidomic Profile Of Placenta Tissues In Response To Endogenous Digitalis Like Factor (EDLF) Exposure

2.1 Abstract

The exact pathogenesis of PE remains enigmatic despite substantial research and many confidently proposed theories. Endogenous inhibitors of the Na^+/K^+ -ATPase or sodium pump (SP) have been associated with hypertension and proteinuria and have also been suggested to contribute to the pathogenesis of preeclampsia (PE). These endogenous SP inhibitors are often referred to as digitalis-like factors (EDLF) because chemical members of the digitalis family are known SP inhibitors. Of these, the most often studied EDLF is ouabain. Maternal obesity is a consistent risk factor for PE and altered lipid metabolism is thought to play an important role in the development of PE. We set out to study whether normal human placenta responded to SP inhibition caused by EDLF with a change in abundance of biomolecules, especially lipids, in the placental cytosol, and if there were such changes, whether there was a characteristic set of lipid changes that could serve as signature for EDLF exposure. Placenta tissues from 20 normal pregnancies were incubated in the presence and absence of ouabain, followed by homogenization, lipid extraction and the study of the extracted lipids by a mass spectrometry-based lipidomics approach. Of the 1207 lipidomic markers surveyed by paired Student t-test, 26 markers were found to have significantly different abundances between cases and control at a false discovery rate (FDR) of 0.05. Using a statistical model built with a sparse partial least squares discriminant analysis method (sPLS-DA) and a bootstrap procedure, a set of 8 lipidomic markers was chosen and subsequently identified or chemically characterized with tandem MS.

This set of lipid markers may then be used to identify placental tissues that have previously been exposed to EDLF with excellent cross-validation performance.

2.2 Introduction

Preeclampsia (PE) is a multisystem disorder that complicates approximately ~5% of all pregnancies. It is characterized by hypertension and proteinuria after 20 weeks gestation. Complications associated with PE include, but are not limited to stroke, liver failure, renal failure, coagulation disorders, neurologic complications, including eclampsia (grand mal seizure), and the HELLP (Hemolysis, Elevated Liver enzymes, and Low Platelet count) syndrome in the mother and fetal growth restriction/small for gestational age babies[9]. PE is also a risk factor of cardiovascular diseases later in the mother's life and also increases risk of several diseases including stroke in adult life for children born to preeclamptic mothers[10]. Though the exact pathophysiology of PE is still incompletely understood, the placenta is thought by most to be the most important organ in its pathogenesis[11, 12]. It is generally believed that the principal cause of PE is abnormal placentation. Cytotrophoblasts (from the placenta) fail to adequately invade and remodel the uterine spiral arteries, leading to the continued presence of low-flow, high-resistance vessels, thus the supply of maternal blood to the fetoplacental unit is inadequate, resulting in underperfusion, hypoxia of the placenta and possibly of the fetus[10]. A central characteristic of PE is the imbalance between proangiogenic factors (VEGF, PlGF, TGF-β and surface endoglin) and antiangiogenic factors (sFlt-1 and sEng) in many of these women. In addition, oxidative stress, EDLF, the immune system, and genetics have been associated with the pathogenesis of PE[11].

DLFs (digitalis-like factors) are cardiac glycosides that consist of plant-derived cardenolides (e.g. ouabain, digoxin and digitalis) and animal-originated bufadienolides (e.g. marinobufagenin and proscillaridin A). Digitalis and related compounds are used to treat congestive heart failure because they specifically inhibit the Na^+/K^+ -ATPase or sodium pump, leading ultimately to enhanced myocardial contractility[13] and better cardiac output. EDLFs appear to cause similar effects but are produced endogenously rather than administered as medications. Increased concentrations of EDLF have been associated with a number of diseases, especially hypertension, but also renal disease, and PE. Significantly higher levels of EDLF have been detected in PE placental homogenates, sera from preeeclamptic women and cord blood from neonates born to women with PE compared with controls[1, 2, 14]. Evidence has suggested that EDLF potentially contributes to the pathogenesis of PE, that the potential source of EDLF is the placenta[1, 15], and that the EDLF detected is structurally similar to ouabain or bufalin[16]. It is believed that the release of EDLF is an attempt on the part of the mother to improve fetal-placental perfusion, but if it persists, it also unfortunately leads to hypertension. EDLF has been associated with a number of abnormalities in PE such as hypoxia, oxidative stress, pro-inflammatory cytokines as well[1].

Lipids are biomolecules that are insoluble in water but soluble in non-polar organic solvents. Lipids have been divided into eight major categories, with further divisions into classes and subclasses. The lipidome refers to the complete collection of chemically distinct lipids within a cell, tissue, organ or a biological system. The functions of lipids vary from membrane formation and energy storage to cell signaling and protein regulation. Lipidomics is defined as the full characterization of lipid molecular species and their biological roles using variable analytical methods such as high-performance liquid chromatography (HPLC) and mass

spectrometry (MS). The link between maternal obesity and PE has been well documented[19, 20]. Altered lipid metabolism is believed to play a key role in the pathogenesis of PE. For example, low-density lipoproteins (LDL) and triglycerides have been suggested to be involved in glomerular lesions which are associated with proteinuria[17]. Upregulated processes in PE such as inflammation and oxidative stress have been associated with higher levels of sphingolipids (SM)[18] and glycerophosphoserine (PS) in serum of women with PE[19].

Despite the belief that EDLF may contribute to the pathogenesis of PE, its exact mechanism still remains unknown, compounded by the fact that the pathophysiology of PE is complex[10-12]. Therefore, we aim to investigate the biochemical signature of placental lipid changes in response to EDLF exposure employing a global lipidomics approach. This is the first attempt to study the lipidomic profile of placenta tissues in response to SP inhibition. No such studies have been reported previously in the literature. Following incubation of placental tissues (20 normal placentas) in the presence or absence of ouabain, a proposed EDLF, lipids were extracted from tissue homogenates and then the extract directly injected into a mass spectrometer for identification and characterization purposes. We hypothesized that the presence of EDLF will lead to specific lipidomic changes in placental tissues, and if there are such changes, that the lipid markers revealed will be identified or chemically characterized using tandem MS. These findings can in turn facilitate the identification of placentas that have previously been exposed to SP inhibition, and furthermore lead to better understanding of the complex pathophysiology of PE.

2.3 Materials and methods

2.3.1 Tissue collection

Lipidomic analysis of placental tissue was the focus of this study. To this end, application was made and Institutional Review Boards at Intermountain Healthcare and Brigham Young University approved this study. No personal or medical information about patients was provided. Twenty placentas were obtained immediately after uncomplicated vaginal or Cesarean section deliveries of healthy individuals. Four to five pieces of tissue (~ 15 mm × 5 mm × 5 mm) taken midway between umbilical cord and edge of placenta were cut off from the intervillous region on the fetal side after fetal membranes were removed. The tissue pieces were then kept on ice and processed within twenty minutes.

2.3.2 Sample processing

The placental tissues were cut into small pieces (~3mm) with sterilized scissors. All visible blood clots and vessels were removed with sterilized tweezers as thoroughly as possible. Then the tissue pieces were washed repeatedly (5+ times) with ice-cold, sterile phosphate-buffered saline (PBS) until all visible blood was removed. The placental tissues were patted dry on a sterilized paper towel. All tissue samples were processed on ice to maintain minimal proteolysis.

Placental tissues (~300mg/well) were incubated in 4mL of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO) for 48 hours at 37°C in an incubator gassed with 5% CO_2, in the absence or presence of ouabain (final concentration 50nM). The culture medium was changed at 24 hrs.

Immediately after incubation, placental explants were removed from their wells and patted dry on sterilized paper towels and further processed separately thereafter. Each placental explant was placed in a 5 mL stainless steel ball mill cylinder (Mikro-dismembrator, Sartorius Stedim, Bohemia, NY) along with ~12 stainless steel balls with the addition of 1.5 mL ice-cold 1X PBS, 20 µL of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 20 µL of 8.87 mM 1, 10-phenanthroline (metallopeptidase inhibitor). This was followed by submersion of the entire cylinder in liquid nitrogen for 4 minutes to allow for thorough freezing of the contents in the cylinder. The cylinder was then placed in the ball mill dismembrator and shaken at 2000 rpm for ~12 minutes. The placental homogenate was transferred to a 15 mL conical tube with the addition of 1.5 mL ice-cold 1X PBS. Next, the sample mixture was vortexed thoroughly and centrifuged for 30 minutes at 4000 rpm (Sorvall RT7 Centrifuge, Thermo Scientific, Waltham, MA) at 4°C. The supernatant was collected, aliquotted and stored at −80°C until further processing and assay[1-4].

The following method of lipid extraction has been found to generate the greatest number of MS features with highest signal to noise ratio[5]. To a glass stoppered, glass vessel 3.6 mL of hexane and isopropanol (3:2, v:v) and 600 µL of 0.5 M KH_2PO_4 were added to 400 µL of homogenate (supernatant) for lipid extraction. Hexane is normally used for the extraction of lipids with low polarity, while isopropanol is better suited to extract lipids that have both polar and non-polar regions. After being vortexed vigorously, the mixture was agitated on a motorized shaker for 1 hr at 80 rpm at room temperature. Then, 1 mL of water was added to the samples for better separation of the organic phase from aqueous phase, followed by centrifugation for 10 min at 2000 rpm. The upper organic phase was collected, dried under nitrogen, and redissolved in 200 µL of chloroform : methanol (3:1) to prevent oxidative degradation. The combination of the

polar solvent methanol and the non-polar solvent chloroform is usually used for lipid storage. The lipid extracts were stored at −80°C until mass spectrometric analysis[5, 6].

2.3.3 Mass spectrometric analysis

Prior to analysis 46 μL of chloroform, 92 μL of methanol, and 28 μL of 100 mM $CH_3CO_2NH_4$ were added to 40 μL of lipid extract. These samples then were injected directly into a mass spectrometer (6230 TOF LC/MS Agilent Technologies, Santa Clara, CA) through an electrospray nebulizer at the flow rate of 10 μL/min for 4 min using a syringe pump. The mass spectrometer (MS) was operated in the positive ion mode. The capillary voltage was at 3500 V. The MS data was collected at the acquisition rate of 1 spectrum/sec from mass to charge ratios (m/z) of 100 to 3000. The drying gas flow rate was set to 5 L/min, and the pressure of the nebulizer gas was 1.03 bar. A total ion current (TIC) chromatogram from m/z 100 to 3000 was generated for each sample and used to extract a MS spectrum, from which a peak list consisting of the m/z of all peaks and their relevant abundances was exported. The TIC counts were calculated to normalize all peaks in each MS spectrum. The MassHunter Qualitative Analysis software (Agilent Technologies, Santa Clara, CA) was used for data analysis.

2.3.4 Statistical analysis

A one tailed Student t-test was used to calculate p-values from the normalized abundances for all monoisotopic peaks. Any peak which showed a significant difference between control and case at a p-value smaller than 0.05 was viewed as a candidate marker. In other words, the set of candidate markers that changed with ouabain incubation will be considered as a biochemical signature for human placentas exposed to EDLF. The percentage of p-values below

0.05 was obtained using the binomial exact method, assuming all the t-tests are independent. In addition, a permutation test was applied for the same calculations. The false discovery rate (FDR) approach was employed to correct for the multiple comparisons made.

We built a statistical model utilizing the sPLS-DA method for the selection of candidate markers as well. Parameter optimization was achieved through cross-validation. The bootstrap procedure was then performed to evaluate the stability regarding the detection of potential markers for EDLF exposure in placenta. Last but not least, leave-one-out validation was applied to estimate the predictive performance of the statistical model.

2.3.5 Identification and chemical characterization of lipids by tandem MS

The candidate markers selected by the statistical model were chemically characterized by tandem MS employing a fragmentation step (6530 Q-TOF MS, Agilent Technologies, Santa Clara, CA). The samples were directly injected at a flow rate of 10 μL/min for at least 4 min into the MS run in the positive ion mode. The capillary voltage was 3500 V. The targeted MS/MS (seg) was applied for the fragmentation of the charged markers using collision-induced dissociation (CID). The isolation mass window was set to narrow (~1.3 m/z). The collision energies for each candidate marker were optimized to allow best fragmentation coverage. The MS/MS spectra were collected at the mass range of 50-1000 m/z, at the acquisition rate of 1 spectra/s.

Exact mass studies were employed to determine the elemental composition of lipids of interest, using an Elemental Composition Calculator and externally added, internal standards having known masses, i.e. m/z 121.0509 and 922.0098. The LIPID MAPS Structure Database (LMSD) was utilized for the prediction of the formula and main class and subclass. The

METLIN Database (The Scripps Research Institute) was used to identify all possible fragments and neutral losses derived from the fragmentation of the parent ion of interest. The MS/MS data was exported and submitted to the following MS/MS databases for possible identification purposes: The LIPID MAPS, METLIN, HMDB (Human Metabolome Database), MassBank (High Quality Mass Spectral Database) using spectrum search, mzCloud (Advanced Mass Spectral Database). Online resources such as Lipidbank, Madison Metabolomics Consortium Database (MMCD), Lipidblast (the m/z lookup function) were used in the analyses as well. In addition, fragmentation patterns and typical fragments of various lipid classes were compared manually with the MS/MS literature data to facilitate chemical characterization of lipids of interest.

2.4 Results

2.4.1 Discovery of candidate lipid markers

Of the 1207 candidate lipidomic markers surveyed by a paired Student t-test, 106 candidate markers were discovered having p-values below 0.05. With a 95% confidence interval of (0.0725, 0.105), the percentage of markers with $p<0.05$ was calculated to be 0.0878 using the binomial exact method, which indicates that there are significantly more significant candidate markers ($p=4.59\times10^{-8}$) than we expected. Using the permutation test, the number of candidate markers was found to be marginally significantly higher ($p=0.098$) than we expected. Then we proceeded to apply the FDR correction and set the cutoff to 0.05. After correction, 26 markers were found to have significantly different abundances between case and control at the FDR=0.05 level (Table 2.1).

Table 2.1 Lipid biomarkers that have significant differences between case and control at the FDR=0.05 level

Markers (m/z)	P-value	Higher in
133.1	6.05e-05	control
137.0944	1.05e-04	control
146.0687	9.14e-04	control
151.1107	3.82e-05	control
163.111	3.50e-04	control
165.0901	2.00e-04	control
165.1226	5.65e-04	control
168.1386	2.56e-09	case
179.1059	6.57e-05	control
186.1462	8.40e-09	case
193.157	2.90e-04	control
196.1333	1.62e-04	control
201.0878	1.42e-04	control
217.1579	2.89e-04	control
219.1734	6.69e-04	control
235.1683	2.53e-04	control
258.1578	8.16e-04	control
264.1693	4.21e-04	control
286.1529	2.40e-04	control
311.2554	7.33e-04	case
316.1001	1.38e-06	control
368.2784	3.52e-04	control
373.2333	1.01e-03	control
409.308	2.24e-04	control
441.3297	6.88e-04	control
787.4522	8.42e-04	control

Four markers comprised the optimized statistical model built using the sPLS-DA method with cross-validation (highlighted in yellow in Table 2.2). The stability of the selection of markers was evaluated utilizing a bootstrap procedure. Among 500 repetitions of the bootstrap, 8 markers appeared more than 25% of the time (Table 2.2). And all 8 markers have significant differences between case and control at the FDR=0.05 level except m/z 189.1621. This indicates that the 8 markers can be considered part of a biochemical signature of human placentas that have been exposed to EDLF (using ouabain as both an EDLF candidate and a known sodium pump inhibitor), though it is possible that m/z 189.1621 provides no information regarding the effect of EDLF on placenta. Using a leave-one-out cross validation, the panel of markers making up the statistical model was evaluated for its predictive ability on future data. Both the ROC curve (Figure 2.1) and the performance estimates (Table 2.3) showed that the performance of the model is statistically significantly better than a random model bearing no useful information.

2.4.2 Identification and Chemical characterization of lipids

It has repeated been suggested by laboratory, animal and clinical experiments that higher levels of EDLF contribute to the pathogenesis of PE and there is evidence that the source of EDLF production is human placenta. Therefore, the investigation of the biochemical signature of placentas exposed to EDLF will likely shed light on the involvement of EDLF in the development of PE. In this study we examined the lipidomic profile of placentas that had been exposed to EDLF employing a global lipidomics strategy. In addition, the discovery of such a lipidomic profile will facilitate the identification of placental tissues that have previously had exposure to EDLF.

Our lipidomic analysis of 20 cases and 20 control placentas yielded 106 candidate

Table 2.2 List of lipid biomarkers that can serve as a biochemical signature indicative of EDLF exposure in human placenta. (The 8 markers were selected using a bootstrap procedure, markers highlighted in yellow were chosen using a sPLS-DA method and cross-validation.)

Marker	Proportion of Times
168.1386	0.960
179.1059	0.269
186.1462	0.852
189.1621	0.289
217.1579	0.301
235.1683	0.347
316.1001	0.473
441.3297	0.265

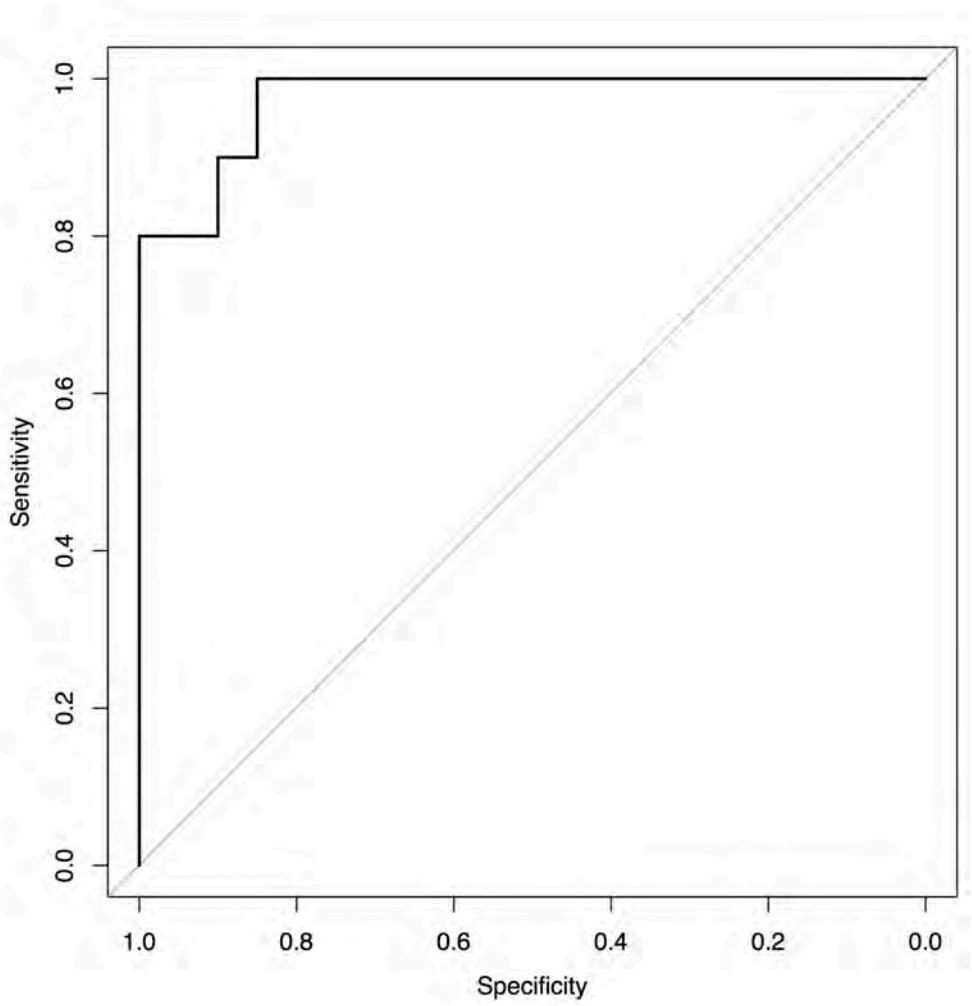

Figure 2.1 ROC Curve of the lipidomic profile indicative of EDLF exposure in human placenta obtained using a leave-one-out cross validation

Table 2.3 Performance estimates of the lipidomic profile indicative of EDLF exposure in human placenta

	Estimate	LB	UB
AUC	0.975	0.925	1
Classification	0.925	0.825	1
Sensitivity	1	1	1
Specificity	0.850	0.696	1

markers that had statistically significant abundance differences. Using a statistical model built through the bootstrap procedure, 8 lipid markers were shown to have much higher performance and provided a biochemical profile of placentas that had been exposed to EDLF. Furthermore, the same list of selected markers showed statistically significantly better predicted performance in its discriminatory ability in future studies. The charge state of all 8 markers was +1, and they were identified or chemically characterized by tandem MS. Searches on LIPID MAPS, METLIN and HMDB using mass and charge usually yielded a large number of isobaric molecules for each of the 8 markers. However, none of them has either the same or a closely similar fragmentation pattern, i.e. MS^2 spectrum. This is not surprising given the very limited archive of lipids available in all current lipid databases. Given that none of the MS^2 spectra generated in this study for the 8 best biomarkers closely resembled archived MS^2 spectra in databases (such as METLIN, HMDB, massbank and mzCloud), none of the 8 compounds could be identified unambiguously. Both the accurate masses of prominent peaks and their intensity ratios in the MS/MS spectra need to be close to enable identification[7]. The 'fragment search' and 'neutral loss search' options[7, 8] on METLIN helped us predict the elemental compositions (Table 2.4) and possible structures (data not shown) of all 8 markers based on available ESI-MS/MS spectra in the database. Consequently, chemical characterization of the markers allowed us to gain insight into the structural compositions of the selected lipid markers but without confirmed final structures.

Database searching and exact mass study suggested that the marker at m/z 168.138 was an ammoniated precursor peak that has an elemental composition of $C_{10}H_{18}NO^+$. It may be a fatty alcohol, fatty aldehyde or isoprenoid. The mass difference between m/z 168.1361 and 150.0291 indicated the loss of H_2O suggesting the possible presence of an –OH group. By the

Table 2.4 Predicted elemental compositions of fragments and neutral losses using METLIN

Lipid marker (m/z)	Collision energy (CE)	Fragment (m/z)	Neutral loss (m/z)
168.1386	10	117.07 ($C_7H_{11}O^+$), 123.11 ($C_9H_{15}^+$)	45.0222 (CH_3NO), 57.058 (C_3H_7N)
	15	43.05 ($C_3H_7^+$), 54.03 ($C_3H_4N^+$), 67.05 ($C_5H_7^+$), 82.07 ($C_5H_8N^+$), 97.05 ($C_5H_7NO^+$), 108.04 ($C_6H_6NO^+$)	28.0268 (C_2H_4), 69.0226 (C_3H_3NO), 82.0423 (C_5H_6O), 86.0635 (C_4H_8NO), 101.0856 ($C_5H_{11}NO$), 114.1053 ($C_7H_{14}O$), 125.0813 ($C_7H_{11}NO$)
	20	41.03 ($C_3H_5^+$), 53.03 ($C_3H_3N^+$), 81.06 ($C_5H_7N^+$), 98.06 ($C_5H_8NO^+$), 108.08 ($C_7H_{10}N^+$)	58.0398 (C_3H_6O), 60.0489 (C_2H_6NO), 70.0764 (C_5H_{10}), 87.066 (C_4H_9NO), 115.1006 ($C_6H_{13}NO$), 127.0947 ($C_7H_{13}NO$)
	25	142.06 ($C_{10}H_8N^+$ or $C_{11}H_{10}^+$), 153.06 ($C_{11}H_7N^+$)	
	40	55.0172 ($C_3H_3O^+$), 78.04 ($C_5H_4N^+$ or $C_6H_6^+$), 82.026 ($C_4H_4NO^+$), 110.0558 ($C_6H_8NO^+$), 130.06 ($C_9H_8N^+$)	58.0822 (C_4H_{10}), 86.1116 (C_6H_{14}), 113.1218 ($C_7H_{15}N$)
179.1059	10	43.0182 ($C_2H_3O^+$), 91.0535 ($C_7H_7^+$), 105.0689 ($C_8H_9^+$), 121.0625 ($C_8H_9O^+$), 133.0995 ($C_{10}H_{13}^+$), 137.094 ($C_9H_{13}O^+$), 151.1092 ($C_{10}H_{15}O^+$), 161.0939 ($C_{11}H_{13}O^+$)	18.0097 (H_2O), 27.9944 (CO), 28.0306 (C_2H_4), 30.009 (CH2O), 30.0467 (C_2H_6), 40.0314 (C_3H_4), 42.01 (C_2H_2O), 42.046 (C_3H_6), 46.0045 (CH_2O_2), 46.0403 (C_2H_6O), 56.025 (C_3H_4O), 58.0415 (C_3H_6O), 60.0557 (C_3H_8O), 70.0404 (C_4H_6O), 74.0351 ($C_3H_6O_2$), 78.0443 (C_6H_6), 108.091 (C_8H_{12}), 118.0757 (C_9H_{10}), 136.0858 ($C_9H_{12}O$)
	20	55.0547 ($C_4H_7^+$), 59.0485 ($C_3H_7O^+$), 67.0548 ($C_5H_7^+$), 77.0382 ($C_6H_5^+$), 146.07 ($C_{10}H_{10}O^+$)	16.0299 (CH_4), 41.0013 (C_2HO), 44.025 (C_2H_4O), 55.0165 (C_3H_3O), 69.0318 (C_4H_5O), 106.0385 (C_7H_6O), 124.0483 ($C_7H_8O_2$)
	40	39.0249 ($C_3H_3^+$), 51.0236 ($C_4H_3^+$), 65.0389 ($C_5H_5^+$), 103.0528 ($C_8H_7^+$), 115.0531 ($C_9H_7^+$), 117.0677 ($C_9H_9^+$), 128.061 ($C_{10}H_8^+$), 145.0609 ($C_{10}H_9O^+$)	16.9999 (OH), 38.0139 (C_3H_2), 62.0363 ($C_2H_6O_2$), 63.0221 (C_5H_3), 74.0139 (C_6H_2), 76.0512 ($C_3H_8O_2$), 77.0374 (C_6H_5), 80.022 (C_5H_4O), 89.0361 (C_7H_5), 94.0373 (C_6H_6O), 114.0651 ($C_6H_{10}O_2$), 128.0804 ($C_7H_{12}O_2$), 140.0791 ($C_8H_{12}O_2$)
186.1462	10	85.0257 ($C_4H_5O_2^+$), 100.1111 ($C_6H_{14}N^+$), 104.0614 ($C_8H_8^+$), 123.0683 ($C_7H_9NO^+$), 140.0664 ($C_7H_{10}NO_2^+$)	16.9981 (HO), 32.0242 (CH_4O), 44.0204 (C_2H_4O), 55.0407 (C_3H_5N), 63.0273 (C_5H_3), 68.0252 (C_4H_4O), 80.0254 (C_5H_4O), 82.0825 (C_6H_{10}), 86.0328 ($C_4H_6O_2$), 94.0787 (C_7H_{10}), 101.1182 ($C_6H_{15}N$), 108.0953 (C_8H_{12}),

Lipid marker (m/z)	Collision energy (CE)	Fragment (m/z)	Neutral loss (m/z)
186.1462	10		126.1029 ($C_8H_{14}O$)
	15	43.0544 ($C_3H_7^+$), 55.0547 ($C_4H_7^+$), 62.0595 ($C_2H_8NO^+$), 71.0519 ($C_4H_7O^+$), 80.0484 ($C_5H_6N^+$), 91.0542 ($C_7H_7^+$), 110.0599 ($C_6H_8NO^+$), 137.099 ($C_9H_{13}O^+$)	27.9975 (CO), 30.0115 (CH_2O), 31.0374 (CH_5N), 46.0448 (C_2H_6O), 58.0765 (C_4H_{10}), 55.0052 (C_2HNO), 66.0471 (C_5H_6), 67.0055 (C_3HNO), 82.0443 (C_5H_6O), 88.088 ($C_5H_{12}O$), 94.0446 (C_6H_6O), 97.0845 ($C_6H_{11}N$), 106.0769 (C_8H_{10}), 113.0817 ($C_6H_{11}NO$), 115.0958 ($C_6H_{13}NO$), 124.0882 ($C_8H_{12}O$), 125.082 ($C_7H_{11}NO$), 131.093 ($C_6H_{13}NO_2$), 143.0933 ($C_7H_{13}NO_2$)
	20	30.0341 (CH_4N^+), 41.0412 ($C_3H_5^+$), 67.0552 ($C_5H_7^+$), 131.0757 ($C_9H_9N^+$)	27.0139 (CHN), 31.0055 (HNO), 44.0104 (CH_2NO), 57.0195 (C_2H_3NO), 63.0206 (C_5H_3), 68.0266 (C_4H_4O), 72.0491 (C_3H_6NO), 74.0277 ($C_2H_4NO_2$), 81.0315 (C_5H_5O), 85.054 (C_4H_7NO), 90.0345 (C_6H_4N), 101.0416 ($C_4H_7NO_2$), 153.0806 ($C_8H_{11}NO_2$)
	25	78.0459 ($C_6H_6^+$), 124.0741 ($C_7H_{10}NO^+$)	
	40	56.0485 ($C_3H_6N^+$), 68.0519 ($C_4H_6N^+$), 130.0587 ($C_9H_8N^+$)	26.0133 (C_2H_2), 63.021 (C_5H_3)
	50	51.027 ($C_4H_3^+$), 56.0268 ($C_3H_4O^+$), 82.0308 ($C_4H_4NO^+$), 103.0557 ($C_8H_7^+$)	
189.1621	10	45.0369 ($C_2H_5O^+$), 55.0566 ($C_4H_7^+$), 69.0674 ($C_5H_9^+$), 95.0835 ($C_7H_{11}^+$), 121.0622 ($C_8H_9O^+$), 133.0998 ($C_{10}H_{13}^+$), 147.1139 ($C_{11}H_{15}^+$ or $C_6H_{15}N_2O_2^+$), 161.0687 ($C_9H_9N_2O^+$ or $C_{10}H_9O_2^+$), 171.1045 ($C_8H_{15}N_2O_2^+$)	17.0027 (OH), 26.0161 (C_2H_2), 29.0403 (C_2H_5), 38.0163 (C_3H_2), 42.0458 (C_3H_6), 43.0544 (C_3H_7), 52.0304 (C_4H_4), 56.0599 (C_4H_8), 59.0226 (CH_3N_2O), 78.0432 (C_6H_6), 88.0629 ($C_3H_8N_2O$), 92.0573 (C_7H_8), 94.0762 (C_7H_{10}), 102.0371 (C_7H_4N, or $C_3H_6N_2O_2$), 102.077 ($C_4H_{10}N_2O$), 116.0479 (C_8H_6N), 120.0923 (C_9H_{12}), 126.0676 ($C_7H_{10}O_2$), 134.1031 ($C_{10}H_{14}$), 144.1228 ($C_7H_{16}N_2O$, or $C_8H_{16}O_2$)

Lipid marker (m/z)	Collision energy (CE)	Fragment (m/z)	Neutral loss (m/z)
189.1621	20	41.0418 ($C_3H_5^+$), 77.0381 ($C_6H_5^+$), 91.0533 ($C_7H_7^+$ or $C_2H_7N_2O_2^+$), 119.0867 ($C_9H_{11}^+$, or $C_5H_{13}NO_2^+$, or $C_4H_{11}N_2O_2^+$), 147.0805 ($C_{10}H_{11}O^+$)	27.9938 (CO), 28.0334 (C_2H_4), 42.0486 (C_3H_6), 56.0272 (C_3H_4O), 70.0424 (C_4H_6O), 70.0641 (C_4H_8N), 106.0387 (C_7H_6O), 112.1127 ($C_7H_{14}N$)
	25	67.0551 ($C_5H_7^+$), 78.0448 ($C_6H_6^+$), 159.1167 ($C_{12}H_{15}^+$, or $C_7H_{15}N_2O_2^+$)	30.0484 (C_2H_6), 92.0616 (C_7H_8), 111.1203 (C_8H_{15}), 122.11 (C_9H_{14})
	40	65.0371 ($C_5H_5^+$), 103.053 ($C_8H_7^+$, or $C_3H_7N_2O_2^+$), 117.067 ($C_9H_9^+$, or $C_4H_9N_2O_2^+$)	38.0159 (C_3H_2), 52.0299 (C_4H_4)
	50	53.0383 ($C_4H_5^+$), 115.053 ($C_9H_7^+$, or $C_4H_7N_2O_2^+$), 128.0605 ($C_{10}H_8^+$, or $C_5H_8N_2O_2^+$), 158.073 ($C_{11}H_{10}O^+$)	30.0125 (CH_2O), 43.02 (C_2H_3O), 75.0222 (C_6H_3), 105.0347 (C_7H_5O)
	60	51.0251 ($C_4H_3^+$), 102.0467 ($C_8H_6^+$, or $C_3H_6N_2O_2^+$)	111.1135 (C_8H_{15})
217.1579	10	119.082 ($C_9H_{11}^+$, or $C_7H_{12}Na^+$), 147.114 ($C_{11}H_{15}^+$, or $C_9H_{16}Na^+$), 157.0994 ($C_{12}H_{13}^+$), 175.1097 ($C_{12}H_{15}O^+$), 189.1604 ($C_{14}H_{21}^+$), 199.1081 ($C_{14}H_{15}O^+$)	18.0103 (H_2O), 27.9957 (CO), 28.032 (C_2H_4), 42.0087 (C_2H_2O), 42.0464 (C_3H_6), 56.0277 (C_3H_4O), 70.043 (C_4H_6O), 70.0784 (C_5H_{10}), 80.0261 (C_5H_4O), 98.075 ($C_6H_{10}O$)
	20	57.0698 ($C_4H_9^+$), 69.0697 ($C_5H_9^+$), 83.0477 ($C_5H_7O^+$, or $C_3H_8ONa^+$), 133.0985 ($C_{10}H_{13}^+$, or $C_8H_{14}Na^+$), 156.0907 ($C_{12}H_{12}^+$), 187.1058 ($C_{13}H_{15}O^+$)	15.027 (CH_3), 30.0512 (C_2H_6), 31.0151 (CH_3O), 46.0421 (C_2H_6O), 69.0343 (C_4H_5O), 76.0287 (C_6H_4), 84.0585 (C_5H_8O), 104.0581 (C_8H_8), 118.0361 (C_8H_6O), 119.0851 (C_9H_{11}), 130.036 (C_9H_6O), 133.0631 (C_9H_9O), 134.1093 ($C_{10}H_{14}$), 148.0873 ($C_{10}H_{12}O$), 160. 0872 ($C_{11}H_{12}O$)
	30	45.0342 ($C_2H_5O^+$), 55.0536 ($C_4H_7^+$), 91.0532 ($C_7H_7^+$, or $C_5H_8Na^+$), 105.068 ($C_8H_9^+$, or $C_6H_{10}Na^+$), 129.0673 ($C_8H_{10}Na^+$, or $C_{10}H_9^+$), 141.069 ($C_9H_{10}Na^+$, or $C_{11}H_9^+$), 159.115 ($C_{12}H_{15}^+$, or $C_{10}H_{16}Na^+$)	30.0477 (C_2H_6), 38.0141 (C_3H_2), 50.0144 (C_4H_2), 54.047 (C_4H_6), 58.042 (C_3H_6O), 68.0618 (C_5H_8), 74.0137 (C_6H_2), 88.0897 ($C_5H_{12}O$), 104.0614 (C_8H_8), 112.089 ($C_7H_{12}O$), 126.1038 ($C_8H_{14}O$), 162.1034 ($C_{11}H_{14}O$)
	40	67.0543 ($C_5H_7^+$, or $C_3H_8Na^+$), 79.0537 ($C_6H_7^+$, or $C_4H_8Na^+$), 117.0658 ($C_9H_9^+$, or $C_7H_{10}Na^+$), 155.087 ($C_{10}H_{12}Na^+$, or $C_{12}H_{11}^+$), 169.0972 ($C_{11}H_{14}Na^+$, $C_{13}H_{13}^+$)	52.0314 (C_4H_4), 76.0333 (C_6H_4), 88.0327 (C_7H_4), 90.0435 (C_7H_6), 100.0912 ($C_6H_{12}O$), 102.0429 (C_8H_6), 138.1033 ($C_9H_{14}O$), 150.1027 ($C_{10}H_{14}O$)
	50	65.0389 ($C_5H_5^+$), 77.0378 ($C_6H_5^+$, or $C_4H_6Na^+$), 115.052 ($C_9H_7^+$, or $C_7H_8Na^+$), 154.0747 ($C_{12}H_{10}^+$)	17.0051 (OH), 38.0142 (C_3H_2), 39.0227 (C_3H_3), 56.0278 (C_3H_4O), 77.0369 (C_6H_5), 89.0358 (C_7H_5), 94.042 (C_6H_6O), 102.105 ($C_6H_{14}O$),

Lipid marker (m/z)	Collision energy (CE)	Fragment (m/z)	Neutral loss (m/z)
217.1579	50		106.0409 (C_7H_6O), 140. 1192 ($C_9H_{16}O$), 152.1181 ($C_{10}H_{16}O$)
	60	51.0239 ($C_4H_3^+$), 128.0602 ($C_{10}H_8^+$, or $C_8H_9Na^+$), 165.0707 ($C_{13}H_9^+$)	77.0363 (C_6H_5), 166.1331 ($C_{11}H_{18}O$)
235.1683	10	57.0701 ($C_4H_9^+$), 109.0996 ($C_8H_{13}^+$, or $C_6H_{14}Na^+$), 123.1145 ($C_9H_{15}^+$, or $C_7H_{16}Na^+$), 137.0938 ($C_9H_{13}O^+$, or $C_7H_{14}ONa^+$), 149.1298 ($C_{11}H_{17}^+$, or $C_9H_{18}Na^+$), 151. 1097 ($C_{10}H_{15}O^+$, or $C_8H_{16}ONa^+$), 161.0933 ($C_{11}H_{13}O^+$, or $C_9H_{14}ONa^+$), 179.1039 ($C_{11}H_{15}O_2^+$), 189.1607 ($C_{14}H_{21}^+$), 217.1547 ($C_{15}H_{21}O^+$)	18.0108 (H_2O), 27.9942 (C=O), 40.0309 (C_3H_4), 42.0101 (C_2H_2O), 46.0048 (CH_2O_2), 52.0295 (C_4H_4), 56.0614 (C_4H_8), 66.045 (C_5H_6), 68.0249 (C_4H_4O), 74.0722 ($C_4H_{10}O$), 80.0237 (C_5H_4O), 80.0609 (C_6H_8), 84.0558 (C_5H_8O), 86.0357 ($C_4H_6O_2$), 98.0717 ($C_6H_{10}O$)
	20	43.0181 ($C_2H_3O^+$), 57.0704 ($C_4H_9^+$), 67.0544 ($C_5H_7^+$, or $C_3H_8Na^+$), 81.0694 ($C_6H_9^+$, or $C_4H_{10}Na^+$), 95.0833 ($C_7H_{11}^+$, or $C_5H_{12}Na^+$), 109.1000 ($C_8H_{13}^+$, or $C_6H_{14}Na^+$), 133.0994 ($C_{10}H_{13}^+$, or $C_8H_{14}Na^+$), 151.1085 ($C_{10}H_{15}O^+$, or $C_8H_{16}ONa^+$), 161.0931 ($C_{11}H_{13}O^+$, or $C_9H_{14}ONa^+$), 179.1023 ($C_{11}H_{15}O_2^+$), 189.1597 ($C_{14}H_{21}^+$), 217.1565 ($C_{15}H_{21}O^+$)	15.0252 (CH_3), 18.0091 (H_2O), 27.9937 (CO), 28.0289 (C_2H_4), 32.025 (CH_4O), 38.0141 (C_3H_2), 41.0382 (C_3H_5), 42.0085 (C_2H_2O), 42.0456 (C_3H_6), 46.0029 (CH_2O_2), 52.03 (C_4H_4), 56.0252 (C_3H_4O), 56.0603 (C_4H_8), 60.0188 ($C_2H_5O_2$), 66.045 (C_5H_6), 69.0319 (C_4H_5O), 70.0023 ($C_3H_2O_2$), 70.0391 (C_4H_6O), 70.0762 (C_5H_{10}), 74.0745 ($C_4H_{10}O$), 76.029 (C_6H_4), 80.0237 (C_5H_4O), 80.0597 (C_6H_8), 83.0478 (C_5H_7O), 84.019 ($C_4H_4O_2$), 84.0541 (C_5H_8O), 93.0313 (C_6H_5O), 94.0387 (C_6H_6O), 94.0764 (C_7H_{10}), 98.0329 ($C_5H_6O_2$), 98.073 ($C_6H_{10}O$), 107.048 (C_7H_7O), 108.0565 (C_7H_8O), 108.0903 (C_8H_{12}), 112.0479 ($C_6H_8O_2$), 116.0841 ($C_6H_{12}O_2$), 118.075 (C_9H_{10}), 122.0319 ($C_7H_6O_2$), 122.0732 ($C_8H_{10}O$), 122.1053 (C_9H_{14}), 126.0676 ($C_7H_{10}O_2$), 132.0893 ($C_{10}H_{12}$), 136.0871 ($C_9H_{12}O$), 140.0843 ($C_8H_{12}O_2$), 145.0609 ($C_{10}H_9O$), 150.1021 ($C_{10}H_{14}O$), 154.0982 ($C_9H_{14}O_2$), 160.0861 ($C_{11}H_{12}O$), 168.1132 ($C_{10}H_{16}O_2$), 174.1384 ($C_{13}H_{18}$), 178.0972 ($C_{11}H_{14}O_2$), 192.1495 ($C_{13}H_{20}O$)

Lipid marker (m/z)	Collision energy (CE)	Fragment (m/z)	Neutral loss (m/z)
235.1683	25	43.0183 ($C_2H_3O^+$), 57.0703 ($C_4H_9^+$), 67.0542 ($C_5H_7^+$, or $C_3H_8Na^+$), 81.0688 ($C_6H_9^+$, or $C_4H_{10}Na^+$), 93.0678 ($C_7H_9^+$, or $C_5H_{10}Na^+$), 109.0992 ($C_8H_{13}^+$, or $C_6H_{14}Na^+$), 119.0833 ($C_9H_{11}^+$, or $C_7H_{12}Na^+$), 133.0996 ($C_{10}H_{13}^+$, or $C_8H_{14}Na^+$), 147.1138 ($C_{11}H_{15}^+$, or $C_9H_{16}Na^+$), 161.0929 ($C_{11}H_{13}O^+$, or $C_9H_{14}ONa^+$), 187.1103 ($C_{13}H_{15}O^+$)	15.0207 (CH_3), 26.0136 (C_2H_2), 28.0305 (C_2H_4), 38.0146 (C_3H_2), 54.0107 (C_3H_2O), 54.046 (C_4H_6), 55.0172 (C_3H_3O), 66.045 (C_5H_6), 68.0251 (C_4H_4O), 80.0596 (C_6H_8), 88.0537 ($C_4H_8O_2$), 90.0435 (C_7H_6), 94.0425 (C_6H_6O), 106.0415 (C_7H_6O), 120.0561 (C_8H_8O), 130.04 (C_9H_6O), 142.0997 ($C_8H_{14}O_2$)
	30	91.0536 ($C_7H_7^+$, or $C_5H_8Na^+$), 105.0689 ($C_8H_9^+$, or $C_6H_{10}Na^+$)	28.029 (C_2H_4), 38.0145 (C_3H_2), 42.0443 (C_3H_6), 56.0241 (C_3H_4O), 56.0609 (C_4H_8), 70.0394 (C_4H_6O), 82.0395 (C_5H_6O), 96.0548 (C_6H_8O)
	40	41.0392 ($C_3H_5^+$), 79.0534 ($C_6H_7^+$, or $C_4H_8Na^+$), 91.0543 ($C_7H_7^+$, or $C_5H_8Na^+$), 159.1134 ($C_{12}H_{15}^+$, or $C_{10}H_{16}Na^+$), 187.1065 ($C_{13}H_{15}O^+$), 205.0986 ($C_{16}H_{13}^+$)	16.0312 (CH_4), 26.0148 (C_2H_2), 27.9931 (CO), 40.0291 (C_3H_4), 68.0222 (C_4H_4O), 76.0536 ($C_3H_8O_2$), 102.0684 ($C_5H_{10}O_2$), 116.0827 ($C_6H_{12}O_2$)
316.1001	10	57.0334 ($C_3H_5O^+$), 75.0432 ($C_3H_7O_2^+$), 85.0631 ($C_5H_9O^+$), 201.0861 ($C_{13}H_{13}O_2^+$), 299.0957 ($C_{17}H_{15}O_5^+$)	17.0006 (OH), 18.0098 (H_2O), 28.0297 (C_2H_4), 144.0527 ($C_{10}H_8O$), 176.0142 ($C_9H_4O_4$), 197.006 ($C_8H_5O_6$), 224.0525 ($C_{14}H_8O_3$), 242.0623 ($C_{14}H_{10}O_4$)
	20	43.0194 ($C_2H_3O^+$), 298.0834 ($C_{17}H_{14}O_5^+$)	18.0148 (H_2O)
	40	91.0538 ($C_7H_7^+$), 104.0605 ($C_8H_8^+$), 119.046 ($C_8H_7O^+$)	13.0036 (CH)
	50	55.054 ($C_4H_7^+$), 81.0706 ($C_6H_9^+$), 109.0603 ($C_7H_9O^+$)	18.0069 (H_2O), 26.0166 (C_2H_2), 27.9897 (C=O)
441.3297	10	119.0851 ($C_9H_{11}^+$, or $C_7H_{12}Na^+$), 175.1334 ($C_9H_{19}O_3^+$), 237.1588 ($C_{14}H_{21}O_3^+$, or $C_{18}H_{21}^+$)	60.0237 ($C_2H_4O_2$), 118.0737 (C_9H_{10}), 178.0974 ($C_{11}H_{14}O_2$), 188.1521 ($C_{14}H_{20}$), 204.1712 ($C_{11}H_{24}O_3$), 266.1966 ($C_{16}H_{26}O_3$), 306.2258 ($C_{19}H_{30}O_3$), 322.2449 ($C_{20}H_{34}O_3$), 366.2495 ($C_{25}H_{34}O_2$)
	40	107.0844 ($C_8H_{11}^+$, or $C_6H_{12}Na^+$)	27.9959 (C=O), 48.0241 (CH_4O_2), 60.0249 ($C_2H_4O_2$), 76.02 ($C_2H_4O_3$), 306.2497 ($C_{20}H_{34}O_2$), 322.2448 ($C_{20}H_{34}O_3$), 334.2456 ($C_{21}H_{34}O_3$)

same token, the mass difference between m/z 168.1356 and 140.067 suggested a CO loss. The presence of m/z 53.0345, 67.0531 and 81.0691 suggested methylation or demethylation (a change of 14 in mass) when the charged marker was fragmented using the collision energy (CE) of 20. And the product ion m/z 125.9757 suggested the neutral loss of CH_3CN (acetylation). Likewise, a neutral loss of 59 suggested the loss of trimethylamine.

The elemental composition of the protonated marker at m/z 179.105 was suggested to be $C_{11}H_{15}O_2$+ by exact mass study. It can be a fatty alcohol, fatty ester, fatty aldehyde or fatty acid. For example, m/z 41, 91, 105 and 161 are usually observed in the MS spectra of fatty esters. The product ion at m/z 143.0829 suggested the loss of two water molecules at the collision energy of 10. At CE 20, the neutral loss of 78.0446 suggested the presence of C_6H_6. The combination of the product ions at m/z 91 and 77 indicated that this marker might be an aromatic compound.

The species at m/z 186.145 was predicted to be an ammoniated fatty acid, fatty aldehyde, fatty ester or isoprenoid with the elemental composition of $C_{10}H_{20}NO_2^+$. The lipid mass spectral prediction tool suggested this marker to be ammoniated FA (10:2) or ammoniated FA (10:1)-cyclo. The presence of the product peaks at m/z 140, 154, and 168 using the CE of 10 indicated that those fragments are likely to be alkenes and/or cycloalkanes. A water loss of 18.0076 was observed in the same fragmentation spectrum.

The charged lipid marker with m/z 189.16 may be an amino fatty acid with the elemental composition of $C_9H_{21}N_2O_2^+$. The precursor ion is probably protonated $[M+H]^+$, although MMCD suggested it might also be ammoniated $[M+NH_4]^+$ or a molecular ion M^+. The predicted elemental composition of all the detected fragments and neutral losses at various CE are listed in Table 4. The peaks at m/z 41, 55, 69 and 83 in the fragmentation spectrum at CE 20 suggested the presence of cycloalkenes. Likewise, the detected product ions at m/z 77 and 91 observed in

the fragmentation spectra of various CE indicated that the marker might be an aromatic compound.

Exact mass studies suggested that the elemental composition of the precursor ion with m/z 217.15 is likely to be either $C_{15}H_{21}O^+$ (protonated) or $C_{13}H_{22}ONa^+$ (sodiated). It may be a fatty acid, or isoprenoid. The peaks with m/z 141, 155 and 169 were predicted to be alkanes according to the their typical fragmentation patterns, and the peaks with m/z 77 and 91 in the MS/MS spectra using a CE of 50 suggested that the precursor ion might be aromatic.

The marker at m/z 235.167 had a mass consistent with the elemental composition of $C_{15}H_{23}O_2^+$ ($[M+H]^+$) or $C_{13}H_{24}O_2Na^+$ ($[M+Na]^+$). It was predicted to be a fatty acid, fatty ester or isoprenoid. The m/z 189.1607 and m/z 161.0923 were obtained from m/z 217.1547 by successively losing CO. The product ions with m/z 43 and 57 are usually observed in the fragmentation pattern of alkanes. The detection of m/z 67, 81, 95 and 109 suggested that alkynes were possibly present.

The marker with m/z 316.098 was predicted to have the elemental composition of $C_{17}H_{16}O_6^+$. It may be a molecular ion or a protonated precursor ion. The peak at m/z 299.09 indicated the loss of a hydroxyl group from the precursor ion. The presence of fragment ions at m/z 43, 57, 70, and 85 suggested that those peaks were alkane fragments. Similarly, the presence of ions at m/z 91 and 77 suggested an aromatic precursor ion. The fragment loss of m/z 71 may be due to the loss of neutral species such as C_5H_{11}. The observed neutral loss of 59 suggested the loss of trimethylamine.

The elemental composition of m/z 441.3 was proposed to be either $C_{29}H_{45}O_3^+$ (protonated) or $C_{27}H_{46}O_3Na^+$ (sodiated) based on database searching and exact mass studies. The mass is consistent with its being a sterol, a secosteroid or bile acid or its derivative. The mass

difference between m/z 441.3 and 425.3 suggested the loss of NH_2 or that hydroxylation or epoxidation occurred during fragmentation. The possible loss of trimethylamine was also observed. The Fragment Search feature on METLIN and Peak Search feature on Massbank yielded a great number of possible identifications for two prominent peaks (m/z 59 and 119) observed on the fragmentation spectra, but the marker remains unidentified.

2.5 Discussion

EDLF has been shown to be an inhibitor of Na, K^+-ATPase (the sodium pump, SP). Increased levels of EDLF have been associated with the pathogenesis of essential hypertension, experimental hypertension and PE[1, 2]. SP inhibition leads to vascular smooth muscle contraction and later if sustained, hypertension. Evidence has also shown a potential link between EDLF and abnormal renal function[2]. It is believed that EDLF may derive from placenta and contribute to the pathogenesis of PE. Statistically significantly increased production and release of EDLF from placenta tissues have been reported under the conditions of hypoxia, oxidative stress (H_2O_2) or the presence of proinflammatory cytokines such as TNF-α, all of which are typical abnormalities seen with PE[1]. Digibind, the Fab fragment that can cross-react with and inactivate EDLF, significantly reduced blood pressure in animal models of hypertension. It has also been demonstrated that Digibind can reduce SP inhibition caused by serum or placental homogenates from preeclamptic pregnancies[2]. A phase II clinical trial of Digibind treatment for women with severe PE greatly reduced SP inhibition and was associated with better maternal and neonatal outcomes[32, 33].

This study was conducted to test the following hypotheses: Firstly, whether normal human placenta responded to SP inhibition caused by EDLF with a change in the abundance of

lipids in the cytosol; and secondly, if there were such changes, whether there was a characteristic set or pattern of responses. Therefore, we incubated placental tissues from 20 normal pregnancies in the presence and absence of EDLF, and then studied the biochemical profile of the placenta tissues using mass spectrometry-based lipidomics method. Indeed, changes in the abundance of lipids in the cytosol were observed. Of the 1207 lipidomic markers surveyed by a paired Student t-test, 26 markers showed statistically significantly different abundances between cases and controls at the false discovery rate of 0.05 (Table 2.1). Using a statistical model built with the sPLS-DA method and the bootstrap procedure, a set of 8 lipidomic markers was chosen (Table 2.2). All eight markers were then identified or chemically characterized using tandem MS (Table 2.4). This set of 8 markers can in return, facilitate the identification of placentas that have previously been exposed to EDLF. The ROC curve of the cross-validated predictions (Figure 2.1) and the performance estimates of this set of lipid markers (Table 2.3) were shown as well.

Lipids play important roles in various cell signaling mechanisms and in the composition of membrane receptors and ion channels. Modified lipid composition in cells can result in severe pathologies. Maternal obesity is considered a potential risk factor for PE[22]. The link between obesity and PE may partly be due to higher levels of pro-inflammatory cytokines triggered by adipose tissue. In addition, the important roles that lipids play in various signaling pathways further relates impaired lipid metabolism to PE[20]. Lipid-laden macrophages have been reported in the arterial lesions at the site of uteroplacental implantation in women with PE. The term "glomerular endotheliosis" describes lipid deposits observed in the renal glomeruli in preeclamptic women. Additionally, the endothelial dysfunction and insufficient trophoblast invasion in PE may result from altered lipid metabolism[17]. It has been suggested that certain classes of lipids are more strongly associated than others with the development of PE[22].

Lipidomics, one of the "omics technologies" in systems biology that also includes genomics, transcriptomics, proteomics, metabolomics and epigenetics, combined with platform technologies and bioinformatics, has enabled the identification and quantitation of bioactive lipids in diseased patients and healthy controls[29]. It is expected that the high-throughput global analysis of the lipidome can provide further insights into the complex pathogenesis of PE, despite the extremely complicated structural diversity of lipids[18, 20]. Using direct infusion, shotgun lipidomics as was done here has the advantages of recording mass spectrum at a constant concentration of lipid extracts and avoiding chromatographic abnormalities and time constraints. A pre-selected internal standard per lipid class is usually sufficient for quantitation[38, 39].

Ouabain is among one of the most studied cardenolides and a proposed EDLF. The effect of SP inhibition on normal human placenta using a global technique such as lipidomics has never been reported previously in literature. One study, however, investigated the effect of sub-lethal concentration of ouabain (36nM) on the lipid contents in PC-3 cells (human prostate cancer cell line) using Fourier transform infrared spectroscopy[21]. It was found that the following lipid species were affected by the presence of ouabain: phosphatidylethanolamines (PE), phosphatidylcholines (PC), phosphatidylglycerol (PG), sphingomyelin (SM) and phosphatidylinositol (PI). In our study of PE, a set of 8 lipid markers were revealed and can be used as a potential biochemical signature predictive of placentas that have been exposed to EDLF. Despite the coverage limitation in current available databases, the possible identities and structures (data not shown) of lipid fragments following tandem MS have been proposed. The lipid classes of the selected markers range from fatty acids and other fatty acyl containing compounds to sterol lipids and prenol lipids. It is our hope that as the capacity of current lipid

databases grows and expands, the markers selected in this study can be identified and further investigated functionally.

An altered serum lipidomic profile in PE has been limitedly studied and reported in literature using either a lipidomics or metabolomics approach. Metabolomics usually surveys the known low molecular weight metabolites or breakdown molecules in blood samples following protein precipitation. A two-phase study of serum lipidomic profile predictive of PE revealed 23 different lipid biomarkers, including fatty acids, fatty alcohol and aldehydes, glycerophosphocholines (PC) and triglycerides[5]. Another two-phase study of plasma metabolomic biomarkers predictive of later PE reported 14 metabolites, among them are a series of fatty acids, steroids and phospholipids[23]. It has been suggested that higher levels of proinflammatory cytokines in PE can lead to lipid dysregulation by various distinct mechanisms including stimulating lipolysis and releasing free fatty acids[30]. Decreased levels of PC in serum, for instance, have been associated with altered choline metabolism, which affects angiogenesis and inflammation[31]. In one metabolomics study of PE, significantly higher levels of lipid were found in first-trimester preeclamptic sera, esp. higher levels of triglycerides that were collectively predictive of PE compared to sera from healthy controls[31]. Lipids such as triglycerides and very low density lipoproteins (VLDL) have been linked to endothelial damage secondary to oxidative stress as reported in women with PE[28, 31, 34]. In one study, increased levels of triglycerides and VLDL were detected in serum of women with PE compared with controls, and the levels of both were significantly correlated with the severity of proteinuria[17, 28]. Further, women with PE have also shown to have statistically significantly different levels of total cholesterol (TC), low density lipoprotein (LDL) and high density lipoprotein (HDL) in serum compared with healthy women[37], although conflicting results also exist[17].

In one study of plasma lipid profiles of women with early-onset PE, the lipid biomarkers found and identified belonged to the classes of PC, glycerophosphoserines (PS), glycerophosphoglycerols, glycosyldiadylglycerols, and glycerophosphates[20, 22, 30]. PS, for instance, a major constituent of lipid signal molecules, are involved in enhanced oxidative stress, coagulation and apoptosis in PE, possibly mediated by reactive oxygen species (ROS) derived from placenta. Disturbed lipid metabolism has also been reported in late-onset PE, the metabolites identified include glycerol, carnitine, methylhistidine and acetone, along with fatty acids and members of some other lipid classes[31]. It was suggested that the transport of fatty acids by carnitine is significantly altered in PE and is associated with lipid peroxidation[19]. Sphingolipids have been suggested as key molecules in the pathogenesis of PE as well and may serve as biomarkers. Increased levels of SM have been associated with endothelial dysfunction in patients with PE[20]. Dysregulated sphingolipid profiles leading to ceramide overload, and thus increased trophoblast autophagy, has also been reported in the context of PE[24, 29]. An association between omega-3 and omega-6 fatty acids and PE has been suggested. A Sudanese study of PE investigating omega-3 and omega-6 fatty acids reported increased levels of arachidonic acid (omega-6) in association with sphingomyelin only and eicosapentaenoic and docosahexaenoic acids (both omega-3) in association with phosphatidylethanolamines, phosphatidylcholines and sphingomyelins[25, 35], although conflicting results regarding omega-3 fatty acids have also been found[26, 27]. All of these studies were in serum.

Proposed predictive biomarkers for PE as reported in the literature to date have been discordant among various studies. As such, it is hard to have confidence in them. Some contradictions have been suggested due to different pathophysiologies concerning different PE phenotypes, such as early onset and late onset PE, or mild and severe PE[18]. This of course could

lead to different biomarker profiles if in fact the explanation is true. Therefore, a single screening test may be unable to predict subsequent PE with the required sensitivity, specificity and cost effectiveness. It is also possible that the predictive PE biomarkers described are artificial differences due to the large numbers of species measured.

Somewhat similarly, despite substantial evidence of increased concentrations of EDLF in preeclamptic women, some women with PE do not have detectable levels of EDLF[33]. Clearly, EDLF does not explain all PE although it appears to contribute substantially to the disease when present and antibody therapies that counteract EDLF have been shown to ameliorate both maternal and fetal pathology. With this in mind, it is critical that the EDLF status of women with PE be considered in any investigation concerning the potential roles of EDLF play in the pathogenesis of PE or the allocation of EDLF targeting therapies in the treatment of PE. Our study can serve that purpose. However, unlike proteomics, the analysis of lipidomics MS/MS spectra is challenging due to the high degree of molecular heterogeneity of lipids, the high number of isobaric lipids and their more diverse and complicated fragmentation mass spectra[39]. Additionally, various factors such as the choice of mass spectrometer, the collision energy used, ion mode and the choice of adduct ions to name a few contribute to the tremendous variability and diversity of the MS/MS spectra of lipids[36]. Lipidomics is still at an early stage, and current databases for lipids are substantially incomplete. More challenging still there are only few searchable fragmentation databases with very limited data available (comparing to the total number of lipids in human) that can be used to identify lipids which is very different than what is available for peptides and proteins. Consequently only lipids that have been included in the databases can possibly be identified. Structural MS^2, often done in the positive mode, can provide fragments representing neutral losses and can indicate the presence of specific head

groups, fatty acids or oxidized fatty acids but cannot indicate the points of connection of the pieces. Sometimes more structural information of the selected markers may be obtained through conducting tandem MS experiments in negative ion mode or employing specialized derivatization techiniques. In addition, the lipids comprising the profile discovered in this study should be further investigated for their biological or pathological significance in the context of PE.

In conclusion, normal human placenta appear to respond to SP inhibition caused by EDLF with a generally consistent set of changes in the abundance of lipids in the cytosol of human placenta, and an optimal set of lipid changes indicative of EDLF exposure were discovered using a mass spectrometry based lipidomics method. The selected lipid markers can now be used to identify placentas that have previously been exposed to EDLF. This study should lead to better understanding of the role of sodium pump inhibition in PE pathogenesis, the altered lipid metabolism observed in PE and facilitate the development of future preventive and therapeutic strategies for PE.

2.6 References

1. Ma, J.; Esplin, M. S.; Adair, C. D.; Mason, L. A.; Graves, S. W., Increasing evidence for and regulation of a human placental endogenous digitalis-like factor. *Reproductive sciences* **2012,** *19* (4), 437-48.

2. Hopoate-Sitake, M. L.; Adair, C. D.; Mason, L. A.; Torres, C.; Kipikasa, J.; Graves, S. W., Digibind reverses inhibition of cellular Rb+ uptake caused by endogenous sodium pump inhibitors present in serum and placenta of women with preeclampsia. *Reproductive sciences* **2011,** *18* (2), 190-9.

3. Kedia, K.; Smith, S. F.; Wright, A. H.; Barnes, J. M.; Tolley, H. D.; Esplin, M. S.; Graves, S. W., Global "omics" evaluation of human placental responses to preeclamptic conditions. *American journal of obstetrics and gynecology* **2016,** *215* (2), 238 e1-238 e20.

4. Kedia, K.; Nichols, C. A.; Thulin, C. D.; Graves, S. W., Novel "omics" approach

for study of low-abundance, low-molecular-weight components of a complex biological tissue: regional differences between chorionic and basal plates of the human placenta. *Analytical and bioanalytical chemistry* **2015,** *407* (28), 8543-56.

5. Anand, S.; Young, S.; Esplin, M. S.; Peaden, B.; Tolley, H. D.; Porter, T. F.; Varner, M. W.; D'Alton, M. E.; Jackson, B. J.; Graves, S. W., Detection and confirmation of serum lipid biomarkers for preeclampsia using direct infusion mass spectrometry. *Journal of lipid research* **2016,** *57* (4), 687-96.

6. Anand, S.; Barnes, J. M.; Young, S. A.; Garcia, D. M.; Tolley, H. D.; Kauwe, J. S. K.; Graves, S. W., Discovery and Confirmation of Diagnostic Serum Lipid Biomarkers for Alzheimer's Disease Using Direct Infusion Mass Spectrometry. *Journal of Alzheimer's disease : JAD* **2017,** *59* (1), 277-290.

7. Zhu, Z. J.; Schultz, A. W.; Wang, J.; Johnson, C. H.; Yannone, S. M.; Patti, G. J.; Siuzdak, G., Liquid chromatography quadrupole time-of-flight mass spectrometry characterization of metabolites guided by the METLIN database. *Nature protocols* **2013,** *8* (3), 451-60.

8. Tautenhahn, R.; Cho, K.; Uritboonthai, W.; Zhu, Z.; Patti, G. J.; Siuzdak, G., An accelerated workflow for untargeted metabolomics using the METLIN database. *Nature biotechnology* **2012,** *30* (9), 826-8.

9. Jeyabalan, A., Epidemiology of preeclampsia: impact of obesity. *Nutrition reviews* **2013,** *71 Suppl 1*, S18-25.

10. Noris, M.; Perico, N.; Remuzzi, G., Mechanisms of disease: Pre-eclampsia. *Nature clinical practice. Nephrology* **2005,** *1* (2), 98-114; quiz 120.

11. Powe, C. E.; Levine, R. J.; Karumanchi, S. A., Preeclampsia, a disease of the maternal endothelium: the role of antiangiogenic factors and implications for later cardiovascular disease. *Circulation* **2011,** *123* (24), 2856-69.

12. Young, B. C.; Levine, R. J.; Karumanchi, S. A., Pathogenesis of preeclampsia. *Annual review of pathology* **2010,** *5*, 173-92.

13. Lingrel, J. B., Na,K-ATPase: isoform structure, function, and expression. *Journal of bioenergetics and biomembranes* **1992,** *24* (3), 263-70.

14. Beyers, A. D.; Odendaal, H. J.; Spruyt, L. L.; Parkin, D. P., The possible role of endogenous digitalis-like substance in the causation of pre-eclampsia. *South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde* **1984,** *65* (22), 883-5.

15. Adair, C. D.; Luper, A.; Rose, J. C.; Russell, G.; Veille, J. C.; Buckalew, V. M., The hemodynamic effects of intravenous digoxin-binding fab immunoglobulin in severe preeclampsia: a double-blind, randomized, clinical trial. *Journal of perinatology: official journal of the California Perinatal Association* **2009,** *29* (4), 284-9.

16. Agunanne, E.; Horvat, D.; Uddin, M. N.; Puschett, J., The treatment of preeclampsia in a rat model employing Digibind. *American journal of perinatology* **2010,** *27* (4), 299-305.

17. Lima, V. J.; Andrade, C. R.; Ruschi, G. E.; Sass, N., Serum lipid levels in pregnancies complicated by preeclampsia. *Sao Paulo medical journal = Revista paulista de medicina* **2011,** *129* (2), 73-6.

18. Acharya, A.; Brima, W.; Burugu, S.; Rege, T., Prediction of preeclampsia-bench to bedside. *Current hypertension reports* **2014,** *16* (11), 491.

19. ElDesouki, R.; Habib, F., Preeclampsia at the moleculer level. *Applied medical research* **2015**, *1* (2), 65-72.

20. Korkes, H. A.; Sass, N.; Moron, A. F.; Camara, N. O.; Bonetti, T.; Cerdeira, A. S.; Da Silva, I. D.; De Oliveira, L., Lipidomic assessment of plasma and placenta of women with early-onset preeclampsia. *PloS one* **2014,** *9* (10), e110747.

21. Gasper, R.; Vandenbussche, G.; Goormaghtigh, E., Ouabain-induced modifications of prostate cancer cell lipidome investigated with mass spectrometry and FTIR spectroscopy. *Biochimica et biophysica acta* **2011,** *1808* (3), 597-605.

22. Oliveira, L. D.; Korkes, H.; Turco, E. L.; Bertola, R.; Sass, N.; Bonetti, T.; Moron, A. F.; Silva, I. D., PP098. Lipidic fingerprinting in women with early-onset preeclampsia: A first look. *Pregnancy hypertension* **2012,** *2* (3), 293.

23. Kenny, L. C.; Broadhurst, D. I.; Dunn, W.; Brown, M.; North, R. A.; McCowan, L.; Roberts, C.; Cooper, G. J.; Kell, D. B.; Baker, P. N.; Screening for Pregnancy Endpoints, C., Robust early pregnancy prediction of later preeclampsia using metabolomic biomarkers. *Hypertension* **2010,** *56* (4), 741-9.

24. Melland-Smith, M.; Ermini, L.; Chauvin, S.; Craig-Barnes, H.; Tagliaferro, A.; Todros, T.; Post, M.; Caniggia, I., Disruption of sphingolipid metabolism augments ceramide-induced autophagy in preeclampsia. *Autophagy* **2015,** *11* (4), 653-69.

25. Bakheit, K. H.; Ghebremeskel, K.; Pol, K.; Elbashir, M. I.; Adam, I., Erythrocyte omega-3 and omega-6 fatty acids profile in Sudanese women with pre-eclampsia. *Journal of obstetrics and gynaecology : the Journal of the Institute of Obstetrics and Gynaecology* **2010,** *30* (2), 151-4.

26. Kesmodel, U.; Olsen, S. F.; Salvig, J. D., Marine n-3 fatty acid and calcium intake in relation to pregnancy induced hypertension, intrauterine growth retardation, and preterm delivery. A case-control study. *Acta obstetricia et gynecologica Scandinavica* **1997,** *76* (1), 38-44.

27. Williams, M. A.; Zingheim, R. W.; King, I. B.; Zebelman, A. M., Omega-3 fatty acids in maternal erythrocytes and risk of preeclampsia. *Epidemiology* **1995,** *6* (3), 232-7.

28. Brown, S. H.; Eather, S. R.; Freeman, D. J.; Meyer, B. J.; Mitchell, T. W., A Lipidomic Analysis of Placenta in Preeclampsia: Evidence for Lipid Storage. *PloS one* **2016,** *11* (9), e0163972.

29. Dobierzewska, A.; Soman, S.; Illanes, S. E.; Morris, A. J., Plasma cross-gestational sphingolipidomic analyses reveal potential first trimester biomarkers of preeclampsia. *PloS one* **2017,** *12* (4), e0175118.

30. Baig, S.; Lim, J. Y.; Fernandis, A. Z.; Wenk, M. R.; Kale, A.; Su, L. L.; Biswas, A.; Vasoo, S.; Shui, G.; Choolani, M., Lipidomic analysis of human placental syncytiotrophoblast microvesicles in adverse pregnancy outcomes. *Placenta* **2013,** *34* (5), 436-42.

31. Austdal, M.; Tangeras, L. H.; Skrastad, R. B.; Salvesen, K.; Austgulen, R.; Iversen, A. C.; Bathen, T. F., First Trimester Urine and Serum Metabolomics for Prediction of Preeclampsia and Gestational Hypertension: A Prospective Screening Study. *International journal of molecular sciences* **2015,** *16* (9), 21520-38.

32. Adair, C. D.; Buckalew, V. M.; Graves, S. W.; Lam, G. K.; Johnson, D. D.; Saade, G.; Lewis, D. F.; Robinson, C.; Danoff, T. M.; Chauhan, N.; Hopoate-Sitake, M.; Porter, K. B.; Humphrey, R. G.; Trofatter, K. F.; Amon, E.; Ward, S.; Kennedy, L.; Mason, L.; Johnston, J. A., Digoxin immune fab treatment for severe preeclampsia. *American journal of perinatology* **2010,** *27* (8), 655-62.

33. Lam, G. K.; Hopoate-Sitake, M.; Adair, C. D.; Buckalew, V. M.; Johnson, D. D.; Lewis, D. F.; Robinson, C. J.; Saade, G. R.; Graves, S. W., Digoxin antibody fragment, antigen binding (Fab), treatment of preeclampsia in women with endogenous digitalis-like factor: a secondary analysis of the DEEP Trial. *American journal of obstetrics and gynecology* **2013,** *209* (2), 119 e1-6.

34. Jin, W. Y.; Lin, S. L.; Hou, R. L.; Chen, X. Y.; Han, T.; Jin, Y.; Tang, L.; Zhu, Z. W.; Zhao, Z. Y., Associations between maternal lipid profile and pregnancy complications and perinatal outcomes: a population-based study from China. *BMC pregnancy and childbirth* **2016,** *16*, 60.

35. Das, U. N., Cytokines, angiogenic, and antiangiogenic factors and bioactive lipids in preeclampsia. *Nutrition* **2015,** *31* (9), 1083-95.

36. Hartler, J.; Tharakan, R.; Kofeler, H. C.; Graham, D. R.; Thallinger, G. G., Bioinformatics tools and challenges in structural analysis of lipidomics MS/MS data. *Briefings in bioinformatics* **2013,** *14* (3), 375-90.

37. Spracklen, C. N.; Smith, C. J.; Saftlas, A. F.; Robinson, J. G.; Ryckman, K. K., Maternal hyperlipidemia and the risk of preeclampsia: a meta-analysis. *American journal of epidemiology* **2014,** *180* (4), 346-58.

38. Han, X.; Gross, R. W., Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts

of biological samples. *Mass spectrometry reviews* **2005,** *24* (3), 367-412.

39. Kofeler, H. C.; Fauland, A.; Rechberger, G. N.; Trotzmuller, M., Mass spectrometry based lipidomics: an overview of technological platforms. *Metabolites* **2012,** *2* (1), 19-38.

Chapter 3 Peptidomic Profile of Placenta Tissues In Response To Endogenous Digitalis-Like Factor (EDLF) Exposure

3.1 Abstract

Ouabain, a known endogenous sodium pump (SP) inhibitor and widely studied digitalis-like factor (EDLF), can contribute to hypertension and has been associated with renal dysfunction and maternal endothelial injury, all major features of preeclampsia (PE). The clinical trials that utilized Digibind, a Fab fragment of anti-digoxin antibody that can bind and inactivate EDLF, showed improved maternal and neonatal outcomes of statistical significance in preeclamptic women during either the antepartum or postpartum period. Not only mounting evidence suggests that the placenta plays a central role in the pathogenesis of PE, but the origin of EDLF implicated in the setting of PE is believed to be the placenta. Endogenous peptides and small proteins are essential to most physiological processes and may regulate or contribute to the pathophysiology of various diseases. In this study, we aim to investigate whether specific and consistent changes in the peptidomic profile of normal placental tissues result from SP inhibition caused by ouabain, and if there are such changes, whether a specific set of peptidomic markers can serve as a signature of placental EDLF exposure and be applied in future studies to PE placenta to assess EDLF exposure. In this study, placental tissues from 20 healthy pregnancies were incubated in the presence and absence of ouabain, followed by homogenization, protein precipitation and the study of the peptides using a mass spectrometry-based peptidomics approach. Of the 275 peptidomic markers evaluated by paired Student t-test, 9 candidate markers showed statistically significant abundance differences between cases and controls. A set of 8 markers was found using a logistic regression model built with the Akaike information criterion

(AIC). However, after applying a false discovery rate (FDR) correction or when using more conservative approaches to overcome over-fitting, no peptidomic markers or set of markers showed specific, statistically different changes in abundance between cases and controls. In addition, the potential of peptidomic markers to add value to previously revealed lipidomic profile was explored through an optimal sparse partial least squares discriminant analysis (sPLS-DA), cross-validation studies and logistic regression models. Results suggested that the formerly revealed lipidomic profile was not statistically improved by the addition of any peptidomic marker and hence peptide differences would not likely help define placentas that had been exposed to EDLF.

3.2 Introduction

Preeclampsia (PE) is a multi-system disorder that is estimated to affect 5-8% pregnancies worldwide[9]. The definition of PE has been changed recently and proteinuria is no longer required, according to both the American College of Obstetrics and Gynecology (ACOG) and the International Society for the Study of Hypertension in Pregnancy (ISSHP). The new diagnostic criteria include the presence of de-novo hypertension after 20 weeks of gestation, proteinuria or other maternal organ damage, ranging from neurological complications to uteroplacental dysfunction[9, 10]. PE is associated with various maternal, fetal, and neonatal morbidities and increased mortality[21]. Postpartum preeclamptic women have increased risks of developing cardiovascular disease, renal disease and metabolic disorders later in life[9]. Children born to preeclamptic women are more likely to be afflicted with cerebral palsy, stroke, diabetes mellitus or bronchopulmonary dysplasia[10, 12]. Management of this polymorphic disease involves carefully weighing the benefits of delayed delivery for the fetus against potential maternal complications

resulting from such a delay[12]. The only cure of PE is the delivery of the placenta. Aspirin has been reported to prevent PE in women at risk but has shown no benefit when used in unselected pregnant women. In addition, calcium supplementation can reduce incidences of PE in women with low dietary calcium intake[12]. However, in a large study of calcium supplementation in pregnant women in the U.S., no benefit was observed[48]. Further evaluation of other preventative interventions such as low molecular weight heparin is needed before definite conclusions can be drawn[11].

The placenta is thought to play a central role in the pathophysiology of PE and it is believed that the primary cause of PE is abnormal placentation. A two-stage hypothesis has been proposed: defective remodeling of uterine spiral arteries leads to chronic placental ischemia (stage 1), followed by secretion of soluble factors into the maternal circulation that results in widespread maternal endothelial damage (stage 2)[9, 11]. Hypertension occurs afterwards to compensate for reduced blood flow through uterine arteries[12]. PE has been divided into two categories by some: placental PE vs. maternal PE and early onset PE vs. late onset PE (after 34 weeks of gestation) by others[9]. It is generally believed that different subtypes of PE have different phenotypes and pathophysiology. In early onset or placental PE, abnormal transformation of uterine spiral arteries leads to underperfusion of the placenta. This subtype is usually associated with significantly higher incidences of maternal and fetal complications. In late onset or maternal PE, however, the placenta is mostly normal, maternal endothelial dysfunction instead contributes to generalized vasoconstriction and reduced blood supply to kidney, brain and heart. One aspect of note is that substantial overlap between the two subtypes can exist and parts of both pathologies are present in most preeclamptic women[9, 11]. The exact pathophysiology of PE is still not understood. A multitude of disease pathways have been

suggested to contribute to the clinical onset of PE. For instance, antiangiogenic factors like soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng), the heme oxygenase pathway, the hydrogen sulfide generating system, the nitric oxide pathway, reactive oxygen species, cytokines (TNF-α, IL-10 and INF-γ among many others) and chemokines, several susceptibility genes, autoantibodies to angiotensin receptor 1, the Notch signaling pathway, and misfolded proteins have all been proposed[9, 11, 13, 14].

Endogenous digitalis-like factors (EDLF) are specific sodium pump (SP) inhibitors. EDLF may contribute to elevated blood pressure in essential and experimental hypertension[1]. SP inhibition caused by EDLF can lead to increased intracellular sodium concentration, membrane depolarization and subsequent increased intracellular calcium levels. The latter can result in vascular smooth muscle contraction, generalized vasoconstriction, chronic hypertension, and also reduced uteroplacental perfusion in PE[28, 29, 31]. EDLF has also been associated with renal dysfunction, and possibly maternal pulmonary edema and neonatal intraventricular hemorrhage in severe PE[21]. The exact structure of EDLF remains elusive, but evidence strongly suggests that the EDLF implicated in the setting of PE is structurally similar to cardiac glycosides such as ouabain, bufalin, or marinobufagenin (MBG)[1]. Digibind, the Fab fragment of an anti-digoxin antibody, can cross-react with, bind and thereby inactivate EDLF, and has been shown to reverse SP inhibition induced by cord blood and plasma of women with severe PE[30], increase uteroplacental circulation, and lower blood pressure in women with PE, in patients with essential hypertension, and in animal models of hypertension and PE by binding a hypertensinogenic EDLF[15-18, 20, 28, 31, 33]. The clinical trials that have evaluated the effect of Digibind administered to women with severe PE during the antepartum or the immediate postpartum period showed statistically significantly decreased blood pressure or better maternal and neonatal outcomes in

the treatment group, providing strong evidence that EDLF contributes to major features of women with PE[19-21].

EDLF has been detected in the maternal plasma, placenta, cord blood and fetal blood[21, 23, 24, 28]. However, evidence strongly suggested that the EDLF implicated in PE originates from the placenta, due to higher quantities of EDLF measured in the placenta than found in plasma, rapid EDLF clearance from maternal plasma after delivery of the placenta and the fact that EDLF was present in fetal plasma for a few days following childbirth[19]. A study concerning the production and regulation of EDLF in the setting of PE provided strong evidence for the presence of a placental EDLF, whose biosynthesis may share steps with the isoprenoid and steroid pathways[1]. For example, the addition of ketoconazole, a drug that inhibits steroid synthesis, caused reduced production and release of EDLF from explanted placental tissue in a dose-dependent manner. Conversely, addition of 17α-hydroxyprogesterone, an intermediate in steroid synthesis, statistically significantly increased production and release of EDLF from placental explants in both a dose-dependent and time-dependent manner. In addition, imposition of several of many proposed pathogenic abnormalities present in PE such as hypoxia, oxidative stress and proinflammatory cytokines has been shown to lead to statistically significant higher EDLF levels, sometimes a doubling of EDLF produced and released from placental explant culture[1, 28].

Endogenous peptides have a variety of biological functions and are indispensible to most, if not all, physiological processes[40, 42, 45]. Examples of small native peptides include some hormones, neuropeptides and other signaling molecules. Peptides may also modulate protein-protein interactions in a cellular system. Peptidomics is defined as the characterization of all peptides in a biological sample, for instance, a cell organelle, cell, tissue, or organ. MS-based peptidomics is currently capable of detection and quantitation of many, although not all, peptides

with identification of many post-translational modifications. In peptidomics studies, peptides are usually generated by endogenous proteolytic enzymes, some with unknown identities. Peptidomics is considered more difficult than proteomics, due to the wide range in size, charge states and occasional hydrophobicity of endogenous peptides[40, 42], and the complexity is amplified by various PTMs of peptides such as methylation, glycosylation and phosphorylation. Peptidomic studies usually aim to investigate the endogenous form of peptides and therefore exclude the use of trypsin. This simplifies sample preparation and yet makes interpretation of MS data complex and increases the rate of false positive identifications, especially regarding larger peptides. More stringent and statistically significant criteria for peptide identification are preferred over protein identification because peptide identity is usually derived from a single MS^2 spectrum.

Given the apparent causal role of EDLF in hypertension and evidence for EDLF participation in major features and pathways of PE, it is of great interest to investigate the effect of EDLF exposure on biological samples. Such a study should shed light on the mechanisms by which EDLF contributes to PE specifically and the pathophysiology of PE in general. The placenta remains the most important organ in the pathogenesis of PE due to the substantial overlap between different pathologies of PE in preeclamptic women and the fact that abnormal placentation is thought to be its primary cause. Moreover, evidence strongly suggests that one source of EDLF in PE is the placenta. Therefore, the biological samples that we employed in this study were fresh placental tissues collected and processed immediately after delivery from uncomplicated human pregnancies. Taking into consideration the importance of the peptidome to most physiological processes, we aim to study the peptidomic profile of placental tissues in response to SP inhibition caused by EDLF exposure using a MS-based peptidomics approach.

Placental tissues (20 normal human placentas) were first incubated in the absence or presence of ouabain, a proposed EDLF, followed by peptide extraction from tissue homogenates, fractionation of peptides and small proteins through reverse phase LC and qualitative and quantitative analysis of eluate using a mass spectrometer. We hypothesized that the presence of EDLF will lead to specific peptidomic changes in placental tissues, and if there were such changes, that the peptidomic markers revealed will be identified or chemically characterized using tandem MS. Results from this study may potentially be used to identify placentas that had been previously exposed to SP inhibition.

3.3 Materials and methods

3.3.1 Tissue collection

Institutional Review Boards at Intermountain Healthcare and Brigham Young University approved this study. No personal or medical information about patients was provided. Twenty placentas were obtained immediately after uncomplicated vaginal or Cesarean section deliveries from women having poor fetal presentations, with failure of labor to progress, or due to fetal distress but without evidence of maternal medical problems. Four to five pieces of placenta (~15 mm × 5 mm × 5 mm), taken midway between the umbilical cord and the edge of each placenta were dissected from the intervillous region on the fetal side after removal of fetal membranes. The tissue pieces were then kept on ice and processed within 20 minutes.

3.3.2 Sample processing

The placental pieces were cut into smaller pieces (~3mm) with sterilized scissors. All visible blood clots and vessels were thoroughly removed with sterilized tweezers. All visible blood was removed by repeatedly (5+ times) washing the tissue pieces with ice-cold, sterile PBS before the tissues were patted dry. All samples were processed on ice to minimize proteolysis.

3.3.3 Placental explant culture

Placenta tissues (~300mg/well) were incubated in 4mL of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO) for 24 hours at 37°C in an incubator gassed with 5% CO_2 and 95% O_2 in the absence and presence of ouabain (final concentration 50nM).

3.3.4 Placental tissue homogenization

Immediately after incubation, placental explants were removed from their wells and patted dry on sterilized paper towels and processed individually thereafter. Each placental explant was placed in a 5 mL stainless steel ball mill cylinder along with ~12 stainless steel balls, 1.5 mL ice-cold 1X PBS, 20 µL of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 20 µL of 8.87 mM 1, 10-phenanthroline (a further protease inhibitor). Then the entire cylinder was submerged in liquid nitrogen for 4 minutes, subsequently placed in the ball mill dismembrator (Mikro-dismembrator, Sartorius Stedim, Bohemia, NY) and shaken at 2000 rpm for ~12 minutes. With the addition of 1.5 mL ice-cold 1X PBS, the tissue homogenate was vortexed thoroughly and centrifuged for 30 minutes at 4000 rpm (Sorvall RT7 Centrifuge, Thermo Scientific, Waltham, MA) at 4°C to remove debris. The supernatant was collected, aliquotted and immediately stored at −80°C until further processing and assay[1-3].

3.3.5 Acetonitrile precipitation

In order to remove uninformative, high-abundance, higher-molecular weight (MW) proteins and obtain greater access to low-abundance, lower-MW small proteins and peptides of interest, the placental homogenate was subjected to a modified technique of acetonitrile (ACN) precipitation[3-6]. 400 μL of high performance liquid chromatography (HPLC)-grade ACN was added to 200 μL of placental homogenate. This was followed by vigorous vortexing for ~10 sec and incubation for 30 min at room temperature. Then the mixture was centrifuged (IEC Micromax RF; Thermo Fisher Scientific, Waltham, MA, USA) at 14,000 rpm for 15 min at 4 °C. The supernatant was separated from the pellet and vortexed briefly following the addition of 300 μL of HPLC-grade water. The total volume was reduced to ~200 μL using a vacuum centrifuge (CentriVap Concentrator; Labconco, Kansas City, MO, USA) at 37 °C to remove the organic solvent. Then the apparent protein concentration of each control or case specimen was measured using a Bradford protein assay kit (Bio-Rad laboratories, CA, USA). A volume of each lyophilized sample equivalent to 10 μg of apparent protein was reduced to 5 μL using the vacuum centrifuge, then immediately stored at −80°C until further analysis.

3.3.6 Capillary liquid chromatography separation

We performed capillary liquid chromatography (cLC) using an LC Packings Ultimate Capillary HPLC pump system to more fully separate peptides and small proteins and other smaller biomolecules including lipids in the samples. The cLC system was controlled by Agilent MassHunter Workstation Software (Agilent Technologies, Santa Clara, CA, USA). 5 μL of 88% formic acid (FA) was added to each of the protein-depleted samples previously processed,

making the final volume 10 µL with a final apparent protein concentration of 1 µg/µL. Each acidified sample was then vortexed briefly and injected into the cLC system through a FAMOS autosampler (Dionex, Sunnyvale, CA, USA) kept at 4 °C. The cLC columns contained a 1 mm (16.2 µm) MicroBore guard column (Upchurch Scientific, Oak Harbor, WA, USA) coupled to a 15 cm×250 µm i.d. in-house packed capillary column. The POROS R1 reversed-phase media (Applied Biosystems, Foster City, CA, USA) was used to dry-pack the guard column and slurry-pack the capillary column. The elution gradient employed an aqueous phase (98% HPLC grade H_2O, 2% ACN, 0.1% FA) and an organic phase (2% H_2O, 98% ACN, 0.1% FA). At a flow rate of 5 µL/min, the gradient started with an isocratic hold of 95% aqueous phase and 5% organic phase for 3 min, then it was increased linearly to 60% organic phase in 24 min, followed by a further linear increase to 95% organic phase over the next 7 mins to allow the elution of more hydrophobic biomolecules. The gradient was held at 95% organic phase for the next 7 min to ensure elution of all analytes, and then quickly reduced to 95% aqueous phase and 5% organic phase over 5 min and held at the same concentration for the following 12 min for re-equilibration of the column. A blank run preceded the analysis of each sample to ensure washout and reproducibility.

3.3.7 Mass spectrometric analysis

Eluate from the capillary column was introduced into a quadrupole time-of-flight mass spectrometer (6530 Accurate-Mass Q-TOF LC/MS, Agilent Technologies, Santa Clara, CA, USA) through a Jetstream electrospray ionization (ESI) source. The ESI source was operated in the positive ion mode and the capillary voltage (VCap) was set at 3500 V. The drying gas flow rate was 5 L/min. The gas temperature was set at 300 °C and the nebulizer pressure was 15 psi.

The MS data was acquired over the mass range of m/z 400-3000 at the acquisition rate of 8 spectra/s using Agilent MassHunter Data Acquisition software. The elution profile for each sample was reported as a total ion chromatogram (TIC) and subsequently analyzed using Agilent MassHunter Qualitative Analysis software.

3.3.8 Elution time normalization

In an effort to minimize day-to-day and potentially run-to-run chromatographic variability, internal time standards were selected and used for the normalization of elution time. Eleven 2-min elution windows were defined throughout the usable part of the cLC chromatogram, excluding the void volume of ~16 min and the washout period. Each elution window was defined around a central endogenous peak best meeting criteria for its appropriateness and used for time normalization[7]. Criteria required the peak to be easily distinguished and measured, baseline resolved from neighboring peaks, occurring at the right time (i.e. 1-2 min after the previous marker) and present in untreated and treated samples. If, however, no central peak meeting all the criteria for time standards was found in one specific window, the earlier and later time standards of 2 neighboring windows were utilized instead to normalize data in that elution window. An extracted ion chromatogram (EIC) for each sample was generated from the TIC, followed by the extraction of MS spectra from the EIC (therefore averaging MS spectra) within each 2-min window defined by the selected internal time alignment peaks.

Following time alignment, the extracted MS spectra for both ouabain-exposed and ouabain-free pieces from the same placenta were color coded (treatment versus control) and recorded. Then all 20 sets of results were overlaid and visually inspected. All the peaks that

appeared to show quantitative differences (~1.5x) between treatment and control were considered candidate markers[2, 4] and their respective abundances (as a peak height) were determined and recorded. The abundances of candidate markers were obtained from the EIC individually extracted, based on the mass-to-charge ratio (m/z) of each marker. In addition, one co-eluting peak whose abundance did not change with ouabain exposure was selected from each time window according to previously described criteria[8] and candidate peak abundances were normalized to its abundance[3, 4]. The normalized abundances of potential markers were submitted for subsequent statistical analysis.

3.3.9 Statistical analysis

A two tailed Student t-test was used to evaluate p-values. Any peak that had a p-value below 0.1 was considered a candidate marker that might represent a significant change in abundance with ouabain exposure. A false discovery rate (FDR) correction was also applied subsequently.

Three logistic regression models were built for further analysis of the MS data. The first two step-wise models used the Akaike information criterion (AIC) and the Bayesian information criterion (BIC), respectively. The third model was built with an aim to achieve the greatest reduction in residual deviance of statistical significance.

Two additional models were built to overcome the overfitting problem: an optimal lasso logistic regression model and an optimal sparse partial least squares discriminant analysis (sPLS-DA) model. N-fold cross validation was used to estimate the predictive performance of the models built.

Lastly, the potential added value of the peptidomic markers to the previously selected lipidomic profiles (See Chapter 2) was investigated. The candidate peptide biomarkers were combined with all the candidate lipidomic markers and submitted to the sPLS-DA algorithm. Cross-validation was also performed. Additionally, we investigated all models comprising lipidomic markers and one peptidomic marker in attempt to find models with the lowest deviance.

3.4 Results

3.4.1 Elution time normalization

The volume of cLC-MS data generated by our peptidomics approach presents a challenge to its in-depth analysis. Additionally, small, but real day-to-day chromatographic variability and modest variation of in-house packed columns necessitated elution time normalization. By aligning MS spectra using a central recognizable, endogenous species as a time alignment standard within each 2-min window, chromatographic variability was effectively minimized, enabling meaningful comparisons of peaks and their responses to ouabain, i.e. EDLF, exposure for each placenta. The following internal time-alignment standards were chosen for such purposes: m/z 695.09 (z=+4) eluted at 18 min, m/z 827.75 (z=+6) eluted at 20.6 min, m/z 474.2 (z=+1) eluted at 22.7 min, m/z 672.36 (z=+3) eluted at 25.2 min, m/z 570.109 (z=+1) eluted at 27.86 min, m/z 409.1543 (z=+1) eluted at 30.12 min, m/z 496.3159 (z=+1) eluted at 36.2 min, m/z 594.36 (z=+1) eluted at 38.2 min, m/z 650.43 (z=+1) eluted at 40.4 min, and m/z 675.53 (z=+1) eluted at 43.4 min.

Endogenous references peaks within each time window were employed to further minimize non-biological variability, due to sample processing or differences in ionization efficiencies. All reference peaks showed comparable abundances between no ouabain exposure and ouabain treatment. The m/z values of the selected reference peaks are listed hereafter: 709.92 (the 2nd time window), 543.23 (3rd window), 401.58 (4th window), 642.49 (5th window), 409.15 (6th window), 533.35 (7th window), 454.28 (8th window), 463.30 (9th window), 672.42 (10th window) and 578.31 (11th window).

3.4.2 Exploration of peptidomic markers indicative of EDLF exposure

In this study we investigated the peptidomic profile of placentas that had been exposed to SP inhibition using a global cLC-MS-based peptidomics approach. Placental tissue explants were incubated in the absence or presence of ouabain, a known SP inhibitor and proposed EDLF. After tissue homogenization, acetonitrile precipitation allowed us greater access to informative peptides and small proteins having low-abundances and lower-molecular weights. Following cLC separation and the MS procedure, we built a variety of statistical models to explore whether the presence of EDLF resulted in specific peptidomic changes in placental tissues.

275 peaks were observed and submitted to statistical analysis using a paired Student t-test. Of these, 9 of the candidate markers (m/z 416.0675, 509.7571, 786.0771, 401.5766, 489.33, 577.39, 424.325, 428.365 and 519.48) had p-values below 0.1, showing statistically or near statistically significant differences in abundances between treatment and control. However, no markers were found to have statistical significance between cases and controls after applying a FDR correction to account for multiple comparisons.

To further model the MS data of peptidomic markers, we built three different logistic regression models. The first model employed the AIC criterion and the markers that had statistically significant differences between treatment and control are listed hereafter: m/z 416.07, 489.33, 564.24, 403.22, 808.56, 544.33, 889.49 and 617.94. The area under curve (AUC), classification rate, sensitivity and specificity of this model appeared to be 1, that is the model could identify correctly placenta exposed to ouabain compared to tissue from the same placenta that had not had ouabain exposure. Nonetheless, this model seemed particularly prone to the overfitting problem, which often suggested poor predicative performance on future data. No peptidomics markers were revealed using the second and third logistic regression models we built, indicating that none of the candidate markers had differences between cases and controls of statistical significance.

An optimal lasso logistic regression model was built to overcome over-fitting, but this more conservative approach found no useful sets of peptidomic markers. However, an optimal sPLS-DA model, also a more conservative approach, trained for the same purpose revealed 2 markers (m/z 416.07 and 489.33) that showed statistical significance. The classification rate, sensitivity and specificity of this model were 0.684, and the AUC was 0.742. The ROC curve (Figure 3.1) was near the diagonal line, suggesting that the sPLS-DA model would perform poorly at predicting future observations. N-fold cross validation was applied to both models for performance estimates, but neither of the penalized methods was capable of clearly distinguishing between treatment and control.

In conclusion, while some peptidomic panels performed well with the limited data, however, after correction for a false discovery rate, no peptidomic marker or set of markers had the clear ability to identify placenta exposed to EDLF/ouabain using conservative statistical

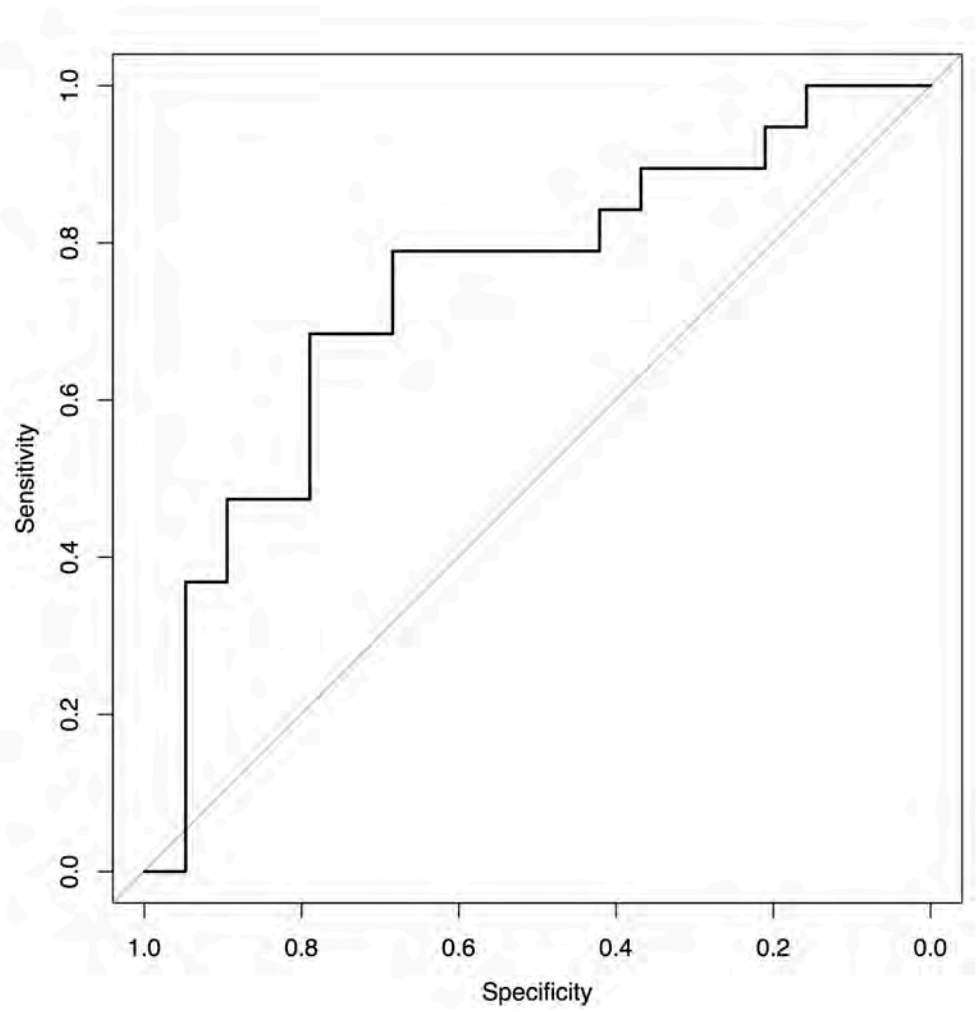

Figure 3.1 ROC curve of selected peptidomic markers using an optimal sPLS-DA model

approaches that more realistically consider the future ability to identify placenta exposed to EDLF. We conclude that EDLF exposure does not lead to any obvious, consistent specific peptidomic changes in normal placental tissues as revealed by this particular peptidomics approach.

3.4.3 Exploration of peptidomic markers with added value to previously selected lipidomic markers

In Chapter 2, we surveyed the lipidomic profile of placental tissue response to EDLF exposure. Here, by combining the 1207 candidate lipid markers and 275 candidate peptidomic markers, we examined the possible added value of combining peptidomic markers to the existing lipidomic profile. However, an optimal sPLS-DA model did not find any peptidomic markers that added value to the lipid biomarker profile. Cross-validation studies yielded similar results. Next, we explored all models that included lipidomic markers and one peptidomic marker, seeking to find a model with minimal deviance. The model that included the peptidomic marker m/z 587.89 and the 4 lipidomic markers selected by the sPLS-DA model (refer to Chapter 2) showed the best deviance of 1.17×10^{-09} and was better than a logistic regression model comprising the 4 lipidomic markers alone that had achieved a very good deviance of 2.6×10^{-09}. However, while the model encompassing one peptidomic marker fit the data better, the improvement was small and not statistically significant. Hence, we conclude that no peptidomic marker added statistically significant value to previously modeled lipidomic profile found, and the predictive performance of the previously modeled lipidomic signature in response to EDLF exposure is unlikely to be improved with the addition of any peptidomic markers observed here.

3.5 Discussion

This study was conducted to test the following hypotheses: Firstly, that normal human placenta responded to SP inhibition caused by EDLF with a change in the abundance of peptides and small proteins in the cytosol; and secondly, if there were such changes, that there would be a characteristic set or pattern of responses. Placental tissues from 20 healthy pregnancies were incubated in the presence and absence of EDLF, followed by the exploration of the biochemical profile of the placental tissues employing a MS-based peptidomics approach. Due to the complexity of the sample extracts, reverse-phase capillary liquid chromatography was utilized for peptide fractionation, followed by elution time normalization using both internal time-alignment standards and endogenous reference peaks prior to statistical analysis.

Of the 275 peptidomic markers observed and surveyed using a paired Student t-test, 9 of the candidate markers showed statistically or near statistically significant quantitative differences between cases and control ($p<0.1$). However, no peptidomic marker had statistical significance after a FDR correction was applied. A logistic regression model built with the AIC criterion revealed 8 peptidomic markers that had statistically significantly different abundances between cases and controls. Yet, after correcting for potential overfitting, none of peptidomic markers continued to show obvious statistical differences. More conservative statistical approaches were utilized to overcome over-fitting, and thereafter no peptidomic marker or set of markers were statistically different. Therefore, we draw the conclusion that SP inhibition caused by ouabain exposure does not result in consistent specific changes in the peptidomic profile of normal placental tissues with EDLF exposure using this specific peptidomics method. In addition, the potential added value of peptidomic markers to previously selected lipidomic markers was assessed. Neither the optimal sPLS-DA model nor cross-validation studies revealed any

candidate markers that added value to the lipidomic profile. Combining 4 lipidomic markers with 1 peptidomic marker, despite one logistic regression model showing very good deviance, did not improve the model significantly. We conclude that the inclusion of peptidomic markers will probably not improve discrimination of ELDF exposure beyond the previously modeled lipidomic profile.

Despite the new diagnostic criteria for PE, hypertension remains the most important symptom of this systemic disease. Additionally, increased vascular permeability and impaired renal hemodynamics, including decreased glomerular filtration rate (GFR), renal plasma flow (RPF) have been associated with PE [26, 31]. It is widely believed that the placenta plays a crucial role in the pathogenesis of PE, due to the fact that delivery of the placenta is the only known treatment of PE, that in the case of hydatidiform mole PE can occur in the absence of fetus but the placenta is required[10, 12], and that immediate uterine curettage can lead to accelerated recovery in women with severe PE[25]. It is also likely that the placenta is a localized target for EDLF associated with PE[28].

EDLFs are endogenous SP inhibitors that may contribute to the pathogenesis of PE. Despite the existence of some contradictory studies, the majority of research has reported elevated concentrations of EDLF in both maternal plasma and placental homogenates from PE patients. This was supported by both functional bioassays indicating the degree of SP inhibition and radioimmunoassays measuring the apparent concentration of EDLF[19, 20, 22, 31, 33]. Statistically significantly increased SP inhibition was also detected in women with severe PE, possibly resulting from elevated circulating levels of EDLF[30]. Ouabain may play important roles in the inhibition of the proliferation, migration and invasion of cytotrophoblasts, cells that are essential to normal placentation during pregnancy[35, 36]. Research suggested that EDLF could also cause

elevated peripheral vascular resistance and vascular fibrosis, and possibly maternal endothelial dysfunction in PE[19]. It has been reported that ouabain can cause endothelial barrier injury and increased permeability in endothelial cells (EC), both of which can be prevented by pretreating samples with Digoxin Immune Fab (DIF) specifically[31]. Digibind has also been shown to reduce the inflammatory response and SP dysfunction induced by TNF-α in vascular endothelium[29].

Proteomics investigates the expression, structure and function of all proteins and their post-translational modifications (PTMs) within a biological system. Although considered a central approach in proteomics in the past, two-dimensional gel electrophoresis combined with MALDI-mass spectrometry (MS) has limited resolving power and was limited to the study of abundant, larger proteins, typically with molecular weights between 15 and 100kDa and with isoelectric points between 4 and 10[37, 46]. Alternatively, both LC-MS and LC-MS/MS enable the detection of proteins with improved dynamic range, accuracy and reproducibility[43]. Two-dimensional, or three-dimensional chromatographic separations of tryptic peptides, in particular, when combined with one-dimensional electrophoresis can provide much better peak capacity[44]. Most proteomic studies employ a "bottom-up" approach, using the proteolytic enzyme trypsin to generate protein-specific peptides that can be sequenced by tandem-MS. Such peptides usually have 10+ residues in length, and a charge state of +2. Improved data quality and sequencing results of tryptic fragments can more than compensate for sample complexity resulting from trypsin digestion of a mixture of proteins[40]. The identification of one protein can be accomplished without identifying all of the tryptic fragments generated from trypsin proteolysis[38]. "Top-down" proteomics, on the other hand, can better characterize protein isoforms and PTMs[43].

Apart from proteins, the production and metabolism of peptides can also reflect physiological and pathological changes within a biological system[37]. Peptidomics is a relatively new field compared with proteomics. It involves the comprehensive qualitative and quantitative analysis of the peptidome within a biological sample. It complements proteomics and enables the detection and identification of small proteins[45] and peptides, smaller than 10kDa. Unlike proteomics, all the peptides of interest under investigation in peptidomics studies are to be identified because peptides produced from the same precursor may have different functions. It is necessary to recognize that though the focus of peptidomics is the study of native or endogenous peptides, protein degradation fragments[38] and sampling artifacts[41] can be simultaneously detected and characterized as well.

If possible, protein degradation should be carefully minimized in peptidomic studies without disturbing the biological matrix because even a small fraction of protein breakdown will render difficult the detection of bioactive peptides that are already at low levels. Methods include the addition of acids, protease inhibitors and/or chelating agents[39], appropriate selection of tissues that contain high levels of peptides, enrichment of peptides of interest such as glycopeptides or phosphopeptides using affinity columns, microwave irradiation of biological samples prior to tissue processing and peptide extraction[38, 42], the use of cold temperature, and employment of highly sensitive mass spectrometers such as LTQ-FT and LTQ-Orbitrap[39].

Peptide extraction is usually performed in studies employing a peptidomics approach because of the presence of proteins, lipids, carbohydrates and salts in biological samples in addition to peptides can limit their analysis. Moreover, low molecular weight peptides are likely to associate with high-abundance proteins, and the signals of any abundant protein also can suppress the signal of low-abundance peptides. Size-exclusion chromatography (SEC) is time-

consuming but can selectively isolate peptides of specific mass ranges. Ultrafiltration membranes are a simple alternative and allow rapid separation of peptide fractions with different molecular weight ranges, however peptide losses and partial contamination can occur. Protein precipitation can also be achieved through the addition of acid, organic solvents or a combination of agents[42]. We employed an acetonitrile (ACN) treatment for protein depletion in this study. Evidence has confirmed that compared to ultrafiltration, ACN precipitation allows for not only the denaturation and thus removal of large, abundant (and typically uninformative proteins), but also the dissociation and release of low-MW, low-abundance proteins and peptides from large carrier proteins with sufficient reproducibility and sensitivity[6]. This approach then enables the detection of many more small proteins and peptides of interest as well as other biomolecules soluble in ACN, providing broader access to the peptidome under investigation.

Three technologies have been more commonly utilized in MS-based peptidomics studies: MALDI-TOF MS, offline LC-MALDI-TOF MS and nanoLC ESI-TOF MS. MALDI-TOF MS has high sensitivity and tolerance against interfering substances and thus can be used for the direct analysis of the peptidome in biological samples such as tissues or peptide extracts. Additional spatial information of peptides inside cells can also be obtained with this approach. The interpretation of the MS spectra is usually simplified due to the production of singly charged peptides from MALDI[37]. Offline LC MALDI-TOF MS, however, is best utilized with automated sample spotting and high throughput instruments[37]. NanoLC ESI-TOF MS has the advantages of high throughput, improved sensitivity and signal-to-noise ratio, therefore enabling the detection of low abundance peptides in complex biological samples[37]. Multidimensional protein identification technology (MudPIT) that employs a biphasic column can be an efficient alternative for the identification of overlooked peptides due to co-elution[38], although sample

preparation is more complex and the reproducibility of this approach is probably limited thus limiting quantitative comparisons[42].

Quantitative peptidomics includes both label-free and labeled quantification of peptides[42]. The approximation of the relative levels of peptides can be obtained using mass spectrometers, although MS technologies are not inherently completely quantitative. Label-free quantification can be done with any analytical platform and requires only simple sample preparation. Examples of label-free quantification are spectral counting and the ion signal intensity approach[42]. The relative quantitation of peptides can be measured using isotopic labeling. Absolute quantitation can be achieved with the addition of known amounts of internal standards that contain heavy stable isotopes. Peptide quantification with isotope labeling can be done without fragmentation, although the resulting spectra are generally more complex. SILAC culture has been reported in peptidomics studies[42]. The isotopic coded affinity tags (ICAT) that can react with the sulfhydryl group are hardly used in quantitative peptidomics due to the fact that endogenous peptides rarely contain cysteines[38]. Isobaric tag reagents such as isobaric tags for relative and absolute quantification (iTRAQ), tandem mass tags (TMT) can be adapted to quantitative peptidomics without difficulty. They have the advantages of improved throughput and more accurate quantitation. In addition, trimethylamino-butyryl (TMAB) tags are considered ideal labels for peptidomics studies due to the presence of a quaternary amine[40].

Successful peptide sequencing in peptidomics studies usually requires a combination of accurate mass and tandem mass spectrum. Collision-induced dissociation (CID) is the most frequently used method for peptide fragmentation except glycopeptides[42]. QTOF with CID has been used to sequence peptides below 3-5kDa, and rarely up to 9kDa. Electron transfer dissociation (ETD) works well for glyco-peptides and provides moderately different results from

CID[42]. Both FTICR with ECD and linear quadrupole ion trap with ETD have the potential for sequencing larger peptides and even smaller proteins[38]. In addition, a combination of CID, ETD and high-energy CID has been reported to generate a more complete fragmentation profile[42].

Most peptide spectral databases widely used in proteomics are rarely applicable to peptidomics studies. In peptidomics, peptides are identified through either database searching that compares experimental tandem mass spectra with theoretical spectra, or de novo sequencing without making reference to a sequence library. De novo sequencing may benefit from chemical derivatization of peptides when sequence information is insufficient for certain organisms. It can also be applied to peptide sequence confirmation[39]. Programs utilized in database searching include Mascot, SEQUEST, X!Tandem, etc, and for de novo sequencing, examples are PepNovo, MS-Tag, and Peaks to name a few[42]. There have also been reports of programs that can combine both identification approaches.

In our study, we investigated the peptidomic profile of normal placental tissues in the presence and absence of SP inhibition caused by ouabain exposure. Despite the discovery of several peptidomic markers that might indicate previous ouabain exposure, we were unable to obtain a set of peptidomic markers that could reliably predict ouabain exposure in future studies. This was likely due in part to very stringent statistical analyses applied and to the specific peptidomics method we employed in this study that only interrogated a few hundred peaks. Perhaps a higher concentration of ouabain would have provided a more dramatic change of peptides and small proteins. Furthermore, fractionation of peptides by multiple chromatography steps may be used to allow for more peptide to be detected[38] or perhaps a more sensitive mass spectrometer would have revealed more. The broader range of concentrations of proteins and peptides present in biological samples that can differ by many orders of magnitude renders it

difficult to overcome the limited dynamic range imposed by current MS technologies in both proteomics and peptidomics studies.

In conclusion, though a small set of peptidomic markers appears to show statistically significant abundance differences between cases and control in response to EDLF exposure in normal placental tissues, no peptidomic marker or set of markers was selected using more stringent methods of statistical analysis that assess the predictive value of markers of interest in future data. We also explored combining potential peptidomic markers with previously revealed lipidomic profiles to improve their ability to reveal EDLF exposure, however, none added statistically better discrimination than the lipid markers alone.

3.6 References

1. Ma, J.; Esplin, M. S.; Adair, C. D.; Mason, L. A.; Graves, S. W., Increasing evidence for and regulation of a human placental endogenous digitalis-like factor. *Reproductive Sciences* **2012,** *19* (4), 437-48.

2. Kedia, K.; Smith, S. F.; Wright, A. H.; Barnes, J. M.; Tolley, H. D.; Esplin, M. S.; Graves, S. W., Global "omics" evaluation of human placental responses to preeclamptic conditions. *American Journal of Obstetrics and Gynecology* **2016,** *215* (2), 238 e1-238 e20.

3. Kedia, K.; Nichols, C. A.; Thulin, C. D.; Graves, S. W., Novel "omics" approach for study of low-abundance, low-molecular-weight components of a complex biological tissue: regional differences between chorionic and basal plates of the human placenta. *Analytical and Bioanalytical Chemistry* **2015,** *407* (28), 8543-56.

4. Shah, D. J.; Rohlfing, F.; Anand, S.; Johnson, W. E.; Alvarez, M. T.; Cobell, J.; King, J.; Young, S. A.; Kauwe, J. S.; Graves, S. W., Discovery and Subsequent Confirmation of Novel Serum Biomarkers Diagnosing Alzheimer's Disease. *Journal of Alzheimer's Disease : JAD* **2016,** *49* (2), 317-27.

5. Anand, S.; Bench Alvarez, T. M.; Johnson, W. E.; Esplin, M. S.; Merrell, K.; Porter, T. F.; Graves, S. W., Serum biomarkers predictive of pre-eclampsia. *Biomarkers in Medicine* **2015,** *9* (6), 563-75.

6. Merrell, K.; Southwick, K.; Graves, S. W.; Esplin, M. S.; Lewis, N. E.; Thulin, C. D., Analysis of low-abundance, low-molecular-weight serum proteins using mass spectrometry. *Journal of Biomolecular Techniques : JBT* **2004,** *15* (4), 238-48.

7. Merrell, K.; Thulin, C. D.; Esplin, M. S.; Graves, S. W., Systematic internal standard selection for capillary liquid chromatography-mass spectrometry time normalization to facilitate serum proteomics. *Journal of Biomolecular Techniques : JBT* **2008,** *19* (5), 320-7.

8. Alvarez, M. T.; Shah, D. J.; Thulin, C. D.; Graves, S. W., Tissue proteomics of the low-molecular weight proteome using an integrated cLC-ESI-QTOFMS approach. *Proteomics* **2013,** *13* (9), 1400-11.

9. Gathiram, P.; Moodley, J., Pre-eclampsia: its pathogenesis and pathophysiolgy. *Cardiovascular Journal of Africa* **2016,** *27* (2), 71-8.

10. Mol, B. W.; Roberts, C. T.; Thangaratinam, S.; Magee, L. A.; de Groot, C. J.; Hofmeyr, G. J., Pre-eclampsia. *Lancet* **2016,** *387* (10022), 999-1011.

11. Gathiram, P.; Moodley, J., Pre-eclampsia: its pathogenesis and pathophysiolgy. *Cardiovascular Journal of Africa* **2016,** *27* (2), 71-8.

12. Uzan, J.; Carbonnel, M.; Piconne, O.; Asmar, R.; Ayoubi, J. M., Pre-eclampsia: pathophysiology, diagnosis, and management. *Vascular Health and Risk Management* **2011,** *7*, 467-74.

13. Naljayan, M. V.; Karumanchi, S. A., New developments in the pathogenesis of preeclampsia. *Advances in Chronic Kidney Disease* **2013,** *20* (3), 265-70.

14. Warrington, J. P.; George, E. M.; Palei, A. C.; Spradley, F. T.; Granger, J. P., Recent advances in the understanding of the pathophysiology of preeclampsia. *Hypertension* **2013,** *62* (4), 666-73.

15. Gusdon, J. P.; Buckalew, V. M.; Hennessy, J. F., A digoxin-like immunoreactive substance in preeclampsia. *American Journal of Obstetrics and Gynecology* **1984**, *150* (1), 83-85.

16. Beyers, A. D.; Odendaal, H. J.; Spruyt, L. L.; Parkin, D. P., The possible role of endogenous digitalis-like substance in the causation of pre-eclampsia. *South African Medical Journal* **1984,** *65* (22), 883-5.

17. The importance of endogenous digoxin-like factors in rats with various forms of experimental hypertension.

18. Adair, C. D.; Buckalew, V.; Taylor, K.; Ernest, J. M.; Frye, A. H.; Evans, C.; Veille, J. C., Elevated endoxin-like factor complicating a multifetal second trimester pregnancy:

treatment with digoxin-binding immunoglobulin. *American Journal of Nephrology* **1996,** *16* (6), 529-31.

19. Adair, C. D.; Luper, A.; Rose, J. C.; Russell, G.; Veille, J. C.; Buckalew, V. M., The hemodynamic effects of intravenous digoxin-binding fab immunoglobulin in severe preeclampsia: a double-blind, randomized, clinical trial. *Journal of perinatology : official journal of the California Perinatal Association* **2009,** *29* (4), 284-9.

20. Adair, C. D.; Buckalew, V. M.; Graves, S. W.; Lam, G. K.; Johnson, D. D.; Saade, G.; Lewis, D. F.; Robinson, C.; Danoff, T. M.; Chauhan, N.; Hopoate-Sitake, M.; Porter, K. B.; Humphrey, R. G.; Trofatter, K. F.; Amon, E.; Ward, S.; Kennedy, L.; Mason, L.; Johnston, J. A., Digoxin immune fab treatment for severe preeclampsia. *American Journal of Perinatology* **2010,** *27* (8), 655-62.

21. Lam, G. K.; Hopoate-Sitake, M.; Adair, C. D.; Buckalew, V. M.; Johnson, D. D.; Lewis, D. F.; Robinson, C. J.; Saade, G. R.; Graves, S. W., Digoxin antibody fragment, antigen binding (Fab), treatment of preeclampsia in women with endogenous digitalis-like factor: a secondary analysis of the DEEP Trial. *American Journal of Obstetrics and Gynecology* **2013,** *209* (2), 119 e1-6.

22. Lopatin, D. A.; Ailamazian, E. K.; Dmitrieva, R. I.; Shpen, V. M.; Fedorova, O. V.; Doris, P. A.; Bagrov, A. Y., Circulating bufodienolide and cardenolide sodium pump inhibitors in preeclampsia. *Journal of Hypertension* **1999,** *17* (8), 1179-87.

23. Seccombe, D. W.; Pudek, M. R.; Whitfield, M. F.; Jacobson, B. E.; Wittmann, B. K.; King, J. F., Perinatal changes in a digoxin-like immunoreactive substance. *Pediatric Research* **1984,** *18* (11), 1097-9.

24. Shrivastav, P.; Gill, D. S.; D'Souza, V.; O'Brien, P. M.; Dandona, P., Secretion of atrial natriuretic peptide and digoxin-like immunoreactive substance during pregnancy. *Clinical Chemistry* **1988,** *34* (5), 977-80.

25. Magann, E. F.; Martin, J. N., Jr.; Isaacs, J. D.; Perry, K. G., Jr.; Martin, R. W.; Meydrech, E. F., Immediate postpartum curettage: accelerated recovery from severe preeclampsia. *Obstetrics and Gynecology* **1993,** *81* (4), 502-6.

26. Moran, P.; Baylis, P. H.; Lindheimer, M. D.; Davison, J. M., Glomerular ultrafiltration in normal and preeclamptic pregnancy. *Journal of the American Society of Nephrology : JASN* **2003,** *14* (3), 648-52.

27. Agunanne, E.; Horvat, D.; Harrison, R.; Uddin, M. N.; Jones, R.; Kuehl, T. J.; Ghanem, D. A.; Berghman, L. R.; Lai, X.; Li, J.; Romo, D.; Puschett, J. B.,

Marinobufagenin levels in preeclamptic patients: a preliminary report. *American Journal of Perinatology* **2011,** *28* (7), 509-14.

28. Hopoate-Sitake, M. L.; Adair, C. D.; Mason, L. A.; Torres, C.; Kipikasa, J.; Graves, S. W., Digibind reverses inhibition of cellular rb+ uptake caused by endogenous sodium pump inhibitors present in serum and placenta of women with preeclampsia. *Reproductive Sciences* **2011,** *18* (2), 190-9.

29. Wang, Y.; Lewis, D. F.; Adair, C. D.; Gu, Y.; Mason, L.; Kipikasa, J. H., Digibind attenuates cytokine TNFalpha-induced endothelial inflammatory response: potential benefit role of digibind in preeclampsia. *Journal of Perinatology* **2009,** *29* (3), 195-200.

30. Adair, C. D.; Haupert, G. T., Jr.; Koh, H. P.; Wang, Y.; Veille, J. C.; Buckalew, V., Erythrocyte sodium/potassium ATPase activity in severe preeclampsia. *Journal of Perinatology* **2009,** *29* (4), 280-3.

31. Wang, Y.; Fan, R.; Gu, Y.; Adair, C. D., Digoxin immune fab protects endothelial cells from ouabain-induced barrier injury. *American Journal of Reproductive Immunology* **2012,** *67* (1), 66-72.

32. Uddin, M. N.; Horvat, D.; Childs, E. W.; Puschett, J. B., Marinobufagenin causes endothelial cell monolayer hyperpermeability by altering apoptotic signaling. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **2009,** *296* (6), R1726-34.

33. Graves, S. W., Sodium regulation, sodium pump function and sodium pump inhibitors in uncomplicated pregnancy and preeclampsia. *Frontiers in Bioscience* **2007,** *12*, 2438-46.

34. Pullen, M. A.; Brooks, D. P.; Edwards, R. M., Characterization of the neutralizing activity of digoxin-specific Fab toward ouabain-like steroids. *The Journal of Pharmacology and Experimental Therapeutics* **2004,** *310* (1), 319-25.

35. Oparil, S.; Schmieder, R. E., New approaches in the treatment of hypertension. *Circulation Research* **2015**, *116* (6), 1074-1095.

36. Uddin, M. N.; Horvat, D.; Glaser, S. S.; Mitchell, B. M.; Puschett, J. B., Examination of the cellular mechanisms by which marinobufagenin inhibits cytotrophoblast function. *The Journal of Biological Chemistry* **2008,** *283* (26), 17946-53.

37. Clynen, E.; De Loof, A.; Schoofs, L., The use of peptidomics in endocrine research. *General and Comparative Endocrinology* **2003,** *132* (1), 1-9.

38. Fricker, L. D.; Lim, J.; Pan, H.; Che, F. Y., Peptidomics: identification and quantification of endogenous peptides in neuroendocrine tissues. *Mass Spectrometry Reviews* **2006,** *25* (2), 327-44.

39. Farrokhi, N.; Whitelegge, J. P.; Brusslan, J. A., Plant peptides and peptidomics. *Plant Biotechnology Journal* **2008,** *6* (2), 105-34.

40. Schrader, M.; Knappe, P. S.; Fricker, L. D., Historical perspective of peptidomics. *EuPA Open Proteomics* **2014**, *3*, 171-182.

41. Romanova, E. V.; Sweedler, J. V., Peptidomics for the discovery and characterization of neuropeptides and hormones. *Trends in Pharmacological Sciences* **2015**, *36* (9), 579-586.

42. Dallas, D. C.; Guerrero, A.; Parker, E. A.; Robinson, R. C.; Gan, J.; German, J. B.; Barile, D.; Lebrilla, C. B., Current peptidomics: applications, purification, identification, quantification, and functional analysis. *Proteomics* **2015,** *15* (5-6), 1026-38.

43. Di Meo, A.; Pasic, M. D.; Yousef, G. M., Proteomics and peptidomics: moving toward precision medicine in urological malignancies. *Oncotarget* **2016,** *7* (32), 52460-52474.

44. Aebersold, R.; Mann, M., Mass spectrometry-based proteomics. *Nature* **2003,** *422* (6928), 198-207.

45. Schrader, M.; Schulz-Knappe, P., Peptidomics technologies for human body fluids. *Trends in Biotechnology* **2001,** *19* (10 Suppl), S55-60.

46. Boonen, K.; Landuyt, B.; Baggerman, G.; Husson, S. J.; Huybrechts, J.; Schoofs, L., Peptidomics: the integrated approach of MS, hyphenated techniques and bioinformatics for neuropeptide analysis. *Journal of Separation Science* **2008,** *31* (3), 427-45.

47. Fricker, L. D., Analysis of mouse brain peptides using mass spectrometry-based peptidomics: implications for novel functions ranging from non-classical neuropeptides to microproteins. *Molecular BioSystems* **2010,** *6* (8), 1355-65.

48. Levine, R. J.; Hauth, J. C.; Curet, L. B.; Sibai, B. M.; Catalano, P. M.; Morris, C. D.; DerSimonian, R.; Esterlitz, J. R.; Raymond, E. G.; Bild, D. E.; Clemens, J. D.; Cutler, J. A., Trial of calcium to prevent preeclampsia. *The New England journal of medicine* **1997,** *337* (2), 69-76.

Chapter 4 Gender-Specific Biomarkers For Alzheimer's Disease

4.1 Abstract

Alzheimer's disease has become a global health crisis. While AD has been extensively studied, it is currently difficult to diagnose, especially in its early stages. This inability has impeded the development of drugs. Part of the challenge may be that AD is actually more heterogeneous in its etiology than previous understood. Research continues to amass that suggests the disease may differ between men and women. It has been reported that women are more susceptible to AD than men, and that higher age-adjusted incidence of AD has been found in women. Besides gender differences in various clinical symptoms of AD, it appears that gender also plays an important role in the pathogenesis, diagnosis and prognosis of the disease. Consequently, as one attempts to develop diagnostic tools to identify those likely to have very early AD and therefore facilitate more efficient drug studies, gender should also be considered in this effort. In this study, we analyzed data obtained from a previous study of diagnostic serum lipid biomarkers for AD with the consideration of potential gender difference. We investigated the interaction between gender and disease stage through analysis of variance (ANOVA) and analysis of covariance (ANCOVA). Markers with significant gender and disease stage interactions were reported for each of the three different statistical models after applying false discovery rate (FDR) correction. Using a lasso logistic regression model with binary classification (control vs. all AD stages), we identified different markers for different genders, the coefficient estimates for different gender were also found to be different. We proceeded to build a new ordinal model that includes a gender-specific marker and compared its diagnostic capability with a previous model using the bootstrap procedure. Results indicated that the new

model performed statistically significantly better than the previous model, thus we conclude that the addition of a gender-specific biomarker improved the predictive performance of a diagnostic model for AD.

4.2 Introduction

Alzheimer's disease (AD) is the most common form of dementia. It is generally characterized by progressive memory impairment, loss of cognitive functions and increased psychosocial incompetence. Among AD patients, women perform substantially worse on various neuropsychological tasks than men[1, 6], the rate of cognitive and functional deterioration is faster in women as well[14]. Women with AD have also been reported to be significantly outperformed by men at the same stage of AD in language, semantic abilities, visuospatial abilities and episodic memory[38], to which gender differences in age, education or disease severity do not seem to contribute. Gender differences in the clinical manifestation of AD seem to be most pronounced among patients with MCI[1]. Additionally, gender differences in neuropsychiatric symptoms of AD have been reported. For example, among patients with AD, apathy and anxiety are more common in men whereas women suffer from delusions more often[35].

More than 5 million people suffer from AD in the United States, approximately two-thirds of them are women[1]. The age-adjusted incidence of AD has also been reported to be higher in women than in men in several studies, although controversial findings exist[3, 4, 34]. It appears that women are more likely to develop AD in their lifetime than men[13]. One study reported that the remaining lifetime risk of developing AD is 6.3% in a 65-year-old man, whereas it is 12% in a woman of the same age[5]. AD has been classified into four different stages: very mild AD or mild cognitive impairment MCI (CDR 0.5), mild AD (CDR 1), moderate AD

(CDR 2), and severe AD (CDR 3). The asymptomatic phase (preclinical AD) may last decades prior to the clinical onset of AD[41]. It has also been reported that the probability of developing MCI at older ages is generally higher in women than in men[12]. Similarly, the rate of progression[11] from MCI to AD is higher in women[34] than in men beyond the age of 80.

The number of AD cases globally has been projected to triple by the year 2050. In 2010, the estimated worldwide cost of dementia has been reported to exceed 1% of global GDP. The economic and social costs of AD are so massive that the issue of AD has reached political attention[34]. Unfortunately, none of the currently available treatments for AD can prevent, slow or halt its progression or reverse it. Interestingly, gender differences have been reported among patients with AD regarding the response to treatments[12, 34]. Evidence also suggested that men are more likely to receive antipsychotic medications than women due to the reported gender differences in non-cognitive behavioral problems among individuals with AD[36].

Neuritic plaques and NFT have long been considered two neuropathological hallmarks of AD. In a longitudinal clinicopathologic cohort study, more neuritic plaques and NFT have been reported in women than men among AD patients after adjusting for age[10, 34]. In another study that examined more than 5,000 autopsy cases, women were found to have more extensive deposition of amyloid plaques at early NFT stage I, II, and III than men[24]. The relation of AD pathology to clinical diagnosis also appears to be gender-specific, with each unit increase in global AD pathology the probability of AD diagnosis in men increased almost 3-fold, however the odds of clinical AD in women increased more than 20-fold, and the correlation between gender and AD pathology remained significant even after adjusting for age and educational levels. In the same study, with each additional unit of AD pathology, more significant reduction in various cognitive function scores has been reported in women than in men[10].

Gender differences in Aβ42 and total tau, two examples of CSF biomarkers for AD have also been reported. One study found that women show 1%-1.5% faster annual rates of brain atrophy than men[8], and the atrophy rates were found to be correlated with CSF changes of Aβ, tau, and APOE ε4 status[34]. More pronounced cognitive deficits have been associated with low Aβ42 levels or high levels of total tau in the CSF of women compared to those in men[1]. Women with low Aβ42 levels have more severe left hippocampal atrophy, worse episodic memory performance, worse executive function performance and greater decrease in functional abilities. Similarly, both the left hippocampal atrophy and longitudinal executive function deficits were more severe in women with high total tau levels[1]. Furthermore, the interaction between gender and CSF biomarkers may be modified by the diagnostic status, education level and APOE ε4 status. One study reported that higher tau levels (near significance) were observed among female MCI patients compared with male MCI patients, after adjusting for APOE ε4 status and age[54].

Evidence showed that there is greater APOE ε4-related risk of developing AD in women than in men[19, 33]. For example, among individuals with APOE ε4 heterozygous genotype, women are two times more likely to develop AD compared with men[20]. The risks of conversion to MCI or conversion from MCI to AD were significantly increased or significantly increased to a greater degree in women than in men among APOE ε4 carriers, respectively[32]. APOE ε4 has been suggested to have the potential capacity to modify gender differences in neuropsychiatric symptoms of AD[33]. In patients with modest to severe AD, evidence has shown that female APOE ε4 carriers had a 7.7-fold and 8.3-fold higher risks of developing disinhibition and irritability than male carriers after adjusting for age and educational levels, respectively[33]. Although the same study also reported that gender differences in neuropsychiatric symptoms do not exist in moderate to severe AD before APOE ε4-stratified analysis, or both before and after

APOE ε4-stratified analysis in mild AD[33]. More pronounced deleterious effects of APOE ε4 genotype have also been reported in women than in men related to the hippocampal pathology, cortical thickness[22], default mode functional connectivity[23, 34] and memory performance. One study showed that women with either one or two APOE ε4 alleles were associated with significantly reduced hippocampal volume, which however, was found only in men with two APOE ε4 alleles[21, 34]. The effects of sex hormones on neuropsychiatric symptoms have been suggested to differ by APOE ε4 status in women with AD as well; yet such regulation or association has not been reported in men with AD[33]. These findings suggest that the interaction between gender and APOE genotype may be detected in the preclinical stage of AD[23]. Apart from APOE ε4, higher risk of AD was also found to be associated with the Met66 allele of brain derived neurotrophic factor (BDNF) gene in females[51]. In addition, SNPs such as the G allele of NSP65 of the peroxisome proliferator-activated receptors gamma and rs688 of the low-density lipoprotein receptor were suggested to be potential risk factors of AD for the male gender and be associated with significantly higher risks of AD in men, respectively[12]. However, no associations between gender differences and familial AD have been reported so far. Familial AD can be caused by mutations in one of the three genes: APP, PSEN1 or PSEN2. Genetics and autosomal dominance have been suggested to prevail over other factors[34].

In addition, gender differences in various sociocultural aspects have also been reported. In one longitudinal study, more pronounced differences in cognitive gains between earlier born cohorts (1886-1913) and later born cohorts (1914-1948) were found in women than in men[29]. It was suggested that the higher male cognitive reserve, which potentially results from higher education and/or occupation, led to differences in gender-related risk for AD[12]. Current cognitive activity may also contribute to gender differences in cognitive aging trajectories[34]. Gender

differences in exercise patterns have also been associated with differential risks for AD between females and males[30]. It is generally believed that the protective effect of exercise against the development of AD is more pronounced in women than in men[12].

Gender differences in the brain physiology have been investigated and reported as well. For example, gender has been suggested to be one of the significant predictors of hippocampal volume since women typically have smaller volumes than men[7]. It has been suggested that men can tolerate more pathology than women because men may have a larger brain reserve. This is supported by evidence that the cerebral metabolic deficits are greater in men than in women with the same cognitive impairment levels[15, 16]. The rate of brain volume decline has been found to be faster in women than in men among patients with MCI and AD, and this difference persisted even after adjusting for the difference in cerebral brain volume between men and women[17, 18]. In another study, not only were the rates of brain atrophy and clinical decline higher in women than men in all cohorts (healthy individuals, individuals with MCI and individuals with AD), but also gender differences demonstrated risk as great as that of the APOE ε4 effect after adjusting for education, ApoE4, age and baseline cognition[9].

It seems that gender plays an important role in the pathogenesis, diagnosis and prognosis of AD[65]. Various factors have been suggested to contribute to gender differences in the prevalence of or susceptibility to AD, including the differences between the female population and male population in life expectancy (age being the risk factor), genetics (APOE ε4 status), sex hormones, prevalence of type 2 diabetes, depression and anxiety (all higher in women), hypertension (esp. higher in women older than 75 years), cognitive detection biases, educational level and sociocultural effects[2, 34]. However, the exact effect of gender on AD is still poorly understood[34]. Among thousands of research articles regarding AD, very few studies were

designed to test gender differences manifested in AD, not to mention the role that gender plays in AD pathophysiology[2]. It has been suggested that gender, a fundamental human variable, should be taken into account in both design and analysis of basic and clinical research at all levels[12].

Biomarkers have the potential to provide early detection, diagnosis, progression monitoring and prognosis of AD, and they may have further value in treatment selection and new drug discovery[53, 55]. Most research on AD biomarkers has focused on amyloid beta protein and tau protein in the CSF with some measure of diagnostic utility in mid- to late-stage disease. These same analytes do not perform well in serum or plasma. However, recently two large drug studies that targeted Aβ and successfully reduced it failed to influence the progression of AD suggesting that Aβ may not be reflective of the early events in AD and hence may be a poor biomarker for very early AD diagnosis. Moreover, studies of AD biomarkers have not considered gender in their analysis. If the disease differs between men and women and classic diagnostic targets may not be useful in early disease, there is a substantial need to find new biomarkers and very importantly to find those that take into account gender differences. We report on efforts to find early stage AD biomarkers that perform better in women or men.

4.3 Data and Methods

All serum specimens were collected as part of a previous study at the Knight Alzheimer's Disease Research Center (ADRC), the Washington University School of Medicine, St. Louis, MO (WUSTL) with approval from Washington University Institutional Review Board (IRB)[40]. We were provided sera from 55 controls and 58 cases (7 cases of CDR=0.5, 4 cases of CDR=1, 19 cases of CDR=2 and 28 cases of CDR=3, none of which has any co-morbidities) with the

approval from the Institutional Review Board for Human Subjects at BYU. All specimens were further processed in the following fashion after being stored at -80^{o}C.

Using our novel method of sample processing, highly abundant proteins were first removed from all specimens by acetonitrile precipitation[40]. The supernatant was collected. Then, with the addition of HPLC grade water to the supernatant, all residual traces of acetonitrile were removed from all samples using vacuum centrifuge. A Bio-Rad microliter plate protein assay was utilized to measure apparent protein concentration. All case and control samples were concentrated separately to 0.2 μg/μL 'protein' and acidified with 88% formic acid (FA). Then all samples were randomly loaded onto and carefully analyzed through a capillary-LC-ESI-QTOF mass spectrometry system. A 1mm microbore guard column and an in-house guard column (both packed with POROS R1 reverse phase media) were used to separate compounds. Information regarding the specific gradient used can be found in our published work[40]. The Analyst QS® software package was used for data collection and analysis. MS data for each specimen was collected at m/z 500-2500 from 5-55 min of gradient elution, followed by time normalization of the data using internal standards. Spectra of all cases and controls were then color coded and overlaid to facilitate selection of candidate AD biomarkers, which were normalized using internal references as well. All the above procedures were performed in a previous study of serum lipid AD biomarkers[40].

All experiments in this study were performed on the data obtained from a previous study of diagnostic serum lipid biomarkers for Alzheimer's disease[40]. Prior to the statistical analysis, one observation was deleted because the gender of the subject was unlisted. Fully conditional specification was used to estimate the few missing biomarker abundances, and the biomarkers

were standardized to have sample mean 0 and sample variance 1. R software and packages were utilized for all analyses in this study.

4.3.1 Analysis of variance and analysis of covariance

In conjunction with Justin Barnes and Dr. Dennis Tolley of the Department of Statistics at BYU, analysis of variance (ANOVA) and analysis of covariance (ANCOVA) for each biomarker were conducted to test the following null hypotheses of equal effects: that the biomarker abundances for the two genders are equal, that the biomarker abundances for different disease stages are equal, and that there is no interaction between disease stage and gender.

For biomarker $l = 1,\ldots, p$, the ANOVA model is defined as follows:

$$Y_{ijk} = \mu + \alpha_i + \tau_j + (\alpha\tau)_{ij} + \varepsilon_{ijk}$$

Y_{ijk} is the marker abundance of the k^{th} observation for stage i and gender j, μ is the overall mean, α_i is the effect of binary disease stage $i = 0,1$ (which corresponds to the group of healthy controls and the group of patients with any stage of AD, respectively), τ_j is the effect of gender $j = 0,1$ (which corresponds to males and females, respectively), $(\alpha\tau)_{ij}$ is the interactive effect of the i^{th} stage and the j^{th} gender, and $\varepsilon_{ijk} \sim$ Normal $(0,1)$ is the residual for the observation. We also created a second type of ANOVA model, in which the disease stages are categorical, therefore $i = 0, 1, 2, 3$ (which corresponds to the group of healthy controls, the group of patients with CDR 0.5, the group of patients with CDR 1, and the group of patients with CDR 2, respectively). In the ANCOVA model, the disease stage effect was treated as a linear variable ($i = 0, 1, 2, 3$) instead of a categorical or factor variable due to the fact that disease stage is ordinal. Lastly, a

false discovery rate (FDR) correction was applied to all three models to correct for multiple comparisons problem.

4.3.2 Gender-specific binary classification models

Because several biomarkers were found to have significant interactions between disease stage and gender, we proceeded to build gender-specific binary (control vs. all AD stages) classification models using a lasso logistic regression model, which was more efficient computationally compared to the Bayesian lasso probit regression model used in a previous study[40]. Data selection was conducted as follows: we excluded biomarkers that have different directionality, and saved biomarkers that have p values below 0.05 in a T-test. Then we saved biomarkers that showed significant interactions between gender and disease stage ($p<0.05$) in an ANOVA model, and biomarkers with both a disease stage $p<0.15$ and a gender*disease stage interaction $p<0.1$ in an ANOVA model. Then, we trained the lasso logistic regression model on all the saved biomarkers.

We calculated the coefficients for both genders in the lasso logistic regression models, and we also compared them to the coefficients of the Bayesian lasso probit regression model used in the previous study[40]. Next, the coefficient differences for both the male and female models were quantified using the bootstrap method. This was achieved by removing biomarkers that showed insignificant coefficient differences for the two genders, followed by the identification of biomarkers that had coefficient estimates that were statistically significantly different using a 95% confidence interval. Then Bonferroni-corrected confidence intervals were applied to solve the multiple comparisons problem. In addition, the proportion of times that the biomarkers were selected was evaluated in the models for both genders across the bootstrap data.

Lastly, we compared the cross-validated performance of both gender-specific and non-gender-specific lasso logistic regression models.

4.3.3 New Ordinal Model

Because it had been shown that there were biomarkers that have significant interaction effects between gender and disease stage, that biomarkers selected for the male and female gender were substantially different, and that the coefficient differences in the male and female models were significantly different at 95% confidence intervals, we proceeded to test whether the addition of a gender specific biomarker would significantly improve the diagnostic performance of a predictive model for AD using Bayesian lasso probit ordinal regression model. The procedure to build the model was identical to that applied in a previous study[40], except that a gender-specific marker was included here. Note that there were three different disease stages in this model: controls, stages 0.5 or 1 and stage 2.

Next, we estimated the performance of the new ordinal model using *n*-fold cross-validation. MSE and classification rate of the current model were calculated. The classification rate was also compared with that of the model created in a previous study[40]. For the purpose of comparison, the classification rate of a model identical to that in the previous study but trained on the data obtained in this study was calculated as well.

Then, the performance difference between the new ordinal model and the model built in the previous study[40] (i.e. Improvement) was quantified using the bootstrap procedure for internal invalidation. We calculated the MSE and classification rate, $CI_{improvement}$ (95% confidence interval of the improvement), %Better (the proportion of times that the new ordinal model performed better than the previous model, i.e. MSE<0 and the classification rate>0) and p-value

for both models. Note that the following null hypothesis was tested: that the new ordinal model showed better performance for 50% of the time, or that it showed worse performance than the previous model.

4.4 Results

4.4.1 ANOVA and ANCOVA

In the first type of ANOVA model with a binary disease stage effect, 3 biomarkers (m/z 724.52, 750.53, and 752.54) were found to demonstrate significant ($p<0.05$) interactions between gender and disease stage at the FDR=0.3 level. The effects of gender on the biomarker abundances were significant or near significant for all 3 biomarkers (m/z 752.54 was the only marker that showed a significant gender effect at the FDR=0.3 level), although no significant disease stage effect was observed. The p-values for each of the 3 biomarkers testing the null hypotheses above are shown in Table 4.1. The gender specific relationships between biomarker abundances and binary disease stage for the 3 biomarkers are shown in Figure 4.1.

In the second type of ANOVA model where the disease stage effect is categorical, 8 biomarkers were found to have significant interactions between gender and disease stage at the FDR=0.3 level. However, only 3 of the 8 biomarkers (m/z 430.37, 514.38 and 630.47) showed significant disease stage effects at the FDR=0.3 level, and they were selected by the following ANCOVA model as well. In addition, several biomarkers showed significant or near significant gender effects, but only 3 markers (m/z 630.47, 724.52 and 752.54) were found to have significant gender effects at the FDR=0.3 level. The p-values for each of the 8 biomarkers testing the null hypotheses above are given in Table 4.2. The gender specific relationships between

Table 4.1 Biomarkers that have significant interactions between gender and biomarker abundances in the ANOVA model with binary disease stage

Marker (m/z):	724.52	750.53	752.54
Disease State Effect	0.175	0.483	0.713
Gender Effect	0.049	0.097	0.017
Interaction Effect	0.027	0.026	0.013

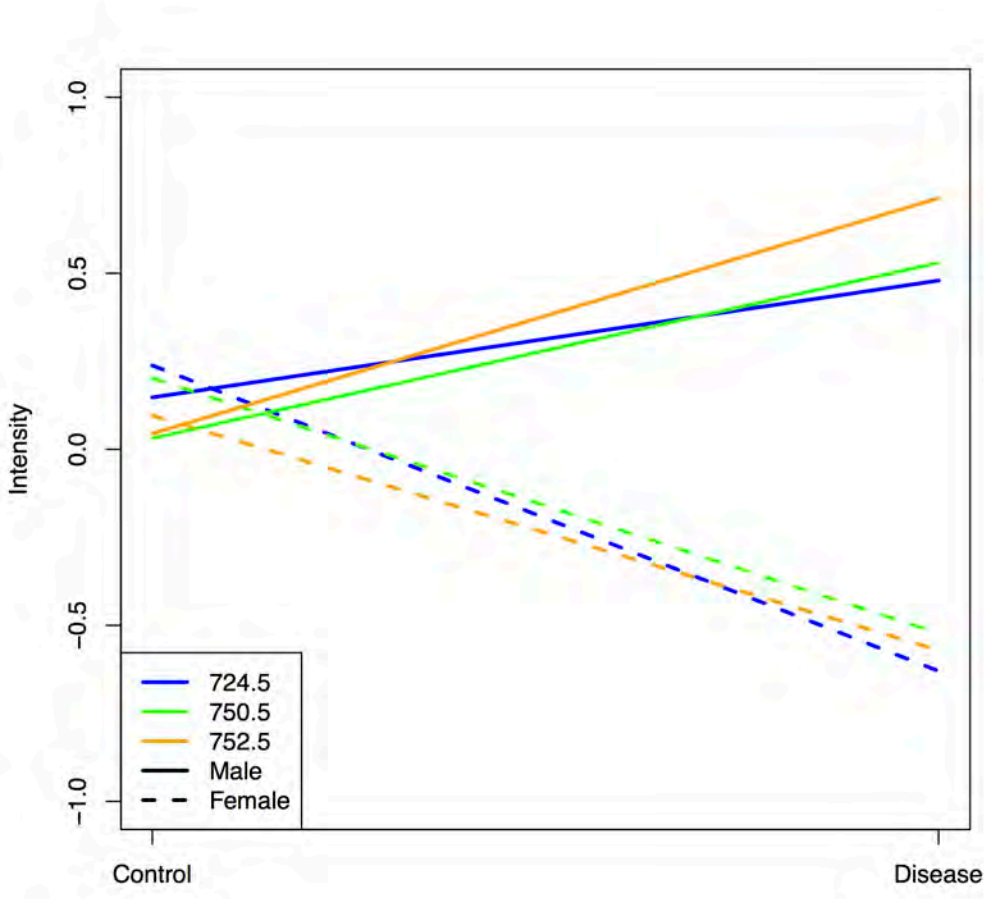

Figure 4.1 Gender specific relationships between biomarker abundances and binary disease stage

Table 4.2 Biomarkers that show significant interactions between gender and biomarker abundances in the ANOVA model with categorical disease stage

Marker (m/z):	430.37	447.35	514.38	630.47	724.52	750.53	752.54	766.56
Stage Effect	0.023	0.544	0.038	0.009	0.484	0.897	0.799	0.415
Gender Effect	0.380	0.949	0.066	0.006	0.043	0.098	0.017	0.311
Interaction Effect	0.032	0.006	0.014	0.001	0.009	0.026	0.009	0.039

categorical disease stage and biomarker abundances for 5 of the biomarkers (m/z 447.35, 724.52, 750.53, 752.54 and 766.56) are shown in Figure 4.2.

In the ANCOVA model, where the disease stage effect is linear, we found 3 biomarkers (m/z 430.37, 514.38 and 630.47) that showed significant interactions between gender and disease stage at the FDR=0.3 level. The disease stage effects for all 3 markers were significant at the FDR=0.3 level as well. Among the 3 markers, only m/z 630.47 showed a significant gender effect at the FDR=0.3 level. The p-values for each of the 3 biomarkers testing the null hypotheses above are listed in Table 4.3. The gender specific relationships between linear disease stage and abundances for the 3 biomarkers are shown in Figure 4.3. It was also observed that, there is a strong monotonic relationship between biomarker abundance and disease stage for males, yet no such relationship seems to be present for females between disease stage and abundance.

4.4.2 Gender-specific binary classification models

After the process of data selection, the gender-specific binary classification models were created based on the data selected. The coefficients for both genders in the gender-specific lasso model are listed in Table 4.4, as are the coefficients obtained from the Bayesian lasso probit regression model used in a previous study[40]. If the coefficient for one biomarker is zero, it means that this marker was not selected to be in the model, i.e. showed no gender effect. Seven biomarkers (m/z 430.37, 514.38, 620.42, 714.6, 752.54, 886.76 and 888.78) were selected for the Male model; the same number of biomarkers (m/z 620.42, 630.47, 724.52, 799.65, 824.62, 860.75 and 862.77) were selected for the Female model. However, only one marker (m/z 620.42) was shared between the two genders. It was also observed that 4 in 7 biomarkers in the Female

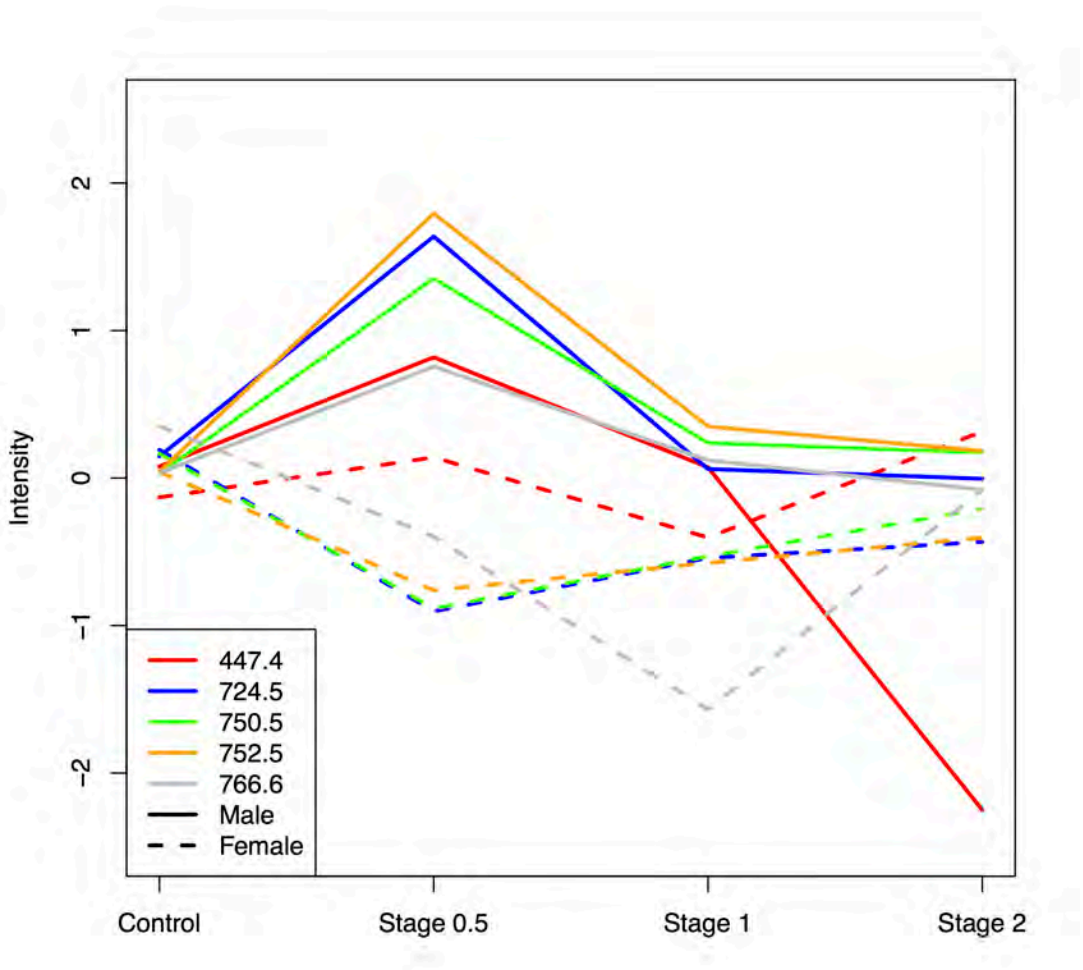

Figure 4.2 Gender specific relationships between biomarker abundances and categorical disease stage

Table 4.3 Biomarkers with significant interactions between gender and marker abundances in the ANCOVA model with linear disease stage

Marker (m/z):	430.37	514.38	630.47
Disease Stage Effect	0.0030	0.0053	0.0016
Gender Effect	0.2925	0.0873	0.0137
Interaction Effect	0.0123	0.0125	0.0073

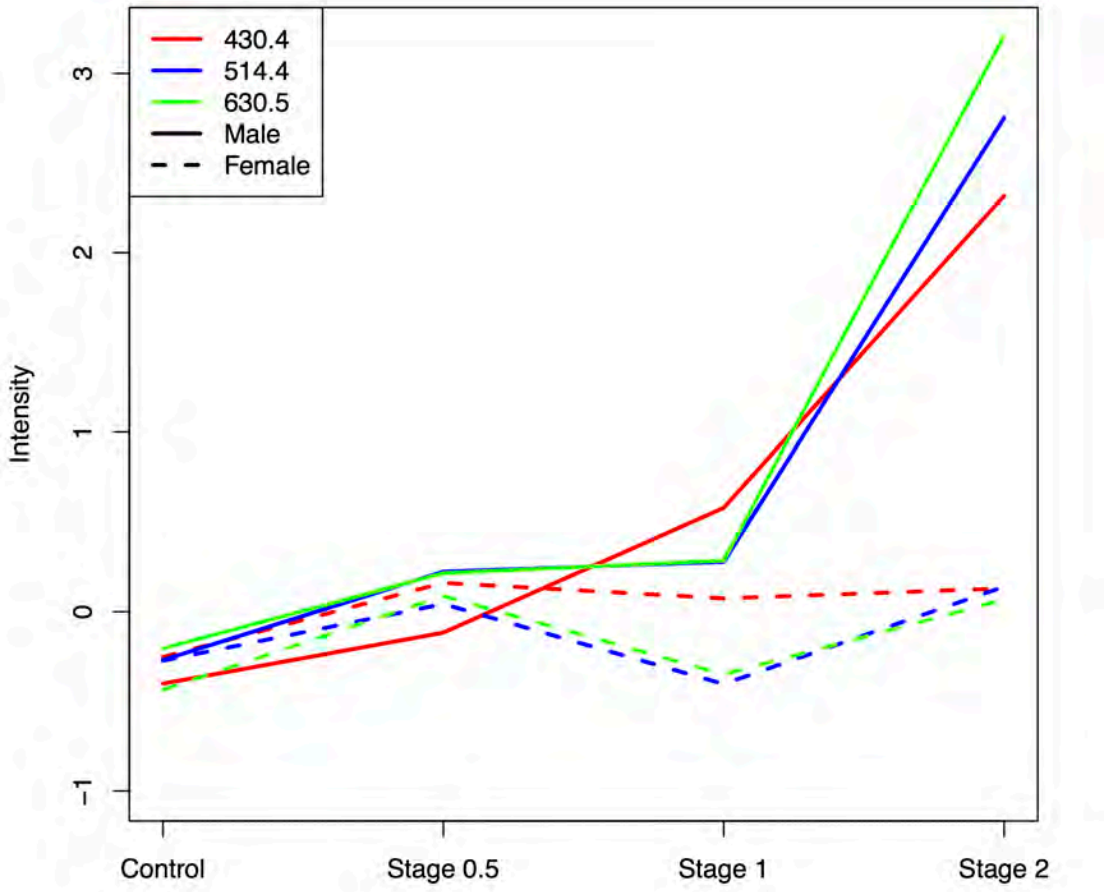

Figure 4.3 Gender specific relationships between biomarker abundances and linear disease stage

Table 4.4 Coefficients for the gender-specific lasso logistic regression models

Marker	Male	Female	Bayesian Model
(Intercept)	-0.581	0.232	0
430.37	0.627	0	0
514.38	0.353	0	0
577.5	0	0	-0.924
603.5	0	0	0.409
620.42	-0.480	-0.541	-0.936
630.47	0	1.347	0.525
714.6	-0.006	0	-0.269
724.52	0	-0.985	0
752.54	0.197	0	0
799.65	0	-0.511	0
824.62	0	0.084	0.430
860.75	0	0.192	0.570
862.77	0	0.516	0
886.76	0.013	0	0
888.78	0.451	0	0

model (m/z 620.42, 630.47, 824.62 and 860.75) were shared with the Bayesian model, but only 2 in 7 biomarkers (m/z 620.42 and 714.6) were shared between the Male model and the Bayesian model, therefore it can be concluded that the biomarkers in the Bayesian lasso probit regression model were more closely aligned with those in the Female lasso logistic regression model compared with the biomarkers in the Male model.

Next, the coefficient differences in the Male and Female models were quantified. 46 biomarkers were selected after removing biomarkers that showed insignificant coefficient differences. Then, 30 biomarkers that had coefficient estimates that were statistically significantly different were identified using a 95% confidence interval. 7 of these biomarkers are listed Table 4.5. A confidence interval was considered to be statistically significant if its lower bound (LB) or upper bound (UB) was zero. After applying the Bonferroni-corrected confidence intervals, 4 biomarkers (m/z 391.27, 488.35, 752.54 and 792.57) that showed significant coefficient differences in the Male and Female models remained, and their coefficient differences were significantly higher in females, males, males and males, respectively (data not shown).

Biomarkers that show the largest differences in the proportion of times selected (a difference greater than 0.2) are listed in Table 4.6. It was observed that the differences between the Male and Female model in the proportion of times that the biomarkers were selected are substantial. Additionally, all the biomarkers selected in the female model showed up in more than 22% of the bootstrap samples, and all the biomarkers in the male model appeared in at least 19.8% of the bootstrap samples (data not shown).

Table 4.5 Coefficient differences in the male and female models using 95% confidence intervals

Marker (m/z)	LB	UB
430.37	0	5.022
577.5	-1.292	0
724.52	0	3.544
752.54	0	3.019
862.77	-1.636	0
886.76	0	2.079
888.78	0	2.039

Table 4.6 Proportion of times that the biomarker was selected in the gender-specific models

Marker (m/z)	Male Model	Female Model
430.37	0.802	0.104
514.38	0.364	0.060
630.47	0.256	0.512
724.52	0.086	0.518
766.56	0.118	0.490
799.65	0.124	0.428
862.77	0.028	0.364
888.78	0.220	0.014
930.83	0.240	0.032

Last but not least, the cross-validated performance of both the gender-specific and non-gender-specific lasso logistic regression models are shown in Table 4.7. The gender-specific model performed fractionally, but significantly better than the non-gender-specific model.

4.4.3 New Ordinal Model

The markers that were selected, the coefficient estimates, CI (95% credible intervals) and probabilities P(|Estimate| ≤ 0) for the new ordinal model are shown in Table 4.8. A coefficient estimate greater than 0 means that the higher the biomarker abundance, the higher of the probability of AD. However, a coefficient estimate less than 0 indicates the opposite trend.

In the attempt to estimate the performance of the new ordinal model, we obtained an MSE of 0.464 and a classification rate of 0.696 (data not shown), whereas the classification rate obtained from the previous model[40] was 0.702. However, the new ordinal model with the inclusion of a gender-specific biomarker showed somewhat better performance than the previous model, due to the fact that a classification rate of 0.679 was achieved from a model identical to the one used in the previous study but trained on the data obtained in this study.

The performance difference between the new ordinal model and the previous model[40] is shown in Table 4.9. It may appear that the performance difference between the two models is not statistically significant, due to the fact that the MSE of the new model is 0.050 lower than that of the previous model and $CI_{improvement}$ includes 0, and that the classification rate of the new model was 0.019 higher than that of the previous model and the $CI_{improvement}$ includes 0 as well. However, the new ordinal model showed better performance than the previous model in 82% of the bootstrap replications based on MSE with a p-value of 7.71×10^{-16}, and in 61% of the bootstrap replications based on the classification rate with a p-value of 0.011. The two p-values

Table 4.7 Cross-validated performance of lasso logistic regression models

Metric	Non-Gender-Specific	Gender-Specific
AUC	0.723	0.739
Classification	0.696	0.750
Sensitivity	0.630	0.741
Specificity	0.759	0.759

Table 4.8 Summary of the bayesian lasso probit ordinal regression model

Marker (m/z)	Coefficient Estimate	CI	p
229.13	-0.144	(-0.584, 0.152)	0.208
430.37	0.053	(-0.188, 0.342)	0.350
514.38	0.107	(-0.214, 0.544)	0.282
602.44	0.107	(-0.208, 0.537)	0.277
603.5	0.068	(-0.220, 0.432)	0.336
620.42	-0.461	(-0.970, -0.031)	0.014
630.47	0.137	(-0.198, 0.613)	0.242
714.6	-0.108	(-0.420, 0.124)	0.211
824.62	0.212	(-0.078, 0.627)	0.108
630.47*Male	0.142	(-0.176, 0.608)	0.228

Table 4.9 Summary of performance improvement in the new ordinal model

Statistic	Improvement	$CI_{Improvement}$	% Better	p-value
MSE	-0.050	(-0.179, 0.071)	82	$7.71 * 10^{-16}$
Classification Rate	0.019	(-0.135, 0.089)	61	0.011

given demonstrate that the opposite of the null hypothesis tested above was true. It can be concluded that the new ordinal model performed statistically significantly better than the model created in the previous study[40]. Consequently, we conclude that the addition of a gender specific biomarker improved the diagnostic performance of a predictive model for AD.

4.5 Discussion

In this study, we proposed that there are gender-specific biomarkers for AD and inclusion of such biomarkers in a multi-marker model of AD can greatly improve diagnostic capability. Prior to discovery of potential gender-specific biomarkers for AD, we set out to study whether there was a significant interaction effect between disease stage and gender. Of the three models we used, it appears that the ANCOVA model worked the best. When testing for a linear disease stage effect, three markers that showing a significant interaction between disease stage and gender were discovered in this model. Though a confirmation study using different data is needed, this finding suggests that biomarkers staging AD may differ with gender. The first ANOVA model selected three markers that had significant interaction effects between disease stage and gender. However, the disease stage effects for the three markers individually were insignificant. Results obtained from the second ANOVA model have few implications due to the fact that the disease stage for this model is ordinal.

Next we attempted to train separate AD diagnostic models for each gender using a lasso logistic regression model. As we expected, the biomarkers selected for AD diagnosis and stage differ considerably between women and men (see Table 4.4). The coefficient differences between the male model and the female model were significantly different (data not shown).

Furthermore, compared with the Bayesian lasso probit regression model, results indicated that the lasso logistic regression model was preferable in terms of gender-specific biomarker studies.

The performance of gender-specific binary (i.e. cases versus controls) classification models was evaluated. The Female model was found to perform better than the Male model based on the classification rates, although both were able to identify cases very well and were not markedly different in model performance despite employing different markers (Table 4.10). It should be remembered that previous binary classification was excellent even when not considering gender. Not surprisingly overall performance was not markedly different when a model developed for one gender was applied to the other ('gender switch', Table 4.11). However, the Female diagnostic model, when applied to males, performed substantially better in almost every aspect than the Male model applied to females.

Last but not least, we built a new ordinal model for AD diagnosis and disease stage that included a gender-specific biomarker using a Bayesian lasso probit ordinal regression model. We also quantified the performance difference between the newly built model and a previous model that excluded the gender specific biomarker. Evidence (% Better and p-value) suggested that the new ordinal model performed statistically significantly better than the previous model. Thus, we can conclude that the diagnostic capability of the predictive model for AD, which was already very good, can likely be improved by adding a gender-specific biomarker.

According to the mean standard error (MSE) and classification rates, a better performance was found in the gender-specific ordinal model for males than for females (Table 4.12). Generally, the female data provided better performance in binary classification, whereas the male data performed better for the staging disease. This better performance of the gender-

Table 4.10 Performance of gender-specific binary model: by gender

Metric	Overall	Female Model (on females)	Male Model (on males)
AUC	0.739	0.708	0.711
Classification	0.750	0.821	0.679
Sensitivity	0.741	0.938	0.727
Specificity	0.759	0.667	0.647

Table 4.11 Performance of models on opposite genders

Metric	Overall	Male Model (on females)	Female Model (on males)
AUC	0.748	0.740	0.829
Classification	0.732	0.714	0.821
Sensitivity	0.630	0.688	0.818
Specificity	0.828	0.750	0.824

Table 4.12 Performance of gender specific ordinal model: by gender

Metric	Overall	Just Females	Just Males
AUC	0.798	0.771	0.834
Classification (Binary)	0.786	0.786	0.786
Sensitivity (Binary)	0.840	0.750	0.824
Specificity (Binary)	0.742	0.812	0.727
MSE	0.464	0.571	0.357
Classification (Ordinal)	0.696	0.643	0.75

specific ordinal model for males suggests the presence of markers (for example, m/z 430.37, 514.38 and 630.47 in Figure 4.3) that might allow marker intensities to specify disease stage. However, the numbers for these studies of the interaction of biomarker abundance and AD stage for each gender were few and additional understanding of their performance awaits further investigation.

Numerous studies have been designed and conducted for the discovery of AD biomarkers, which include genomic biomarkers, biochemical biomarkers (for instance, CSF, blood and urinary biomarkers) and various neuroimaging techniques. However, none or very few of them took into consideration the potential role that gender plays in the pathological cascade of AD. Even so, several of the many AD biomarkers discovered have been found to be gender-specific as well.

One article[53] reported the discovery of a new plasma biomarker for AD diagnosis using LC-API-CI-MS. The new biomarker was identified as desmosterol. The study found that both the desmosterol level and the desmosterol/cholesterol ratio in plasma were significantly decreased in patients with AD and MCI compared to healthy controls, and this result was confirmed using 109 clinical samples in the same study as well. Interestingly though, gender differences regarding this new plasma biomarker was observed. Both the desmosterol level and the desmosterol/cholesterol ratio in plasma were more significantly reduced in the female patients with AD than male patients. In addition, higher discrimination power of the biomarker desmosterol/cholesterol ratio was observed in female AD patients. Similar results were also observed among female patients with MCI. Furthermore, gender differences in the correlation between desmosterol level or desmosterol/cholesterol ratio and Mini-mental state examination scores were reported.

Another study[59] reported that transthyretin (TTR) might be a candidate CSF biomarker for AD that is also gender specific. It was observed that the TTR level in plasma was significantly lower in female patients with MCI or AD compared with male patients with MCI or AD, and healthy female controls. In addition, the estradiol level in the plasma was reduced in both genders. A correlation between disease stage and TTR level was also observed[55]. Thus it was concluded that the modulation of AD by TTR might be gender specific.

The protein, progranulin (PGRN), has also been suggested to be a candidate biomarker for both AD and another neurodegenerative disorder[55]. It was proposed that the contribution of PGRN to AD pathogenesis is related to gender. PGRN level was reported to be increased (although not significantly) in women compared to men. Moreover, the PGRN level was found to be positively correlated with age among female AD patients[60]. Another potential biomarker for AD or MCI, plasma leptin, was also found to have approximately twice as high concentration in the female population as that among the male patients[61].

One study that was designed to discover predictive plasma biomarkers that can differentiate between patients with stable MCI (S-MCI) and those with progressive MCI (P-MCI) reported a model of 12 abundance plasma proteins that showed an accuracy of approximated 79% in predicting the outcomes of MCI[62]. Interestingly, several gender specific protein markers were also found. Among them, for example, alpha-2-macrogloblin (A2M) showed a strong correlation with MCI progression in women, yet such correlation was not found among male MCI patients. One article published recently reported a significant association between serum A2M concentration and concentrations of tau and p-tau in CSF. It was found that a potential specific mechanism centered on the role of A2M that associates systemic inflammation with AD pathogenesis is gender specific[64]. In addition, the well acknowledged fact

that gender differences exist in abundant proteins of blood proteome[63] puts in doubt various studies of AD biomarkers without considering the effect of gender. Therefore, it was suggested that gender specific models for potential AD biomarkers should be studied and used for the validation of numerous studies employing sex-unified models as well.

Apart from the discovery of several AD biomarkers that also seem to be gender-specific, one study focused on the identification of homogeneous subpopulations of AD patients using a multi-layer clustering method (clinical descriptors of AD as the first layer and biological descriptors as the second layer). Gender specific differences in AD were reported[66]. Among AD patients and patients with MCI, there are significant differences in the clusters constructed between female and male subpopulations. To be specific, two clusters (M1 and M2) were constructed for the male population yet only one cluster (F1) was constructed for the female population. AD patients in the M2 cluster are characterized, surprisingly, with statistically significantly increased ICV and whole brain volume compared with those in the M1 cluster, and such M2 cluster was not found in the female population[1]. The above results, as the authors suggested, might be due to an artifact in the data processing procedures, but it is also possible that such findings result from the presence of gender specific differences in AD. And the construction of the M2 cluster indicates that it is likely that two different biological pathways for AD exist in the male population only, which necessitates the segmentation of patient population in studies of AD biomarkers and other related areas. Although the authors pointed out that the clusters constructed in this study are small and only includes a small percentage of AD patients, and the practical segmentation of AD population in a non ad-hoc way seems impossible as of now.

It seems that "biomedical research in general, and neuroscience in particular, has been built on a false assumption that one may safely ignore potential sex influences."[38, 39]. In particular, sexual dimorphism of the brain (including neurogenesis, synaptogenesis and axon guidance[12], etc.) have been largely ignored in a multitude of studies investigating AD biomarkers. Here we provide a brief summary of some of the many aspects of the sexual dimorphism of the brain among healthy population. In the general population, evidence suggested that men perform significantly better on tasks concerning their visuospatial abilities such as mental rotation, spatial perception and spatial visualization[45, 46]. Gender differences in verbal abilities (including lexical fluency, category fluency and confrontation naming) appear to be less robust than visuospatial abilities[38]. In the healthy elderly population, elderly men seems to perform better on tasks regarding mental rotation and spatial perception than elderly women, although variable results were reported for spatial visualization tasks[47, 48]. Gender differences in verbal abilities and memory among the elderly are however inconsistent[38]. Sexual dimorphism is also present in terms of brain structure. Women seem to have smaller brain volumes, bigger gray matter volume and greater cortical depth compared with men. Robust gender differences were also observed in the white matter of the brain[25].

It has been suggested that both genetic differences (X and Y chromosomes) and the differences between female hormones and male hormones contribute to sexual dimorphism of the human brain[34, 49]. At the genetic level, evidence suggested that the X-chromosome is more susceptible to neurodegeneration in AD[2]. Disproportionally increased susceptibility to AD in female may result from X-inactivation patterns at the epigenetic level as well[44]. It had been found that certain brain areas that show greater sexual dimorphism are associated with developmentally greater amounts of sex hormone receptors[50]. However, the exact mechanism

concerning the effects of sex steroid hormones on brain structure remains elusive[25]. Although it had been reported that sexual dimorphism of the brain may also result from the functions of both PSEN1 and PSEN2, which are thought to be involved in familial AD[57].

Gender differences in hormones have been studied in the context of AD. It was suggested that the severe estrogen deficiency after menopause in women is associated with higher risk of MCI and AD[37]. Men, however, do not experience significant estrogen loss. Since estrogen can be converted from testosterone in men, their testosterone declines are less significant than those in women[12]. Estrogen has been reported previously to be capable of increasing the activity of choline acetyltransferase[26], preventing neural tau hyperphosphorylation[31], reducing Aβ aggregation and Aβ-induced apoptosis of neurons[27], enhancing neural survival through a variety of mechanisms including improving mitochondrial function (mitochondrial bioenergetic deficits is one of many characteristics of AD), maintaining calcium homeostasis and promoting Aβ clearance[12, 28]. Hormone replacement therapy (HRT) has also been associated with reduced risk of AD in women following menopause or after bilateral oophorectomy. However, controversial findings do exist. It was reported that the risk of AD was not lowered in women who initiated HRT 5 years or later after menopause, and surprisingly the risk of AD was two times higher among women who started HRT at or after 65 years old[52]. It has been suggested that the discrepancies between observational studies and clinical trials might potentially result from differences in the timing of HRT regarding "a window of opportunity" or mixing of effects[12, 37]. Additionally, findings from transgenic animal studies also suggested that AD pathogenesis could be regulated by sex hormones from both genders[58].

So far very little research has been done regarding gender differences among AD patients, despite the reports on sexual dimorphism of the human brain among health population

and on a number of gender differences in the following aspects ranging from clinical symptoms, prevalence, risk factors of AD to responses to treatment and drug discovery. Even though few contradicting findings have been reported, it is only reasonable to take gender differences in AD biomarkers into consideration based on the results of the majority of neuroscientific studies concerning AD. Indeed, evidence has suggested that a number of processes in AD can be affected by gender[2]. One study reported that the glucose metabolism in the major brain areas related to AD was significantly greater in women than men[56]. Understandably, our study is not the only one to have suggested that biomarkers for AD may vary by gender[55].

One aspect we could have taken into consideration in this study is the APOE ε4 status of the subjects. APOE ε4 allele has been confirmed to be the known strongest known genetic risk factor for late onset AD[34]. However, it has been suggested that there is a strong interaction between APOE and gender. For example, the total tau levels in CSF are significantly different between women with APOE ε4 genotype and women with homozygous APOE ε3 genotype, yet the same difference was not found in men[24, 34]. The large gender differences in deposition of senile plaques at early NFT stage I, II, and III are found to be specifically linked to women with APOE ε4 genotype as well[24]. In addition, a significant APOE ε4-by-sex interaction on CSF levels of total tau, p-tau and tau-Aβ-ratio was reported among APOE ε4 carriers with MCI, with more AD-like changes in women[32]. We suggest that future studies on gender-specific biomarkers of AD should take into account the APOE ε4 status of the subjects. Additionally, population studies and longitudinal multicenter studies on the effects of gender on AD can be beneficial since most of the current few studies on gender specific biomarkers of AD are post hoc and exploratory in nature. It is our belief that more research efforts should be focused on the gender differences in AD, and gender-specific biomarkers in particular. Not only the study of gender-

specific biomarker can facilitate accurate diagnosis of AD, but also substantially improve our understanding of the molecular and genetic pathogenesis of AD, including the potential mechanism behind gender differences. Thus, clinical trials of AD can be designed to tailor to each individual gender, and better prognosis of AD (esp. MCI) can possibly be achieved.

4.6 Conclusion

This study investigated possible gender-specific serum lipid biomarkers for AD. Using data from a previous set of studies on the discovery and validation of biomarkers for AD diagnosis and staging, we first examined whether there was a significant interaction effect between gender and AD and further between gender and disease stage. Using an ANCOVA we discovered three AD biomarkers that showed a significant ($p<0.05$) interaction effect between disease stage and gender. Next, we created gender-specific AD case versus control classification models using a lasso logistic regression model. Only one in seven markers was found to be shared between the two gender-specific panels. Coefficient differences between the two gender-specific models appeared to be significantly different as well. Lastly, we built a new ordinal model that included a gender-specific AD biomarker using a Bayesian lasso probit ordinal regression model. We found that the new model had a statistically significantly better performance than a previous model, which was built using the same method but without the addition of a gender-specific biomarker. We concluded that the diagnostic performance of a predictive model of AD can be potentially improved with the inclusion of a gender-specific biomarker. Taken together the results of this study provide strong preliminary evidence of gender-specific biochemical changes in women versus men with AD. Our results suggest that gender-specific serum lipid biomarkers exist that allow for diagnosis of AD and the staging of

disease. Our results suggest that inclusion of gender in the development of biomarker panels for AD will improve the performance of biomarkers which may allow for earlier and more accurate diagnosis of AD, allowing for smaller, more targeted drug trials and the potential to identify gender-specific, disease specific biomarkers that will predict response to AD therapeutics. Clearly, further studies powered to explore gender and AD stage are needed but appear promising.

4.7 References

1. Koran, M. E. I.; Wagener, M.; Hohman, T. J.; Alzheimer's Neuroimaging, I., Sex differences in the association between AD biomarkers and cognitive decline. *Brain imaging and behavior* **2017,** *11* (1), 205-213.

2. Lin, K. A.; Doraiswamy, P. M., When Mars Versus Venus is Not a Cliche: Gender Differences in the Neurobiology of Alzheimer's Disease. *Frontiers in neurology* **2014,** *5*, 288.

3. Andersen, K.; Launer, L. J.; Dewey, M. E.; Letenneur, L.; Ott, A.; Copeland, J. R.; Dartigues, J. F.; Kragh-Sorensen, P.; Baldereschi, M.; Brayne, C.; Lobo, A.; Martinez-Lage, J. M.; Stijnen, T.; Hofman, A., Gender differences in the incidence of AD and vascular dementia: The EURODEM Studies. EURODEM Incidence Research Group. *Neurology* **1999,** *53* (9), 1992-7.

4. Hebert, L. E.; Scherr, P. A.; McCann, J. J.; Beckett, L. A.; Evans, D. A., Is the risk of developing Alzheimer's disease greater for women than for men? *American journal of epidemiology* **2001,** *153* (2), 132-6.

5. Seshadri, S.; Wolf, P. A.; Beiser, A.; Au, R.; McNulty, K.; White, R.; D'Agostino, R. B., Lifetime risk of dementia and Alzheimer's disease. The impact of mortality on risk estimates in the Framingham Study. *Neurology* **1997,** *49* (6), 1498-504.

6. Irvine, K.; Laws, K. R.; Gale, T. M.; Kondel, T. K., Greater cognitive deterioration in women than men with Alzheimer's disease: a meta analysis. *Journal of clinical and experimental neuropsychology* **2012,** *34* (9), 989-98.

7. Apostolova, L. G.; Dinov, I. D.; Dutton, R. A.; Hayashi, K. M.; Toga, A. W.; Cummings, J. L.; Thompson, P. M., 3D comparison of hippocampal atrophy in amnestic

mild cognitive impairment and Alzheimer's disease. *Brain : a journal of neurology* **2006,** *129* (Pt 11), 2867-73.

8. Hua, X.; Hibar, D. P.; Lee, S.; Toga, A. W.; Jack, C. R., Jr.; Weiner, M. W.; Thompson, P. M.; Alzheimer's Disease Neuroimaging, I., Sex and age differences in atrophic rates: an ADNI study with n=1368 MRI scans. *Neurobiology of aging* **2010,** *31* (8), 1463-80.

9. Holland, D.; Desikan, R. S.; Dale, A. M.; McEvoy, L. K.; Alzheimer's Disease Neuroimaging, I., Higher rates of decline for women and apolipoprotein E epsilon4 carriers. *AJNR. American journal of neuroradiology* **2013,** *34* (12), 2287-93.

10. Barnes, L. L.; Wilson, R. S.; Bienias, J. L.; Schneider, J. A.; Evans, D. A.; Bennett, D. A., Sex differences in the clinical manifestations of Alzheimer disease pathology. *Archives of general psychiatry* **2005,** *62* (6), 685-91.

11. Petersen, R. C.; Roberts, R. O.; Knopman, D. S.; Geda, Y. E.; Cha, R. H.; Pankratz, V. S.; Boeve, B. F.; Tangalos, E. G.; Ivnik, R. J.; Rocca, W. A., Prevalence of mild cognitive impairment is higher in men. The Mayo Clinic Study of Aging. *Neurology* **2010,** *75* (10), 889-97.

12. Mielke, M. M.; Vemuri, P.; Rocca, W. A., Clinical epidemiology of Alzheimer's disease: assessing sex and gender differences. *Clinical epidemiology* **2014,** *6*, 37-48.

13. Gao, S.; Hendrie, H. C.; Hall, K. S.; Hui, S., The relationships between age, sex, and the incidence of dementia and Alzheimer disease: a meta-analysis. *Archives of general psychiatry* **1998,** *55* (9), 809-15.

14. Tschanz, J. T.; Corcoran, C. D.; Schwartz, S.; Treiber, K.; Green, R. C.; Norton, M. C.; Mielke, M. M.; Piercy, K.; Steinberg, M.; Rabins, P. V.; Leoutsakos, J. M.; Welsh-Bohmer, K. A.; Breitner, J. C.; Lyketsos, C. G., Progression of cognitive, functional, and neuropsychiatric symptom domains in a population cohort with Alzheimer dementia: the Cache County Dementia Progression study. *The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry* **2011,** *19* (6), 532-42.

15. Perneczky, R.; Drzezga, A.; Diehl-Schmid, J.; Li, Y.; Kurz, A., Gender differences in brain reserve : an (18)F-FDG PET study in Alzheimer's disease. *Journal of neurology* **2007,** *254* (10), 1395-400.

16. Perneczky, R.; Diehl-Schmid, J.; Forstl, H.; Drzezga, A.; Kurz, A., Male gender is associated with greater cerebral hypometabolism in frontotemporal dementia: evidence for sex-related cognitive reserve. *International journal of geriatric psychiatry* **2007,** *22* (11), 1135-40.

17. Skup, M.; Zhu, H.; Wang, Y.; Giovanello, K. S.; Lin, J. A.; Shen, D.; Shi, F.; Gao, W.; Lin, W.; Fan, Y.; Zhang, H.; Alzheimer's Disease Neuroimaging, I., Sex differences in grey matter atrophy patterns among AD and aMCI patients: results from ADNI. *NeuroImage* **2011,** *56* (3), 890-906.

18. Luders, E.; Gaser, C.; Narr, K. L.; Toga, A. W., Why sex matters: brain size independent differences in gray matter distributions between men and women. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2009,** *29* (45), 14265-70.

19. Farrer, L. A.; Cupples, L. A.; Haines, J. L.; Hyman, B.; Kukull, W. A.; Mayeux, R.; Myers, R. H.; Pericak-Vance, M. A.; Risch, N.; van Duijn, C. M., Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *Jama* **1997,** *278* (16), 1349-56.

20. Payami, H.; Zareparsi, S.; Montee, K. R.; Sexton, G. J.; Kaye, J. A.; Bird, T. D.; Yu, C. E.; Wijsman, E. M.; Heston, L. L.; Litt, M.; Schellenberg, G. D., Gender difference in apolipoprotein E-associated risk for familial Alzheimer disease: a possible clue to the higher incidence of Alzheimer disease in women. *American journal of human genetics* **1996,** *58* (4), 803-11.

21. Fleisher, A.; Grundman, M.; Jack, C. R., Jr.; Petersen, R. C.; Taylor, C.; Kim, H. T.; Schiller, D. H.; Bagwell, V.; Sencakova, D.; Weiner, M. F.; DeCarli, C.; DeKosky, S. T.; van Dyck, C. H.; Thal, L. J.; Alzheimer's Disease Cooperative, S., Sex, apolipoprotein E epsilon 4 status, and hippocampal volume in mild cognitive impairment. *Archives of neurology* **2005,** *62* (6), 953-7.

22. Liu, Y.; Paajanen, T.; Westman, E.; Wahlund, L. O.; Simmons, A.; Tunnard, C.; Sobow, T.; Proitsi, P.; Powell, J.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Muehlboeck, S.; Evans, A.; Spenger, C.; Lovestone, S.; Soininen, H.; AddNeuroMed, C., Effect of APOE epsilon4 allele on cortical thicknesses and volumes: the AddNeuroMed study. *Journal of Alzheimer's disease : JAD* **2010,** *21* (3), 947-66.

23. Damoiseaux, J. S.; Seeley, W. W.; Zhou, J.; Shirer, W. R.; Coppola, G.; Karydas, A.; Rosen, H. J.; Miller, B. L.; Kramer, J. H.; Greicius, M. D.; Alzheimer's Disease Neuroimaging, I., Gender modulates the APOE epsilon4 effect in healthy older adults: convergent evidence from functional brain connectivity and spinal fluid tau levels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2012,** *32* (24), 8254-62.

24. Corder, E. H.; Ghebremedhin, E.; Taylor, M. G.; Thal, D. R.; Ohm, T. G.; Braak, H., The biphasic relationship between regional brain senile plaque and neurofibrillary

tangle distributions: modification by age, sex, and APOE polymorphism. *Annals of the New York Academy of Sciences* **2004,** *1019*, 24-8.

25. Sacher, J.; Neumann, J.; Okon-Singer, H.; Gotowiec, S.; Villringer, A., Sexual dimorphism in the human brain: evidence from neuroimaging. *Magnetic resonance imaging* **2013,** *31* (3), 366-75.

26. Luine, V. N., Estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. *Experimental neurology* **1985,** *89* (2), 484-90.

27. Nilsen, J.; Chen, S.; Irwin, R. W.; Iwamoto, S.; Brinton, R. D., Estrogen protects neuronal cells from amyloid beta-induced apoptosis via regulation of mitochondrial proteins and function. *BMC neuroscience* **2006,** *7*, 74.

28. Yao, J.; Brinton, R. D., Estrogen regulation of mitochondrial bioenergetics: implications for prevention of Alzheimer's disease. *Advances in pharmacology* **2012,** *64*, 327-71.

29. Gerstorf, D.; Ram, N.; Hoppmann, C.; Willis, S. L.; Schaie, K. W., Cohort differences in cognitive aging and terminal decline in the Seattle Longitudinal Study. *Developmental psychology* **2011,** *47* (4), 1026-41.

30. Hogervorst, E.; Clifford, A.; Stock, J.; Xin, X.; Bandelow, S.; Exercise to prevent cognitive decline and Alzheimer's disease: for whom, when, what, and (most importantly) how much? *Journal of Alzheimer's Disease & Parkinsonism* **2012**, *2*, e117.

31. Alvarez-de-la-Rosa, M.; Silva, I.; Nilsen, J.; Perez, M. M.; Garcia-Segura, L. M.; Avila, J.; Naftolin, F., Estradiol prevents neural tau hyperphosphorylation characteristic of Alzheimer's disease. *Annals of the New York Academy of Sciences* **2005,** *1052*, 210-24.

32. Altmann, A.; Tian, L.; Henderson, V. W.; Greicius, M. D.; Alzheimer's Disease Neuroimaging Initiative, I., Sex modifies the APOE-related risk of developing Alzheimer disease. *Annals of neurology* **2014,** *75* (4), 563-73.

33. Xing, Y.; Tang, Y.; Jia, J., Sex Differences in Neuropsychiatric Symptoms of Alzheimer's Disease: The Modifying Effect of Apolipoprotein E epsilon4 Status. *Behavioural neurology* **2015,** *2015*, 275256.

34. Laws, K. R.; Irvine, K.; Gale, T. M., Sex differences in cognitive impairment in Alzheimer's disease. *World journal of psychiatry* **2016,** *6* (1), 54-65.

35. Xing, Y.; Wei, C.; Chu, C.; Zhou, A.; Li, F.; Wu, L.; Song, H.; Zuo, X.; Wang, F.; Qin, W.; Li, D.; Tang, Y.; Jia, X. F.; Jia, J., Stage-specific gender differences in cognitive and neuropsychiatric manifestations of vascular dementia. *American journal of Alzheimer's disease and other dementias* **2012,** *27* (6), 433-8.

36. Ott, B. R.; Lapane, K. L.; Gambassi, G., Gender differences in the treatment of behavior problems in Alzheimer's disease. SAGE Study Group. Systemic Assessment of Geriatric drug use via Epidemiology. *Neurology* **2000,** *54* (2), 427-32.

37. Carter, C. L.; Resnick, E. M.; Mallampalli, M.; Kalbarczyk, A., Sex and gender differences in Alzheimer's disease: recommendations for future research. *Journal of women's health* **2012,** *21* (10), 1018-23.

38. Laws, K. R.; Irvine, K.; Gale, T. M., Sex differences in cognitive impairment in Alzheimer's disease. *World journal of psychiatry* **2016,** *6* (1), 54-65.

39. Cahill, L., Fundamental sex difference in human brain architecture. *Proceedings of the National Academy of Sciences of the United States of America* **2014,** *111* (2), 577-8.

40. Anand, S.; Barnes, J. M.; Young, S. A.; Garcia, D. M.; Tolley, H. D.; Kauwe, J. S. K.; Graves, S. W., Discovery and Confirmation of Diagnostic Serum Lipid Biomarkers for Alzheimer's Disease Using Direct Infusion Mass Spectrometry. *Journal of Alzheimer's disease : JAD* **2017,** *59* (1), 277-290.

41. Yang, H.; Lyutvinskiy, Y.; Herukka, S. K.; Soininen, H.; Rutishauser, D.; Zubarev, R. A., Prognostic polypeptide blood plasma biomarkers of Alzheimer's disease progression. *Journal of Alzheimer's disease : JAD* **2014,** *40* (3), 659-66.

42. Lyutvinskiy, Y.; Yang, H.; Rutishauser, D.; Zubarev, R. A., In silico instrumental response correction improves precision of label-free proteomics and accuracy of proteomics-based predictive models. *Molecular & cellular proteomics : MCP* **2013,** *12* (8), 2324-31.

43. Forlenza, O. V.; Radanovic, M.; Talib, L. L.; Aprahamian, I.; Diniz, B. S.; Zetterberg, H.; Gattaz, W. F., Cerebrospinal fluid biomarkers in Alzheimer's disease: Diagnostic accuracy and prediction of dementia. *Alzheimer's & dementia* **2015,** *1* (4), 455-63.

44. Ferrari, R.; Dawoodi, S.; Raju, M.; Thumma, A.; Hynan, L. S.; Maasumi, S. H.; Reisch, J. S.; O'Bryant, S.; Jenkins, M.; Barber, R.; Momeni, P., Androgen receptor gene and sex-specific Alzheimer's disease. *Neurobiology of aging* **2013,** *34* (8), 2077 e19-20.

45. Cohen, J. *Statistical power analysis for the behavioral sciences*, 2nd ed.; Lawrence Erlbaum Associates: New York, 1988.

46. Li, R.; Singh, M., Sex differences in cognitive impairment and Alzheimer's disease. *Frontiers in neuroendocrinology* **2014,** *35* (3), 385-403.

47. Duff, K.; Schoenberg, M. R.; Mold, J. W.; Scott, J. G.; Adams, R. L., Gender differences on the Repeatable Battery for the Assessment of Neuropsychological Status subtests in older adults: baseline and retest data. *Journal of clinical and experimental neuropsychology* **2011,** *33* (4), 448-55.

48. Campos, A.; Perez-Fabello, M. J.; Gomez-Juncal, R., Gender and age differences in measured and self-perceived imaging capacity. *Personality and individual difference* **2004**, *37* (7), 1383-1389.

49. van Amelsvoort, T.; Compton, J.; Murphy, D., In vivo assessment of the effects of estrogen on human brain. *Trends in endocrinology and metabolism: TEM* **2001,** *12* (6), 273-6.

50. Goldstein, J. M.; Seidman, L. J.; Horton, N. J.; Makris, N.; Kennedy, D. N.; Caviness, V. S., Jr.; Faraone, S. V.; Tsuang, M. T., Normal sexual dimorphism of the adult human brain assessed by in vivo magnetic resonance imaging. *Cerebral cortex* **2001,** *11* (6), 490-7.

51. Sohrabji, F.; Miranda, R. C.; Toran-Allerand, C. D., Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. *Proceedings of the National Academy of Sciences of the United States of America* **1995,** *92* (24), 11110-4.

52. Shao, H.; Breitner, J. C.; Whitmer, R. A.; Wang, J.; Hayden, K.; Wengreen, H.; Corcoran, C.; Tschanz, J.; Norton, M.; Munger, R.; Welsh-Bohmer, K.; Zandi, P. P.; Cache County, I., Hormone therapy and Alzheimer disease dementia: new findings from the Cache County Study. *Neurology* **2012,** *79* (18), 1846-52.

53. Sato, Y.; Suzuki, I.; Nakamura, T.; Bernier, F.; Aoshima, K.; Oda, Y., Identification of a new plasma biomarker of Alzheimer's disease using metabolomics technology. *Journal of lipid research* **2012,** *53* (3), 567-76.

54. Holland, D.; Desikan, R. S.; Dale, A. M.; McEvoy, L. K.; Alzheimer's Disease Neuroimaging, I., Higher rates of decline for women and apolipoprotein E epsilon4 carriers. *AJNR. American journal of neuroradiology* **2013,** *34* (12), 2287-93.

55. Pietraforte, D.; Straface, E.; Piscopo, P.; Vona, R.; Confaloni, A., Sex-related biomarkers in cardiovascular and neurodegenerative disorders. *Annali dell'Istituto*

superiore di sanita **2016,** *52* (2), 230-9.

56. Perneczky, R.; Drzezga, A.; Diehl-Schmid, J.; Li, Y.; Kurz, A., Gender differences in brain reserve : an (18)F-FDG PET study in Alzheimer's disease. *Journal of neurology* **2007,** *254* (10), 1395-400.

57. Piscopo, P.; Canterini, S.; Carletti, V.; Rosa, P.; Crestini, A.; Fiorenza, M. T.; Confaloni, A.; Sex effect on presenilins expression in post-natal rat brain. *Advances in Bioscience and Biotechnology* **2013**, *4*, 1086-1094.

58. Rosario, E. R.; Carroll, J. C.; Oddo, S.; LaFerla, F. M.; Pike, C. J., Androgens regulate the development of neuropathology in a triple transgenic mouse model of Alzheimer's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2006,** *26* (51), 13384-9.

59. Ribeiro, C. A.; Santana, I.; Oliveira, C.; Baldeiras, I.; Moreira, J.; Saraiva, M. J.; Cardoso, I., Transthyretin decrease in plasma of MCI and AD patients: investigation of mechanisms for disease modulation. *Current Alzheimer research* **2012,** *9* (8), 881-9.

60. Piscopo, P.; Rivabene, R.; Galimberti, D.; Crestini, A.; Talarico, G.; Vanacore, N.; Scarpini, E.; Bruno, G.; Confaloni, A., Gender effects on plasma PGRN levels in patients with Alzheimer's disease: a preliminary study. *Journal of Alzheimer's disease : JAD* **2013,** *35* (2), 313-8.

61. Johnston, J. M.; Hu, W. T.; Fardo, D. W.; Greco, S. J.; Perry, G.; Montine, T. J.; Trojanowski, J. Q.; Shaw, L. M.; Ashford, J. W.; Tezapsidis, N.; Alzheimer's Disease Neuroimaging, I., Low plasma leptin in cognitively impaired ADNI subjects: gender differences and diagnostic and therapeutic potential. *Current Alzheimer research* **2014,** *11* (2), 165-74.

62. Yang, H.; Lyutvinskiy, Y.; Herukka, S. K.; Soininen, H.; Rutishauser, D.; Zubarev, R. A., Prognostic polypeptide blood plasma biomarkers of Alzheimer's disease progression. *Journal of Alzheimer's disease : JAD* **2014,** *40* (3), 659-66.

63. Miike, K.; Aoki, M.; Yamashita, R.; Takegawa, Y.; Saya, H.; Miike, T.; Yamamura, K., Proteome profiling reveals gender differences in the composition of human serum. *Proteomics* **2010,** *10* (14), 2678-91.

64. Varma, V. R.; Varma, S.; An, Y.; Hohman, T. J.; Seddighi, S.; Casanova, R.; Beri, A.; Dammer, E. B.; Seyfried, N. T.; Pletnikova, O.; Moghekar, A.; Wilson, M. R.; Lah, J. J.; O'Brien, R. J.; Levey, A. I.; Troncoso, J. C.; Albert, M. S.; Thambisetty, M., Alpha-2 macroglobulin in Alzheimer's disease: a marker of neuronal injury through the RCAN1 pathway. *Molecular psychiatry* **2017,** *22* (1), 13-23.

65. Ronquillo, J. G.; Baer, M. R.; Lester, W. T., Sex-specific patterns and differences in dementia and Alzheimer's disease using informatics approaches. *Journal of women & aging* **2016,** *28* (5), 403-11.

66. Gamberger, D.; Zenko, B.; Mitelpunkt, A.; Lavrac, N.; Identification of gender specific biomarkers for Alzheimer's disease. *Brain Informatics and Health* **2015**, LNAI 9250, 57-66.

Chapter 5 Concluding Remarks

5.1 Summary

5.1.1 Summary: Chapter 2

Endogenous digitalis-like factors (EDLF) are sodium pump (SP) inhibitors that have been associated with a number of diseases including preeclampsia (PE). Altered lipid metabolism is thought to play an important role in the development of PE, even though the exact pathogenesis of PE remains enigmatic. In this study involving placental tissues from 20 normal pregnancies and ouabain, the most often studied EDLF, we found that normal human placenta seems to respond to SP inhibition caused by ouabain with a generally consistent set of changes in the abundance of lipids in the placenta cytosol, we also discovered a characteristic set of lipid changes indicative of EDLF exposure using a mass spectrometry-based lipidomics method. Among the 1207 lipidomic markers surveyed by Student t-test, a set of 8 lipidomic markers was chosen after applying a statistical model built with a sparse partial least squares discriminant analysis method (sPLS-DA) and a bootstrap procedure, followed by chemical characterization with tandem MS. The selected lipid markers could potentially then be used to identify placentas that have been previously exposed to EDLF.

5.1.2 Summary: Chapter 3

Endogenous peptides and small proteins are important to most physiological processes and may regulate or contribute to the pathophysiology of PE. It has been suggested that not only the placenta plays a central role in the pathogenesis of PE, but it produces and releases EDLF

itself. EDLF as a SP inhibitor has been associated with several features of PE. Using placenta tissues from 20 healthy pregnancies, we tested the following hypotheses: firstly, normal human placenta responds to SP inhibition caused by ouabain, this EDLF studied with a change in the abundance of peptides and small proteins in the cytosol; and secondly, if such changes occur, that there would be a characteristic set or pattern of responses. A modified procedure of acetonitrile (ACN) precipitation was employed to remove high abundance, high molecular weight (MW) proteins, followed by a global cLC-MS-based peptidomics approach. 275 candidate peptidomic markers were observed and evaluated using the Student t-test. However, no peptidomic marker or set of markers was revealed by this particular peptidomics approach after correction for a false discovery rate or using more conservative statistical models. Evidence also suggested that the predictive performance of the existing lipidomic profile in response to EDLF exposure is unlikely to benefit from the addition of any peptidomic markers studied here.

5.1.3 Summary: Chapter 4

It is believed that Alzheimer's disease (AD) is a biologically and biochemically complex disease of the cerebral cortex. Despite its potentially important role in the pathobiology, clinical symptoms and diagnosis of AD, gender has largely been overlooked in the majority of the AD research conducted in the past. We investigated the potential gender differences in serum lipid biomarkers for AD using data obtained from a previous set of studies, with the attempt to improve diagnostic tools for early AD to allow for more efficient drug studies. We created ANOVA (Analysis of Variance) and ANCOVA (Analysis of Covariance) models to test three hypotheses, the first of which was the hypothesis that there is no interaction between gender and AD disease stage. Biomarkers that demonstrated significant ($p<0.05$) gender interaction with

disease stage at the FDR=0.3 level were reported for each of three models. Next, we created binary gender-specific AD cases vs. controls classification models using lasso logistic regression models, followed by additional numerical studies. Several markers showed different expression in AD subjects that were gender specific. The coefficient differences between the two gender-specific multi-marker models also appeared to be significantly different, indicating significantly different biochemical profiles in AD cases between the two genders. Last but not least, we tested whether the addition of a gender-specific marker would improve the diagnostic performance of a predictive model of AD using a Bayesian lasso probit ordinal regression model. We found that the new ordinal model performed statistically significantly better than the previous model which did not consider gender.

5.2 Limitations

5.2.1 Limitations: Chapter 2

In an attempt to gain a better understanding of the pathogenesis of PE, we selected and chemically characterized a set of 8 lipidomic placental biomarkers that were indicative of EDLF exposure using a mass spectrometry-based lipidomics approach. Despite the low abundances of these markers, we obtained good quality fragmentation spectra for all 8 markers. However, as with most lipidomic MS results, none of the 8 markers was uniquely identified due to several reasons. The most prominent of these is that there are only very limited archives of lipids in all current databases. These archives are not searchable by fragmentation data by rely only on mass. Another very significant problem is that many lipids share the same or very nearly the same mass with other lipids making identification difficult. Based on our experience using the available

databases for lipid identification, searches that use accurate mass only almost always result in a large number of various lipid species of different classes that have shared accurate mass. Another problem is that of overlapping peaks. This often confounds fragmentation data making it problematic to know which fragments are derived from one compound. In addition, fragmentation spectra can vary somewhat depending on the choice of the collision energy, adduct ion, type of mass spectrometer[5], etc. As a result, the chemical identity and hence biological function of these markers in the pathogenesis of PE remain unknown even though we were able to predict the elemental compositions and possible structural features of each of the 8 lipid markers. Confirmation studies of new samples should be conducted to confirm the selected markers that can indicate EDLF exposure in the placenta.

5.2.2 Limitations: Chapter 3

We tested whether normal human placenta responds to SP inhibition caused by ouabain, an EDLF, with changes in the abundance of peptides. We used a MS based peptidomics approach. While some peptides had values at $p<0.05$, none displayed significant changes in abundance with ouabain exposure after correcting for multiple comparisons. This result could mean that the concentration of ouabain, which was only tested at 50 nM, may have been too low to produce a statistically significant difference[14]. It may be that the tissues may have not been exposed for a long enough period of time. It may be that tissue processing may be somewhat irreproducible obscuring smaller changes, considering the anatomical complexity of the placenta. We attempted to collect tissues in the intervillous region, it is possible that a small part of the chorionic plate or the basal plate was collected as well. Variation in placenta morphology might also contribute to the lacking of peptidomic markers discovered in this study. It may be that the

tissues were unresponsive to ouabain, which more selectively inhibits the α3 isoform of the SP. There may have been limitations in only running the mass spectrometer in the positive ion mode[13]. The lack of an internal time alignment standard between the 33-35 min time window may have increased the chromatographic variability, limiting the detection of candidate markers. We used acetonitrile precipitation to remove high abundance and high MW proteins present in the placenta homogenates, therefore limiting our study to low abundance, low MW peptides only. It would be interesting and potentially more informative to investigate a broader range of biomolecules using a proteomics approach.

5.2.3 Limitations: Chapter 4

We discovered and identified gender-specific markers by analyzing data acquired from a previous study of diagnostic serum lipid biomarkers for AD[21]. The inclusion of gender-specific markers also improved the predictive performance of an AD diagnostic model significantly statistically. However, the data obtained in this discovery phase must be confirmed to ensure that the gender-specific markers observed continue to show diagnostic utility. A lack of reproducibility may result from technical variability, sample preparation, and biological variability[17]. Therefore, a confirmatory study designed and powered appropriately is needed using other serum specimens from a new set of AD patients.

It was suggested that lipid metabolism disorders are related to AD and lipids have a role in the pathophysiology of AD[18]. Additionally, a large multitude of proteins and peptides may contribute to AD pathology[19]. In fact, a previous study published in our lab discovered and confirmed 11 low abundance and low molecular weight (MW) serum peptide markers that allowed for the diagnosis of AD in most subjects[20]. It is of interest to analyze the same data and

examine the confirmed 11 markers with the consideration of gender. Again, additional studies should be conducted to determine if any previously found serum peptide AD marker as well as novel markers are gender-specific.

5.3 Future objectives

5.3.1 Future research objective: Chapter 2

Clearly further efforts are needed to identify those placental lipid markers that were indicative of EDLF exposure. Use of a more mass accurate mass spectrometer might help. This should allow for greater confidence in the elemental composition. It is possible that using an instrument with different ionization methods might produce different fragments allowing for a more complete understanding of the molecular components making the parent molecule. Having MS^n likewise using ion trap or FTMS might produce smaller identifiable constituents. We can also benefit from the huge collection of MS^n spectra available in the NIST MS Search database, although the differences between the fragmentation rules for electron ionization and those for ESI should be considered.

Gas chromatography mass spectrometry (GC-MS) may be used although derivatization steps are needed for nonvolatile lipids like fatty acids and sterol[7, 8]., but this approach is unlikely to work and allow complete structural determination because it assumes that we know minimally the class of the molecule and functional groups that can be derivatized.

Ion-mobility spectrometry (IMS) coupled to MS is traditionally used to character peptides, proteins and small molecules[25]. Recently a few studies have shown its application in the characterization and identification of lipids in complex biological samples using the shotgun

lipidomics method[24]. IMS-MS can potentially enhance sensitivity, spectral clarity, overall detection limits, and allow selective analysis of lipid species that have the same accurate mass and improved lipid identification[26]. Therefore, it is of interest to utilize IMS-MS to elucidate further the class and structure of the lipid markers that were indicative of EDLF exposure in our study. IMS-MS may also allow the separation of isomeric and isobaric lipid species although instrument with better resolving power may be needed. The differentiation of lipid classes and subclasses contributes to the improved structural annotation of unknown lipid markers. In addition, computational modeling supported by IMS-MS instrument may complement lipid marker identification achieved through UHPLC IMS MS studies[25].

Another possible approach involves the use of reported data in the literature. Since the fragmentation spectra, such are we obtained, are not included in any major databases like LIPID MAPS structure database and HMDB among many others[5], we may look for lipids that have similar fragmentation patterns as published in the literature instead of focusing on direct identification of the markers by comparing MS/MS spectra alone. Shared fragmentation pathways usually indicate structural similarity between known lipids and unidentified ones[4, 5, 7]. However, this requires that the someone else has started with the compound of interest and submitted it to MS-MS fragmentation studies. However, one can learn a great deal even from fragmentation spectra of structurally related lipids. If a hypothesized structure can be determined, it may be possible to synthesize it, if it is not commercially available, especially since the number of commercially available lipid standards is limited. Then, we will compare the MS/MS spectra of the synthetic standard with those of the selected markers. We may also test whether the synthetic standard will elute with the same retention times as the endogenous markers using LC-MS. Combined with exact mass studies and possibly co-elution studies, this

approach will potentially enable us to confirm the final structures of the markers[2]. However, this particular method is labor intensive by its nature, requires a degree of luck and may require the collaboration with organic chemists.

The advances of bioinformatics technology may also enable the identification of novel lipids in our study. This approach requires not only a thorough understanding of the biochemical aspect of the study, but computational skills and the ability to use cheminformatics and variable statistics tools. For example, we may build in silico MS/MS libraries based on the fragmentation patterns of external reference standards, fragmentation rules, and computer-generated structures to facilitate the identification of novel lipids[12].

Compared to direct infusion used in shotgun lipidomics, LC-MS analysis of complex lipid mixtures improves sensitivity, enables the separation of isomeric lipid species, and increases the likelihood of successfully identification by adding the parameter of retention time. Therefore, the addition of chromatographic separation of lipid extracts might improve structure elucidation of already selected markers and reveal new candidate lipid markers that can indicate EDLF exposure in placenta. Additionally, we could run the same samples in negative ion mode (ESI-MS/MS), with a variety of collision energies, thus making full use of all the fragmentation spectra available in different databases. However, while it is possible that new lipid markers belonging to different classes may be revealed, structure elucidation of new markers is not guaranteed and it will be nearly impossible to differentiate newly discovered markers from previously selected ones because it is likely to get a completely different set of peaks in the negative mode for the same molecule.

Another possible approach is LC-NMR, which stands for liquid chromatography nuclear magnetic resonance[1]. Despite its high reproducibility, NMR has poor resolving power and low

sensitivity compared with MS[8]. Samples need to be purified and or enriched prior to the analysis using NMR spectrometry, and a substantially larger amount and significantly purer sample is usually required for NMR analysis when it is compared to mass spectrometry[25]. However, successful structural elucidation of unknown lipids in complex mixtures without isolating individual lipids had been reported by combining chromatographic separation methods such as LC with high resolution NMR spectroscopy, in addition to mass spectrometry[3, 8, 10, 11]. It was also suggested that the elucidation of the exact structure of lipids still requires MS^n experiments, in addition to large-scale biochemical isolation and NMR[4]. The complexity of our sample and the low abundances of selected markers may limit the use of this approach. It has been suggested that LC-NMR is more preferable when a lot of information regarding the sample is already known if the lipids under analysis have lower percentage[25], however, the approach may provide complementary structural information if we use it at the right time.

5.3.2 Future research objective: Chapter 3

It might worth running the same samples in negative ion mode (ESI-MS). Additional candidate peptidomic markers that could indicate EDLF exposure in the placenta may be revealed, especially acidic peptides that are more likely to form deprotonated molecules. In addition, cleaner fragmentation spectra have been reported in the negative ion mode that might improve the possibility of successfully identification[27].

Peptides and small proteins are indispensible to most physiological processes, therefore it is worth investigating the peptidome in placental cytosol in response to a higher final concentration of EDLF. A broader range of peptides, small proteins and metabolites can be surveyed if we run the MS in both positive and negative ion mode.

In addition to focusing on low abundance and low MW peptides, we can also employ a more global proteomic approach to survey the full length proteins present in human placental cytosol, one such method is termed MudPIT, short for Multidimensional Protein Identification Technology. Unlike 2D-gel electrophoresis that focuses more on high abundance proteins, MudPIT allows for a more comprehensive, non-biased study of placental proteome when coupled with effective sample fractionation methods such as LC or ultracentrifugation. In one study, differential sucrose gradient ultracentrifugation enabled the proteins in tissue homogenates to be extracted into separate subcellular fractions, therefore substantially reducing sample complexity and allowed for the detection of both low abundance and high abundance proteins[15]. The MudPIT analysis of each fraction can be repeated more than once to increase detection coverage. Following tissue homogenization and protein extraction, proteins are digested with trypsin, the resulting peptide mixtures are separated using reverse-phase and strong cation exchange (SCX) columns[16]. After chromatographic separation of the peptides, proteins can be identified by comparison to protein databases.

5.3.3 Future research objective: Chapter 4

The candidate gender-specific serum lipid AD markers need to be confirmed to insure that they are reproducible and useful. Previously unanalyzed serum specimens from a different group of AD patients will be analyzed with the same MS based untargeted serum lipidomics approach[21]. New samples will be processed following the same procedure. If different lipid markers for different genders were both discovered and confirmed, it is reasonable to move on to targeted evaluation of gender-specific markers in larger cohorts of AD patients in a clinical validation phase[22]. Both the diagnostic accuracy and predictive ability of the markers will be

assessed in different study populations[23]. Multi-marker panels can be modeled based on replicating markers.

Low abundance proteins and peptides had also been associated with AD pathology. Therefore, it is equally interesting to explore whether any of the confirmed peptide markers for AD diagnosis are gender specific. A confirmatory study would then be necessary if any promising gender-specific peptide AD markers.

If gender-specific lipid/peptide markers can be discovered and confirmed, it might be interesting to investigate gender-specific markers for different disease stages of AD (CDR 0.5, CDR1, CDR2, and CDR3). Results obtained from the ANOVA model (where the disease stage effect is categorical) and the ANCOVA model (where the disease stage effect is linear) have suggested this possibility. The large number of gender differences in various aspects of AD such as symptoms, risks of developing the disease and brain physiology may potentially result from different pathophysiology related to gender, and possibly gender-specific path of disease progression. This could consequently impact the effective diagnosis and treatment of the disease for different genders.

5.4 References

1. Mehta, B. K.; Kori, P.; Mehta, D.; Misra, H., Novel lipid constituents identified from the leaves of *Artabotrys odoratissimus* (R.Br). *Arabian journal of chemistry* **2017**, *10*, S742-S746.

2. Garrett, T. A.; Raetz, C. R.; Richardson, T.; Kordestani, R.; Son, J. D.; Rose, R. L., Identification of phosphatidylserylglutamate: a novel minor lipid in Escherichia coli. *Journal of lipid research* **2009,** *50* (8), 1589-99.

3. Mehta, B. K.; Verma, M.; Gupta, M., Novel lipid constituents identified in seeds of *Nigella sativa* (Linn). *J. Braz. Chem. Soc.* **2008**, *19* (3), 458-462.

4. Papan, C.; Penkov, S.; Herzog, R.; Thiele, C.; Kurzchalia, T.; Shevchenko, A., Systematic screening for novel lipids by shotgun lipidomics. *Analytical chemistry* **2014,** *86* (5), 2703-10.

5. Hartler, J.; Triebl, A.; Ziegl, A.; Trotzmuller, M.; Rechberger, G. N.; Zeleznik, O. A.; Zierler, K. A.; Torta, F.; Cazenave-Gassiot, A.; Wenk, M. R.; Fauland, A.; Wheelock, C. E.; Armando, A. M.; Quehenberger, O.; Zhang, Q.; Wakelam, M. J. O.; Haemmerle, G.; Spener, F.; Kofeler, H. C.; Thallinger, G. G., Deciphering lipid structures based on platform-independent decision rules. *Nature methods* **2017,** *14* (12), 1171-1174.

6. Fiehn, O., Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling. *Current protocols in molecular biology* **2016,** *114*, 30 4 1-30 4 32.

7. Li, L.; Han, J.; Wang, Z.; Liu, J.; Wei, J.; Xiong, S.; Zhao, Z., Mass spectrometry methodology in lipid analysis. *International journal of molecular sciences* **2014,** *15* (6), 10492-507.

8. Alexandri, E.; Ahmed, R.; Siddiqui, H.; Choudhary, M. I.; Tsiafoulis, C. G.; Gerothanassis, I. P., High Resolution NMR Spectroscopy as a Structural and Analytical Tool for Unsaturated Lipids in Solution. *Molecules* **2017,** *22* (10).

9. Kliman, M.; May, J. C.; McLean, J. A., Lipid analysis and lipidomics by structurally selective ion mobility-mass spectrometry. *Biochimica et biophysica acta* **2011,** *1811* (11), 935-45.

10. Willmann, J.; Thiele, H.; Leibfritz, D., Combined reversed phase HPLC, mass spectrometry, and NMR spectroscopy for a fast separation and efficient identification of phosphatidylcholines. *Journal of biomedicine and biotechnology* **2011**, *2011*, Article ID 385786, 1-8.

11. Elshafie, H. S.; Viggiani, L.; Mostafa, M. S.; El-Hashash, M. A.; Camele, I.; Bufo, S., Biological activity and chemical identification of ornithine lipid produced by *Burkholderia gladioli* pv. *agaricicola* ICMP 11096 using LC-MS and NMR analyses. *Journal of biological research* **2017**, *90*:6534.

12. Ma, Y.; Kind, T.; Vaniya, A.; Gennity, I.; Fahrmann, J. F.; Fiehn, O., An in silico MS/MS library for automatic annotation of novel FAHFA lipids. *Journal of cheminformatics* **2015,** *7*, 53.

13. Schrader, M.; Knappe, P. S.; Fricker, L. D., Historical perspective of peptidomics. *EuPA open proteomics* **2014**, *3*, 171-182.

14. Ma, J.; Esplin, M. S.; Adair, C. D.; Mason, L. A.; Graves, S. W., Increasing evidence for and regulation of a human placental endogenous digitalis-like factor. *Reproductive sciences* **2012,** *19* (4), 437-48.

15. Kislinger, T.; Gramolini, A. O.; MacLennan, D. H.; Emili, A., Multidimensional protein identification technology (MudPIT): technical overview of a profiling method optimized for the comprehensive proteomic investigation of normal and diseased heart tissue. *Journal of the American Society for Mass Spectrometry* **2005,** *16* (8), 1207-20.

16. Maor, R.; Jones, A.; Nuhse, T. S.; Studholme, D. J.; Peck, S. C.; Shirasu, K., Multidimensional protein identification technology (MudPIT) analysis of ubiquitinated proteins in plants. *Molecular & cellular proteomics : MCP* **2007,** *6* (4), 601-10.

17. Knepper, M. A., Common sense approaches to urinary biomarker study design. *Journal of the American Society of Nephrology : JASN* **2009,** *20* (6), 1175-8.

18. Zarrouk, A.; Debbabi, M.; Bezine, M.; Karym, E. M.; Badreddine, A.; Rouaud, O.; Moreau, T.; Cherkaoui-Malki, M.; El Ayeb, M.; Nasser, B.; Hammami, M.; Lizard, G., Lipid Biomarkers in Alzheimer's Disease. *Current Alzheimer research* **2018,** *15* (4), 303-312.

19. Thambisetty, M.; Lovestone, S., Blood-based biomarkers of Alzheimer's disease: challenging but feasible. *Biomarkers in medicine* **2010,** *4* (1), 65-79.

20. Shah, D. J.; Rohlfing, F.; Anand, S.; Johnson, W. E.; Alvarez, M. T.; Cobell, J.; King, J.; Young, S. A.; Kauwe, J. S.; Graves, S. W., Discovery and Subsequent Confirmation of Novel Serum Biomarkers Diagnosing Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* **2016,** *49* (2), 317-27.

21. Anand, S.; Barnes, J. M.; Young, S. A.; Garcia, D. M.; Tolley, H. D.; Kauwe, J. S. K.; Graves, S. W., Discovery and Confirmation of Diagnostic Serum Lipid Biomarkers for Alzheimer's Disease Using Direct Infusion Mass Spectrometry. *Journal of Alzheimer's disease : JAD* **2017,** *59* (1), 277-290.

22. Lescuyer, P.; Hochstrasser, D.; Rabilloud, T., How shall we use the proteomics toolbox for biomarker discovery? *Journal of proteome research* **2007,** *6* (9), 3371-6.

23. Rifai, N.; Gerszten, R. E., Biomarker discovery and validation. *Clinical chemistry* **2006**, *52* (9), 1635-1637.

24. Paglia, G.; Kliman, M.; Claude, E.; Geromanos, S.; Astarita, G., Applications of ion-mobility mass spectrometry for lipid analysis. *Analytical and bioanalytical chemistry* **2015,** *407* (17), 4995-5007.

25. Alsante, K. M.; Baertschi, S. W.; Coutant, M.; Marquez, B. L.; Sharp, T. R.; Zelesky, T. C., Degradation and impurity analysis for pharmaceutical drug candidates. *Separation science and technology* **2011**, *10*, 59-169.

26. Hinz, C.; Liggi, S.; Griffin, J. L., The potential of Ion Mobility Mass Spectrometry for high-throughput and high-resolution lipidomics. *Current opinion in chemical biology* **2018,** *42*, 42-50.

27. Gao, J.; Cassady, C. J., Negative ion production from peptides and proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid communications in mass spectrometry : RCM* **2008,** *22* (24), 4066-72.