의학박사 학위논문

Discovery of Peripheral Biomarkers for Major Depressive Disorder Using Proteomics and Heart Rate Variability Analysis

단백체 분석 및 심박변이도 분석을 통한 주요우울증의 말초 바이오마커 발굴

2016년 2월

서울대학교 대학원
의학과 정신과학 전공
김 은 영

Discovery of Peripheral Biomarkers for Major Depressive Disorder Using Proteomics and Heart Rate Variability Analysis

by

Eun Young Kim

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Psychiatry) at Seoul National University College of Medicine

December 2015

Approved by Thesis Committee:

Professor _____Chairman

Professor _____Vice chairman

Professor _____

Professor _____

Professor _____

단백체 분석 및 심박변이도 분석을 통한 주요우울증의 말초 바이오마커 발굴

지도교수 안 용 민

이 논문을 의학박사 학위논문으로 제출함

2015 년 10 월

서울대학교 대학원

의학과 정신과학 전공

김 은 영

김은영의 의학박사 학위논문을 인준함

2015 년 12 월

위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

Abstract

Eun Young Kim
Medical Science (Psychiatry)
The Graduate School
Seoul National University

Introduction: Major depressive disorder (MDD) is a systemic and multifactorial disorder involving complex interactions between genetic predisposition and disturbances of various molecular pathways. Its underlying molecular pathophysiology remains unclear, and no valid and objective diagnostic tools for the condition are available. This study performed a quantitative proteomic analysis using serum samples and heart rate variability (HRV) analysis for the identification of novel peripheral markers for depression. Additionally, we attempted to improve classification accuracy by combining proteomic and HRV markers using machine learning methods.

Methods: The study subjects consisted of 25 drug-free female MDD patients and 25 age- and sex- matched healthy controls. First, quantitative serum proteome profiles were obtained and analyzed by liquid chromatography-tandem mass spectrometry using serum samples from 10 MDD patients and 10 healthy controls. Next, candidate biomarker sets were verified using multiple-reaction monitoring in 25 patients and 25 healthy controls. The panel of potential protein biomarkers was selected using logistic regression with lasso regularization. We also analyzed 22 linear and nonlinear HRV parameters and selected important features for discriminating patients with MDD from healthy controls using a support vector machine with recursive feature eliminations. Finally, we identified combined biomarker panels consisting of proteins and HRV indexes with maximum classification

accuracy.

Results: Among several linear and nonlinear HRV parameters, seven indexes, including RMSSD, SDNN, pNN10, LF, ApEn, SampEn, and CSE20, were able to classify patients with MDD versus normal controls with 64% diagnostic accuracy. The proteomic analysis identified a serum biomarker panel consisting of six proteins, including apolipoprotein D, apolipoprotein B, group-specific component, ceruloplasmin, hormerin, and profilin1, with 67% classification accuracy. In the combined classification analysis, a separation between MDD and normal controls could be achieved using five parameters, apolipoprotein D, group-specific component, ceruloplasmin, RMSSD, and SampEn, with 80% classification accuracy.

Conclusions: In this study, we were able to find peripheral biomarker candidates that changed quantitatively in patients with MDD. The HRV analysis identified several classifiers that were associated with sympathetic dominance and reduced complexity of heart rate dynamics. The proteomic analysis revealed altered serum proteins that were related to the modulation of the immune and inflammatory systems, oxidative system, and lipid metabolism in MDD. Better classification accuracy can be achieved by combining HRV and proteomic data compared with using either alone. Further studies using larger, independent cohorts are needed to verify the role of these candidate biomarkers for the diagnosis of MDD.

Keywords: Major depressive disorder, Proteomics, Heart rate variability, Biomarker, Machine learning

***Student Number*:** 2011-30541

Tables of contents

List of tables

List of figures

List of abbreviation

AD: Alzheimer's disease

ANOVA: Analyses Of Variance

ApEn: Approximate entropy

Apo-B: Apolipoprotein B

Apo-D: Apolipoprotein D

BAI: Beck Anxiety Inventory

BBB: Blood-brain barrier

BDNF: Brain-derived neurotrophic factor

BHS: Beck Hopelessness Scale

BIS: Barratt Impulsivity Scale

BMI: Body mass index

BSI: Beck Scale for Suicide Ideation

CAN: Central autonomic network

CSEx: Corrected Shannon entropy

CSF: Cerebrospinal fluid

CV: Cross-validation

CVD: Cardiovascular disease

DALYs: Disability adjusted life years

DAVID: Visualization and Integrated Discovery

DEP: Differentially Expressed Protein

DFA: Detrended fluctuation analysis

ECG: Electrocardiogram

ESI: Electrospray ionization

FASP: Filter Aided Sample Preparation

GOBP: Gene Ontology Biological Process

HAMD: Hamilton Rating Scale for Depression

HDL: High-density lipoproteins

HF: High frequency

HFnu: HF normalized unit

HPA: Hypothalamic–pituitary–adrenal

HRV: heart rate variability

IDS-SR: Inventory of Depressive Symptomatology-Self-report version

iTRAQ: Isobaric tags for relative and absolute quantitation

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LDAEP: loudness dependence of auditory evoked potentials

LDL: Low density lipoproteins

LF: Low frequency

LF/HF: Ratio of LF to HF power

LFnu: LF normalized unit

LODs: limits of detection

MANOVA: Multivariate analysis of variance

MALDI: Matrix-assisted laser desorption/ionization

MDD: Major Depressive Disorder

M.I.N.I.: Mini-International-Neuropsychiatric Interview

MMP-9:Matrix metallo-proteinase 9

MRM: Multiple Reaction Monitoring

m/z : mass to charge ratio

PFC: Prefrontal cortex

QqQ: Triple Quadrupole

RMSSD: Square root of the mean squared differences of successive normal sinus intervals

RSD: Relative standard deviation

SampEn: Sample entropy

SDNN, Standard deviation of all RR intervals

SRM: Selected Reaction Monitoring

SVM: Support vector machines

TMT: Tandem mass tags

VDP: Vitamin D binding protein

VLDL:Very low density lipoprotein

1. Introduction

Major depressive disorder (MDD) is a serious brain disorder characterized by symptoms including low mood, low self-esteem, inappropriate guilt, thoughts of death and suicide, diminished concentration, loss of interest or pleasure in normally enjoyable activities, and disturbance of sleep and appetite. The prevalence has typically been cited as being approximately 10% of the population worldwide with a lifetime prevalence of 17% (1). Prospective studies of MDD have described frequent relapses and chronicity of major depressive episodes during the course of MDD; 20% of patients with at least 2 years follow-up were never free of depressive symptoms for even 1 week (2). Furthermore, the lifetime risk among patients with depressive disorders for suicide attempts is estimated to be 8–17% (3-7) and the lifetime risk of completed suicide is approximately 4–15% for patients with depressive disorders (8-10). The effects of MDD are wide-ranging, including a negative impact on families, work, and relationships, and have been associated with debilitating co-morbidities such as general ill health and substance abuse. A recent analysis of the Global Burden of Disease Study 2010 indicated that depressive disorder was the second-leading cause of global disability (11) and accounted for 40% of all disabilities caused by mental and substance use disorders (12). By 2030, unipolar depressive disorder is predicted to be the leading cause of lost disability adjusted life years (DALYs) in high-income countries (13). Furthermore, MDD is a risk factor for non-compliance with medical treatment (14). Increased symptom burdens of depressive disorders are aggravated by a lack of accurate diagnostic tools, which cause long delays

between the onset of symptoms and clinical intervention, and result in a more severe illness that is consequently more difficult to treat. This most likely due to current practices where diagnosis and treatment of psychiatric diseases are dependent on the outcome of psychiatric interviews based on clusters of symptoms and other clinical features. In this regard, the development of biomarkers for diagnosis and classification of MDD subtypes are a necessary step in the development of sensitive, specific, and reliable diagnostic tests.

However, diagnostic tests based on biomarkers for MDD studied to date appear to have limitations in terms of diagnostic accuracy (15). The development of biomarkers for MDD is complicated by the fact that the diagnostic procedures for depression are themselves phenomenological in nature and difficult to quantify. Moreover, depression is a polygenic, systemic, and multifactorial disorder involving complex interactions between genetic predisposition (16), and the disturbance of key molecular pathways, including neurotransmitter systems, synaptic plasticity, and neuroendocrinological systems (17), along with the impact of environmental factors such as stressful life events (18). Multiple biological processes are known to be involved in the pathophysiology of MDD, including deficiencies in monoamine transmitters and their metabolites, a reduction in the production of neurotrophins, mainly brain-derived neurotrophic factor (BDNF) (19, 20), and altered neuronal synaptic plasticity (20). Recently, several studies have suggested that chronic systemic inflammation (i.e., increased pro-inflammatory cytokines) (21) involving oxidative stress and neuroinflammation (22) as a key mechanism in depression. There are also robust findings of altered endocrine factors (e.g., hypothalamic-pituitary-adrenal [HPA], thyroid, sex steroids) and metabolic

dysregulation (e.g., insulin resistance) in depression (23). In addition to biochemical factors, sleeping and waking electroencephalography (EEG) architecture (24), several specific structural and functional neuroimaging patterns (25) as well as genetic factors, such as common variants of the promoter of the serotonin transporter (26) have been studied as biological markers of depression. Although a large number of biomarkers for MDD have been suggested, a clinically applicable test has not yet been developed, as each biomarker individually contributes a very modest proportion of the variance in depression risk, and the tests are limited by a lack of sensitivity and specificity (15).

Although most previous studies have been conducted using single biochemical measures, several recent studies have developed composite biomarker panels to increase the predictive power of these measures using an aggregate score or predictive algorithm to diagnose and classify MDD subtypes, as well as measure treatment responses (27). These approaches could be advantageous in the development of biomarkers by profiling a diverse array of peripheral/serum growth factors, cytokines, hormones, and metabolic markers to provide coverage of multiple biological abnormalities that contribute to the heterogeneity of MDD. Domenici et al. (2010) performed multi-analyte profiling that allowed for the evaluation of up to 79 proteins, including a series of cytokines, chemokines, and neurotrophins previously suggested to be involved in the pathophysiology of depression; they identified several proteins with high discriminant power, such as insulin and matrix metalloproteinase 9 (MMP-9) (28). Similarly, Papakostas et al. (2013) presented a multi-assay, serum-based test by selecting nine serum-

based markers from biochemical domains previously associated with MDD (e.g., inflammation, neurotrophin, HPA axis, and metabolism) and combined these mathematically into a single measure that yielded maximum overall sensitivity and specificity (15). In that study, application of a test using an MDD score involving nine molecules resulted in an overall sensitivity and specificity of 91% and 81%, respectively, for MDD. However, these studies were limited in discovering novel biomarkers because they used a restricted number of candidate molecules from the known hypothetical pathophysiology of depression.

On the other hand, the study of biomarkers and the implementation of biomarker evaluation utilizing "-omics" methods, such as proteomics and metabolomics, is considered to be powerful for discovering novel biomarker molecules involved in the pathophysiology of disease. A key advantage to these approaches is the analysis of potential biomarkers in an unbiased manner without a required hypothesis to guide and restrict the study. These global approaches may develop more robust and objective assessment criteria in disease diagnosis (29, 30), improve understanding of the etiology/pathology of disorders, and develop better observation of treatment responses through biomarker profiles (31). These have been employed in a wide variety of disorders, including cancer, cardiovascular disease (CVD), and psychiatric illness (29, 32, 33). Although genomic and transcriptomic approaches can provide information about genetic factors and a wide range of gene products influencing depression pathophysiology, proteomics is required to demonstrate functional abnormalities, given the heterogeneous manifestation and multifactorial, complex nature of depression (34).

Proteomics takes our understanding of the brain closer to a functional level, and therefore, disease-determining endpoint. Taken together, proteomic analysis enables the detection of protein biomarkers related to functional abnormalities involved in the pathophysiology of MDD in an unbiased manner without the need for a hypothesis to guide and/or restrict the analysis (34).

Biomarker studies of psychiatric disorders including depression present particular hurdles, such as the inherent difficulties in accessing relevant biological materials, since the main pathophysiology appears to be in the brain. Sampling of brain tissue or cerebrospinal fluid (CSF) would be the most suitable method for biochemical analysis, such as '-omics' analysis, to identify disease biomarkers. However, these samples are not practically accessible in living humans due to the high costs and invasiveness of brain biopsy and lumbar puncture.

Other options include structural and/or functional neuroimaging and EEG, which can directly measure brain function, but there are limitations in practical clinical use due to the high facility cost and time-consuming nature of these modalities. Moreover, the heterogeneity of brain tissue may present challenges, considering, for example, that decreases in synaptic proteins could result from cell-specific mechanisms or a functional change, such as lack of input to a particular brain region. On the other hand, peripheral blood samples can be easily obtained with minimal invasiveness. Moreover, approximately 500 mL of CSF is absorbed into the blood every day (35) and blood-brain barrier (BBB) hyperpermeability related to oxidative stress and neuroinflammation has been reported in MDD (22, 36, 37), which implies that

protein exchange may occur between the brain and the peripheral circulation. Therefore, peripheral blood may be a promising source for identifying depression-related biomarkers (38)

Although several proteomic tools have been applied to MDD, most prior studies have used post-mortem brain tissue or animal models (29, 34). A few proteomic studies exist that have used clinically accessible samples from patients with MDD. Xu, et al. (2012) analyzed plasma samples from 21 depressed patients and 21 healthy controls using a quantitative proteomic approach based on isobaric tags for relative and absolute quantitation (iTRAQ) and multi-dimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) (38). They found that the functions of the altered proteins are primarily involved in lipid metabolism and immunoregulation, suggesting that early perturbation of lipid metabolism and immunoregulation may be involved in the pathophysiology of MDD. Another study by AlAwam et al. (2012) performed serum protein and peptide profiling in 39 male patients and 30 male controls with ages ranging from 35 to 50 years using a Matrix-assisted laser desorption/ionization–mass spectrometry (MALDI-MS) (39). Although they found no protein signals that differentiated the patients from the controls, and principle component analysis of the entire peptide profile did not allow for distinct clustering of the two groups, further analysis of individual peptides identified three peptide signals whose intensities were significantly different between the patients and the control subjects. However, the serum proteome affected by MDD requires further investigation and the results should be validated using advanced techniques. In addition, multiparametric data analyses are necessary to account for the complexity of

disease mechanisms in psychiatric disorders (40, 41).

In the context of polygenic diseases, a valid biomarker may turn out to be a combination of different observations, possibly different methodologies, rather than a single one. Convincing evidence indicates that depression affects entire organ systems, including the endocrinological, immunological, and autonomic nervous systems, through interactions between the brain and the body (42). Previous research has provided a plethora of evidence for an autonomic imbalance in patients suffering from MDD (43). In this regard, heart rate variability (HRV) could be one plausible and complimentary biomarker for depression. HRV is defined as the amount of fluctuation from the mean heart rate in an electrocardiogram (ECG), and the beat-to-beat variation in heart rate is dependent on the balance between sympathetic activation mediated by cardiac noradrenergic receptors and tonic inhibition mediated by cholinergic vagal input. As a reliable and sensitive measure to quantify cardiac autonomic control, HRV has been applied as a research and monitoring tool to assess various medical conditions. Because parasympathetic vagal inputs enable biological systems to respond flexibly to environmental changes, decreased HRV, which is characterized by sympathetic dominance, indicates a maladaptive response to physical or psychological demands in the environment (44). Reduced HRV is associated with diverse cardiovascular and endocrine abnormalities, and increased risk of the development of diabetes and metabolic syndrome in adults (45-50). Abnormal values of several linear and non-linear HRV indexes are important predictors of mortality in elderly people (51) as well as in diverse clinical populations (52, 53). Impaired HRV is also related to psychiatric disorders.

Recent meta-analyses suggested that depression and anxiety disorders are associated with reduced HRV and that HRV is inversely related to the severity of depression (43, 54). The central autonomic network (CAN), a network including the prefrontal cortex (PFC), cingulate, insula, amygdala, and brainstem structures, has been suggested to be a key player in psychiatric symptoms and reduced HRV. Depressive symptoms can be indicated by a failure of inhibition in affective, cognitive, physiological, and behavioral responses implicated in the CAN, which results in decreased vagal outflow and reduced HRV (54). This implicates a possible use of HRV parameters as a diagnostic marker of depressive symptoms in clinical populations. Although the linear parameters of the time and frequency domains are currently used as standard HRV measures, HRV is a high-dimensional dynamic system with multiple time scales, as it is generated from complex interactions between hemodynamic, electrophysiological, and humeral variables, as well as by autonomic regulation via the CAN (55). It was also documented that different components of HRV decline at different rates with age, indicating a shift in the balance between sympathetic and parasympathetic components (56). Previous studies suggest significant sex differences and age-sex interactions (57, 58). For example, female subjects tended to have lower low-frequency (LF) but higher high-frequency (HF) outputs than males, suggesting that men were more sympathetic-dominant than women, although these sex differences in some linear HRV measures weakened with age. These findings highlight the need for age- and sex-stratified reference values of HRV.

In this study, serum samples from patients with MDD and healthy controls were analyzed using a quantitative proteomic approach for the identification

of novel peripheral markers for depression. In addition to molecular biomarkers, this study investigated linear and nonlinear HRV parameters as potential electrophysiological biomarkers for depression. Finally, we attempted to improve the classification accuracy by combining proteomic and HRV markers using a machine learning algorithm and to provide preliminary data regarding combinational biomarkers.

2. Materials and methods

2.1. Study subjects

The study subjects consisted of 25 female MDD patients with current major depressive episode, and 25 age- and sex-matched healthy controls from Seoul National University Hospital. Patients who met the criteria for MDD according to the Fourth Edition of the Diagnostic and Statistical Manual for Mental Disorders, as diagnosed using the Mini International Neuropsychiatric Interview, were considered for inclusion. MDD patients diagnosed with other comorbid psychiatric diseases or who had taken psychotropic medications (including anxiolytics, antidepressants, antipsychotic medications, and anticonvulsants) during the past 8 weeks were excluded from the study. Healthy controls had no current or past diagnosis of MDD and no family history of any psychiatric disorder. None of the subjects were taking medication that could affect variation in cardiac rhythm or alter the blood levels of relevant factors (such as nonsteroidal anti-inflammatory agents and steroids), and none had suffered from chronic or acute diseases, such as cardiovascular disease, pulmonary disease, hypertension, endocrine abnormalities, rheumatic diseases, or cerebrovascular disease. Subjects who

were pregnant, nursing, or menstruating were also excluded.

The protocol was approved by the Ethics Committee of Seoul National University Hospital, and this study was conducted in accordance with the latest version of the Declaration of Helsinki. Written informed consent was obtained from each patient prior to enrollment.

2.2. Clinical assessment of affective symptoms

2.2.1. Depressive symptoms

The objective severity of depression was measured using the 17-item Hamilton Rating Scale for Depression (HAMD) (59). The item response options are on a 3-point scale, ranging from 0–2 for insomnia, gastrointestinal somatic symptoms, general somatic symptoms, genital symptoms, loss of weight, and insight, and on a 5-point scale ranging from 0–4 for other symptoms. The total score ranges from 0 to 52 and higher scores reflect severe depressive symptoms. We divided the items on the HAMD into somatic and psychological domains (60, 61). The somatic domain of the HAMD included questions addressing somatic-gastrointestinal issues, weight loss, insomnia (early, middle, late), retardation, agitation, anxiety-somatic, and somatic-general symptoms. The psychological domain included questions measuring depressed mood, insight, guilt, suicide, loss of interest, hypochondriasis, anxiety-psychic issues, and genital symptoms.

The Inventory of Depressive Symptomatology-Self Report (IDS-SR) was used as a self-rating instrument for depressive symptoms (62). The IDS is composed of 30 items with a total score ranging from 0 to 84. All items are rated on a scale from 0 (symptom is not present) to 3 (strongest impairment).

Twenty-eight of the 30 items are summed to a standard total score, ranging from 0 to 84 (as only appetite and weight increase or decrease is scored). Previous studies demonstrated that a 3-factor model consisting of 'mood/cognition,' 'anxiety/arousal,' and 'sleep' factors was found to fit well across different groups of MDD patients, including current MDD, remitted MDD, and healthy controls (62, 63). Among these, the 'mood/cognition' factor including 11 items (i.e., feeling sad, reactivity of mood, quality of mood, appetite disturbance, concentration/decision making, self-criticism and blame, future pessimism, suicidal thoughts, energy/fatigability, interest in sex, and interpersonal sensitivity) and the 'anxiety/arousal' factor including 8 items (i.e., feeling irritable, psychomotor agitation, psychomotor retardation, aches and pains, sympathetic arousal, panic/phobic symptoms, constipation/diarrhea, and leaden paralysis) can be adapted for use as specific subscales in both clinical practice and scientific research (63). Therefore, we adopted these two reliable subscales as well as total scores for association analysis.

2.2.2 Anxiety

The severity of self-reported anxiety symptoms was measured using the Beck Anxiety Inventory (BAI) (64). The item response options are on a 4-point scale ranging from absence of that symptom (0) to severe or persistent expression of that symptom (3) in the past week. This scale is a 21-item instrument and the total score ranges from 0 to 63, with higher scores reflecting severe anxiety symptoms. Beck et al. suggested three subscales of the BAI representing subjective, somatic, and panic symptoms through cluster analysis in psychiatric outpatients with anxiety disorders (65).

2.2.3 Hopelessness

The Beck Hopelessness Scale (BHS) is a self-report instrument designed to measure three aspects of hopelessness (66): feelings about the future, loss of motivation, and expectations during the past week. Each of the 20 statements is scored as 0 or 1. The scale also has good reliability (test-retest, r = 0.81) (67).

2.2.4 Suicidal ideation

The Beck Scale for Suicide Ideation (BSI) was used to measure suicidal ideation (68). The BSI is a 19-item self-report instrument for detecting and measuring the current intensity of the patient's attitudes, behaviors, and the specificity of a patient's thoughts about dying by suicide during the past week. Respondents rate the intensity of suicidality (wish to die, desire to attempt or commit suicide, duration and frequency of thoughts, deterrents) using a 3-point Likert-type scale ranging from 0 to 2. The responses are then summed to yield a total score of 0–38 and higher scores on the scale indicate greater suicidal intent. The scale has demonstrated good internal consistency (α = 0.87–0.97) and moderate test-retest reliability (r = 0.54) (69).

2.2.5. Impulsivity

Impulsivity is significantly correlated with the severity of hopelessness and anhedonia in depression (70, 71). Subjects completed the Barratt Impulsiveness Scale (BIS) (72), a 30-item self-report measure designed to assess impulsivity with regard to attention, motor activity, and planning.

Individual items are rated on scales ranging from 1 to 4, yielding total scores from 30 to 120, with higher scores indicating greater impulsivity. The internal consistency coefficient for the BIS total score is reportedly 0.83 for general psychiatric patients (72).

2.3. HRV analysis

2.3.1. Data acquisition and preprocessing of ECG recordings

Before ECG recording, all participants fasted and refrained from smoking and caffeine for more than 2 hours and alcohol for more than 12 hours. ECGs were obtained in a quiet, dim room maintained at a comfortable temperature. Testing times were from 9:30 to 11:30 in the morning, and 1:00 to 4:30 in the afternoon. Subjects were instructed to minimize their movement and breathe regularly with eyes closed in a recumbent position using a two-channel ECG monitor. After a test session of approximately 5 minutes to stabilize the signal, a 10-minute ECG was acquired for 50 subjects with a sampling frequency of 1,000 Hz, and a 7-minute ECG was acquired for another group of 5 subjects with a sampling frequency of 300 Hz at complete rest. To reduce the non-stationary effect, the artifact-free 5-minute ECG data were chosen for the HRV analysis. This analysis window is standard for short-term HRV (73). Normal RR intervals ((where R is a point corresponding to the peak of the QRS complex of the ECG wave; and RR is the interval between successive Rs) were extracted using a standard procedure (74). RR intervals were filtered using an adaptive filter algorithm to replace and interpolate ventricular premature beats and artifacts (75). Both linear and non-linear measures were estimated as indices of HRV.

2.3.2. Time and frequency domain parameters of HRV

Several linear measures of HRV were computed according to current guidelines (73). As the time domain measures, the mean length of all RR intervals (Mean RR), the standard deviation of all RR intervals (SDNN), the square root of the mean squared differences of successive normal sinus intervals (RMSSD), and the percentage of successive RR interval differences whose absolute value x exceeded 10, 20, 30, 50 ms (pNNx) were calculated (76). The SDNN shows parasympathetic activity as well as the sympathetic activity of heart function, while RMSSD and pNNx reflect predominantly vagal function. In the frequency domain, spectral analysis was performed using a standard autoregressive algorithm, after detrending and resampling the irregularly time-sampled recording of consecutive RR intervals. The power spectrum density was conventionally estimated for two major frequency ranges: the LF band (0.04–0.15 Hz) and the HF band (0.15–0.4 Hz). The LF normalized unit (LFnu) and HF normalized unit (HFnu) were also calculated as relative spectrum density. The LF is considered to be an index of sympathetic and parasympathetic modulation and the HF reflects parasympathetic modulation. The ratio of LF to HF power (LF/HF) was computed to evaluate sympathovagal balance (73).

2.3.3. Nonlinear measures of HRV

As a nonlinear measure to quantify the randomness or degree of self-similarity of heart rate patterns at different time scales, detrended fluctuation analysis (DFA) was used to derive alpha 1 or alpha 2 as the short-term or

long-term quantification of the fractal scaling properties of RR intervals (77). Alpha 1 reflects the scaling rate of short-term temporal fluctuations in the heart rate time series; a DFA1 of 0.5 would indicate a totally random signal and a DFA1 of 1.5 would indicate one that is totally correlated. A DFA1 of 1 indicates a healthy subject. As a measure of the nonlinear correlation or 'regularity' of the fluctuations in a time series, approximate entropy (ApEn) and sample entropy (SampEn) were also calculated. SampEn is free from the bias induced by the finite length of physiological data and self-matches in calculating the ApEn (78). Lastly, the corrected Shannon entropy (CSEx) was calculated using binary symbols generated from a coarse-graining process (79), based on a threshold x, which is the absolute difference of successive RR intervals (80). A higher CSEx value reflects a higher degree of short-term complexity of symbolic patterns generated from the binary sequences (81).

2.3.4. Statistical analysis

The normality of the distributions of each HRV parameter was tested using the one-sample Shapiro-Wilk normality test. HRV parameters that did not pass the normality test were log-transformed. Independent *t*-tests for each of the autonomic variables were conducted to ascertain whether a univariate difference was present between the groups. The partial correlation analyses, adjusted for age and body mass index (BMI), performed between each HRV parameter and each scale score for affective symptoms were employed for assessing whether specific autonomic variables were associated with specific symptomatology in MDD subjects.

In addition, we performed support vector machines (SVM) with recursive

feature elimination (RFE) to select HRV parameters for the classification between MDD and normal controls. SVM classifiers have been used as popular and powerful tools for classification, due to their strong theoretical origin in statistical learning theory as well as their good performance in practical applications (82). To address the problem of selection of a small subset of features from broad patterns of gene expression data, Guyon et al. proposed an RFE approach utilizing SVM [reference]. We used linear SVM, generalized to non-separable training data. The optimal hyperplane was identified using L1-regularization. Optimal parameters were determined using a sequential minimal optimization (SMO) algorithm. For unbiased classification and feature selection, we performed 5-fold cross-validation (CV). In each fold of the CV, the individuals were grouped into disjoint training and testing sets such that there were no subjects used for both training and testing in a single fold. The 5-fold CV experiment was repeated 10,000 times and the number of selection times for each HRV parameter was calculated to determine the relative importance of each feature to the discrimination between the MDD and control groups. The average classification accuracy was then calculated. The optimal number of features was determined at the maximum classification accuracy of the test data. All p-values were two-tailed, and a value of $p < 0.05$ was taken to indicate statistical significance. Statistical analyses were performed using the Statistical Package for the Social Sciences v. 20.0 for Windows (SPSS, Inc., Chicago, IL, USA) and R software.

2.4. Proteomics analysis

2.4.1. Sample collection

Upon meeting all of the inclusion and none of the exclusion criteria, blood samples were obtained from subjects after an overnight fast (at least 12 h). Sampling times were from 9:30 to 11:30 AM. Venipuncture was performed on site to obtain serum for biological tests, from which plasma was separated within 1 h of collection. The aliquoted serum samples were stored at -78°C until analysis.

2.4.2. Immunodepletion of high-abundance serum proteins

Equal volumes (40 μL) of serum from 10 depressed patients and 10 healthy controls were pooled. Six high-abundance proteins (albumin, IgG, antitrypsin, IgA, transferrin, and haptoglobin) in the pooled samples were depleted using an Agilent Multiple Affinity Removal Spin Cartridge (Agilent Technologies Inc., USA). Desalting, buffer exchange and concentration were performed using Amicon® Ultra-0.5 Centrifugal Filter Devices (3K NMWL; EMD Millipore, Germany). The protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc., USA).

2.4.3. Protein digestion, Tandem mass tags (TMT)-labeling and peptide fractionation

Serum proteins were digested using the filter-aided sample preparation method (82). Proteins (50 μg) were diluted with denaturation buffer (4% SDS, 0.1 M DTT, and 0.1 M Tris/HCl; pH 7.6) and reduced at 37°C for 45 min, followed by 7 min at 100°C. The buffer was changed to 8 M urea in 0.1 M

Tris/HCl (pH 8.5) and the proteins were alkylated with 50 mM iodoacetamide at room temperature for 20 min. Then trypsin was added (trypsin: protein = 1: 50 [w/w]) and the samples were incubated at 37°C for 12 h. The digested peptides of the MDD and control groups were labeled with compounds TMT6-128 and TMT6-130 of the Tandem Mass Tag™ 6-plex (TMTsixplex™) Reagents (Thermo Fisher Scientific Inc., USA), respectively. The labeled peptides were divided into 12 fractions using a 3100 OFFGEL fractionator with a low-resolution kit, pH 3–10 (Agilent Technologies Inc., USA) (83). The peptides in each fraction were collected and desalted using a C18 spin column (Pierce® C18 Spin Column; Thermo Fisher Scientific Inc., USA). The desalted peptides were dried using a SpeedVac system for 3 h (Thermo Fisher Scientific Inc., USA) and resuspended in aqueous 0.1% formic acid prior to LC-MS/MS analysis.

2.4.4. LC-MS/MS analysis

The peptide samples were analyzed using a Q Exactive™ mass spectrometer (Thermo Fisher Scientific Inc., Germany) coupled with an Easy-nLC UPLC system (Easy-nLC 1000; Thermo Fisher Scientific Inc., Denmark) and EASY-Spray column (C18, 2 μm particle size, 100 Å pore size, 75 μm id × 50 cm length; Thermo Fisher Scientific Inc., USA) in duplicate. The column temperature was maintained at 60°C. The peptide samples were eluted using a linear gradient of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), where the percentage of the latter mobile phase was increased from 3% to 50% for 151 min, then to 65% for 14 min at a flow rate of 270 nL/min. The separated peptide ions eluted from the analytic

column were entered into the mass spectrometer at an electrospray voltage of 2 kV. MS precursor ion scans were acquired in profile mode with an AGC target value of 1×10^5, a mass resolution of 70,000 at 200 m/z, a maximum ion accumulation time of 60 ms, and a mass range of 400–1600 m/z. The mass spectrometer was operated in the data-dependent tandem MS mode. The 10 most abundant ions detected in a precursor MS scan were selected for MS/MS experiments. To prevent repetitive acquisition of the same peptides, the dynamic exclusion option was applied with an exclusion duration of 20 s. The selected precursor ions were isolated using an isolation mass width of 2 Da and fragmented in an HCD cell with 30% normalized collision energy.

2.4.5. Protein identification

All tandem mass spectra were searched against the Universal Protein Resource human protein database (UniProt release 2013_03, 89,430 entries) using SEQUEST® in Proteome Discoverer 1.4 (Thermo Fisher Scientific Inc., Germany). The tolerance was set to 10 ppm for precursor ions and 0.8 Da for fragment ions. Only fully tryptic ends were considered and two missed cleavage sites were allowed. Fixed modification options were used for carbamidomethylation (+57.021460 Da) on cysteine and TMT tag (+229.162932 Da) on lysine and the N-terminus. The oxidation (+15.994920 Da) of methionine was set as a variable modification. The search result files were imported into Scaffold Q+ (version 4.3.2, Proteome Software Inc., USA) (84). To validate peptide and protein identifications, the Peptide Prophet and Protein Prophet algorithms were applied (85, 86). The thresholds of peptide probability > 95% and protein probability > 99% were used and protein

identifications with at least two distinct peptides were considered correct. The identified protein accessions were then mapped to Entrez Gene information to obtain non-redundant Gene IDs.

2.4.6. Protein quantification and identification of differentially expressed proteins

The quantification of protein abundances was performed using the Scaffold Q+ and isobar R packages (84, 87). The overall procedure using Scaffold Q+ was as follows. First, the tag intensities from two tags were normalized to an equal median. Then the intensity of each protein was estimated using kernel density estimation with normalized tag intensities from spectra of the corresponding protein. Finally, proteins whose intensities were altered more than 1.5-fold between the two groups were selected as DEPs. The overall procedure using the isobar R package involved normalizing each tag to equal the median intensity, and then estimating the protein ratio based on the weighted average of the ratios of normalized tag intensities and the ratio's variance. Such variance indicates the accuracy of the ratio and is reported as a signal p-value. DEPs with signal p-values $\leq$ 5% and absolute fold changes $\geq$ 1.5 were considered significant. Fifty proteins were selected from the profiling data analysis. In addition, 37 proteins were included from the LC/MS experiments using serum samples from patients with MDD (38, 88) and from the literature via PubMed searches. In total, 87 protein targets were selected.

2.4.7. Selection of MRM transitions for target proteins

The MRM methods were developed using Skyline 2.5 (89). For each protein, the target peptides were selected based on the following criteria: fully tryptic peptides, with no missed cleavages, unique to a particular protein, with a length between 6 and 20 amino acids, and with no cysteine or methionine residues. For each peptide, the three most intense fragment y-ions were selected. The peptides were ranked based on the sum of selected fragment ion intensities in the spectrum for a given peptide and the top three peptides for each protein were selected. The National Institute of Standards and Technology (NIST) Library of Peptide Ion Fragmentation Spectra was obtained from PeptideAtlas and was used as the reference spectral library (90). For the proteins not included in the NIST spectral library, the MRM transitions were selected from the SRMAtlas database (91). For each protein, the peptides with an adjusted suitability score ≥ 0.7 were selected and a list of the fragment y-ions with an m/z larger than the precursor m/z was determined for each peptide. Collision energies (CEs) were determined according to the following equations: $CE = 0.031 \times (m/z) + 1$ and $CE = 0.036 \times (m/z) - 4.8$ (m/z) for doubly and triply charged precursor ions. The list of transitions of Q1 and Q3 m/z at unit resolution (FWHM 0.7 Da) with CE was exported. Protein samples from pooled serum of each group were prepared using the method described above. MRM experiments were performed on a 6490 triple-quadrupole mass spectrometer (Agilent Technologies Inc., USA) coupled with a 1290 Infinity LC system (Agilent Technologies Inc., USA). The digested peptides (8 µg) were separated on a Zorbax Rapid Resolution High Definition Eclipse Plus C18 column (150 × 2.1 mm, 1.8 µm particles; P/N. 959759-902; Agilent Technologies Inc., USA) at 0.4 mL/min over 30 min. The mobile

phases of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) were used and the latter mobile phase was increased from 3% to 39% over 25 min. The spray voltage was set at 2.5 kV, and the temperature of the nitrogen drying gas was set at 250°C. The temperature of the column and the autosampler of 1290 Infinity system (Agilent Technologies Inc., USA) were maintained at 50°C and 4°C, respectively. A post-run isocratic step was used for column re-equilibration for 5 min. By performing triplicate experiments, the most intense and reproducibly detected transitions were selected as the final set of MRM results.

2.4.8. LC-MRM/MS analysis

The serum samples of 50 subjects were depleted using the method described above, and protein from each sample (50 μg) was spiked with 0.5 pmol LFTGHPETL*EK (synthetic *Bos taurus* myoglobin peptide) as a standard peptide for normalization. The leucine residue indicated by an asterisk (*) was labeled with 13C6 isotope. The protein samples were digested using the methods described above and then 8 μg digested peptide was analyzed using the abovementioned LC-MRM/MS method. A total of 108 transitions, including 105 transitions for 35 target proteins and 3 transitions for the spiked standard peptide, were measured simultaneously using dynamic MRM mode (500 ms cycle time and 1.5 min retention time window) in a single LC/MS run. Triplicate samples were run in a randomized manner.

2.4.9. Statistical analysis for the identification of candidate serum biomarker panel from MRM assay

Raw data were processed using Skyline 2.5 to quantify peak areas of fragment ions from target peptides (89). The intensities of fragment ions from 150 runs (triplicates of 50 samples) were normalized to the spiked standard peptide and were summarized as protein levels using the MSStats R package (92). To select important proteins for distinguishing between MDD and controls, we applied logistic regression with lasso regularization (93). For unbiased classification and feature selection, we performed fivefold cross-validation (CV), which was repeated 1000 times; the number of times selected for each protein was computed to determine the relative importance of each protein to the discrimination of MDD patients and controls. Then the average classification accuracy was computed. Receiver operating characteristic (ROC) curves and the areas under the curves (AUC) were calculated by logistic regression to evaluate the discriminatory performance of the selected proteins (94).

2.5. Combined classification using HRV indexes and proteomic data

2.5.1. Statistical analysis

To select important serum proteins and the HRV indexes for classification of MDD and healthy controls, we performed SVM with RFE. The contribution of each feature was evaluated using 5-fold CV. The cross-validation approach was repeated 1,000 times and the number of selection times for each feature was calculated. The optimal number of features was determined at the maximum classification accuracy of test the data. Finally,

with the selected features, the 5-fold CV experiment was repeated 10,000 times and the average classification accuracy was calculated.

3. Results

3.1. HRV analysis

3.1.1. Demographic and clinical characteristics of study subjects

The baseline characteristics of study participants are summarized in **Table 1.1.** The mean (SD) age of subjects was 38.6 (13.0) years in patients with MDD and 37.5 (11.1) years in normal controls; this difference was not significant. No BMI difference was observed. There were significant differences between the MDD and normal control groups regarding several affective symptoms scales, including the HAMD, IDS-SR, BAI, BHS, and SSI, but excluding the mean scores of the BIS motor and nonplanning subscales.

3.1.2. Independent *t*-tests

Table 1.2 presents the relationship between mood state and several HRV parameters. No significant differences in the univariate analyses were observed for the HRV parameters.

3.1.3. Partial correlation analysis between the HRV parameters and the affective symptom scales in patients with MDD

In MDD patients, the HAMD somatic subscale scores showed a significant positive correlation with lnApEn (r = 458, p-value = 0.032), and the IDS-SR

anxiety/arousal subscale scores showed a significant negative correlation with SampEn (r = −0.444, p-value = 0.044) (**Tables 1.3** and **1.4**). BAI total score was significantly correlated with several linear and nonlinear parameters (r = −0.602, p-value = 0.005 for lnpNN10; r = −0.524, p-value = 0.018 for Pnn20; r = 0.582, p-value = 0.007 for LFnu; r = −0.582, p-value = 0.007 for HFnu; r = 0.593, p-value = 0.006 for lnLF/HF; r = −0.585, p-value = 0.007 for SampEn; r = −0.446, p-value = 0.048 for lnCSE30; r = 0.552, p-value = 0.012 for DFA alpha1, and r = −0.471, p-value = 0.036 for lnDFA crossover). Attention in impulsivity was significantly positively correlated with lnLF/HF (r = 0.448, p-value = 0.042), while nonplanning was significantly correlated with several linear and nonlinear parameters (r = −0.582, p-value = 0.006 for lnpNN10; r = 0.544, p-value = 0.011 for LFnu; r = −0.544, p-value = 0.011 for HFnu; r = 0.558, p-value = 0.009 for lnLF/HF; r = −0.464, p-value = 0.034 for SampEn).

3.1.4. Selection parameters of HRV using SVM

To select the important HRV indexes for classification of MDD and healthy controls, we performed SVM with recursive feature elimination (**Figure 1.1**). The contribution of each HRV index was evaluated by 5-fold CV (**Table 1.5**). The CV approach was repeated 10,000 times and the number of selection times of each HRV index was calculated. Based on 5-fold CV and the 10,000 randomization experiment, a separation between MDD and normal conditions could be achieved by 7 parameters: RMSSD, CSE20, LF, pNN10, SDNN, ApEn, and SampEn (**Figure 1.2** and **Table 1.5**). An average classification accuracy of 63.8% was achieved by these features. The

sensitivity, specificity, and positive predictive value were 55.3%, 72.8%, and 67.9%, respectively.

3.2. Proteomic analysis

3.2.1. Serum proteome profiling

We first selected 10 samples from each group and pooled equal volumes of sera. For each pooled sample, TMT-tag was labeled and then LC-MS/MS analysis was performed in duplicate. The peptides sequences were identified using the SEQUEST search algorithm, and the search results were further validated using the PeptideProphet and ProteinProphet algorithms. **Figure 2.1.** was a schematic of the approach used. In each experiment, 703 and 653 proteins with 6,507 and 6,065 peptides were identified; a total of 905 proteins were thus identified. Of the 905 proteins, 852 were mapped to Gene IDs. To identify DEPs between the MDD and normal groups, we applied two methods: Scaffold Q+ and isobar R. Among the 852 proteins, 50 were identified as DEPs using the two methods (**Figure 2.2A** and **Table 2.1**). To investigate the different expression patterns of the 50 selected DEPs, we applied an enrichment analysis using the functional annotation tool of the Database for Annotation, Visualization and Integrated Discovery (95). The Gene Ontology Biological Processes (GOBPs) represented by the DEPs with $p < 0.1$ (default cutoff) were selected and are depicted in **Figure 2.2B**. The DEPs were mainly involved in acute inflammatory responses ($p = 0.03$) and lipid transport ($p = 0.05$).

3.2.2. MDD-associated proteins (MAPs) reported previously

In addition to the 50 DEPs identified in the profiling experiment, we compiled a list of proteins whose changes in abundance in MDD patients were reported in previous studies. We identified 38 proteins whose abundance differed in the serum of MDD patients from two large-scale LC/MS studies and previous reports in PubMed (38, 88). In total, 87 MAPs were selected for MRM assay.

3.2.3. Monitoring candidate biomarkers by MRM in a large patient cohort

We developed an MRM assay for the 87 MAPs using peptides and empirical evidence from the NIST spectral library and computationally predicted peptides from SRMAtlas. Based on the NIST spectral library and SRMAtlas, we developed MRM assays for 55 and 24 proteins (in total, 79 of the 87 MAPs). Following triplicate evaluations involving LC-MRM/MS and manual inspection, MRM assays that resulted in low-quality results were removed. Then, an optimized MRM assay was determined for 35 target proteins (40% of the MAPs), and we applied it to 50 serum samples. Sixteen proteins were quantified in at least 70% of the samples

3.2.4. Multiparametric serum biomarker panel

To develop a multiparametric serum biomarker panel for MDD, it is important to evaluate the relative contributions of the 16 DEPs to the discrimination of MDD patients and normal individuals. Individual proteins could not be used to classify samples correctly due to the marginal differences in their abundances between the MDD and normal groups (**Table 2.2**).

Therefore, their collective contribution was evaluated. To this end, we applied logistic regression with lasso regularization to the intensities of the 16 proteins (93) (**Figure 2.3A**). According to a fivefold CV and 1000 randomizations experiment, discrimination between MDD patients and normal individuals was achieved using six proteins (APOB, APOD, CP, GC, HRNR, and PFN1) (**Figure 2.3B**). Then a logistic regression model was generated using the intensities of the six selected biomarkers. An average classification accuracy of 68% was achieved by the final model, and the sensitivity and specificity were 67% and 69%, respectively. ROC curves for classification using the six selected candidate proteins are shown in Fig. 3c. The minimum, median, and maximum of AUC out of 1000 times were 0.6080, 0.7376, and 0.8080. Taken together, these results indicate that the six selected biomarkers are predictive for MDD.

3.3. Combined classification with HRV and protein

Based on the 5-fold cross-validation and the 10,000 randomization experiment, a separation between MDD and normal conditions could be achieved using 5 parameters (**Figure 3.1** and **Table 3.1**): APOB, GC, CP, RMSSD, and SampEn. An average classification accuracy of 80.1% could be achieved by these features. The sensitivity, specificity, and positive predictive value were 70.2%, 89.9%, and 89.4%, respectively.

Table 1.1. Baseline demographic and clinical characteristics

Variables	Major depressive disorder (N=25)		Normal control (N=25)		P Value*
	Mean	SD	Mean	SD	
Age, years	38.6	13	37.5	11.1	1.000
BMI, kg/m2	21.5	2.2	21.2	1.9	1.000
Clinical characteristics					
HAMD total	16.6	2.9	4.5	3.5	<0.001
Somatic	8.8	2	2.5	1.8	<0.001
Psychological	7.7	1.8	2	2.1	<0.001
IDS-SR total	31.8	9.3	8.6	8.6	<0.001
Mood/cognition	13.8	4.8	3	4.1	<0.001
Anxiety/arousal	7.5	3.1	2	2	<0.001
Beck Anxiety Inventory	16.1	10.6	5.3	6.5	<0.001
Beck Hopelessness Scale	6.4	5.6	1.4	1.8	<0.001
Scale for Suicide Ideation	9.5	7.2	2.4	3.2	<0.001
Barratt Impulsivity Scale					
Attention	16	3	13.2	3.1	0.012
Motor	20	4.1	19.3	2.7	1.000
Nonplan	26	5.5	24.3	3.5	0.610

* P-value for ANOVA

Table 1.2. Difference of heart rate variability parameters among groups

	Major depressive disorder (N=25)		Normal control (N=25)		
Variables	Mean	SD	Mean	SD	P Value*
Linear parameters					
ln Mean RR interval (ms)	6.745	0.125	6.723	0.113	0.349
lnSDNN (ms)	3.822	0.409	3.59	0.283	0.064
lnRMSSD (ms)	3.457	0.601	3.21	0.369	0.301
lnpNN10 (%)	4.134	0.323	4.158	0.234	1.000
pNN20 (%)	43.838	20.451	41.185	17.786	1.000
pNN30 (%)	29.248	20.492	23.989	16.624	1.000
pNN50 (%)	14.526	3.229	7.297	3.102	0.332
lnLF (ms^2)	6.229	0.982	5.661	0.813	0.074
LFnu (v)	0.565	0.213	0.522	0.192	1.000
lnHF (ms^2)	5.902	1.19	5.526	0.922	0.660
HFn	0.435	0.213	0.478	0.192	1.000
lnLF/HF	0.327	0.983	0.135	0.94	1.000
Non-linear parameters					
lnApEn	0.037	0.137	0.103	0.082	0.074
SampEn	1.434	0.347	1.545	0.204	0.493
CSE10	2.039	0.673	2.205	0.551	1.000
lnCSE20	0.791	0.309	0.906	0.195	0.367
lnCSE30	0.635	0.534	0.626	0.655	1.000
CSE50	1.367	0.813	1.117	0.784	0.829
DFA alpha1	1.098	0.316	1.056	0.258	1.000
DFA alpha2	0.864	0.133	0.914	0.157	0.648
lnDFAcrossover	-0.198	0.354	-0.128	0.359	1.000

* P-value for ANOVA

Table 1.3. Correlation affective symptoms scale with linear measures in heart rate variability in patients with major depressive disorder

	lnMean	lnSDNN	lnRMSSD	lnpNN10	pNN20	pNN30	pNN50	lnLF	Lfnu	lnHF	Hfnu	lnLF/HF
HAMD total	-0.276	-0.132	-0.070	0.054	-0.135	-0.144	-0.110	-0.091	-0.001	-0.051	0.001	-0.023
Somatic	-0.298	-0.217	-0.133	0.141	-0.195	-0.256	-0.267	-0.220	0.012	-0.151	-0.012	-0.021
Psychological	-0.118	0.025	0.032	-0.066	-0.003	0.048	0.115	0.094	-0.015	0.084	0.015	-0.015
IDS-SR total	-0.202	0.040	-0.051	-0.320	-0.170	-0.082	-0.010	0.137	0.270	-0.112	-0.270	0.279
Mood/cognition	0.137	0.246	0.224	-0.031	0.162	0.209	0.270	0.301	0.066	0.184	-0.066	0.079
Anxiety/arousal	-0.307	-0.037	-0.184	-0.399	-0.324	-0.248	-0.173	0.086	0.349	-0.216	-0.349	0.352
Beck Anxiety Inventory	-0.385	-0.048	-0.406	**0.602**	**0.524**	-0.375	-0.263	0.120	**0.582**	-0.424	**-0.582**	**0.593**
Beck Hopelessness Scale	-0.054	0.099	-0.062	-0.309	-0.123	0.007	0.023	0.341	0.401	-0.077	-0.401	0.395
Scale for Suicide Ideation	-0.381	-0.186	-0.267	-0.418	-0.301	-0.190	-0.126	0.008	0.220	-0.170	-0.220	0.228
Barratt Impulsivity Scale	-0.038	0.095	-0.121	-0.367	-0.133	-0.007	0.133	0.286	0.430	-0.143	-0.430	**0.448**
Attention	-0.269	-0.210	-0.252	**-0.477**	-0.382	-0.303	-0.182	-0.181	0.236	-0.357	-0.236	0.258
Motor	-0.068	-0.049	-0.207	**-0.582**	-0.377	-0.236	-0.055	0.112	**0.544**	-0.362	**-0.544**	**0.558**

Text in **Bold** means p-value <0.05

Table 1.4. Correlation affective symptoms scale with non-linear measures in heart rate variability in patients with major depressive disorder

	lnApEn	SampEn	CSE10	lnCSE 20	lnCSE 30	CSE50	DFA alpha1	DFA alpha2	lnDFA crossover
HAMD total	0.302	0.050	0.212	0.215	-0.001	-0.032	-0.023	0.163	0.055
Somatic	**0.458**	0.132	0.293	0.255	-0.017	-0.164	-0.025	0.247	0.093
Psychological	-0.014	-0.063	0.020	0.066	0.018	0.128	-0.010	-0.007	-0.013
IDS-SR total	0.201	-0.389	0.246	0.235	0.083	0.030	0.183	0.114	-0.093
Mood/cognition	0.074	-0.095	-0.094	0.041	0.142	0.245	-0.013	-0.103	0.023
Anxiety/arousal	0.323	**-0.444**	0.321	0.303	0.065	-0.069	0.294	0.188	-0.181
Beck Anxiety Inventory	0.019	**-0.585**	0.415	0.115	**-0.446**	-0.275	**0.552**	0.137	**-0.471**
Beck Hopelessness Scale	0.050	-0.314	0.103	-0.021	-0.137	0.050	0.338	-0.152	-0.396
Scale for Suicide Ideation	0.279	-0.157	0.023	-0.013	-0.040	-0.100	0.264	0.047	-0.267
Barratt Impulsivity Scale	-0.082	-0.066	-0.112	-0.354	-0.253	-0.060	0.334	-0.207	-0.409
Attention	-0.053	-0.261	0.077	-0.156	-0.279	-0.297	0.127	0.326	0.061
Motor	-0.152	**-0.464**	0.284	-0.165	-0.389	-0.303	0.371	0.093	-0.259

Text in **Bold** means p-value <0.05

Table 1.5. Selection frequency of each heart rate variability index computed in fivefold cross-validation experiments 1000 times of support vector machine with recursive feature elimination

Features	**Selection frequency (%)**	**7 selected features**
RMSSD	70.12%	O
CSE20	41.06%	O
LF	37.88%	O
pNN10	37.70%	O
STD	34.84%	O
ApEn	33.78%	O
SampEn	29.90%	O
VLF	24.96%	
CSE30	23.86%	
HF	23.46%	
pNN50	22.02%	
pNN30	19.30%	
pNN20	19.08%	
CSE50	17.30%	
DFAcrossover	15.60%	
Mean	15.46%	
HFnu	14.76%	
DFAalpha1	13.76%	
LFHF	12.12%	
CSE10	12.06%	
DFAalpha2	11.96%	

Table 2.1. List of differentially expressed proteins (DEPs).

Gene Symbol	Fold-change from Scaffold Q+	Fold-change from isobar
AKD1	UP	----
BPIFB1	UP	----
CA2	DOWN	DOWN
CRELD1	DOWN	----
CRP	DOWN	DOWN
DDR2	UP	----
DDX10	DOWN	----
DNAH14	UP	----
ENKUR	DOWN	----
F8	UP	----
FAM53B	DOWN	----
GC	----	UP
H2AFJ	UP	----
H2BFS	UP	----
HHIPL1	UP	----
HIST1H2AD	UP	UP
HIST1H4A	UP	----
HIST2H2BF	UP	----
HPR	DOWN	DOWN
HRNR	DOWN	DOWN
KAT6A	DOWN	----
KCP	DOWN	----
LBP	----	DOWN
LPA	UP	UP
LRP4	----	UP

MAN2B1	----	UP
MYL6	UP	----
MYOM3	DOWN	----
PF4	DOWN	DOWN
PFN1	DOWN	----
PLCE1	DOWN	----
POF1B	DOWN	----
PPBP	----	DOWN
PRUNE2	----	DOWN
PTPN5	DOWN	----
PVRL3	UP	----
PXDNL	DOWN	----
S100A14	DOWN	----
SBSN	DOWN	----
SGOL2	DOWN	----
SLC26A6	DOWN	----
SOGA2	UP	----
SPRR1A	DOWN	----
SRGAP1	UP	UP
TACC2	DOWN	----
TGM3	----	DOWN
TIE1	UP	----
TUBGCP5	UP	----
VLDLR	UP	----
YWHAB	UP	UP

UP and DOWN denote up- and downregulation in MDD patients, respectively.

Table 2.2. Selection of a multiparametric serum biomarker panel using Multiple Reaction Monitoring. The sources of candidate proteins are denoted as follows: DEP, MS, and Others indicate DEPs from the profiling experiment, two previous LC/MS studies, and manually curated from previous reports by searching PubMed, respectively. Log2-transformed fold-change (log2FC), p-value (p-value), and adjusted p-value (adj.p-value) were obtained from MSStats. The Benjamini-Hochberg procedure was used for multiple hypothesis correction. The six selected proteins are denoted by O.

ProteinID	Gene Symbol	Gene ID	Category	Fold-change from profiling expreriment	log2FC	p-value	adj.p-value	6 biomarker panel
P02774	GC	2638	DEP	UP	0.17	0.00	0.00	O
Q86YZ3	HRNR	388697	DEP	DOWN	0.02	0.42	0.49	O
P07737	PFN1	5216	DEP	DOWN	-0.26	0.00	0.00	O
P04114	APOB	338	MS	----	0.17	0.00	0.00	O
P05090	APOD	347	MS	----	0.36	0.00	0.00	O
P00450	CP	1356	MS	----	-0.05	0.20	0.27	O
Q9NQS3	PVRL3	25945	DEP	UP	0.19	0.00	0.00	----
Q8WVV4	POF1B	79983	DEP	DOWN	-0.03	0.71	0.71	----
P02775	PPBP	5473	DEP	DOWN	-0.13	0.00	0.00	----
P04217	A1BG	1	MS	----	0.03	0.02	0.03	----
P01023	A2M	2	MS	----	-0.02	0.43	0.49	----
P43652	AFM	173	MS	----	0.13	0.00	0.00	----
P04196	HRG	3273	MS	----	-0.07	0.00	0.00	----
P02656	APOC3	345	Others	----	0.24	0.00	0.00	----
P23560	BDNF	627	Others	----	-0.03	0.62	0.66	----
P14780	MMP9	4318	Others	----	0.04	0.16	0.23	----

Table 3.1. Selection frequency of each heart rate variability index and Multiple Reaction Monitoring data computed in fivefold cross-validation experiments 1000 times of support vector machine with recursive feature elimination.

Features	Selection frequency (%)	5 selected features
CP	75.00%	O
APOB	74.20%	O
GC	73.60%	O
SampEn	59.80%	O
RMSSD	59.60%	O
HRNR	54.00%	
CSE20	37.80%	
ApEn	37.60%	
STD	35.40%	
MMP9	35.00%	
APOD	34.40%	
LF	28.20%	
HRG	26.00%	
AFM	25.20%	
A2M	24.80%	
Mean	24.40%	
POF1B	22.40%	
PFN1	21.20%	
pNN10	19.00%	
VLF	17.20%	
pNN50	16.00%	
PVRL3	16.00%	
APOC3	15.60%	
DFA crossover	14.60%	

A1BG	14.40%
CSE10	13.40%
CSE30	13.00%
BDNF	12.60%
PPBP	12.00%
LFHF	10.80%
DFA alpha2	10.60%
CSE50	10.20%
HFnu	10.00%
pNN30	8.40%
pNN20	7.60%
HF	7.40%
LFnu	5.80%
DFA alpha1	5.80%

Figure 1.1. Overview of the Support vector machine with recursive feature elimination procedure.

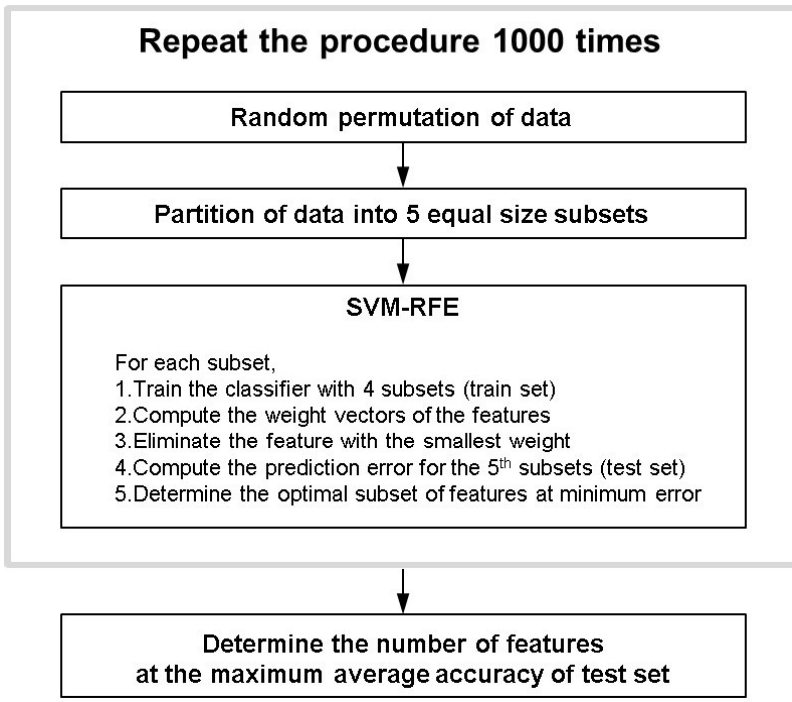


Figure 1.2. Classification accuracy achieved by the specified number of candidate heart rate variability indexes in order of selection frequency computed in fivefold cross-validation experiments 1000 times.

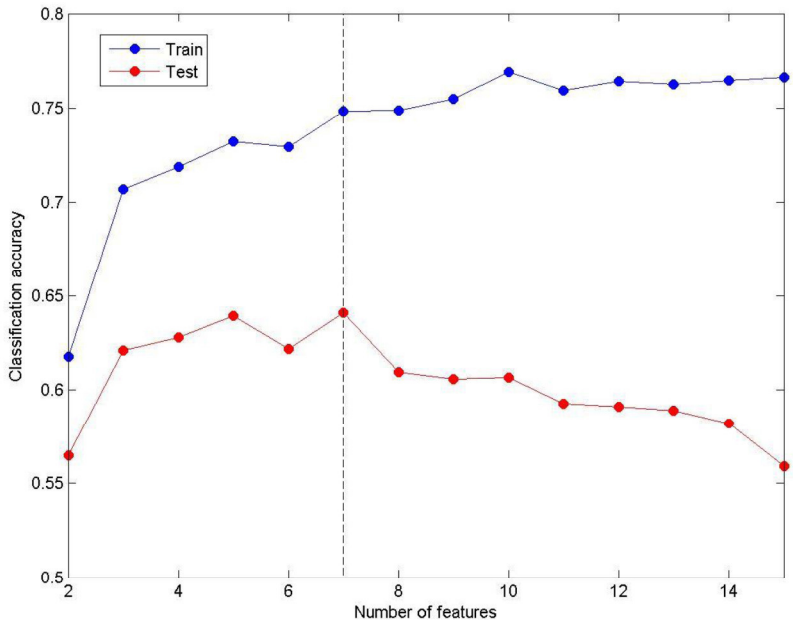


Figure 2.1. Procedure for the development of a serum biomarker panel for diagnosis of major depressive disorder.

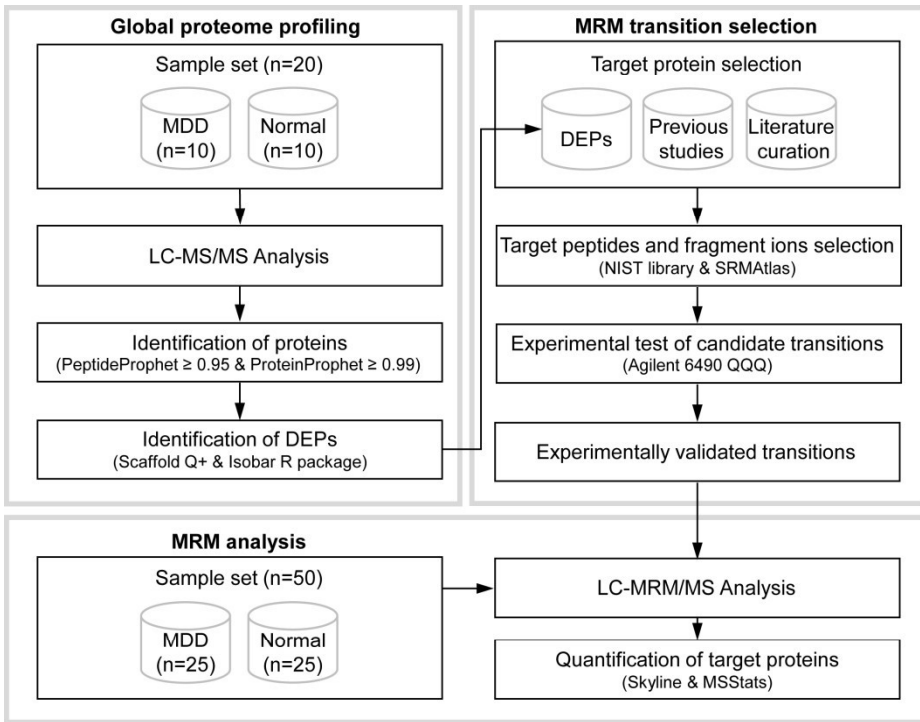


Figure 2.2. Differentially expressed proteins (DEPs) identified in the profiling experiment. a. Venn diagram depicting the DEPs identified by Scaffold Q+ and isobar. b. GOBPs in which DEPs were involved. The black dashed line denotes a p-value of 0.1.

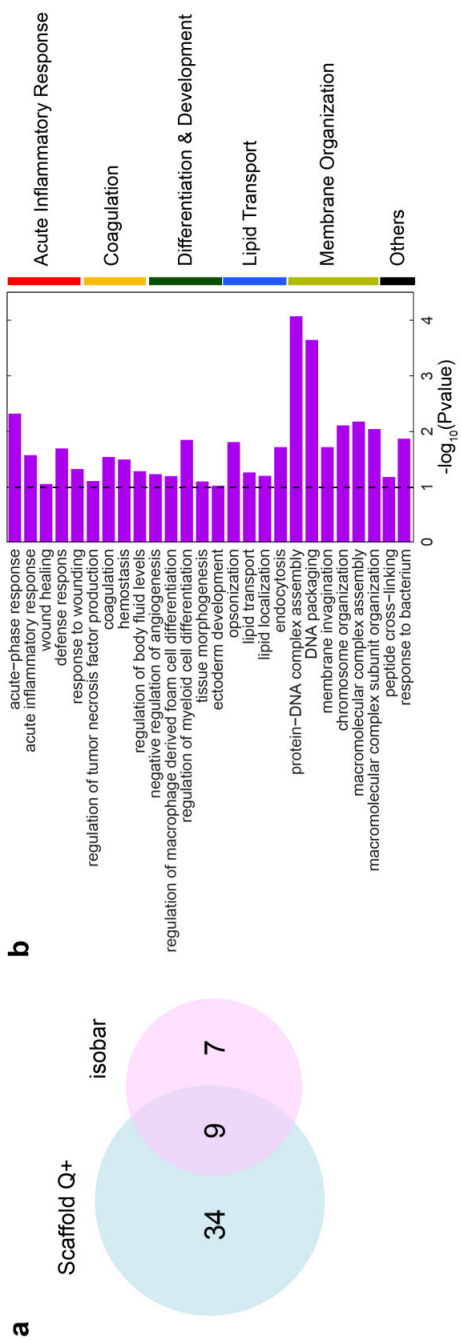


Figure 2.3. Selection of a multiparametric serum biomarker panel. a. Overview of the Multiple Reaction Monitoring data analysis procedure. b. Classification accuracy achieved by the specified number of candidate proteins in order of selection frequency computed in fivefold cross-validation experiments 1000 times. Dots and vertical bars denote the calculated medians and standard deviations of the accuracy, respectively. Lines with circles denote smoothed accuracy curves calculated by moving-average smoothing. c. ROC curves for classification using the six selected candidate proteins. The minimum, median, and maximum of AUCs of 1000 times were 0.6080, 0.7376, and 0.8080, respectively.

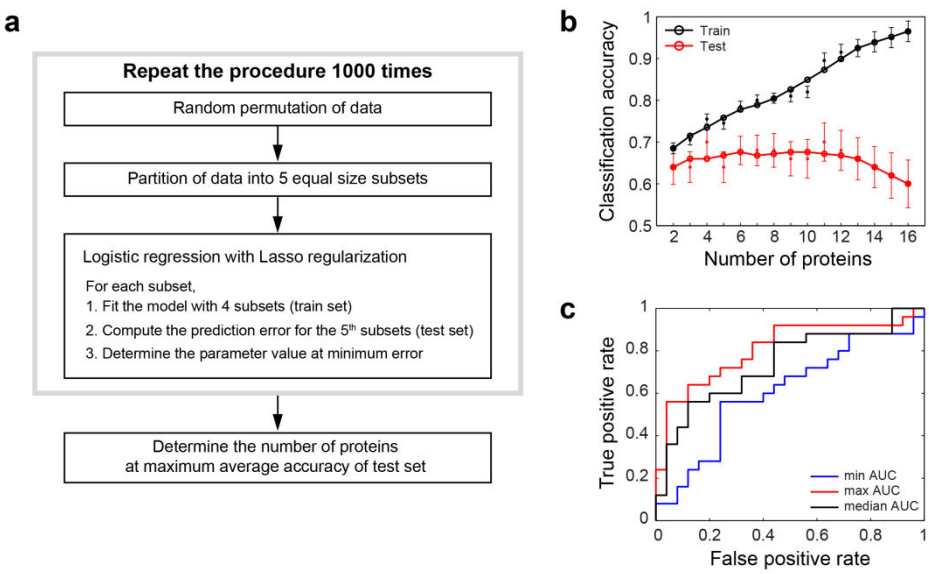
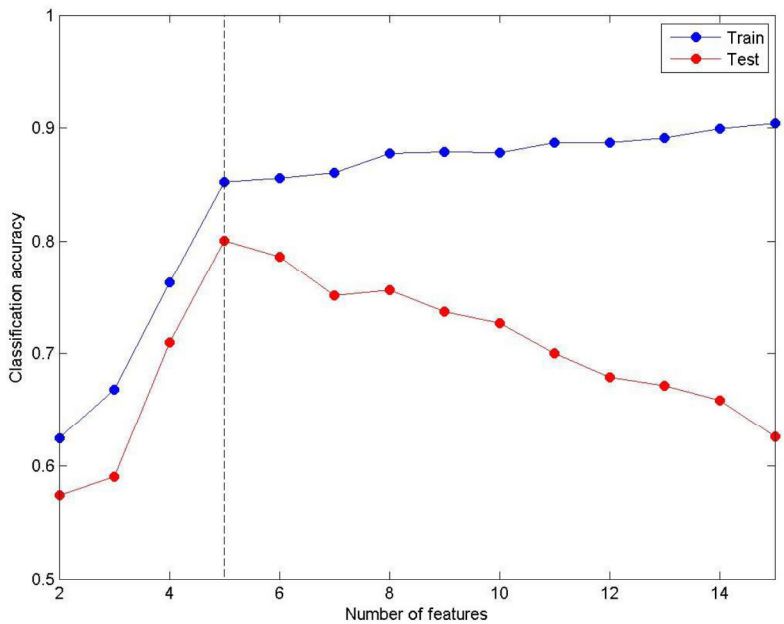


Figure 3.1. Classification accuracy achieved by the specified number of candidate heart rate variability indexes and proteomic data in order of selection frequency computed in fivefold cross-validation experiments 1000 times.



4. Discussion

In this study, we investigated multidimensional peripheral biomarkers for MDD using electrophysiological and molecular approaches including proteomics, which have an advantage of minimal invasiveness. Among several linear and nonlinear HRV parameters, five indexes, including SDNN, LF, ApEn, SampEn, and CSE20, can discriminate MDD subjects from normal controls with 64% overall diagnostic accuracy using a machine learning algorithm, suggesting low complexity of heart rate dynamics and decreased parasympathetic activity over the cardiovascular system in MDD subjects. In proteomic analysis by quantitative serum proteome profiling using LC-MS/MS and verification using MRM, we identified a serum biomarker panel consisting of six proteins, including Apo-D, Apo-B, VDP, ceruloplasmin, hormerin, and profilin1, with a 67% classification accuracy. These proteins were associated with modulation of the immune, inflammatory, and oxidative systems, as well as lipid metabolism. Finally, the combined classification analysis suggested that five parameters (APOB, GC, CP, RMSSD, and SampEn) could discriminate MDD patients from normal controls with an 80.1% overall classification accuracy. Our analyses included only female patients and age-, sex-, and BMI-matched controls to maximize sample homogeneity, and all subjects were drug-naïve to avoid confounding factors due to psychotropic medications.

4.1. HRV analysis

The present study assessed differences in several linear and nonlinear HRV

parameters and identified seven important features for the diagnosis of MDD: RMSSD, SDNN, pNN10, LF, ApEn, SampEn, and CSE20. Despite no significance in the univariate analysis, the severity of anxiety symptoms was found to be significantly correlated with several HRV parameters, indicating reduced parasympathetic activities (pNNx, LFnu, HFnu, and lnLF/HF) and complexity of heart rate dynamics (SampEn). Overall, our findings suggest that low complexity of heart rate dynamics and decreased parasympathetic activity over the cardiovascular system may be involved in the pathophysiology of MDD.

A primary strength of the present study was the inclusion of several clinically valuable non-linear HRV parameters. The irregular variability of the normal heartbeat in the present study suggests that complex nonlinear dynamics, including neural and non-neural mechanisms, are involved in the genesis of HRV. Although the linear measures reflect the magnitude of the RR interval fluctuation as an index of sympathovagal activity, the nonlinear indices reveal the complexity and correlational properties of the RR interval that are dependent upon the functional integrity of autonomic control mechanisms (96, 97). Several studies demonstrated that altered short-term ApEn, SampEn, and fractal components of heart rate fluctuations, such as DFA alpha1, are useful measures for the prediction of pathological states and mortality in diverse clinical and normal populations (98-101).

In this study, although we found no index to separate patients with MDD from healthy controls in the univariate analysis, the multivariate analysis using SVM showed that the collective contribution of seven variables could act as a possible biomarker set for classification. Since the pattern of cardiac

dynamics is the result of interactions among autonomic, humeral, and intrinsic influences on heart rate (102), proper use of multivariate statistical techniques may be necessary to integrate the HRV measures of multiple domains (103).

In this study, SampEn and ApEn were important features for discriminating patients with MDD from normal controls. These findings suggest that these symptoms are associated with lower adaptability and flexibility during neurocardiac regulation. A low level of entropy implies a high degree of regularity and predictability in heart rate dynamics, and a reduction in the flexibility of autonomic control over heart rate responses to external and internal stimuli (104, 105); this is indicative of a maladaptive regulatory system. Low ApEn and SampEn values are associated with mortality in trauma patients (98) and with postoperative cardiac complications in cardiac patients (106), which may be linked to greater cardiovascular risk and mortality levels in MDD patients. In our correlation analysis of patients with MDD, ApEn was also negatively associated with the somatic domains of clinician-rated depressive symptoms. These findings are partially in accordance with previous findings that somatic symptoms of MDD (i.e., sleeping difficulties, changes in appetite, fatigue) tend to be associated with lower HRV to a greater extent than cognitive symptoms of depression (i.e., anhedonia, poor concentration, feelings of worthlessness, suicidal ideation) (107). This study also found that reduced SampEn was associated with the severity of anxiety/arousal domain of self-reported depressive symptoms. Overall, our findings suggest that the distinct complexity index of HRV could be related to specific domains of depressive symptoms. Although the specific origin of the influence of depressive symptoms on the complexity and

symbolic measures has yet to be fully characterized, given the heterogeneity of depressive phenomenology, integration of several meaningful parameters should be considered for the discovery of biomarkers in MDD.

RMSSD, pNN10, LF, and SDNN were also consistently selected as important classifiers for MDD. This suggests sympathetic dominance and decreased parasympathetic outflow, in accordance with previous studies (43). A body of research has demonstrated that depression is associated with an overall reduction in HRV. HRV reflects a measure of an individual's capacity for parasympathetic inhibition of autonomic arousal in emotional expression and regulation (108). A recent study reported that young adults with higher resting state HRV showed more adaptive self-regulation and more social engagement than those with lower resting state HRV (58), supporting the role of HRV as a marker of a person's capacity for self-regulation and psychological flexibility (46). The key component for understanding the association between reduced HRV and depression is the CAN. The CAN regulates and integrates autonomic, endocrine, emotional, and behavioral responses in adaptation to environmental influences (109) HRV reflects the function of the CAN, particularly the inhibitory role of the prefrontal cortex for vagally mediated cardiovascular function. Depressive symptoms can be indicated by a failure of inhibition in affective, cognitive, physiological, and behavioral responses implicated in the CAN, which results in decreased vagal outflow and reduced HRV (54).

Interestingly, the severity of anxiety symptoms in patients with MDD was significantly correlated with several linear and nonlinear parameters, which revealed decreased vagal control and sympathetic dominance indicated by

increased LFnu, decreased HFnu, and increased LF/HF ratio, along with reduced entropy such as SampEn, short-term fractal scaling exponents, such as DFA alpha1 and lnDFA crossover, and complexity, such as CSE30. Anxiety symptoms have been repeatedly found to be related to reductions in HRV, particularly in the HF band (54), Patients with generalized anxiety states exhibit greater threat-related attention biases that are likely associated with a failure of inhibitory function due to the vagally mediated PFC pathway in the CAN, which leads to activation of the sympathetic nervous system due to withdrawal of parasympathetic activity, which, in turn, results in diminished HRV (54). Chronic worriers display poor autonomic cardiac regulation in response to nonthreatening cues (110), and high levels of worry result in a 2- to 3-fold increase in the risk of future myocardial infarctions (111), which may be another linking mechanism between cardiovascular risk and mortality levels in patients with MDD. Given that both depressive and anxiety symptoms and abnormal HRV function can predict adverse cardiovascular outcomes and mortality, future prospective studies should confirm the observed associations between the autonomic correlates of depression or anxiety (i.e., reduced vagal tone and alterations in complexity measures) and cardiovascular risk.

4.2. Proteomic analysis

To the best of our knowledge, our study is the first to use an MRM assay and serum proteome profiling to quantify a multiparametric biomarker panel for MDD. Of the DEPs identified in the profiling experiment and the literature-curated proteins, 16 proteins were quantified using MRM. We

identified a serum biomarker set comprising six proteins that could be used to differentiate MDD patients from controls with 67% sensitivity, 69% specificity, and 68% overall classification accuracy. The results suggested that modulation of the immune and inflammatory systems and lipid metabolism were involved in the pathophysiology of MDD.

To date, two previous proteomics analyses have been performed in MDD subjects (38, 88). The primary strength of our study was the comprehensive protein coverage and multiparametric data analysis using an MRM assay. The proteome coverage values of previous studies were relatively low. Xu et al. identified 94 proteins, of which 9 were differentially expressed in the profiling experiment. Stelzhammer et al. reported the identification of an average of 419 proteins from two cohorts; of these, 169 proteins were detected in both cohorts. Among 169 overlapping proteins, 2 were consistently different in both cohorts. The low proteome coverage in those profiling experiments could have been responsible for their identification of a small number of DEPs. In addition, to verify the discovered DEPs, Xu et al. (2012) reused the samples used in the profiling experiment, which resulted in the verification of only two proteins (A2M and APOB) using ELISA . Stelzhammer et al. (2014) reported two DEPs consistently detected in both cohorts using a single LC-MS^E method; the changes were not validated using another method. In contrast, we expanded our biomarker candidate proteins by including those suggested in previous studies, and thus performed the first and largest quantitative targeted proteomics investigation of serum samples from MDD patients. The six proteins that we identified for classification were APOD, APOB, GC, CP, HRNR, and PFN1.

APOD levels were altered in patients with MDD. APOD was initially isolated from human plasma high-density lipoproteins as a multifunctional transporter for small hydrophobic ligands (112). APOD is highly expressed in the nervous system, including peripheral nerves and the brain (113), and is the major protein in a variety of body fluids, such as cerebrospinal fluid and urine (114). These characteristics are in contrast to those of other apolipoproteins, which are produced in the liver and intestinal epithelium. Increasing evidence suggests that APOD exerts neuroprotective effects by attenuating oxidative stress-induced lipid peroxidation associated with aging, injury, and neurodegenerative processes (113, 115) and by regulating inflammatory cascades in an anti-inflammatory manner, with a focus on eicosanoid and phospholipid metabolism (116). Increased APOD expression in the brain has been reported in neurodegenerative diseases, such as Alzheimer's disease (117) and multiple sclerosis (118), and in aging individuals (113). Furthermore, APOD is significantly upregulated in the brains of schizophrenic and bipolar patients (116) and increased in the plasma of first-episode schizophrenic patients, in whom its increase presumably occurs prior to the first clinical symptoms (119). Thus, elevated serum APOD levels in drug-free depressed patients may reflect a neuroprotective response to oxidative stress and systemic inflammation together with neurovascular dysfunction and blood-brain barrier hyperpermeability, which are implicated in the pathophysiology of MDD (22).

CP is a copper-containing protein with ferroxidase activity (120) that is synthesized mainly by hepatocytes, and to a lesser extent, in the testis, spleen, lungs, and central nervous system. CP plays an important role in iron

metabolism. The protein is an acute-phase reactant that increases in response to inflammation and, as part of the innate immune system (121), CP antagonizes oxidative damage in the central nervous system (122). Its deficiency in neuronal cells may lead to damage secondary to decreased mitochondrial energy production, increased lipid peroxidation, and an increase in oxygen free radicals associated with iron accumulation (123). Our findings are consistent with a recent proteomic study that found decreased CP levels in patients with MDD (88). These results may reflect high levels of oxidative stress in depressed patients, as has been reported previously (22). However, increased CP levels have been observed in patients with schizophrenia (124) and MDD (125), suggesting that oxidative damage may trigger a neurodegenerative process.

GC is a multi-functional plasma protein and a member of the albumin superfamily of binding proteins that includes albumin, α-albumin, and α-fetoprotein, and it is synthesized predominantly in the liver and secreted into the bloodstream (126). In the present study, GC levels were increased in subjects with MDD, consistent with a prior proteomic profiling study (38). GC plays a role in the transport and storage of vitamin D metabolites and control of bone development, but also is implicated in many important biological functions, most importantly, modulation of immune and inflammatory responses such as extracellular actin scavenging, leukocyte C5a-mediated chemotaxis, macrophage activation, and stimulation of osteoclasts (127, 128). Preclinical microarray studies of brain and blood have suggested that the gene encoding vitamin D-binding protein is a potential candidate for involvement in bipolar disorder and psychosis (129, 130).

Increased GC levels have been reported in schizophrenia, suggesting that GC is an acute-phase response linked with the pathophysiology of schizophrenia (131). Similarly, increased GC levels may be associated with abnormalities of the immune and inflammatory responses and the serum lipid profile of MDD patients (38).

APOB levels were increased in MDD patients, in accordance with previous reports (38, 132) APOB is present in very-low-density lipoprotein (VLDL), intermediate-density lipoprotein, and low-density lipoprotein (LDL), and has important roles in peripheral lipid regulation. In particular, lipoproteins that contain APOB is associated with the formation of atherogenic plaque and the development of atherosclerosis (133). APOB levels are considered to be a predictor of cardiocerebrovascular events (134). Furthermore, in a previous study, it was superior to LDL-C and non-high-density lipoprotein cholesterol for predicting cardiovascular events (135) and was a more informative marker of the adequacy of statin therapy than cholesterol markers (136). Elevated APOB levels may underlie the link between depression and increased morbidity and premature mortality, especially from CVD, among depressive patients (137). A previous study reported no significant relationships between ApoB levels and depressive symptoms; however, that study included only male subjects (138).

We observed altered PFN1 levels in MDD subjects. PFN1 is a ubiquitously expressed actin monomer-binding protein associated with mRNA splicing, gene transcription, and multiple cellular functions, including proliferation, survival, motility, endocytosis, and membrane trafficking (139). PFN1 plays a prominent effector role in cardiovascular disease and has been suggested to

link the actin cytoskeleton and its dynamics directly to vascular inflammation (140, 141). Although the mechanism underlying the association with depression remains unclear, a preclinical study proposed the actin-associated molecular pathway as an underlying mechanism of mood disorders, suggesting that the balance of actin dynamics might be altered towards actin depolymerization in mood disorders, with upregulation of the PFN1 gene in the frontal cortex of mice prone to depression (142).

In addition to the aforementioned proteins, HRNR levels were altered in MDD patients in this MRM analysis. HRNR is an intermediate filament-associated protein. Although it was slightly downregulated in the initial profiling analysis using pooled samples, its levels were not significantly different between the two groups. This could have been due to the intrinsic variability of proteomic analyses. In addition, as MDD is a complex mental disorder with a poorly understood multifactorial etiology, the possibility of inter-individual variability cannot be excluded.

A limitation of our study is the small sample size, as is often the case with proteomics studies of depression; generally, 10 to 50 samples are used given the large number of variables in the analysis and short period between the preclinical discovery and verification phases (143). Thus, our results should be considered exploratory. Although our study is the most comprehensive investigation of protein profiling to date, we were not able to quantify proteins with low serum levels, such as cytokines, because the MRM limits of quantification are 0.3 to 1.0 μg/mL (144, 145). Nonetheless, changes in pro-inflammatory cytokine levels have been consistently linked with depression.

4.3. Combined classification

We performed a machine learning analysis on HRV data and proteomic data to separate individuals with MDD from healthy controls. We identified five important classifiers for MDD, APOB, GC, CP, RMSSD and SampEn, which could be used to differentiate patients with MDD from controls with 70.2% sensitivity, 89.9% specificity, and 80.1 % overall classification accuracy.

Better classification accuracy can be achieved by combining HRV and proteomic data compared with using either alone. Protein markers, including APOB, GC, and CP, suggested that modulation of the immune and inflammatory systems and lipid metabolism were primarily involved in the pathophysiology of MDD. HRV markers, including RMSSD and SampEn, reflect the involvement of parasympathetic vagal activity and the complexity of heart rate dynamics in MDD pathology. The results suggest that even though abnormal cardiac autonomic control mechanisms and specific biochemical pathway dysfunction are both related to a clinical diagnosis of depression, they reflect different aspects of MDD pathophysiology, and cannot replace each other in terms of reflecting the disease. Overall, 80% accuracy was achieved, suggesting that combining heart rate dynamics and proteomic information best represents the majority of symptomatic information used currently to arrive at a clinical diagnosis.

Further investigation on larger, independent cohorts will be necessary to verify the potential role of these five candidates as complimentary biomarkers applicable to diagnosis. Along with this validation, it should be evaluated whether these markers represent state markers or trait markers for MDD. Trait

markers are related to the susceptibility or prediction of MDD. On the other hand, state markers can be used to monitor disease progression and early treatment responses to drugs in a patient. For this reason, a prospective study in a homogenous population is necessary.

One of the major issues in discovering biomarkers for psychiatric disorders is the heterogeneity of the current diagnostic category (e.g., DSM). The current diagnostic system allows for several combinations of symptoms to be included in one diagnosis. This heterogeneity results in a debate about subtypes within MDD (e.g., melancholia, atypical depression). Other issues include the delimitation from other disorders (e.g., MDD vs. bipolar disorder; MDD vs. schizophrenia) and diagnostic transition among disorders (e.g., from diagnosis of MDD to bipolar disorder). As DSM constructs do not necessarily reflect underlying pathophysiological pathways, there is the possibility of common pathologic alterations among different psychiatric disorders. Therefore, candidate biomarkers in this study need to be validated via better design of clinical trials focused on the sub-classification of MDD and a differential diagnosis from bipolar disorder or schizophrenia.

5. Conclusion

In this study, we were able to identify peripheral biomarker candidates that changed quantitatively in MDD through HRV analysis and a serum proteomic approach. HRV analysis identified several classifiers that were associated with sympathetic dominance and reduced complexity of heart rate dynamics. In the proteomic analysis, several altered serum proteins related to modulation of the

immune, inflammatory, and oxidative system were found in patients with MDD using LC-MS/MS and MRM proteomic approaches, demonstrating the utility of this method for blood-based biomarker discovery in MDD. Furthermore, better classification accuracy can be achieved by combining HRV and proteomic data than using either alone, suggesting that they reflect different aspects of MDD pathophysiology.

'-omics' approaches may yield unbiased, hypothesis-free insight into the pathophysiologic underpinnings of MDD. However, the application of high-throughput proteome profiling to MDD in humans has mostly been restricted to postmortem brain tissue and animal studies (34). Circulating blood comprises a highly complex system that communicates with every tissue and organ in the body. In addition, collection of peripheral blood samples, as well as HRV analysis is a minimally invasive procedure as compared with collection of tissue samples by biopsy.

This was a preliminary study with a small sample size and a limited number of proteomic and HRV data aimed at demonstrating the power of combining biochemical function with cardiac autonomic dynamics applied in the classification framework. For full validation, the proposed model will need to be applied to a much larger group of subjects, including other age groups and males. Future work will also focus on differentiation of sub-groups in MDD, prediction of treatment response, and early diagnosis at the time of first presentation.

References

1. Fava M, Kendler KS. Major depressive disorder. Neuron. 2000 Nov;28(2):335-41. PubMed PMID: 11144343. Epub 2001/01/06. eng.

2. Judd LL, Akiskal HS, Maser JD, Zeller PJ, Endicott J, Coryell W, et al. A prospective 12-year study of subsyndromal and syndromal depressive symptoms in unipolar major depressive disorders. Archives of general psychiatry. 1998 Aug;55(8):694-700. PubMed PMID: 9707379. Epub 1998/08/26. eng.

3. Hawton K, van Heeringen K. Suicide. Lancet. 2009 Apr 18;373(9672):1372-81. PubMed PMID: 19376453. Epub 2009/04/21. eng.

4. Isometsa ET, Henriksson MM, Aro HM, Heikkinen ME, Kuoppasalmi KI, Lonnqvist JK. Suicide in major depression. The American journal of psychiatry. 1994 Apr;151(4):530-6. PubMed PMID: 8147450. Epub 1994/04/01. eng.

5. Riihimaki K, Vuorilehto M, Melartin T, Haukka J, Isometsa E. Incidence and predictors of suicide attempts among primary-care patients with depressive disorders: a 5-year prospective study. Psychological medicine. 2014 Jan;44(2):291-302. PubMed PMID: 23570583. Epub 2013/04/11. eng.

6. Sokero TP, Melartin TK, Rytsala HJ, Leskela US, Lestela-Mielonen PS, Isometsa ET. Prospective study of risk factors for attempted suicide among patients with DSM-IV major depressive disorder. The British journal of psychiatry : the journal of mental science. 2005 Apr;186:314-8. PubMed PMID: 15802688. Epub 2005/04/02. eng.

7. Vuorilehto MS, Melartin TK, Isometsa ET. Suicidal behaviour

among primary-care patients with depressive disorders. Psychological medicine. 2006 Feb;36(2):203-10. PubMed PMID: 16420714. Epub 2006/01/20. eng.

8. Blair-West GW, Mellsop GW, Eyeson-Annan ML. Down-rating lifetime suicide risk in major depression. Acta psychiatrica Scandinavica. 1997 Mar;95(3):259-63. PubMed PMID: 9111861. Epub 1997/03/01. eng.

9. Inskip HM, Harris EC, Barraclough B. Lifetime risk of suicide for affective disorder, alcoholism and schizophrenia. The British journal of psychiatry : the journal of mental science. 1998 Jan;172:35-7. PubMed PMID: 9534829. Epub 1998/04/16. eng.

10. Nordentoft M, Mortensen PB, Pedersen CB. Absolute risk of suicide after first hospital contact in mental disorder. Archives of general psychiatry. 2011 Oct;68(10):1058-64. PubMed PMID: 21969462. Epub 2011/10/05. eng.

11. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012 Dec 15;380(9859):2163-96. PubMed PMID: 23245607. Epub 2012/12/19. eng.

12. Whiteford HA, Degenhardt L, Rehm J, Baxter AJ, Ferrari AJ, Erskine HE, et al. Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. Lancet. 2013 Nov 9;382(9904):1575-86. PubMed PMID: 23993280. Epub 2013/09/03. eng.

13. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS medicine. 2006 Nov;3(11):e442. PubMed

PMID: 17132052. Pubmed Central PMCID: Pmc1664601. Epub 2006/11/30. eng.

14. DiMatteo MR, Lepper HS, Croghan TW. Depression is a risk factor for noncompliance with medical treatment: meta-analysis of the effects of anxiety and depression on patient adherence. Archives of internal medicine. 2000 Jul 24;160(14):2101-7. PubMed PMID: 10904452. Epub 2000/07/25. eng.

15. Papakostas GI, Shelton RC, Kinrys G, Henry ME, Bakow BR, Lipkin SH, et al. Assessment of a multi-assay, serum-based biological diagnostic test for major depressive disorder: a pilot and replication study. Molecular psychiatry. 2013 Mar;18(3):332-9. PubMed PMID: 22158016.

16. McGuffin P, Katz R. The genetics of depression and manic-depressive disorder. The British journal of psychiatry : the journal of mental science. 1989 Sep;155:294-304. PubMed PMID: 2692760. Epub 1989/09/01. eng.

17. Harrison PJ. The neuropathology of primary mood disorder. Brain : a journal of neurology. 2002 Jul;125(Pt 7):1428-49. PubMed PMID: 12076995. Epub 2002/06/22. eng.

18. Lundberg P, Cantor-Graae E, Rukundo G, Ashaba S, Ostergren PO. Urbanicity of place of birth and symptoms of psychosis, depression and anxiety in Uganda. The British journal of psychiatry : the journal of mental science. 2009 Aug;195(2):156-62. PubMed PMID: 19648549. Epub 2009/08/04. eng.

19. Manji HK, Quiroz JA, Sporn J, Payne JL, Denicoff K, N AG, et al. Enhancing neuronal plasticity and cellular resilience to develop novel,

improved therapeutics for difficult-to-treat depression. Biological psychiatry. 2003 Apr 15;53(8):707-42. PubMed PMID: 12706957. Epub 2003/04/23. eng.

20. Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. Neurobiology of depression. Neuron. 2002 Mar 28;34(1):13-25. PubMed PMID: 11931738. Epub 2002/04/05. eng.

21. Lopresti AL, Maker GL, Hood SD, Drummond PD. A review of peripheral biomarkers in major depression: the potential of inflammatory and oxidative stress biomarkers. Progress in neuro-psychopharmacology & biological psychiatry. 2014 Jan 3;48:102-11. PubMed PMID: 24104186. Epub 2013/10/10. eng.

22. Najjar S, Pearlman DM, Devinsky O, Najjar A, Zagzag D. Neurovascular unit dysfunction with blood-brain barrier hyperpermeability contributes to major depressive disorder: a review of clinical and experimental evidence. Journal of neuroinflammation. 2013;10:142. PubMed PMID: 24289502. Pubmed Central PMCID: 4220803.

23. Hendrickx H, McEwen BS, Ouderaa F. Metabolism, mood and cognition in aging: the importance of lifestyle and dietary intervention. Neurobiology of aging. 2005 Dec;26 Suppl 1:1-5. PubMed PMID: 16290269. Epub 2005/11/18. eng.

24. Steiger A, Kimura M. Wake and sleep EEG provide biomarkers in depression. Journal of psychiatric research. 2010 Mar;44(4):242-52. PubMed PMID: 19762038. Epub 2009/09/19. eng.

25. Savitz JB, Drevets WC. Imaging phenotypes of major depressive disorder: genetic correlates. Neuroscience. 2009 Nov 24;164(1):300-30. PubMed PMID: 19358877. Pubmed Central PMCID: Pmc2760612. Epub

2009/04/11. eng.

26. Collier DA, Stober G, Li T, Heils A, Catalano M, Di Bella D, et al. A novel functional polymorphism within the promoter of the serotonin transporter gene: possible role in susceptibility to affective disorders. Molecular psychiatry. 1996 Dec;1(6):453-60. PubMed PMID: 9154246. Epub 1996/12/01. eng.

27. Schmidt HD, Shelton RC, Duman RS. Functional biomarkers of depression: diagnosis, treatment, and pathophysiology. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2011 Nov;36(12):2375-94. PubMed PMID: 21814182. Pubmed Central PMCID: Pmc3194084. Epub 2011/08/05. eng.

28. Domenici E, Wille DR, Tozzi F, Prokopenko I, Miller S, McKeown A, et al. Plasma protein biomarkers for depression and schizophrenia by multi analyte profiling of case-control collections. PloS one. 2010;5(2):e9166. PubMed PMID: 20161799. Pubmed Central PMCID: Pmc2820097. Epub 2010/02/18. eng.

29. Taurines R, Dudley E, Grassl J, Warnke A, Gerlach M, Coogan AN, et al. Proteomic research in psychiatry. Journal of psychopharmacology (Oxford, England). 2011 Feb;25(2):151-96. PubMed PMID: 20142298. Epub 2010/02/10. eng.

30. Dudley E, Hassler F, Thome J. Profiling for novel proteomics biomarkers in neurodevelopmental disorders. Expert review of proteomics. 2011 Feb;8(1):127-36. PubMed PMID: 21329432. Epub 2011/02/19. eng.

31. Carboni L, Becchi S, Piubelli C, Mallei A, Giambelli R, Razzoli M, et al. Early-life stress and antidepressants modulate peripheral biomarkers in a

gene-environment rat model of depression. Progress in neuro-psychopharmacology & biological psychiatry. 2010 Aug 16;34(6):1037-48. PubMed PMID: 20580919. Epub 2010/06/29. eng.

32. Alaiya A, Al-Mohanna M, Linder S. Clinical cancer proteomics: promises and pitfalls. Journal of proteome research. 2005 Jul-Aug;4(4):1213-22. PubMed PMID: 16083271. Epub 2005/08/09. eng.

33. Van Eyk JE. Overview: the maturing of proteomics in cardiovascular research. Circulation research. 2011 Feb 18;108(4):490-8. PubMed PMID: 21335431. Pubmed Central PMCID: Pmc3500592. Epub 2011/02/22. eng.

34. Martins-de-Souza D, Harris LW, Guest PC, Turck CW, Bahn S. The role of proteomics in depression research. European archives of psychiatry and clinical neuroscience. 2010 Sep;260(6):499-506. PubMed PMID: 19997739. Pubmed Central PMCID: Pmc2940035. Epub 2009/12/10. eng.

35. Hye A, Lynham S, Thambisetty M, Causevic M, Campbell J, Byers HL, et al. Proteome-based plasma biomarkers for Alzheimer's disease. Brain : a journal of neurology. 2006 Nov;129(Pt 11):3042-50. PubMed PMID: 17071923. Epub 2006/10/31. eng.

36. Hampel H, Kotter HU, Moller HJ. Blood-cerebrospinal fluid barrier dysfunction for high molecular weight proteins in Alzheimer disease and major depression: indication for disease subsets. Alzheimer disease and associated disorders. 1997 Jun;11(2):78-87. PubMed PMID: 9194954. Epub 1997/06/01. eng.

37. Niklasson F, Agren H. Brain energy metabolism and blood-brain barrier permeability in depressive patients: analyses of creatine, creatinine, urate, and albumin in CSF and blood. Biological psychiatry. 1984

Aug;19(8):1183-206. PubMed PMID: 6498242. Epub 1984/08/01. eng.

38. Xu HB, Zhang RF, Luo D, Zhou Y, Wang Y, Fang L, et al. Comparative proteomic analysis of plasma from major depressive patients: identification of proteins associated with lipid metabolism and immunoregulation. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP). 2012 Nov;15(10):1413-25. PubMed PMID: 22717272. Epub 2012/06/22. eng.

39. Alawam K, Dudley E, Donev R, Thome J. Protein and peptide profiling as a tool for biomarker discovery in depression. Electrophoresis. 2012 Dec;33(24):3830-4. PubMed PMID: 23161603. Epub 2012/11/20. eng.

40. Pajer K, Andrus BM, Gardner W, Lourie A, Strange B, Campo J, et al. Discovery of blood transcriptomic markers for depression in animal models and pilot validation in subjects with early-onset major depression. Translational psychiatry. 2012;2:e101. PubMed PMID: 22832901. Pubmed Central PMCID: 3337072.

41. Le-Niculescu H, Kurian SM, Yehyawi N, Dike C, Patel SD, Edenberg HJ, et al. Identifying blood biomarkers for mood disorders using convergent functional genomics. Molecular psychiatry. 2009 Feb;14(2):156-74. PubMed PMID: 18301394.

42. Thase ME. Mood Disorders:Neurobiology. In: Sadock BJ, Sadock VA, eds., editors. Comprehensive Textbook of Psychaitry 7th Edition. Philadephia: Lippincott Williams & Wilkins.; 2000. p. 1318–28.

43. Kemp AH, Quintana DS, Gray MA, Felmingham KL, Brown K, Gatt JM. Impact of depression and antidepressant treatment on heart rate

variability: a review and meta-analysis. Biological psychiatry. 2010 Jun 1;67(11):1067-74. PubMed PMID: 20138254. Epub 2010/02/09. eng.

44. Carney RM, Freedland KE, Veith RC. Depression, the autonomic nervous system, and coronary heart disease. Psychosomatic medicine. 2005 May-Jun;67 Suppl 1:S29-33. PubMed PMID: 15953797. Epub 2005/06/15. eng.

45. Dekker JM, Crow RS, Folsom AR, Hannan PJ, Liao D, Swenne CA, et al. Low heart rate variability in a 2-minute rhythm strip predicts risk of coronary heart disease and mortality from several causes: the ARIC Study. Atherosclerosis Risk In Communities. Circulation. 2000 Sep 12;102(11):1239-44. PubMed PMID: 10982537. Epub 2000/09/12. eng.

46. Kemp AH, Quintana DS. The relationship between mental and physical health: insights from the study of heart rate variability. International journal of psychophysiology : official journal of the International Organization of Psychophysiology. 2013 Sep;89(3):288-96. PubMed PMID: 23797149. Epub 2013/06/26. eng.

47. Koskinen T, Kahonen M, Jula A, Mattsson N, Laitinen T, Keltikangas-Jarvinen L, et al. Metabolic syndrome and short-term heart rate variability in young adults. The cardiovascular risk in young Finns study. Diabetic medicine : a journal of the British Diabetic Association. 2009 Apr;26(4):354-61. PubMed PMID: 19388964. Epub 2009/04/25. eng.

48. Licht CM, de Geus EJ, Penninx BW. Dysregulation of the autonomic nervous system predicts the development of the metabolic syndrome. The Journal of clinical endocrinology and metabolism. 2013 Jun;98(6):2484-93. PubMed PMID: 23553857. Epub 2013/04/05. eng.

49. Soares-Miranda L, Sandercock G, Vale S, Santos R, Abreu S, Moreira C, et al. Metabolic syndrome, physical activity and cardiac autonomic function. Diabetes/metabolism research and reviews. 2012 May;28(4):363-9. PubMed PMID: 22238216. Epub 2012/01/13. eng.

50. Windham BG, Fumagalli S, Ble A, Sollers JJ, Thayer JF, Najjar SS, et al. The Relationship between Heart Rate Variability and Adiposity Differs for Central and Overall Adiposity. Journal of obesity. 2012;2012:149516. PubMed PMID: 22649714. Pubmed Central PMCID: Pmc3357556. Epub 2012/06/01. eng.

51. Tsuji H, Venditti FJ, Jr., Manders ES, Evans JC, Larson MG, Feldman CL, et al. Reduced heart rate variability and mortality risk in an elderly cohort. The Framingham Heart Study. Circulation. 1994 Aug;90(2):878-83. PubMed PMID: 8044959. Epub 1994/08/01. eng.

52. Thayer JF, Yamamoto SS, Brosschot JF. The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors. International journal of cardiology. 2010 May 28;141(2):122-31. PubMed PMID: 19910061. Epub 2009/11/17. eng.

53. Kleiger RE, Miller JP, Bigger JT, Jr., Moss AJ. Decreased heart rate variability and its association with increased mortality after acute myocardial infarction. The American journal of cardiology. 1987 Feb 1;59(4):256-62. PubMed PMID: 3812275. Epub 1987/02/01. eng.

54. Chalmers JA, Quintana DS, Abbott MJ, Kemp AH. Anxiety Disorders are Associated with Reduced Heart Rate Variability: A Meta-Analysis. Frontiers in psychiatry. 2014;5:80. PubMed PMID: 25071612. Pubmed Central PMCID: Pmc4092363. Epub 2014/07/30. eng.

55. Costa MD, Peng CK, Goldberger AL. Multiscale analysis of heart rate dynamics: entropy and time irreversibility measures. Cardiovascular engineering (Dordrecht, Netherlands). 2008 Jun;8(2):88-93. PubMed PMID: 18172763. Epub 2008/01/04. eng.

56. O'Brien IA, O'Hare P, Corrall RJ. Heart rate variability in healthy subjects: effect of age and the derivation of normal ranges for tests of autonomic function. British heart journal. 1986 Apr;55(4):348-54. PubMed PMID: 3964501. Pubmed Central PMCID: Pmc1236737. Epub 1986/04/01. eng.

57. Abhishekh HA, Nisarga P, Kisan R, Meghana A, Chandran S, Trichur R, et al. Influence of age and gender on autonomic regulation of heart. Journal of clinical monitoring and computing. 2013 Jun;27(3):259-64. PubMed PMID: 23297094. Epub 2013/01/09. eng.

58. Geisler FC, Kubiak T, Siewert K, Weber H. Cardiac vagal tone is associated with social engagement and self-regulation. Biological psychology. 2013 May;93(2):279-86. PubMed PMID: 23466587. Epub 2013/03/08. eng.

59. Hamilton M. A rating scale for depression. Journal of neurology, neurosurgery, and psychiatry. 1960 Feb;23:56-62. PubMed PMID: 14399272. Pubmed Central PMCID: Pmc495331. Epub 1960/02/01. eng.

60. Duberstein PR, Heisel MJ. Personality traits and the reporting of affective disorder symptoms in depressed patients. Journal of affective disorders. 2007 Nov;103(1-3):165-71. PubMed PMID: 17331588. Epub 2007/03/03. eng.

61. Uher R, Farmer A, Maier W, Rietschel M, Hauser J, Marusic A, et al. Measuring depression: comparison and integration of three scales in the

GENDEP study. Psychological medicine. 2008 Feb;38(2):289-300. PubMed PMID: 17922940. Epub 2007/10/10. eng.

62. Rush AJ, Gullion CM, Basco MR, Jarrett RB, Trivedi MH. The Inventory of Depressive Symptomatology (IDS): psychometric properties. Psychological medicine. 1996 May;26(3):477-86. PubMed PMID: 8733206. Epub 1996/05/01. eng.

63. Wardenaar KJ, van Veen T, Giltay EJ, den Hollander-Gijsman M, Penninx BW, Zitman FG. The structure and dimensionality of the Inventory of Depressive Symptomatology Self Report (IDS-SR) in patients with depressive disorders and healthy controls. Journal of affective disorders. 2010 Sep;125(1-3):146-54. PubMed PMID: 20074811. Epub 2010/01/16. eng.

64. Beck AT, Epstein N, Brown G, Steer RA. An inventory for measuring clinical anxiety: psychometric properties. Journal of consulting and clinical psychology. 1988 Dec;56(6):893-7. PubMed PMID: 3204199. Epub 1988/12/01. eng.

65. Beck AT, Steer RA, Beck JS. Types of self-reported anxiety in outpatients with DSM-III-R anxiety disorders. Anxiety, Stress, & Coping: An International Journal. 1993;6(1):43-55.

66. Beck AT, Steer RA. Manual for the Beck Hopelessness Scale. . Psychological Corporation; 1988.

67. Bouvard M, Charles S, Guerin J, Aimard G, Cottraux J. [Study of Beck's hopelessness scale. Validation and factor analysis]. L'Encephale. 1992 May-Jun;18(3):237-40. PubMed PMID: 1299593. Epub 1992/05/01. Etude de l'echelle de desespoir de Beck (hopelessness scale). Validation et analyse factorielle. fre.

68. Beck AT, Steer RA. Manual for the Beck Scale for Suicide Ideation. San Antonio, TX: Psychological Corporation; 1991.

69. Steer RA, Rissmiller DJ, Ranieri WF, Beck AT. Dimensions of suicidal ideation in psychiatric inpatients. Behaviour research and therapy. 1993 Feb;31(2):229-36. PubMed PMID: 8442750. Epub 1993/02/01. eng.

70. Corruble E, Benyamina A, Bayle F, Falissard B, Hardy P. Understanding impulsivity in severe depression? A psychometrical contribution. Progress in neuro-psychopharmacology & biological psychiatry. 2003 Aug;27(5):829-33. PubMed PMID: 12921916. Epub 2003/08/19. eng.

71. Lewis M, Scott J, Frangou S. Impulsivity, personality and bipolar disorder. European Psychiatry. 2009 Oct;24(7):464-9. PubMed PMID: 19793639. Epub 2009/10/02. eng.

72. Patton JM, Stanford MS, Barratt ES. Factor Structure of the Barratt Impulsiveness Scale. Journal of Clinical Psychology. 1995;51:768-74.

73. Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Circulation. 1996 Mar 1;93(5):1043-65. PubMed PMID: 8598068. Epub 1996/03/01. eng.

74. Pan J, Tompkins WJ. A real-time QRS detection algorithm. IEEE transactions on bio-medical engineering. 1985 Mar;32(3):230-6. PubMed PMID: 3997178. Epub 1985/03/01. eng.

75. Lippman N, Stein KM, Lerman BB. Comparison of methods for removal of ectopy in measurement of heart rate variability. The American journal of physiology. 1994 Jul;267(1 Pt 2):H411-8. PubMed PMID: 7519408.

Epub 1994/07/01. eng.

76. Mietus JE, Peng CK, Henry I, Goldsmith RL, Goldberger AL. The pNNx files: re-examining a widely used heart rate variability measure. Heart (British Cardiac Society). 2002 Oct;88(4):378-80. PubMed PMID: 12231596. Pubmed Central PMCID: Pmc1767394. Epub 2002/09/17. eng.

77. Rajendra Acharya U, Paul Joseph K, Kannathal N, Lim CM, Suri JS. Heart rate variability: a review. Medical & biological engineering & computing. 2006 Dec;44(12):1031-51. PubMed PMID: 17111118. Epub 2006/11/18. eng.

78. Bornas X, Llabres J, Noguera M, Pez A. Sample entropy of ECG time series of fearful flyers: preliminary results. Nonlinear dynamics, psychology, and life sciences. 2006 Jul;10(3):301-18. PubMed PMID: 16762174. Epub 2006/06/10. eng.

79. Kurths J, Voss A, Saparin P, Witt A, Kleiner HJ, Wessel N. Quantitative analysis of heart rate variability. Chaos (Woodbury, NY). 1995 Mar;5(1):88-94. PubMed PMID: 12780160. Epub 1995/03/01. Eng.

80. Park KT, Yi SH. Accessing physiological complexity of HRV by using threshold-dependent symbolic entropy. J Korean Phys Soc. 2004;44:569-76.

81. Steuer R, Ebeling W, Russell DF, Bahar S, Neiman A, Moss F. Entropy and local uncertainty of data from sensory neurons. Physical review E, Statistical, nonlinear, and soft matter physics. 2001 Dec;64(6 Pt 1):061911. PubMed PMID: 11736214. Epub 2001/12/12. eng.

82. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nature methods. 2009

May;6(5):359-62. PubMed PMID: 19377485.

83. Horth P, Miller CA, Preckel T, Wenz C. Efficient fractionation and improved protein identification by peptide OFFGEL electrophoresis. Molecular & cellular proteomics : MCP. 2006 Oct;5(10):1968-74. PubMed PMID: 16849286.

84. Searle BC. Scaffold: a bioinformatic tool for validating MS/MS-based proteomic studies. Proteomics. 2010 Mar;10(6):1265-9. PubMed PMID: 20077414.

85. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Analytical chemistry. 2002 Oct 15;74(20):5383-92. PubMed PMID: 12403597.

86. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. Analytical chemistry. 2003 Sep 1;75(17):4646-58. PubMed PMID: 14632076.

87. Breitwieser FP, Muller A, Dayon L, Kocher T, Hainard A, Pichler P, et al. General statistical modeling of data from protein relative expression isobaric tags. Journal of proteome research. 2011 Jun 3;10(6):2758-66. PubMed PMID: 21526793.

88. Stelzhammer V, Haenisch F, Chan MK, Cooper JD, Steiner J, Steeb H, et al. Proteomic changes in serum of first onset, antidepressant drug-naive major depression patients. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP). 2014 Oct;17(10):1599-608. PubMed PMID: 24901538.

89. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics. 2010 Apr 1;26(7):966-8. PubMed PMID: 20147306. Pubmed Central PMCID: 2844992.

90. Desiere F, Deutsch EW, King NL, Nesvizhskii AI, Mallick P, Eng J, et al. The PeptideAtlas project. Nucleic acids research. 2006 Jan 1;34(Database issue):D655-8. PubMed PMID: 16381952. Pubmed Central PMCID: 1347403.

91. Kusebauch U, Deutsch EW, Campbell DS, Sun Z, Farrah T, Moritz RL. Using PeptideAtlas, SRMAtlas, and PASSEL: Comprehensive Resources for Discovery and Targeted Proteomics. Current protocols in bioinformatics / editoral board, Andreas D Baxevanis [et al]. 2014;46:13 25 1-13 25 8. PubMed PMID: 24939129.

92. Choi M, Chang CY, Clough T, Broudy D, Killeen T, MacLean B, et al. MSstats: an R package for statistical analysis of quantitative mass spectrometry-based proteomic experiments. Bioinformatics. 2014 Sep 1;30(17):2524-6. PubMed PMID: 24794931.

93. Hastie T, Tibshirani R, J. F. The Elements of Statistical Learning: Data Mining, Inference, and Prediction. Second Edition ed: Springer Science & Business Media; 2009.

94. Fawcett T. An introduction to ROC analysis. Pattern Recogn Lett. 2006 Jun;27(8):861-74. PubMed PMID: WOS:000237462800002. English.

95. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols. 2009;4(1):44-57. PubMed PMID: 19131956.

96. Goldberger AL. Non-linear dynamics for clinicians: chaos theory, fractals, and complexity at the bedside. Lancet. 1996 May 11;347(9011):1312-4. PubMed PMID: 8622511. Epub 1996/05/11. eng.

97. Lombardi F. Chaos theory, heart rate variability, and arrhythmic mortality. Circulation. 2000 Jan 4-11;101(1):8-10. PubMed PMID: 10618296. Epub 2000/01/05. eng.

98. Batchinsky AI, Cancio LC, Salinas J, Kuusela T, Cooke WH, Wang JJ, et al. Prehospital loss of R-to-R interval complexity is associated with mortality in trauma patients. The Journal of trauma. 2007 Sep;63(3):512-8. PubMed PMID: 18073594. Epub 2007/12/13. eng.

99. Huikuri HV, Makikallio TH, Peng CK, Goldberger AL, Hintze U, Moller M. Fractal correlation properties of R-R interval dynamics and mortality in patients with depressed left ventricular function after an acute myocardial infarction. Circulation. 2000 Jan 4-11;101(1):47-53. PubMed PMID: 10618303. Epub 2000/01/05. eng.

100. Stein PK, Barzilay JI, Chaves PH, Mistretta SQ, Domitrovich PP, Gottdiener JS, et al. Novel measures of heart rate variability predict cardiovascular mortality in older adults independent of traditional cardiovascular risk factors: the Cardiovascular Health Study (CHS). Journal of cardiovascular electrophysiology. 2008 Nov;19(11):1169-74. PubMed PMID: 18631274. Pubmed Central PMCID: Pmc3638897. Epub 2008/07/18. eng.

101. Tapanainen JM, Thomsen PE, Kober L, Torp-Pedersen C, Makikallio TH, Still AM, et al. Fractal analysis of heart rate variability and mortality after an acute myocardial infarction. The American journal of cardiology. 2002 Aug

15;90(4):347-52. PubMed PMID: 12161220. Epub 2002/08/06. eng.

102. Appelhans BM, Luecken LJ. Heart rate variability and pain: associations of two interrelated homeostatic processes. Biological psychology. 2008 Feb;77(2):174-82. PubMed PMID: 18023960. Epub 2007/11/21. eng.

103. Seely AJ, Macklem PT. Complex systems and the technology of variability analysis. Critical care (London, England). 2004 Dec;8(6):R367-84. PubMed PMID: 15566580. Pubmed Central PMCID: Pmc1065053. Epub 2004/11/30. eng.

104. Chang JS, Yoo CS, Yi SH, Hong KH, Oh HS, Hwang JY, et al. Differential pattern of heart rate variability in patients with schizophrenia. Progress in neuro-psychopharmacology & biological psychiatry. 2009 Aug 31;33(6):991-5. PubMed PMID: 19427888. Epub 2009/05/12. eng.

105. Hautala AJ, Karjalainen J, Kiviniemi AM, Kinnunen H, Makikallio TH, Huikuri HV, et al. Physical activity and heart rate variability measured simultaneously during waking hours. American journal of physiology Heart and circulatory physiology. 2010 Mar;298(3):H874-80. PubMed PMID: 20023121. Epub 2009/12/22. eng.

106. Fleisher LA, Pincus SM, Rosenbaum SH. Approximate entropy of heart rate as a correlate of postoperative ventricular dysfunction. Anesthesiology. 1993 Apr;78(4):683-92. PubMed PMID: 8466069. Epub 1993/04/01. eng.

107. de Jonge P, Mangano D, Whooley MA. Differential association of cognitive and somatic depressive symptoms with heart rate variability in patients with stable coronary heart disease: findings from the Heart and Soul Study. Psychosomatic medicine. 2007 Nov;69(8):735-9. PubMed PMID:

17942844. Pubmed Central PMCID: Pmc2776660. Epub 2007/10/19. eng.

108. Porges SW. The Polyvagal Theory: phylogenetic contributions to social behavior. Physiology & behavior. 2003 Aug;79(3):503-13. PubMed PMID: 12954445. Epub 2003/09/05. eng.

109. Thayer JF, Hansen AL, Saus-Rose E, Johnsen BH. Heart rate variability, prefrontal neural function, and cognitive performance: the neurovisceral integration perspective on self-regulation, adaptation, and health. Annals of behavioral medicine : a publication of the Society of Behavioral Medicine. 2009 Apr;37(2):141-53. PubMed PMID: 19424767. Epub 2009/05/09. eng.

110. Berntson GG, Bigger JT, Jr., Eckberg DL, Grossman P, Kaufmann PG, Malik M, et al. Heart rate variability: origins, methods, and interpretive caveats. Psychophysiology. 1997 Nov;34(6):623-48. PubMed PMID: 9401419. Epub 1997/12/24. eng.

111. Kubzansky LD, Kawachi I, Spiro A, 3rd, Weiss ST, Vokonas PS, Sparrow D. Is worrying bad for your heart? A prospective study of worry and coronary heart disease in the Normative Aging Study. Circulation. 1997 Feb 18;95(4):818-24. PubMed PMID: 9054737. Epub 1997/02/18. eng.

112. McConathy WJ, Alaupovic P. Isolation and partial characterization of apolipoprotein D: a new protein moiety of the human plasma lipoprotein system. FEBS letters. 1973 Dec 1;37(2):178-82. PubMed PMID: 4128506. Epub 1973/12/01. eng.

113. Dassati S, Waldner A, Schweigreiter R. Apolipoprotein D takes center stage in the stress response of the aging and degenerative brain. Neurobiology of aging. 2014 Jul;35(7):1632-42. PubMed PMID: 24612673.

Pubmed Central PMCID: Pmc3988949. Epub 2014/03/13. eng.

114. Rassart E, Bedirian A, Do Carmo S, Guinard O, Sirois J, Terrisse L, et al. Apolipoprotein D. Biochimica et biophysica acta. 2000 Oct 18;1482(1-2):185-98. PubMed PMID: 11058760. Epub 2000/11/04. eng.

115. Bajo-Graneras R, Ganfornina MD, Martin-Tejedor E, Sanchez D. Apolipoprotein D mediates autocrine protection of astrocytes and controls their reactivity level, contributing to the functional maintenance of paraquat-challenged dopaminergic systems. Glia. 2011 Oct;59(10):1551-66. PubMed PMID: 21688324. Epub 2011/06/21. eng.

116. Thomas EA, Copolov DL, Sutcliffe JG. From pharmacotherapy to pathophysiology: emerging mechanisms of apolipoprotein D in psychiatric disorders. Current molecular medicine. 2003 Aug;3(5):408-18. PubMed PMID: 12942994. Epub 2003/08/29. eng.

117. Martinez E, Navarro A, Ordonez C, Del Valle E, Tolivia J. Oxidative stress induces apolipoprotein D overexpression in hippocampus during aging and Alzheimer's disease. Journal of Alzheimer's disease : JAD. 2013;36(1):129-44. PubMed PMID: 23568103. Epub 2013/04/10. eng.

118. Reindl M, Knipping G, Wicher I, Dilitz E, Egg R, Deisenhammer F, et al. Increased intrathecal production of apolipoprotein D in multiple sclerosis. Journal of neuroimmunology. 2001 Oct 1;119(2):327-32. PubMed PMID: 11585636. Epub 2001/10/05. eng.

119. Mahadik SP, Khan MM, Evans DR, Parikh VV. Elevated plasma level of apolipoprotein D in schizophrenia and its treatment and outcome. Schizophrenia research. 2002 Nov 1;58(1):55-62. PubMed PMID: 12363390. Epub 2002/10/05. eng.

120. Vassiliev V, Harris ZL, Zatta P. Ceruloplasmin in neurodegenerative diseases. Brain research Brain research reviews. 2005 Nov;49(3):633-40. PubMed PMID: 16269323. Epub 2005/11/05. eng.

121. Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. Nature reviews Immunology. 2003 Oct;3(10):791-800. PubMed PMID: 14502271.

122. Patel BN, Dunn RJ, Jeong SY, Zhu Q, Julien JP, David S. Ceruloplasmin regulates iron levels in the CNS and prevents free radical injury. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002 Aug 1;22(15):6578-86. PubMed PMID: 12151537. Epub 2002/08/02. eng.

123. Yoshida K, Kaneko K, Miyajima H, Tokuda T, Nakamura A, Kato M, et al. Increased lipid peroxidation in the brains of aceruloplasminemia patients. Journal of the neurological sciences. 2000 Apr 15;175(2):91-5. PubMed PMID: 10831768. Epub 2000/06/01. eng.

124. Wolf TL, Kotun J, Meador-Woodruff JH. Plasma copper, iron, ceruloplasmin and ferroxidase activity in schizophrenia. Schizophrenia research. 2006 Sep;86(1-3):167-71. PubMed PMID: 16842975.

125. Kaya MC, Bez Y, Selek S, Fatih Karababa I, Bulut M, Savas HA, et al. No effect of antidepressant treatment on elevated serum ceruloplasmin level in patients with first-episode depression: a longitudinal study. Archives of medical research. 2012 May;43(4):294-7. PubMed PMID: 22704847. Epub 2012/06/19. eng.

126. Song YH, Ray K, Liebhaber SA, Cooke NE. Vitamin D-binding protein gene transcription is regulated by the relative abundance of hepatocyte

nuclear factors 1alpha and 1beta. The Journal of biological chemistry. 1998 Oct 23;273(43):28408-18. PubMed PMID: 9774468. Epub 1998/10/17. eng.

127. Gomme PT, Bertolini J. Therapeutic potential of vitamin D-binding protein. Trends in biotechnology. 2004 Jul;22(7):340-5. PubMed PMID: 15245906. Epub 2004/07/13. eng.

128. White P, Cooke N. The multifunctional properties and characteristics of vitamin D-binding protein. Trends in endocrinology and metabolism: TEM. 2000 Oct;11(8):320-7. PubMed PMID: 10996527. Epub 2000/09/21. eng.

129. Le-Niculescu H, McFarland MJ, Mamidipalli S, Ogden CA, Kuczenski R, Kurian SM, et al. Convergent Functional Genomics of bipolar disorder: from animal model pharmacogenomics to human genetics and biomarkers. Neuroscience and biobehavioral reviews. 2007;31(6):897-903. PubMed PMID: 17614132. Pubmed Central PMCID: 3313450.

130. Niculescu AB, 3rd, Segal DS, Kuczenski R, Barrett T, Hauger RL, Kelsoe JR. Identifying a series of candidate genes for mania and psychosis: a convergent functional genomics approach. Physiological genomics. 2000 Nov 9;4(1):83-91. PubMed PMID: 11074017.

131. Wan C, La Y, Zhu H, Yang Y, Jiang L, Chen Y, et al. Abnormal changes of plasma acute phase proteins in schizophrenia and the relation between schizophrenia and haptoglobin (Hp) gene. Amino acids. 2007 Jan;32(1):101-8. PubMed PMID: 16897611. Epub 2006/08/10. eng.

132. Sarandol A, Sarandol E, Eker SS, Karaagac EU, Hizli BZ, Dirican M, et al. Oxidation of apolipoprotein B-containing lipoproteins and serum paraoxonase/arylesterase activities in major depressive disorder. Progress in neuro-psychopharmacology & biological psychiatry. 2006 Aug

30;30(6):1103-8. PubMed PMID: 16716479. Epub 2006/05/24. eng.

133. Olofsson SO, Boren J. Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis. Journal of internal medicine. 2005 Nov;258(5):395-410. PubMed PMID: 16238675. Epub 2005/10/22. eng.

134. Benn M. Apolipoprotein B levels, APOB alleles, and risk of ischemic cardiovascular disease in the general population, a review. Atherosclerosis. 2009 Sep;206(1):17-30. PubMed PMID: 19200547. Epub 2009/02/10. eng.

135. Sniderman AD, Williams K, Contois JH, Monroe HM, McQueen MJ, de Graaf J, et al. A meta-analysis of low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol, and apolipoprotein B as markers of cardiovascular risk. Circulation Cardiovascular quality and outcomes. 2011 May;4(3):337-45. PubMed PMID: 21487090. Epub 2011/04/14. eng.

136. Thanassoulis G, Williams K, Ye K, Brook R, Couture P, Lawler PR, et al. Relations of change in plasma levels of LDL-C, non-HDL-C and apoB with risk reduction from statin therapy: a meta-analysis of randomized trials. Journal of the American Heart Association. 2014;3(2):e000759. PubMed PMID: 24732920. Pubmed Central PMCID: Pmc4187506. Epub 2014/04/16. eng.

137. Vaccarino V, McClure C, Johnson BD, Sheps DS, Bittner V, Rutledge T, et al. Depression, the metabolic syndrome and cardiovascular risk. Psychosomatic medicine. 2008 Jan;70(1):40-8. PubMed PMID: 18158378. Epub 2007/12/26. eng.

138. Lehto SM, Ruusunen A, Niskanen L, Tolmunen T, Voutilainen S, Viinamaki H, et al. Elevated depressive symptoms and compositional changes

in LDL particles in middle-aged men. European journal of epidemiology. 2010 Jun;25(6):403-9. PubMed PMID: 20414796. Pubmed Central PMCID: Pmc3249261. Epub 2010/04/24. eng.

139. Ding Z, Bae YH, Roy P. Molecular insights on context-specific role of profilin-1 in cell migration. Cell adhesion & migration. 2012 Sep-Oct;6(5):442-9. PubMed PMID: 23076048. Pubmed Central PMCID: 3496682.

140. Horrevoets AJ. Profilin-1: an unexpected molecule linking vascular inflammation to the actin cytoskeleton. Circulation research. 2007 Aug 17;101(4):328-30. PubMed PMID: 17702977.

141. Romeo GR, Moulton KS, Kazlauskas A. Attenuated expression of profilin-1 confers protection from atherosclerosis in the LDL receptor null mouse. Circulation research. 2007 Aug 17;101(4):357-67. PubMed PMID: 17615372.

142. Nakatani N, Ohnishi T, Iwamoto K, Watanabe A, Iwayama Y, Yamashita S, et al. Expression analysis of actin-related genes as an underlying mechanism for mood disorders. Biochemical and biophysical research communications. 2007 Jan 19;352(3):780-6. PubMed PMID: 17141188. Epub 2006/12/05. eng.

143. Surinova S, Schiess R, Huttenhain R, Cerciello F, Wollscheid B, Aebersold R. On the development of plasma protein biomarkers. Journal of proteome research. 2011 Jan 7;10(1):5-16. PubMed PMID: 21142170.

144. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Molecular & cellular proteomics : MCP. 2006 Apr;5(4):573-88. PubMed PMID: 16332733.

145. Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nature biotechnology. 2009 Jul;27(7):633-41. PubMed PMID: 19561596. Pubmed Central PMCID: 2855883.

국문초록

서론: 우울증은 유전적 소인과 여러 생물학적 경로의 복잡한 상호작용에 의해 발생하는 다차원적 질환이다. 그러나 생물학적 기전이 명확하게 밝혀지지 않아 객관적이고 신뢰도 있는 진단도구가 개발되지 않은 상태이다. 본 연구에서는 정량적 단백체 분석과 심박변이도 분석을 통해 우울증의 말초 바이오마커를 발굴하고자 하였다. 또한, 분류정확도를 높이기 위해 기계학습 분석으로 단백체 정보와 심박변이도 지표를 조합한 통합적 바이오마커 패널을 구성하고자 하였다.

방법: 본 연구대상은 약물을 복용하고 있지 않은 25명의 여성 우울증 환자와 나이, 체질량지수가 매칭된 25명의 여성 정상 대조군을 포함하였다. 단백체 분석에서는 각 10명의 우울증 환자와 정상인의 혈청에 대한 단백체 프로파일링을 수행하여, 환자군과 정상인에서 발현량이 다른 단백질(differentially expressed proteins, DEP)를 동정하였다. 이후 각 25명의 환자군 및 정상인에서 multiple reaction monitoring을 이용한 후보 단백질의 정량적 분석을 수행하였다. 로지스틱 회귀분석과 라소정규화 방법을 이용하여 최종적인 단백질 바이오마커 패널을 구성하였다. 심박변이도 분석에서는 총 22개의 선형, 비선형 심박변이도 지표에 대한 support vector machine (SVM)과 recursive feature eliminations (RFE) 분석을 통하여 주요 우울증 진단을 위한 바이오마커 패널을 구성하였다. 마지막으로 단백질 바이오마커 및 심박변이도 지표를 통합하여 분류정확도가 최대가 되는 바이오마커 패널을 선정하였다.

결과: 기계학습 알고리듬을 적용하였을 때, 심박변이도 지표 중 RMSSD, SDNN, LF, ApEn, SampEn, CSE20을 포함한 7 개의 지표가 우울증을 64%의

정확도로 진단하는 것으로 나타났다. 단백체 분석에서는 67%의 정확도로 우울증을 진단하는 Apo-D, Apo-B, VDP, ceruloplasmin, hormerin, 및 profilin1으로 구성된 바이오마커 패널을 동정하였다. 단백체 정보와 심박변이도 지표를 통합하여 분석하였을 때, Apo-B, VDP, ceruloplasmin, RMSSD, SampEn의 다섯 개의 지표로 80%의 진단정확도를 가진 바이오마커 패널이 구성되었다.

결론: 본 연구에서는 우울증 진단과 관련하여 심박동역학에서 부교감신경계 활성 저하와 복잡도 감소와 관련된 여러 분류기를 동정하였다. 단백체 분석에서는 면역 및 염증계, 산화계 및 지질 대사와 관련된 단백질이 우울증과 관련있는 것으로 밝혀졌다. 단백질 지표와 심박변이도 지표를 조합하였을 때, 진단정확도가 증가하는 것이 관찰되었다. 본 연구에서 발굴된 바이오마커는 향후 더 큰, 독립적인 코호트에서 검증되어야 한다.

핵심어: 주요우울증, 단백체, 심박변이도, 말초 바이오마커, 기계학습

학번: 2011-30541