Peripheral Biochemical Changes in Major Depressive Disorder using Breathomics analysis: New Insights into Molecular Psychiatry

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Abstract

Major depressive disorder (MDD) is one of the most prevalent and disabling psychiatric illnesses worldwide, contributing significantly to the global burden of disease. Despite extensive research, no single biological mechanism fully explains its heterogeneity. Moreover, the clinical management of MDD remains challenging due to limited therapeutic success, imprecise subjective diagnostic criteria, and an incomplete understanding of the underlying molecular pathology.

The metabolome represents the integrated output of complex interactions among the genome, transcriptome, proteome, microbiome, and environmental exposome. From this system-level perspective, we hypothesized that breath-based metabolomics, or breathomics, could non-invasively capture distinct, multi-parametric metabolic signatures associated with the heterogeneous pathophysiology of MDD. The primary aim of this study was to investigate the potential and limitations of the breath gas analysis in identifying known alterations in metabolic pathways implicated in MDD and to support the development of an objective, non-invasive, systems-level molecular diagnostic test, thereby contributing to the advancement of precision psychiatry. Particular attention was paid to post-awakening metabolite dynamics, given the critical role of this time window in activating stress-responsive biological systems such as hypothalamic–pituitary–adrenal axis.

A total of 153 breath samples were collected at awakening, 30- and 60-minutes post awakening from 26 patients with MDD and 25 HCs. Exhaled volatile organic compounds (VOCs) were profiled using the proton transfer reaction mass spectrometry (PTR-MS). Candidate metabolites were initially preselected using the Wilcoxon tests and Student's t-tests. Metabolomic pathway analysis and hierarchical clustering analysis (HCA) were then performed to group significant VOCs according to shared temporal dynamics and biological functions. Neighborhood component analysis was applied to rank VOCs according to their importance in classifying MDD and HCs. Forward feature selection was used as an iterative procedure to build multivariable predictive models and identify potential breathprint combinations. Four machine learning models: support vector machine (SVM), kernel SVM (K-SVM), Naïve Bayes (NB), and logistic regression (LR), were employed to evaluate the classification performance of individual VOCs and breathprints. Model accuracy was validated through Bootstrapped receiver operating characteristics (ROC) analysis.

In total, 26 exhaled VOCs were significantly altered in MDD patients compared to HCs. These VOCs were enriched in five key metabolic pathways, including valine, leucine and isoleucine leucine biosynthesis (p = 5.50 10^{-4}), pyruvate metabolism (p = 7.97 10^{-4}), glycolysis and gluconeogenesis (p = 0.0015), nicotinate and nicotinamide metabolism (p = 0.005) and valine, leucine and isoleucine degradation (p = 0.005). HCA revealed distinct VOC clusters based on their biological functions, reflecting the interaction between host metabolome, exposome, and microbiome. Notably, VOCs of microbial origin exhibited the most pronounced alterations through various fermentation and metabolic pathways. Moreover, the combination of methylpyridine, isoprene, butylamine at their most significant awakening response times represents the most promising 3-variable breathprint across all models (AUC > 0.85, Sensitivity > 0.80, Specificity > 0.85). The addition of trimethylamine further improved model performance, while expanding to five-marker signatures by incorporating propionic acid or benzene further enhanced specificity, supporting their utility for phenotype-specific diagnostics. Furthermore, substituting components of the core breathprint VOCs with similar biological functions yielded comparable high diagnostic performance, suggesting metabolic redundancy, and offering flexibility in breathprint design.

This study provides proof-of-concept that breathomics offers novel insights into the peripheral biochemical alterations associated with MDD, and holds strong potential for developing robust, non-invasive molecular breath signatures capable of distinguishing MDD from HCs with clinically meaningful accuracy. By capturing the multifaceted metabolic alterations underlying MDD and reflecting the complex interactions between host metabolome, gut microbiome and exposome, breathomics supports systems-level diagnostics and aligns with the goals of precision medicine in psychiatry. Notably, the identified breath biomarkers and associated pathways demonstrated strong concordance with findings from previous metabolomics studies using other biomatrices, further reinforcing their biological validity and translational relevance.

Zusammenfassung

Die Major Depression (MD) zählt zu den weltweit häufigsten und belastendsten psychiatrischen Erkrankungen und trägt maßgeblich zur globalen Krankheitslast bei. Trotz intensiver Forschung konnte bisher kein einzelner biologischer Mechanismus vollständig die Heterogenität der Erkrankung erklären. Die klinische Versorgung von Patienten mit MD bleibt zudem eine Herausforderung – bedingt durch den begrenzten therapeutischen Erfolg, subjektive und unscharfe Diagnostikkriterien sowie ein unvollständiges Verständnis der zugrunde liegenden molekularen Pathophysiologie.

Das Metabolom stellt das integrierte Endprodukt komplexer Wechselwirkungen zwischen Genom, Transkriptom, Proteom, Mikrobiom und Umwelt (Exposom) dar. Aus dieser systemischen Perspektive heraus wurde die Studienhypothese aufgestellt, dass die atembasierte Metabolomik („Breathomics“) in der Lage ist, nicht-invasiv spezifische, multiparametrische metabolische Signaturen zu erfassen, die mit der heterogenen Pathophysiologie der MD assoziiert sind. Ziel dieser Studie war es, das Potenzial und die Grenzen der Atemgasanalyse bei der Identifikation bekannter metabolischer Veränderungen bei MD zu untersuchen – mit dem langfristigen Ziel, einen objektiven, nicht-invasiven, molekularbasierten Atemtest zur Diagnose von MD zu entwickeln und damit zur Weiterentwicklung einer personalisierten, präzisionsmedizinischen Psychiatrie beizutragen. Ein besonderer Fokus lag auf der Analyse der zeitlichen Dynamik flüchtiger Metaboliten im Zeitraum vom Aufwachen bis 60 Minuten danach, da diese Phase als kritisch für die Aktivierung stressreaktiver biologischer Systeme wie der Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA-Axis) gilt.

Insgesamt wurden 153 Atemproben zu drei Zeitpunkten (Aufwachen, 30 und 60 Minuten danach) von 26 Patienten mit MD und 25 gesunden Kontrollpersonen erhoben. Die Analyse flüchtiger organischer Verbindungen (FOVs, engl. VOCs – volatile organic compounds) erfolgte mittels Protonen Transfer Reaktions-Massenspektrometrie (PTR-MS). Die Identifikation statistisch signifikanter VOCs erfolgte mittels Wilcoxon-Tests und Student t-Tests. Eine hierarchische Clusteranalyse (HCA) und eine Metabolom-Pfadweganalyse dienten der funktionellen Gruppierung signifikanter VOCs nach zeitlicher Dynamik und biologischer Relevanz. Zur Bewertung der diagnostischen Aussagekraft einzelner VOCs wurde eine Merkmalsgewichtung mittels Nachbarschaftskomponentenanalyse (NKA) durchgeführt. Die schrittweise Merkmalsauswahl (Forward feature Selection) diente zur Identifikation optimaler VOC-Kombinationen („Breathprints“). Vier maschinelle Lernverfahren kamen zum Einsatz: Support-Vektor-Maschine (SVM), Kernel-SVM (nichtlinear), Naive-Bayes-Klassifikator (NB) und logistische Regression (LR). Die Modellgüte wurde mithilfe von bootstrapping-basierten ROC-Kurvenanalysen evaluiert.

Insgesamt wurden 26 VOCs identifiziert, die bei MD-Patienten signifikant verändert waren. Diese Metaboliten waren angereichert in fünf biologischen Pfadwegen: Valin-, Leucin- und Isoleucin-Biosynthese ($p = 5{,}50 \times 10^{-4}$), Pyruvatstoffwechsel ($p = 7{,}97 \times 10^{-4}$), Glykolyse und Glukoneogenese ($p = 0{,}0015$), Nikotinat- und Nikotinamidstoffwechsel ($p = 0{,}005$) sowie Abbau von Valin, Leucin und Isoleucin ($p = 0{,}005$). Die HCA zeigte funktionell unterschiedliche Cluster, die das Zusammenspiel von Wirtsmetabolom, Mikrobiom und

Umwelt-Exposom widerspiegelten. Besonders VOCs mikrobiellen Ursprungs zeigten deutliche Veränderungen. Eine Kombination aus Methylpyridin, Isopren und Butylamin – jeweils zu ihrem diagnostisch optimalen Messzeitpunkt – erwies sich als leistungsfähiges 3-Marker-Breathprint mit hoher diagnostischer Genauigkeit (AUC > 0,85, Sensitivität > 0,80, Spezifität > 0,85). Die Hinzunahme von Trimethylamin verbesserte die Modellleistung leicht; eine Erweiterung auf fünf Marker durch Propionsäure oder Benzol erhöhte die Spezifität weiter und ermöglichte eine differenziertere Phänotypisierung von MD. Der Austausch von VOCs im Haupt-Breathprint durch funktionell ähnliche Verbindungen führte zu vergleichbarer Klassifikationsgenauigkeit – was auf metabolische Redundanz hinweist und die Flexibilität bei der Gestaltung diagnostischer Tests erhöht.

Diese Studie liefert einen Machbarkeitsnachweis dafür, dass Breathomics neue Einblicke in periphere biochemische Veränderungen bei MD ermöglicht und das Potenzial für die Entwicklung robuster, nicht-invasiver molekularer Atemsignaturen mit klinisch relevanter diagnostischer Genauigkeit bietet. Durch die Erfassung vielschichtiger metabolischer Veränderungen und die Berücksichtigung von Wirt-Mikrobiom-Exposom-Interaktionen leistet Breathomics einen Beitrag zur systemmedizinischen Diagnostik und fördert die Ziele der Präzisionspsychiatrie. Die identifizierten VOCs und deren zugehörige Stoffwechselwege stimmen mit Ergebnissen früherer Metabolomik-Studien aus anderen biologischen Matrices überein und bestätigen ihre biologische Relevanz und ihr translationales Potenzial.

Contents

Glossary of Terms

ANS	Autonomic Nervous System
BCAAs	Branched Chain Amino Acids
BCFAs	Branched chain fatty acids
BDI-II	Beck Depression Inventory second edition
BDNF	Brain-Derived Neurotrophic Factor
BET	Buffered End Tidal
BMI	Body Mass Index
CI	Confidence Interval
CNS	Central Nervous System
COI	Costs-of-Illness
CRP	C-reactive protein
DALY	Disability-Adjusted Life Year
DNA	Destroy Deoxyribonucleic Acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5th Edition
EB	Exhaled Breath
EBA	Exhaled Breath Aerosol
EBC	Exhaled Breath Condensate
EBG	Exhaled Breath Gas
ENS	Entering nervous system
GABA	Gamma-Aminobutyric Acid
GBA	Gut-Brain-Axis
GBD	Global Burden of Disease
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HAMD	Hamilton Depression Rating Scale
HCA	Hierarchical Cluster Analysis
HCs	Healthy Controls
HPA	Hypothalamic-pituitary-adrenal
ICD	International Classification of Diseases
IL	Interleukin

LOD	Limit of Detection
LR	Logistic Regression
MCAR	Missing Completely at Random
MAR	Missing at Random
MDA	Malondialdehyde
MDD	Major Depressive Disorders
MNAR	Missing Not at Random
MPA	Metabolic Pathway Analysis
MS	Mass Spectrometry
m/z	Mass-to-charge ratio
NB	Naive Bayes
NOS	Nitrosative Oxygen Species
NS	Nitrosative Stress
OECD	Organization for Economic Co-operation and Development
O&NS	Oxidative and Nitrosative Stress
OS	Oxidative Stress
PA	Proton Affinity
PCA	Principal Component Analysis
ppb	Parts-per-Billion
ppm	Parts-per-Million
ppt	Parts-per-Trillion
PTR-QMS	Proton Transfer Reaction Quadrupole Mass Spectrometry
PTR-MS	Proton Transfer Reaction Mass Spectrometry
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
SCFAs	Short Chain Fatty Acids
SVM	Support Vector Machine
TA	Topological Analysis
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
TMAU	Trimethylaminuria
TNF	Tumor Necrosis Factor
TOF	Time-of-Flight
VOCs	Volatile Organic Compound

"The complexity and wonders of the human body, governed by intricate chemical and physical laws, remain largely unexplored. Advanced research and the integration of artificial intelligence continue to reveal new layers of understanding, yet every discovery reminds us how far we still have to go in unraveling the mysteries of human biology."

1 INTRODUCTION

Mental disorders represent a major public health problem due to their high prevalence, heterogeneous presentation, and their association with disproportionately higher rates of disability, accounting for 16% of global burden of disease in the year 2019 (Arias, Saxena, and Verguet 2022), as well as the lack of understanding of the complex psychopathology underlying these conditions. Almost one billion people worldwide suffer from a mental disorder (The Lancet Global Health 2020; Ferrari et al. 2022). The economic consequences are drastically large. In total, mental disorders were estimated to cost the world economy approximately US$2.5 trillion in 2010, and projected to rise to at least US$6 trillion by 2030 (Bloom et al. 2011). The World Health Organization (WHO) estimated the cumulative global impact of mental disorders between 2011 and 2030 to amount to US$16.3 trillion (World Health Organization 2021; Bloom et al. 2011).

The common psychiatric disorders can generally be categorized into five groups: (1) anxiety, (2) mood, (3) schizophrenia and psychotic disorders, (4) dementia, and (5) eating disorders. Major depressive disorder (MDD)[1] is a common mood disorder that significantly affects a person’s behaviors, emotions, perceptions, and thinking. It is associated with serious socio-economic impacts. The Global Burden of Diseases study (GBD) conducted in 2019 showed that MDD is among the largest causes of disability worldwide, accounting for 4.3% of the global burden of disease and 11% of all years lived with disability globally (World Health Organization 2021). Nowadays, MDD is a public health priority. An urgent understanding of the molecular changes underlying the heterogenous pathophysiology of MDD is needed for precision psychiatry, in order to ensure effective and adequate therapy for all MDD-subtypes.

This chapter serves as an introduction to the research topic of the present thesis. First, a short overview of the research problems, including the clinical issues and socio-economic burdens associated with MDD, will be provided. Second, the research hypothesis and the clinical significance of applying breathomics as a new approach for molecular markers discovery for MDD will be presented. Then, the main objectives of the doctoral thesis will be outlined. Finally, the structure of the present thesis will be provided.

1.1 Clinical Problems of Major Depression

MDD is a complex and heterogeneous mental disorder in its presentation and pathophysiology. It is interconnected to a wide range of clinical problems including high prevalence globally, a high misdiagnosis rate due to the subjective diagnostic and overlapping in symptoms that mimic other disorders, delayed therapeutic onset, a high rate of relapse and recurrence, as well as a high rate of associated morbidity, disability, and mortality.

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[1] Major depressive disorder is also known as clinical depression or unipolar depressive disorder.

High prevalence of MDD globally. — MDD is one of the most prevalent mood disorders, affecting around 3.4% of people worldwide (WHO 2017; Ferrari et al. 2022; Santomauro 2021). The lifetime incidence of depression is more than 12% in men and 20% in women, and is projected to become the leading cause of disability worldwide by 2030 (Gutiérrez-Rojas et al. 2020; P.J. Kennedy, J.F. Cryan , T.G. Dinan 2018; Otte et al. 2016; .4% to 8.3%, and the lifetime prevalence is approximately 10% to 28% (Hauenstein 2003; Shorey, Ng, and Wong 2022). Girls are twice as likely as boys to suffer from MDD. Further, according to newly published statistics, the number of people affected with depression rose dramatically in 2020 globally because of the impact of the COVID-19 pandemic on people's mental health and well-being, as illustrated in Figure 1-1. The GBD study in 2020 estimated approximately 53.2 million (95% CI: 44.8 to 62.9 million) additional cases with MDD, corresponding to an increase of 27,6% in just one year (World Health Organization 2022a; Santomauro 2021). Especially, females and younger people between 20 and 24 years were more affected than other groups. Approximately 35.2 million additional cases among women were estimated in 2020 compared to 2019. This is in accordance with the fact that females are more susceptible to depression and anxiety disorders compared to males, with a twofold increased risk of developing MDD. Further, the Organization for Economic Co-operation and Development (OECD) analysis reported that the depression prevalence for 2022 in OECD countries increased by at least 20% compared to the COVID-19 pre-pandemic period, and in some countries, it more than doubled or even tripled the pre-pandemic rate (OECD/European Union 2022).

Multifactorial etiology of MDD. — The etiology of MDD is multifactorial. The heterogenous presentation of MDD is the outcome of a complex interaction between biological, societal, environmental, and psychological factors (Otte et al. 2016; Remes, Francisco, and Templeton 2021). Several studies in the last decade have proven evidence suggesting that socio-environmental factors play an important role in MDD etiology. Alteration in epigenetic mechanisms could favor MDD progress in response to stressful experiences and environmental factors (Saavedra et al. 2016). Further, several biological mechanisms have been hypothesized

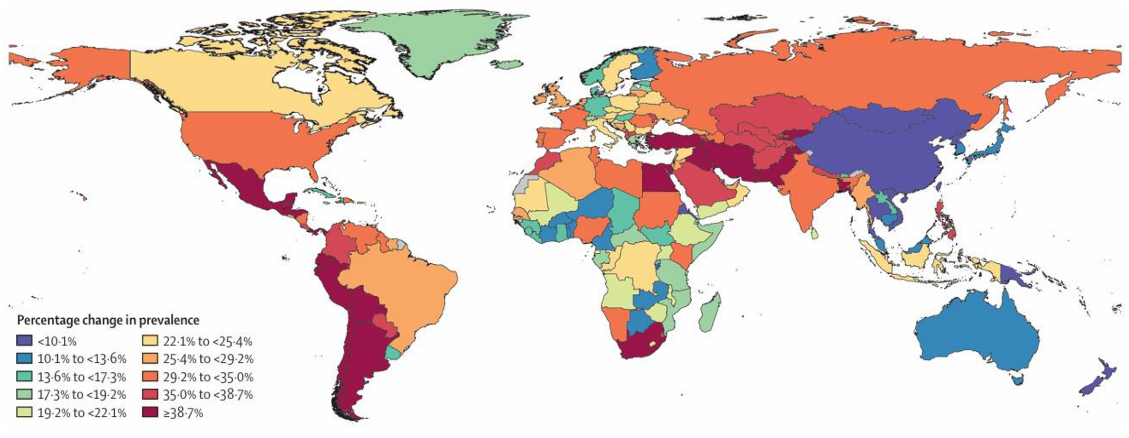

***Figure 1-1| The global distribution of the change rate in the prevalence of major depressive disorder (MDD) after adjustment for the COVID-19 pandemic, 2020**: The prevalence of MDD rose globally between Jan 1, 2020, and Jan 29, 2021 due to the impact of COVID-19 pandemic. The change rate was widely variable across countries. Source: Santomauro, 2021.*

to be associated with MDD pathophysiology including monoamine deficits, inflammatory and oxidative stress pathways activation (Sowa-Kućma et al. 2018; Maes, Galecki, et al. 2011; T. Liu et al. 2015a), neurodegenerative alterations (Moylan et al. 2013; Otte et al. 2016), hypothalamic-pituitary-adrenal (HPA) axis dysfunction (Dedovic and Ngiam 2015b; Jarcho et al. 2013; Otte et al. 2016), gut microbiota dysregulation (Cryan and Dinan 2012; Barandouzi et al. 2020; Cheung et al. 2019), energy metabolism deficiency (Zuccoli, Saia-cereda, and Nascimento 2017; Gu et al. 2021b), and the kynurenine pathway activation (Arteaga-Henriquez et al. 2021; Badawy, Dawood, and Bano 2023). Nevertheless, despite the advances in the neurobiology of MDD, no single established mechanism can explain all aspects of this heterogenous mood disorder.

Subjective diagnosis of MDD. — Even today, diagnosing major depression with an objective and quantitative method remains challenging. Currently, the available diagnostic systems heavily rely on subjective patient self-reports through screening questionnaires, physician assessments, and psychiatric interviews, potentially leading to inaccuracies in expert decision-making, and improper treatment strategies (M. Marty and D. Segal 2017). Nevertheless, these subjective diagnostic criteria are widely applied in clinical settings, research, and routine practices such as policy settings and insurance reimbursement systems. Furthermore, MDD is often misdiagnosed as other mental health disorders such as bipolar disorder. Previous individual studies reported that 50% of patients with depressive disorder are misdiagnosed in primary and specialized care settings (Schulberg and Burns 1988; Ayano et al. 2021). However, globally, there are few studies on misdiagnosis rate of mental health conditions. Several factors contribute to a considerable high magnitude of misdiagnosis. Firstly, MDD manifests in various forms due to the inconsistent physiological dysfunctions observed in patients suffering from this disorder. Secondly, depression symptoms can mimic other physiological conditions or mental disorders, and often coexisting conditions can confound an accurate diagnosis.

Treatment and disease course of MDD. — Despite the elevated prevalence and disability rates associated with MDD, there exists a significant gap between the number of people needing treatment and those actually receiving therapy, particularly among individuals with low socio-economic status (Evans-Lacko et al. 2017). The prevalence of treatment seeking is widely variable across countries, and limited information is available on treatment rates, especially in low- and middle-income countries, as depicted in Figure 1-2 (Moitra et al. 2022; Thornicroft et al. 2017; Mekonen et al. 2021). Different statistics are available depending on the number of the involved persons and countries. A systemic review and meta-analysis in 84 countries between 2000 and 2019 reported that minimally adequate treatment rates for major depression ranged from 23% in high-income countries to 3% in low- and lower middle-income countries. Treatment coverage or mental health service use ranged from 33% in high-income countries to 8% in low- and lower middle-income countries. The World Mental Health Surveys estimated that only a median global percentage of 16.5% of people with MDD are receiving minimal adequate treatment with 22.4%, 11.4% and 3.7% in high-, upper-middle-, and low-/lower-middle income countries respectively (Thornicroft et al. 2017). Furthermore, a global median of 40.3% of people with MDD are receiving mental health service, among them 18.2% in lower-middle-income countries, 31,1% in upper-middle-income countries, 50.6% in high-income countries (Jaeschke et al., 2021; WHO, 2021). A recent meta-analysis, comprising 1.1 million participants from 79 countries and territories, reported that the treatment rates were 48.3% (95%CI: 43.0-53.6%) in high-income countries, 21.4% (95% CI:15.1-27.7%) in middle-income countries, and 16.8% (11.3, 23.0%) in low-income countries (Mekonen et al. 2021). The global distribution of treatment rates of depression according to this meta-analysis are illustrated in Figure 2. Additionally, the culture of stigma and discrimination surrounding mental health conditions remain dominant barriers to accessing health care services (Lazowski et al. 2012; Brown 2010; Yimer et al. 2023).

The course of MDD is manifold, with high variability in response to antidepressant drugs, treatment resistance, remission, and recurrence. High symptom severity, comorbidity with other mental and/or physiological conditions, and a history of childhood trauma are predictors of poor disease course. Indeed, individuals with stressful events in the childhood generally show higher symptom severity, a poorer disease course and elevated treatment resistance (Otte et al. 2016). A meta-analysis showed that 56–60% of people getting treatment respond well to active treatment with antidepressants such as selective serotonin reuptake inhibitors and tricyclic antidepressants (Gerritsen 2010). However, approximately one-third of depressed individuals do not achieve remission from MDD, even after several treatment attempts. Furthermore, the largest randomized trials showed that the remission rate from the first line of treatment was only 28%. Unfortunately, the remission rate in outpatient care settings is less favorable compared to inpatients. Only 25% remit within 6 months and 50% of patients still have MDD after 2 years (Otte et al. 2016).

Depending on the severity of depression, pharmacotherapy, psychopharmacotherapy, and social intervention are recommended as effective and efficient strategies for MDD management. Medications are the mainstay treatment of moderate-to-severe depression. However, potential side-effects are very common, leading to missed doses or premature discontinuation, which can result in relapse, delayed treatment, and limited efficacity of medication in mild cases (Khawam, Laurencic, and Malone 2006). Additionally, psychotherapy is also available, but inaccessible to many individuals who may require assistance. Despite extensive research, the treatments targeting the causal factors of depression are not known yet.

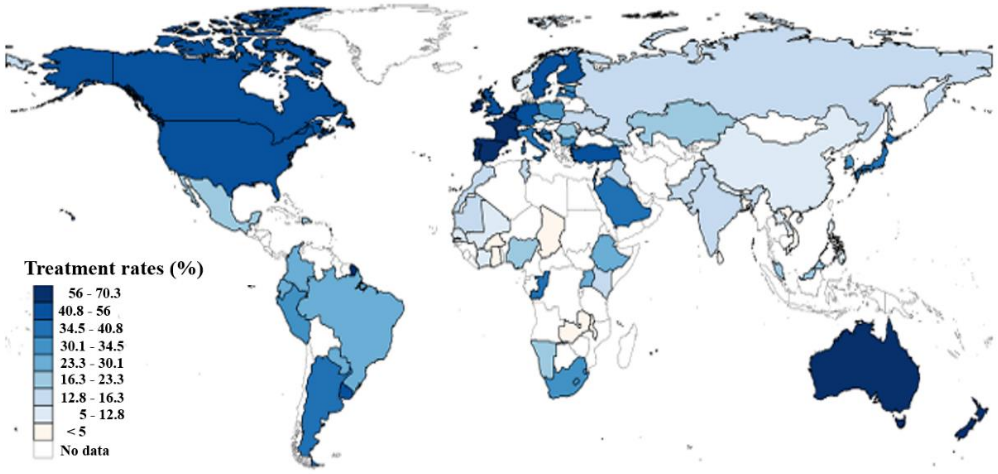

Figure 1-2| Global distribution of treatment rates for depression: *The prevalence of treatment rate is widely variable across countries, and limited information is available on treatment rates, especially in low- and middle-income countries. Source: T. Mekonen et al.,2021*

Mortality and morbidity associated with MDD. — Approximately 40% to 60% of people affected by MDD are at a greater risk of dying prematurely than the general population (World Health Organization 2021). Failure in the early diagnosis and treatment of MDD can be associated with a high suicidal tendency rate. Up to half of patients under therapy relapse despite the progress of antidepressant drugs (Otte et al. 2016). The risk of suicide is 20-fold higher in MDD patient compared to general population (Chesney, Goodwin, and Fazel 2014). Approximately 50% of suicide deaths annually occur within a depressive episode (Al-Qinna and Jaber 2013). Suicide is the second most common cause of death among young people worldwide (World Health Organization 2021). In particular, adolescents with severe depression are even 30 times more likely to commit suicide (Stringaris 2017). During the COVID-19 pandemic the anxiety and depression symptoms increased, and suicide ideation increased significantly in some OECD/EU countries, particularly among young people.

Furthermore, there is a bidirectional relationship between MDD and chronic somatic diseases. Persons with chronic medical conditions such as heart disease, stroke and diabetes mellitus are at greater risk of depression (Ng, How, and Ng 2016; Clarke and Currie 2009). Co-existing MDD is associated with the complications related to these conditions. Similarly, meta-analyses of longitudinal studies reported that individuals with MDD face an increased relative risk (RR) of various diseases compared to those without MDD, particularly in midlife and older adulthood, as shown in Figure 1-3. Further, a large body of evidence from epidemiological studies reported a strong association between depression and other mental disorders such as anxiety disorders and substance use disorders (Steffen et al. 2020). Approximately, 50-60% of individuals with a lifetime history of depression also report a lifetime history of at least one anxiety disorder. Additionally, approximately 10% to 15% of adolescents with recurrent MDD

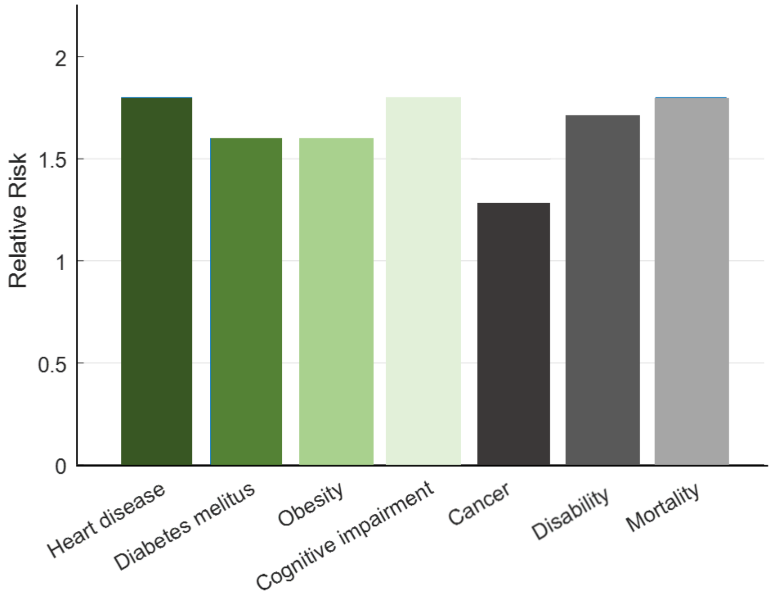

Figure 1-3| The relative risk of comorbidity, disability, and mortality associated with major depressive disorder (MDD): *MDD is associated with high relative risk (RR) to develop chronic somatic diseases, and increased rate of disability and mortality compared to general population without MDD. Patients suffering from MDD have a RR=1.8, 1.6, 1.6, 1.8, and 1.3 to develop heart disease, diabetes mellitus, obesity, and cognitive impairment respectively. Additionally, the people with MDD have a RR for disability and mortality estimated to 1.7 and 1.8 respectively. Adapted from (Otte et al., 2016).*

will subsequently experience bipolar disorder (Hauenstein 2003).

1.2 Social and Economic Burden of Major Depression

MDD is one of the most burdensome illnesses globally, with a heavy global socio-economic impact. It is associated with impaired personal functioning, reduced work productivity, and a high cost-of-illness (COI) (P. S. Wang, Simon, and Kessler 2003; Proudman, Greenberg, and Nellesen 2021; Ronald C. Kessler 2012; P. S. Wang, Simon, and Kessler 2003; Q. Liu et al. 2020; Santomauro 2021). Early onset of MDD has adverse effects on a person's course of life, critical developmental transitions such as educational attainment, marital timing and stability, childbearing, as well as on social role functioning such as marital or parental quality, work performance, and financial success (Ronald C. Kessler 2012). Several studies showed that early onset of MDD is significantly associated with a high failure rate to complete secondary school, estimated to be around 60%, and a high probability of either never marrying or experiencing late marriage. Longitudinal studies reported a bidirectional association between marital dissatisfaction and depressive symptoms (Iyer and Khan 2012). Furthermore, the premarital history of depression has been found as predictor for subsequent marital violence and even divorce.

The GBD study in the year 2019 quantified the disability and mortality burdens imposed by diseases in terms of disability adjusted life years (DALYs), defined as cumulative number of years lived with disability and years of life lost (YLL) due to early death. The GBD study 2019 revealed that among 369 diseases and injuries, mental disorders were leading causes of the global health-related burden with 418 million DAILYs (16% of global DALYs). Among all these conditions, MDD is the greatest cause of disability accounting for 32.9% of all mental health DALYs globally corresponding to 37.2 million global DALYs in 2019 (Metrics 2020; Bloom et al. 2011; Arias, Saxena, and Verguet 2022), and the highest in all age groups except the age group between 0 and 14 years, as highlighted in Figure 1-4A. Additionally, MDD was ranked the 13th leading individual cause of DALYs globally (4th and 6th rank in the age group 10-24 years and 25-49 years respectively) (Institute for Health Metrics and Evaluation 2020), and the third cause of years lived with disability worldwide (König and Konnopka 2020). Further, the global prevalence and health burden of MDD increased during the COVID-19 pandemic leading to additional DALYs globally. Between January 1, 2020, and January 29, 2021 approximately 10.7 million (95% CI: 7·21 – 14·9) additional DALYs were estimated in 204 countries (Santomauro 2021), as presented by age groups and sex in Figure 1-4B.

MDD is associated with excess costs in all subgroups. The economic impact of MDD can be assessed in term of COI, which can be roughly categorized into:

1. Direct costs due to healthcare resource utilization (e.g., inpatients hospitalization, outpatient’s visits, caregivers, medications, and other treatments, etc.).
2. Indirect costs arising from increased premature mortality attributed to the illness, and losses in work productivity due to absenteeism or reduced work productivity despite presenteeism.
3. Suicide costs.

An accurate estimation of the global costs of MDD is not available. On one hand, data for costs are not available for most countries. On the other hand, the available costs are likely to significantly underestimate the true costs due to the lack of data available around some key areas. Nevertheless, several psychiatric epidemiological studies have been conducted in many developed and developing countries (Chodavadia et al. 2023). The total economic burden of MDD among adults in the United States alone was estimated to be at least US$ 326.2 billion (in 2020 values), with 35% attributed to direct costs, 61% to indirect workspace costs, and 4% to suicide-related costs (Greenberg et al. 2021). König et *al*. reported that the annual costs of depression amounted to be €5047 (95% CI: 3214-6880) per patient in Germany. Whereas the highest excess costs were estimated to €9560 and €7955 for the age groups 30–44, and 18–29-year-olds respectively (König and Konnopka 2020). Additionally, anxiety and depression cost the global economy US$1 trillion each year (World Health Organization 2022b; The Lancet Global Health 2020).

Despite the high estimated economic burden of MDD, the costs of MDD as comorbidity with other physical and mental conditions tend to be underestimated. This finding highlights that excessive COI associated with MDD will be a future challenge for health care and policymakers (Proudman, Greenberg, and Nellesen 2021).

1.3 Research Questions, Significance and Hypothesis

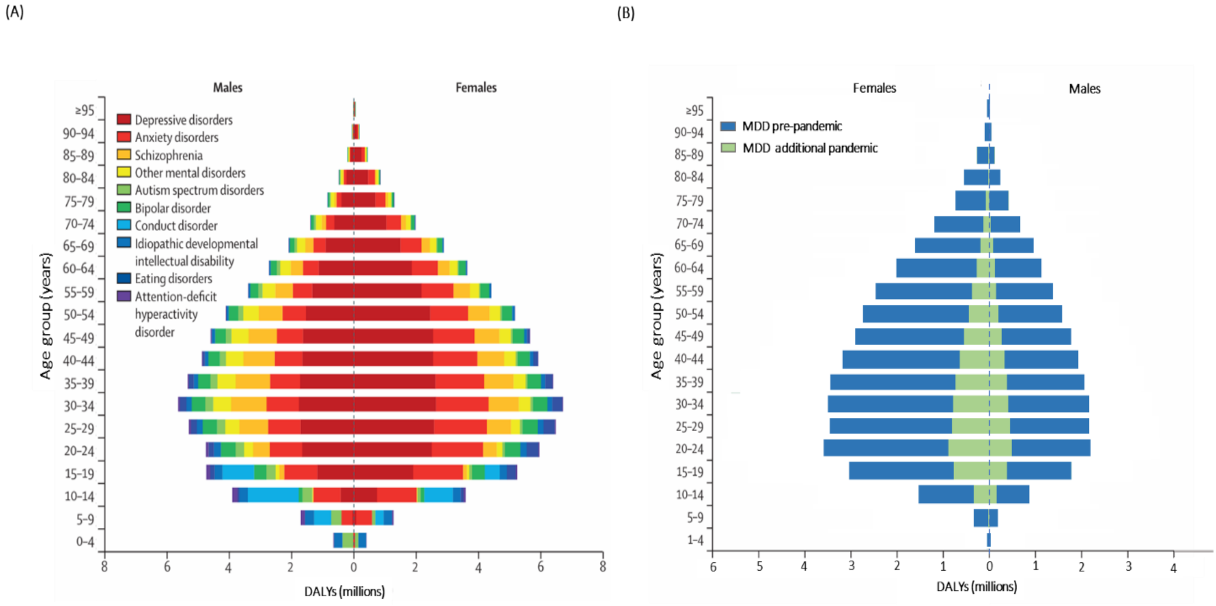

Figure 1-4 | Global *burden of major depressive disorder in term of disability-adjusted-live-years (DALYS), by age and sex:* *(A) MDD is the greatest cause of disability among the mental disorders in 2019 (dark reed), and among all age groups, except the age groups between 0 and 14, (B) Additional DALYS due to COVID-19 pandemic (green) compared to the estimated DAYLs in pre-pandemic period in 2019 (blue). The woman showed higher disability in term of DAYLs relative to males in both, pre-pandemic and during COVID-19 pandemic periods. Sources: (A)* (Ferrari et al. 2022)*, and (B) adapted from Santomauro, 2021*

Research questions of the study. — The research of this study was built on the following main research questions:

1. Could volatile metabolites in the human exhaled breath open new insights into the peripheral molecular changes underlying the pathophysiology of MDD, allowing for

precise and objective molecular diagnostic of MDD, resulting in adequate treatment and better outcomes?

2. What are the distinctive features of applying breath metabolites in MDD research compared to the metabolome of other biomatrices?

Significance of breathomics application in MDD research. — The treatment of MDD remains a significant challenge, in part due to the limited therapeutic success resulting from imprecise diagnostic criteria, and an incomplete understanding of the molecular pathology involved. The pathophysiology of MDD depends on alterations of a wide range of biological systems and pathways that interact with each other to produce the disease's symptoms. Thus, dysregulation of the mechanisms underlying the pathophysiology of MDD can only be successfully assessed by a multi-parametric biomarker signature. In this context, metabolomics, coupled to high-throughput analytical technologies, advanced computational approaches, and cheminformatics tools, could offer completely new insight into the heterogeneous pathophysiology of MDD, opening new horizons into precision psychiatry, as visualized in Figure 1-5. Particularly since the metabolome represents downstream products from the pathophysiological processes occurring at the genetic, transcriptional, and translational levels, as well as byproducts of the gut microbiome metabolism and the global metabolic response to environmental influences. Indeed, metabolomics based on different biomatrices (e.g., blood, urine, plasma, etc.) has recently emerged as a particularly potential qualitative and quantitative tool that provides specific biomarker signatures reflecting alterations in several biochemical processes that underly MDD (Bot et al. 2020; Homorogan et al. 2021; S.-W. Gui et al. 2018; Bot et al. 2020; Sowa-Kućma et al. 2018; Maes, Noto, and Brietzke 2015; Du et al. 2021; Pan et al. 2018), and the effects of drugs therapies on those biological processes (Bhattacharyya et al. 2019; Thorn and Greenman 2012; H. Zhu et al. 2013; Ferrari et al. 2022).

In this thesis, we investigate breathomics as one of the newest branches of metabolomics to explore the metabolite changes that underpin MDD. Compared to other metabolomics biomatrices, breathomics offers practical advantages. Breath samples are inexhaustible, easy to collect, suitable for long-term and frequent monitoring of biomarkers related to disease progression, treatment response or treatment resistance, and can be analyzed in real time making it rapid and cost-effective. Moreover, exhaled breath analysis offers the possibility of untargeted analysis, allowing for the global capturing of metabolites within a sample, and providing qualitative identification and relative quantification of a broad range of metabolites that could mirror dysregulation in an array of biochemical processes in MDD. Further, due to advanced throughput-sensitive mass spectrometry devices, breathomics is rapidly becoming a discovery tool for drug metabolism, environmental exposure, early screening, diagnostics, disease monitoring in a wide range of contexts. These include viral (Houspie et al. 2011; Yan et al. 2017; Milton et al. 2013), and bacterial infectious diseases (Sohrabi et al. 2014), metabolic conditions (Souvik Das, Pal, and Mitra 2016), cancer (Michael Phillips et al. 2003; Michael Phillips et al. 2010; W. Zhou et al. 2017; P. Fuchs et al. 2010; Hakim et al. 2011; Gruber et al. 2014; Sakumura et al. 2017; Nardi-Agmon and Peled 2017), and neuropsychological conditions (Marion et al. 2002; Michael Phillips et al. 2003; C. Jiang et al. 2022), respiratory diseases

(Ibrahim et al. 2021; Finamore, Scarlata, and Incalzi 2019), physical and mental stress (Tonacci et al. 2019; Edwards and Beale 2011; Ritz et al. 2015).

Hypothesis of the thesis research. — Similar to the other metabolomics biomatrices, the human exhaled breath is a complex mixture of small metabolites that originate from various sources. These include exogenous sources such as environmental factors and microbiome, as well as endogenous biochemical processes occurring within the body, and at different biological levels (e.g., genes, proteins, etc.). Regardless of their origin, metabolites can cross from the blood stream to the lung lining fluid, where they are then exchanged into the breath through alveolar gas exchange mechanisms. Depending on their properties, such as solubility and volatility, metabolites can appear either in exhaled breath gas or in the exhaled breath condensate. Therefore, we hypothesize in the present study that:

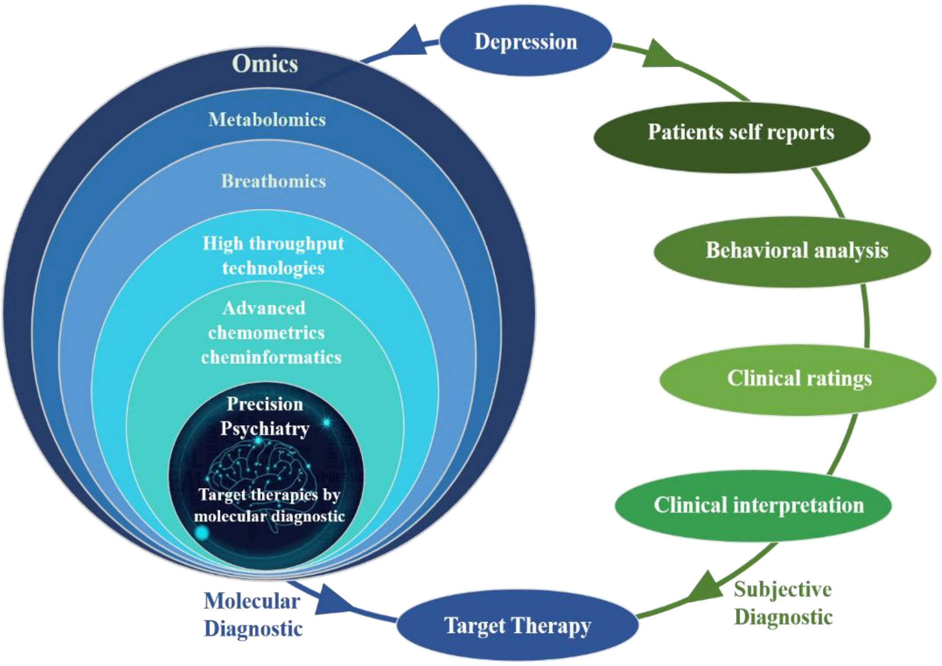

Figure 1-5| Target therapy by molecular diagnostics using breathomics analysis: *The profiling of breath-based metabolomics (Breathomics) data using high-throughput analytical technologies, combined with advanced computational approaches and cheminformatics tools, has the potential to provide completely new insight into the molecular changes associated with MDD. This approach could pave the way for novel molecular diagnostics and precision psychiatry. In additional to the traditional subjective diagnostic methods, such as psychological interventions, molecular diagnostic could enable more precise pharmacotherapy, ultimately leading to improved outcomes for patients.*

1. The volatile metabolites in the exhaled breath of MDD patients could provide a qualitative or relatively quantitative multi-parametric breathprint that mirrors dysregulation of several biological pathways and mechanisms underlying pathophysiologies of MDD.

2. The breathomics analysis could yield a concise breathprint of potential markers for MDD, which may be valuable for developing a low-cost MDD breath test in clinical settings.

1.4 Key Objectives of the Thesis

To the best of our knowledge, this is the first breathomics study to explore alteration of biochemical patterns and metabolic pathways in patients with MDD relative to healthy controls (HCs) using the analytical device proton transfer reaction-mass spectrometry (PTR-MS). The study is still ongoing in cooperation with the Department of Psychiatry, Psychotherapy and Psychosomatics, University Hospital of the RWTH Aachen, Germany. The present thesis represents a proof-of-concept application of breathomics in MDD research, and primarily focus on the biomarker discovery phase of the study. Thus, the main purposes of the present work are:

1. To investigate possibilities and limitations to identify and characterize potential volatile markers in the exhaled breath discriminating between patients with MDD and HCs, aiming to establish a potential breathprint associated with MDD.
2. To identify clusters of volatile compounds with similar awakening response patterns from baseline (awakening time) to 60 minutes after awakening, potentially involved in related biological pathways or networks
3. To identify the affected pathways by MDD using compounds with related awakening stimulus responses, and employing metabolomics tools to rank compounds with related biological functions.
4. To compare the detected potential breath metabolite signature and pathways with those identified in other metabolomics biomatrices.
5. To explore the biological relevance of the identified biomarkers and pathways by linking them to known mechanisms involved in MDD, such as oxidative stress, inflammation, and alterations in the gut microbiome.
6. To evaluate the impact of confounding factors such as smoking, age, gender, and BMI on the breathomics data, ensuring the robustness and reliability of the identified biomarkers.
7. To investigate the association between identified breath biomarkers and the severity of MDD, assessing their potential utility in reflecting the extent of depression.

In order to fulfill these objectives and identify a wide range of peripheral potential markers associated with an array of biological mechanisms and systems, we conducted untargeted breathomics profiling of MDD patients and healthy volunteers by PTR-MS combined with different advanced computational techniques, including artificial intelligence algorithms. The analysis focused on volatile and some semi-volatile organic compounds in the exhaled breath of the participants.

1.5 Thesis Structure

To address the above-mentioned purposes, the present PhD thesis is structured into five chapters:

1. **Introduction:** This chapter provides a brief overview of the doctoral thesis. The clinical and socio-economic research problems are outlined, along with the hypothesis and the

clinical significance of breathomics analysis as a novel diagnostic tool for MDD. The main objectives of the study are also presented.

2. **Theoretical Background:** In this chapter, the theoretical concepts of the breath gas analysis (BGA) and MDD are explained. The chapter begins with an overview of breathomics as novel omics-science, highlighting its current applications in experimental and medical research. The fundamental principles of BGA are presented, followed by a review of state-of-the-art analytical techniques used in breathomics profiling. In the second part, the etiology, diagnosis, and treatment options for MDD are summarized. Additionally, an overview of the current state of peripheral metabolomics markers for MDD is provided, along with a discussed breathomics markers associated with other mental health conditions that may present with symptoms similar to those of MDD.

3. **Methods and materials:** In this section, the methods and materials used in this breathomics study are described. This includes details on the characteristics and selection criteria for cohort groups, the methods for breath sample collection, and the analytical instrumentations employed for breathomics profiling. Additionally, the statistical methods and chemometrics tools used for features selection and performance classification are outlined. Finally, potential confounding factors that could introduce bias into the breathomics analysis are discussed and the association between the potential markers and depression severity is examined.

4. **Results:** In this chapter, the results of the breathomics analysis are presented. This includes the identification of potential markers and affected pathways in patients with MDD, the most relevant cofounding factors affecting the accuracy of the results, and the association between the MDD severity and the potential breath markers. Additionally, the biological relevance of the significant markers is discussed.

5. **Discussion and conclusion:** In this chapter, the results of the breathomics analysis are explained and interpreted within the context of this thesis and related to existing metabolomics markers in the literature. The strengths and limitations of the study are discussed, and the research questions posed in the thesis are addressed. Additionally, the key findings are summarized, and future directions are suggested to overcome the limitations and enhance the validation of the breathomics studies in larger populations.

2 THEORETICAL BACKGROUNDS

2.1 Metabolomics

In recent years, bioanalytical chemistry has witnessed significant advancements in the development of high-throughput platforms and strategies suitable for the analysis of analytes from different biological samples. These analytical techniques have gained popularity for their ability to extract and identify many thousands of potential markers associated with physiological and pathological states of biological systems. This progress has given rise to "omics"-sciences including genomics, transcriptomics, proteomics, microbiomics, exposomics and metabolomics, which have become highly relevant disciplines in biosystems. Metabolomics targets the quantitative and qualitative characterization of all metabolites found within various biological samples such as blood, plasma, feces, urine, cells, exhaled breath (EB) gas, or exhaled breath condensate (EBC). Metabolomics studies aim to assess the interaction and the response of metabolites to physiological conditions, diseases, disease progression, therapeutic intervention, genetic modifications, or environmental effects.

Metabolites are downstream products of genetic transcription and translation, as well as protein metabolism. They are affected by environmental exposures, lifestyle, dietary habits, and interactions with both commensal and pathogenic organisms, as highlighted in Figure 2-1. Unlike the genome, which typically remains relatively consistent over a lifespan, the metabolome[2], as end-products of the omics-science cascade, undergoes constant change and provides an integrated view into the dynamics of metabolic networks, regulatory processes, and pathological changes. Consequently, the metabolome directly reflects the underlying biochemical activity and state of cells and tissues (e.g., gene mutation, enzyme defect). Metabolites can offer early insight into emerging disease states because metabolic changes occur at the earliest stages of disease progression. These changes have the potential to reflect perturbations at lower genomic, transcriptomic and proteomic levels several thousand-fold (Raamsdonk et al. 2001). Several studies have identified genetic alterations associated with tumor growth, or enzyme defects resulting from genetic mutation or non-benign polymorphisms. These perturbations can lead to changes in the production of VOCs in the macroenvironment of the cell, including the production of diseases specific substrates, the reduction or accumulation of specific metabolites that cannot be metabolized normally (Davies et al. 2014; Kwak and Preti 2011). For example, in healthy individuals the trimethylamine (TMA) is oxidized to the odorless trimethylamine N-oxide (TMAO) in the liver. However, in patients with the metabolic disorder trimethylaminuria (TMAU), the monooxygenase enzyme 3 has limited capacity to oxidize TMA, resulting in its excretion in various biomatrices such as urine, sweat, saliva, and breath (Gątarek and Czaplińska 2021).

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[2] The metabolome represents the complete set of metabolites in a biological cell, tissue, organ, or organism.

Nowadays, extracting and analyzing the entire metabolome in a single step remain highly complex due to the large size and the diversity of chemical and physical properties of the metabolites. Consequently, achieving a truly comprehensive understanding of the metabolome remains partly elusive and necessitates the development of advanced technical inventions. Nevertheless, current analytical devices enable the effective identification, and relative quantification of hundreds of metabolites. The main analytical platforms in metabolomics are magnetic resonance (NMR) and mass spectrometry (MS) (A. Alonso, Marsal, and Julià 2015). NMR provides a relatively fast and non-invasive analysis, although it is less sensitive compared to MS. Currently, MS coupled with separation instrumentations such as gas chromatography (GC) and high-performance liquid chromatography are the most commonly applied technologies in metabolomics. They allow for relative quantitative and qualitative analyses of a broad range of organic molecules in different biological matrices with high specificity and sensitivity, throughput using a relatively low number of samples. Nowadays, metabolomics has increasingly been utilized in various health applications and medical research fields, including identification of diagnostic biomarkers for diseases, preclinical drug trials including discovery of new drug targets and prediction of drug responses, toxicology (Steuer, Brockbals, and Kraemer 2019), transplant monitoring, and newborn screening. Metabolomics disturbances have been associated with several human diseases, including metabolic disorders (Gonzalez-Covarrubias, Martínez-Martínez, and Bosque-Plata 2022), Trimethylaminuria (TMAU) (Chhibber-goel and Gaur 2016), cardiovascular diseases (McGarrah et al. 2018),

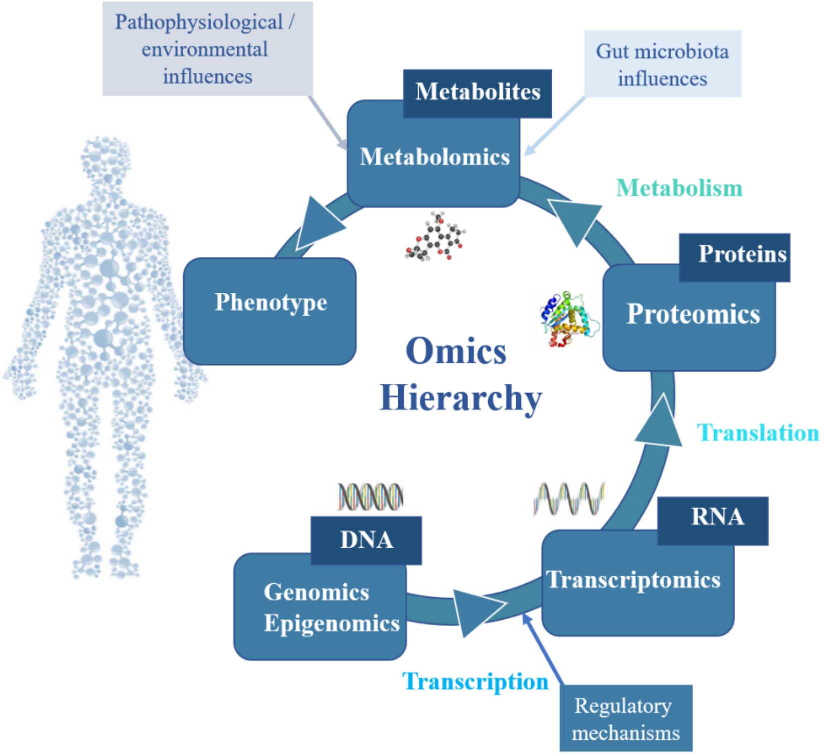

Figure 2-1| Omics-sciences cascade and interaction: *Regulatory processes at genetic level affect the expression of the downstream molecules at other biological omics-levels, including transcriptome, proteome, and then the metabolome levels. Genetic variants in DNA sequence (genomics), non-DNA sequence alteration such as histone modification and methylation (epigenomics), structural changes in RNA (transcriptomics), modification in protein content and enzymatic activities (proteomics) lead to alteration in metabolome profile and function. The metabolome profile is also affected by environmental factors, pathophysiological conditions, and microbiome metabolism. Thus, the metabolome is changing constantly and can provide an integrated view into the dynamics of metabolic networks, regulatory processes, and pathological changes occurring in disease, allowing a better presentation of the phenotype than other omics.*

neurodegenerative disorders (Quintero, Pontes, and Tasic 2021), age-related macular degeneration (Hou, Wang, and Pan 2020), psychiatric disorders (Shih 2019), and cancers (Schmidt et al. 2021).

2.2 Breathomics

2.2.1 History of breathomics

Breath-based metabolomics, known as breathomics, involve the multidimensional analysis of exhaled analytes, including identification and quantification of volatile organic and inorganic patterns, as well non-volatile substances in EBC such as aerosol particles, or even viruses and bacteria. Breath analysis is one of the oldest diagnosis forms that dates back to the time of ancient Greece and Rome. Early skilled physicians from early civilizations recognized that certain diseases were characterized by distinct smells. For instance, diabetes could be diagnosed by the sweet and fruity smell of acetone, liver disease by the smell of musty-fishy odor, kidney failure by the urine-like smell, and lung abscess by the putrid stench (Souvik Das, Pal, and Mitra 2016).

Interestingly, several species with superior olfactory systems compared to humans, such as dogs and rats, can recognize the smellprint of disease. In light of this evidence, olfactory characteristics of animals have been utilized for diagnosing a variety of diseases in the last decade. Trained dogs, for instance, have shown remarkable abilities to distinguish subjects with different stages of pulmonary carcinoma from controls with a high accuracy of 99%, merely by smelling the subject’s breath samples (Mazzatenta, Giulio, and Pokorski 2013a). They have also successfully identified patients with Parkinson’s disorder (Gao et al. 2022), prostate cancer, ovarian cancer, and various infectious diseases from cell lines, bacterial cultures samples, or bio-fluids samples with high specificity (Cambau and Poljak 2020). Apart from dogs, previous investigations suggested that trained African giant pouched rats (*Cricetomys ansorgei*) can sensitively detect pathogens causing pulmonary tuberculosis by smelling the VOCs in human sputum samples. The rats’ sensitivity may even exceed that of sputum smear microscopy diagnostic performed as part of routine TB screening (Reither et al. 2015; Mahoney et al. 2012; Kanaan et al. 2021; Fiebig et al. 2020). It took centuries to identify and quantify the biomolecules responsible for these odors sensed by human and animal olfaction. Recently, several research groups have utilized the biological olfactory of the animals as models to build artificial olfactory systems, known as electronic noses, which are fundamentally different from devices for identification and quantification of individuals metabolites. With the progress of high-throughput analytical technologies, researchers have identified and quantified thousands of VOCs sources in the human breath, most of them in trace concentrations. In 1970s, the Nobel prize winner in chemistry Linus Pauling detected about 250 substances in human breath using GC (Pauling et al. 1971). This contribution provided new insights into breathomics analysis for clinical purposes and spurred further research into the breath gas analysis (BGA). In 1997, Phillips and coworkers estimated 1259 VOCs from 20 healthy subjects using GC coupled to mass spectrometry (GC-MS) (Michael Phillips 1997). In 1999, Phillips *et al.* detected 3481 VOCs in the EB of 50 participant, with an average number of about 200 VOCs detectable in all individuals’ breath (Souvik Das, Pal, and Mitra 2016). A recent review reported a total of 4,412 volatile compounds that have been found in the breath and biofluids of healthy subjects, with 1,488 in breath, 623 in skin, 549 in saliva, 444 in urine, 443 in feces, 379 in blood, 290 in milk and 196 in semen (Drabińska et al. 2021). Nowadays, the advanced computational tools and technological contribution has given new insights into human biochemical metabolism through non-invasive BGA. Different diseases have been characterized by specific breathomics profiles, which have been captured by exhaled breath, known as “breathprints” (Mazzatenta, Giulio, and Pokorski 2013b; Mule, Patil, and Kaur 2021).

Recently, thousands of VOCs have been detected in the exhaled breath and linked to a broad range of pathophysiological conditions and environmental exposures (Pleil 2008). Breath-testing is non-invasive and easy to use. Unlike other metabolomics bio-matrices like blood and urine, breath is inexhaustible and could be of potential interest for frequency and longitudinal monitoring by VOCs profile changes as response to both, external and biological stimulus including disease progression, drug dynamic, and therapy response.

2.2.2 Gas exchange in the lungs

The lungs serve as a crucial interface between the environment and the human body. Various biological processes in the body leave their breathprints by releasing gas molecules, which are transported by blood to the lungs and released into the exhaled breath through the alveoli. Gas exchange takes place in about 300 million alveolar sacs grouped together into acini (~ 8000 to 9000 alveoli per acinus), which are supplied by a terminal bronchiole (Hughes 2007), as illustrated in Figure 2-2. All gases involved in the gas exchange cross the blood gas barrier by passive diffusion, driven by the pressure gradient between the gas in the alveolar space and the plasma of the capillary. According to Dalton’s law, each gas molecule in a gas mixture exerts its own pressure on the surface with which it is in contact. The total pressure exerted by the gas mixture is the sum of the partial pressures of all gas molecules in the mixture, and is proportional to the concentration of the gas mixture. Partial pressures play an important role in the movement of gas molecules, which move into areas with lower pressure than their partial pressures. Further, the greater the pressure gradient, the more rapid the movement of the gas molecules.

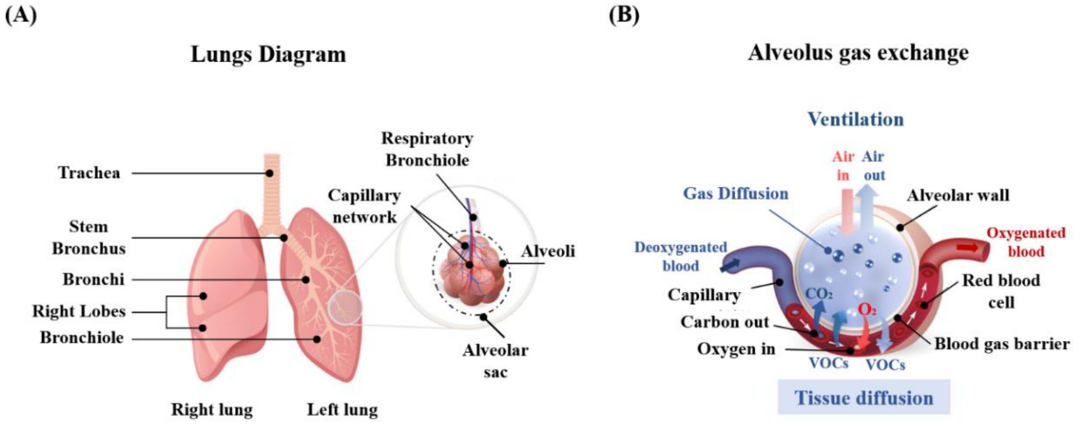

Figure 2-2| Principle of the gas exchange between the pulmonary blood supply and the alveolar sacs of the lungs: *(A) Schematic of the lungs anatomy and alveoli, and (B)Simplified principle of gas exchange at alveoli. The gas exchange occurs in alveolar sacs, which are supplied by a terminal bronchiole. Diffusion of gases happens rapidly across the blood gas barrier, driven by the pressure gradient between the gas in the alveolar space and the blood in the capillaries. Volatile organic compounds (VOCs) (grey) are transported into the exhaled breath following the same principle*

The anatomy of the lung is optimized to maximize the diffusion of gases. Gas exchange occurs across a highly permeable respiratory membrane in alveolar sacs in the depths of the lung with a total thickness of 0.2 - 0.4 µm. The surface area of separating membrane between the alveolar and the blood is very large, ranging from 50 to 100 m^2. The diffusion of gases is governed by Flick's law, which states that the rate of diffusion V_{gas} of a gas through a tissue layer is proportional to the surface area *A* of the tissue, the diffusion coefficient *D*, and the difference of the partial pressure between both sides P_1-P_2, and inversely proportional to the tissue thickness *T:*

$$V_{gas} = \frac{A.D.(P_1 - P_2)}{T} \qquad \textbf{(2.1)}$$

D depends on the characteristics of gas molecules and the tissue according:

$$D = \frac{S_{gas}}{M_w} \qquad \textbf{(2.2)}$$

where S_{gas} and M_w are solubility and molecular weight of the gas respectively.

2.2.3 Molecular profile and application area of breathomics

A single sample of human EB contains thousands of substances from both endogenous and exogenous sources that penetrate from the blood to the lungs' lining fluid, and depending on their volatility and solubility, appear in the EB gas or EBC. The molecular profile of human breath has been extensively characterized in terms of composition and concentration in both physiological and pathological conditions. EB consists mainly of nitrogen (~78%), oxygen (~16%), carbon dioxide (~ 4 - 5%), and condensed water vapor, along with inert gases (Mazzatenta, Giulio, and Pokorski 2013a). The EB contains more carbon dioxide and less oxygen than inhaled air because oxygen is utilized to generate energy during respiration, while carbon dioxide is produced as a by-product of the energy production process. As visualized in Figure 2-3, the residual small fraction represents a rich medium of gases, and potential non-volatile hydrophobic and water-soluble molecules in EBC derived from respiratory droplets (< 1% of EBC) (Horváth et al. 2005), which mirror the person's health and metabolic processes. These potential compounds in EB are chemically very diverse and are commonly classified into:

1. VOCs such as pentane, isoprene, and aldehydes, available in trace concentrations ranging from parts-per-million (ppm, 10^{-6}) to parts-per-trillion (ppt, 10^{-12}).
2. Inorganic compounds such as nitric oxide (NO), carbon dioxide (CO_2), carbon monoxide (CO), hydrogen cyanide (HCN), hydrogen sulfide (H_2S) and ammonia.
3. Non-volatile organic compounds (NOCs) measured in EBC, which vary from small inorganic ions to larger organic molecules such as urea, organic acids, amino acids, and their derivatives, as well as peptides, proteins, and macromolecules

More than 1000 VOCs, representing functional activity of both microbiome and host metabolomes, have been identified in human EB and classified based on their physical (e.g., volatility, solubility, density, odour, vapor pressure, reactivity, etc.), and chemical properties. VOCs consist of carbon elements bounded to one or more of functional groups, which include elements such as oxygen, phosphorus, nitrogen, silicon, halogen, or sulphur. VOCs are characterized by their low boiling point, high saturation vapor pressure, and high volatility. The common chemical classes of VOCs include hydrocarbons (e.g., alkanes, alkenes, alkynes, and aromatic hydrocarbons, etc.), oxygen-containing compounds (e.g., alcohols, ketones, organic acids, aldehydes, esters), nitrogen-containing compounds (e.g., amine such as dimethylamine, trimethylamine, and pyridine), and sulfur-containing compounds (e.g., mercaptans and sulfides).

Over the last decade, the analysis of exhaled VOCs has gained considerable attention due to its potential for non-invasive observation of the biochemical processes within the human body. Compared to non-volatile compounds and volatile metabolites found in other bio-matrices, exhaled VOCs could offer several practical advantages. VOCs are suitable for long-term and frequent monitoring of biomarkers related to physiological conditions, disease progression, treatment response or treatment resistance. Moreover, they are easy to measure particularly beneficial for children and elderly, and can be monitored in real time without sample pre-concentration, and can be analyzed within minutes making it rapid, and cost-effective.

VOCs are mainly produced through biochemical pathways in the body or airway, and can be detected in exhaled breath (34%), urine (11%), blood (6%), saliva (14%), feces (15%) or emitted by skin (20%) (Broza et al. 2015). The breath represents a rich source of VOCs

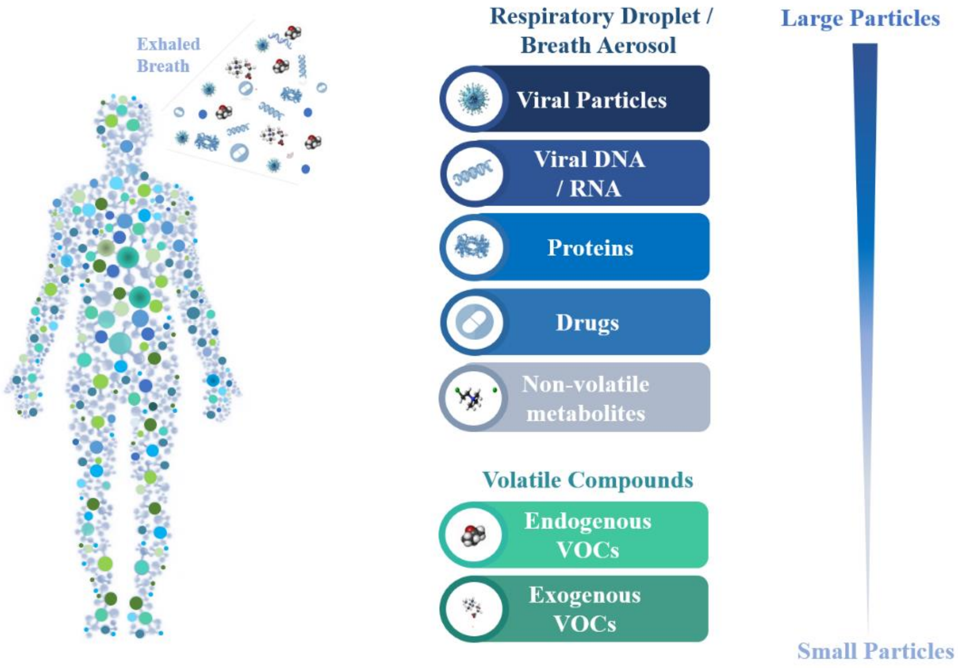

Figure 2-3| Compounds of the human exhaled breath: *In addition to the compounds available in the air, including nitrogen, oxygen, and carbon dioxide, the human exhaled breath represents a rich medium of endogenous and exogenous volatile organic compounds (VOCs) in trace concentrations. These VOCs, along with respiratory droplets that are exhaled as breath aerosols, form a complex mixture that can provide valuable diagnostic information. Moreover, the exhaled breath condensate contains various non-volatile metabolites and macromolecules. These non-volatile compounds may originate from both upper and lower respiratory airways, including lipids, proteins, and macromolecules. Together, these volatile and non-volatile compounds hold significant potential as biomarkers for the diagnosis and treatment of diseases, offering insights into underlying pathophysiological processes and enabling more personalized medical approaches.*

originating from all part of the body making the exhaled VOCs applicable to a wide range of pathophysiological conditions; These include early detection and precision medicine for inflammatory diseases such as respiratory diseases like asthma and COPD (Ferraro et al. 2020), liver cirrhosis and tumors (Miller-Atkins et al. 2020), chronic disease kidney (Bevc et al. 2017), breast cancer (Mandy et al. 2012; Frank Kneepkens, Lepage, and Roy 1994), lung cancer (Nardi-Agmon and Peled 2017; J. Zhou et al. 2017; Machado et al. 2005; Wehinger et al. 2007), oral cancer (Hartwig et al. 2017; Gruber et al. 2014), Gastrointestinal cancer (Xiang et al. 2021), psychological disorders such schizophrenia (M. Phillips, Sabas, and Greenberg 1993; B. M. Ross, Shah, and Peet 2011; Henning et al. 2023; Dietrich-Muszalska and Kontek 2010), neurological disorders such as autism (El-Baz and Suri 2020) and Alzheimer's disease (Tiele et al. 2020), as well as metabolic disorders (Souvik Das, Pal, and Mitra 2016). In addition to their diagnostics and therapeutic management potential, VOCs offer valuable information for dynamic assessments of normal physiological function e.g., physical stress test such as exercise (King et al. 2009), monitoring of drugs' effect in an intra-operative setting (Kamysek et al. 2011), or in a sleep lab (King 2012), pharmacokinetics (J. Beauchamp, Kirsch, and Buettner 2010), environmental exposure (Pleil 2008).

The endogenous gaseous inorganic such as NO, CO, HCN, and H_2S are involved in the regulation of many chemical processes (Belizário, Faintuch, and Malpartida 2021). Horváth *et al.* reported that NO and carbon monoxide could be linked to oxidative stress (Horváth et al. 2005).

EBC mainly consists of condensed water vapor (> 99%) and a small fraction of airway lining fluid, which contains various molecules with diagnosis potential of diseases in the lungs or systemically (Ahmadzai et al. 2013). Currently, a wide range of NOVs have been detected in EBC, ranging from small inorganic molecules such as anions to larger organic molecules (e.g., urea, organic acids, amino acids and their derivatives, etc.), peptides, proteins, surfactants, and macro-particles such as remnants of viruses and bacteria. Additionally, a very small amount of volatile molecules has been identified in EBC, especially compounds with high solubility in aqueous solutions (Ahmadzai et al. 2013; Amann and Smith 2013; Lourenço and Turner 2014; Khoubnasabjafari et al. 2022; Rahimpour et al. 2018; Wallace, Pfeil, and Madden 2019). EBC is a rich biological medium with robust markers used for a wide variety of environmental and clinical biomonitoring applications. It contains many inflammatory mediators such as cytokines, as well as biomarkers of oxidative and nitrosative stress (e.g., 8-isoprostane, 3-nitrotyrosine, S-nitrosothiols, etc.). However, the mechanism explaining the origin of NOCs in human EBC remains disputed and controversial (Horváth et al. 2005). It is believed that proteins and DNA in EBC may originate from either inhaled or respiratory tract- generated aerosols, which contain bacteria and fungi. The small number of epithelial cells and DNA available in EBC is believed to be linked to oxidative stress to cells in the lower respiratory tract, which may cause DNA mutations. This may lead to changes in cell cycling, growth promotion, DNA repair, apoptosis, and can induce angiogenesis (Ahmadzai et al. 2013). Increasing evidence suggests that water-soluble compounds and EB aerosols have been indicated as biological markers for the diagnosis of oxidative stress and inflammation in the airways, carcinogens, and microbial or viral infections contributing to public health such as influenza (Milton et al. 2013), COVID-19 pandemics (Ma et al. 2020), and tuberculosis (Chen, Bryden, and Wood 2020). Additionally, evidence suggests that cells of the inflammatory and pulmonary systems are capable of producing different cytokines, which have been detected in EBC including IL-4, IL-

8, and interfon-γ, although some of them are at the lower limit of detection of the available EB devises (Horváth et al. 2005; Wyse et al., 2004).

None of the EBC biomarkers have been sufficiently validated for clinical use. The reproducibility of EBC biomarkers remains a complex task, as each biomarker needs to be considered individually due to the large range of the compounds present in this biological medium, spanning from protons to DNAs. Additionally, the diversity of their physical and chemical features, such as volatility, solubility, variability, molecular weight, or amount, contribute to this complexity. Currently, the available analytical techniques are not sensitive enough to detect the low concentration of many biomarkers in EBC. Moreover, the collection of the EBC samples requires specialized equipment, coolant to freeze the breath condensate, laboratory space to process the frozen condensate, and subjects should perform breathing maneuvers (Wallace, Pfeil, and Madden 2019; Khoubnasabjafari et al. 2022). The main focus of the present study was on the analysis of the exhaled VOCs. A more in-depth review of NOCs in EBC background and the analytical methods used can be found elsewhere (Wallace, Pfeil, and Madden 2019; Ahmadzai et al. 2013; Horváth et al. 2005).

2.2.4 Origins of exhaled VOCs

The volatile fraction of an individual's EB comprises of compounds of various origins, broadly categorized as endogenous and exogenous, as detailed in Figure 2-4 (Pleil, Stiegel, and Risby 2013). Endogenous VOCs originate from host metabolism, encompassing routine metabolism processes, as well as imbalance in the body associated with diseases or disfunctions (e.g., organ disfunction, cell mutation, enzyme defect, etc.). Typical altered metabolism processes include inflammatory processes, oxidative and nitrosative stress, and Warburg effect. In healthy persons, the regular metabolism processes drive the production and distribution of endogenous VOCs. However, pathological conditions can up- or downregulate these processes, resulting in alterations in the composition and concentrations of VOCs, which are subsequently manifested in exhaled breath (Mazzatenta, Giulio, and Pokorski 2013b; Ruzsany et al. 2023). Considering this evidence, exhaled metabolites offer an integrated view into the upstream pathophysiological processes, potentially serving as unique breathprints associated with specific diseases or physiological conditions across the entire body.

On the contrary, exogenous VOCs (EVOCs) originate from non-host organisms, including commensal and pathogenic microorganisms in the body microbiome. They may also be introduced from various sources in the individual's environment, including pollutants, cleaning products, personal care products, or biogenic VOCs emitted from plants, forests, and anaerobic moors. Additionally, EVOCs can arise from nutrition, drug metabolites during therapeutic interventions, or induced through various lifestyle factors such as smoking and alcohol consumption (Moura, Raposo, and Vassilenko 2023; David and Niculescu 2021). EVOCs generally enter the body through inhalation of indoor and outdoor ambient compounds, ingestion of food, beverage and drugs, or through topical contact (eyes, mouth, and skin). EVOCs themselves or their volatile downstream products are eliminated from the body through exhalation, urine, feces, saliva, emitted by skin or even other bio-fluids such as tears and breast milk (Broza et al. 2015).

EVOCs are not necessarily considered as contaminants that distort the chemical content in the EB. In the last decade, EVOCs have been investigated as potentially useful and non-invasive biomarkers for various clinical applications and environmental exposure (Pleil 2008;

Gaude et al. 2019). Microbial VOC (MVOC) signatures have been utilized in the diagnosis and monitoring of infections, dysbiosis, and antimicrobial treatment and resistance (Belizário, Faintuch, and Malpartida 2021; Agnieszka Smolinska et al. 2018; Clemente et al. 2013). Diverse microbiome communities inhabit the human body and play a substantial and crucial role for maintaining overall health and proper physiological functioning (Clemente et al. 2013). Among these, the gut microbiota contains the most abundant microbial community, which is significantly influenced by several factors such as diet, medication, and lifestyle.

The gut microbiota and its metabolites play a vital role in energy metabolism, including the harvest and storage of energy from the diet. Additionally, it contributes to the production of neurotransmitters and several volatile metabolites, which are involved in various metabolic functions, inflammatory processes, and immune system pathways. Alteration (dysbiosis) in the relative diversity and abundance of gut microbiome network, due to removal of commensal

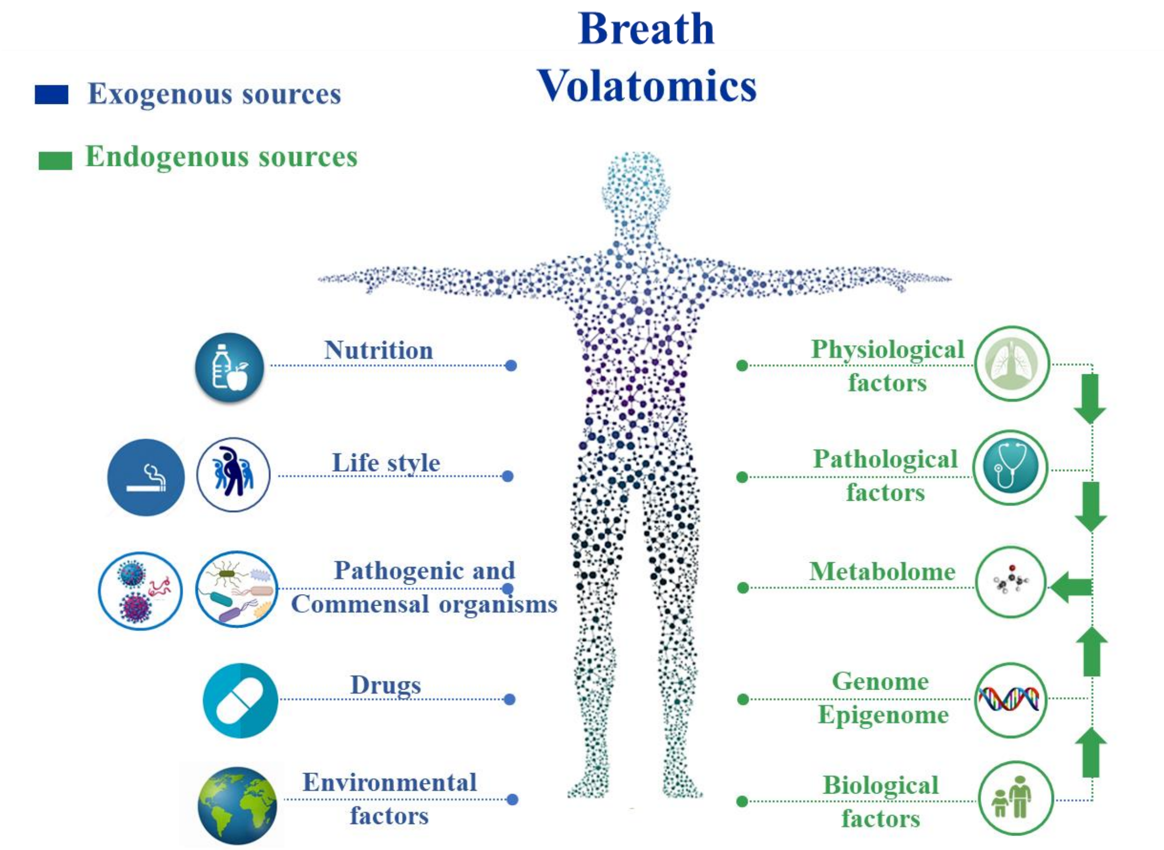

Figure 2-4| Exogenous and endogenous sources of exhaled volatile organic compounds: *Endogenous exhaled VOCs are products of routine physiological and pathological of host metabolism processes (green) that are transported through the blood stream (systemic metabolites) into the lung and then exhaled in the breath. VOCs originating from the airway are directly exhaled in the breath. Several biological factors such age, gender, and gene can significantly influence the composition and level of the systemic and airway born metabolome, impacting the profile of the exhaled VOCs. The exogenous sources (blue) are metabolism products of non-host organisms, including pathogenic (e.g., virus, fungus) and commensal organisms, or downstream products of compounds introduced from the environment into the body (e.g., pollutant, cleaning products), drugs, and nutrition. These compounds themself or their metabolized VOCs circulate in the bloodstream, and are partly eliminated from the body into several biofluids, and breath during gas exchange in the alveoli or in the airways.*

species and overgrowth of pathogenesis species has been associated with many diseases. These include inflammatory bowel disease (Venegas et al. 2019), neurological disorders such as autism (Simeng Liu et al. 2019), psychiatric disorders (Amirkhanzadeh Barandouzi et al. 2020; Zalar, Haslberger, and Peterlin 2018), autoimmune disorders like rheumatoid arthritis, allergy (Clemente et al. 2013), and gastrointestinal diseases (Agnieszka Smolinska et al. 2018). Additionally, the microbiome of the respiratory tract (lung, oral- and nasal cavity) plays an important role in major respiratory diseases and inflammatory conditions. On the other hand, several forms of cancer, including lung, oral and even prostate cancer, have been associated with chronic exposure to carcinogenic VOCs (cVOCs) such as benzene and formaldehyde. Furthermore, the endogenous metabolism of administered EVOCs in vivo could be of great interest for elucidating the toxicokinetic properties of specific EVOCs in the human body, allowing for the assessment of organ function, enzyme activity, or the evaluation of pharmacokinetics of drugs to monitor drug metabolism and optimize therapeutic effect (J. Beauchamp, Kirsch, and Buettner 2010). Moreover, inflammatory and autoimmune diseases have been linked to human exposure to certain EVOCs in the environment (Ogbodo et al., 2022). EVOCs probes can be introduced into the body through oral ingestion, intravenous, or via inhalation, undergo distribution and endogenous metabolism. Finally, EVOCs themselves, or their downstream VOCs are excreted via exhaled breath.

Expired VOCs are generally end products of carbohydrate and lipide metabolism, as well as direct and/or indirect oxidative and nitrosative stress products (Broza et al. 2015). They also are result from cytochrome p450 enzymes metabolism in human cells, or are products of aerobic and anaerobic fermentation processes of gut microbiomes (Belizário, Faintuch, and Malpartida 2021). Oxidative stress (OS) is characterized by an imbalance between the cellular production of prooxidants and antioxidant systems. Oxidative phosphorylation occurs in the mitochondria and serves as a primary source of ATP in aerobic organisms. As by-products it generates free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals are highly reactive substances that can initiate chain reactions. Under physiological conditions, ROS act as natural defense mechanisms against microbial attacks or inflammation in specific regions, and they are balanced by antioxidant protection within the body. However, in pathological or stress conditions, ROS can be overproduced, potentially causing oxidative damage at cellular structures such as phospholipid membranes, proteins, and DNA. ROS can also block enzymes' activity and impair energy generation, or are implicated in toxicity processes that result in cell death (Broza et al. 2015; Calenic et al. 2015; Cao and Duan 2006; Sharifi-Rad et al. 2020). Lipide peroxidation (LPO) is the oxidative degradation of polyunsaturated fatty acids and is implicated in many pathological conditions such as chronic diseases (Sharifi-Rad et al. 2020), and neuropsychiatric disorders (Dietrich-Muszalska and Kontek 2010; Joshi and Praticò 2014). LPO results in a variety of breakdown products, including alkanes, aldehydes, alcohols, ethers, ketones, and hydrocarbons (Sutaria et al. 2022). Moreover, LPO can be monitored by measuring its intermediate or end products. Hydrocarbons, as stable end products of LPO, exhibit low solubility in blood, and are therefore expelled from the body within minutes of their formation in tissues. Exhaled hydrocarbons, such as ethane and n-pentane, have been considered both in vivo and in vitro as LPO markers in the human body (B. M. Ross, Shah, and Peet 2011). Ethane and pentane are emitted when cell cultures are exposed to ROS. Additionally, a good correlation between LPO markers such as malondialdehyde (MAD) and exhaled VOCs such as pentane and pentane has been detected. Moreover, numerous aldehydes, such as acetaldehyde, propanal, butanal, hexanal and 2/3-

hexenal, have been also used as markers for LPO in plants, food products, and clinical studies (Sutaria et al. 2022). However, the emission of aldehydes by biological samples is complex and their concentrations are usually very low (Marco M.L. Steeghs et al. 2006). Ketones are downstream products of fatty acids produced by the liver and oxidized in the Krebs cycle by peripheral tissues. Alcohols are absorbed through the stomach and intestines, and are primarily metabolized by oxidative and non-oxidative pathways, mostly in the liver. Only a small fraction (<10%) of alcohols is eliminated through breath and other biofluids (Hyun et al. 2021). The origins of ethanol and methanol are not fully understood. These alcohols are believed to originate mostly from microbial fermentation of carbohydrates in the gastrointestinal tract or have exogenous origins such as food. Aldehydes can have exogenous sources (tobacco by-products, or dietary sources), or can be endogenously formed by LPO, carbohydrates metabolism, amine oxidases, etc. (O'Brien, Siraki, and Shangari 2005). On the other hand, the indirect OS can be generated by the exposure to EVOCs (e.g., aromatic smoking VOCs, pollutant VOCs, cVOCs etc.). Aromatic and nitrile VOCs are stored in the fatty tissues and are highly reactive resulting in peroxidative damage.

2.2.5 Factors affecting the exhaled VOC profile

Despite promising results revealing diagnostic properties of different biomarkers, BGA is still in its infancy. The introduction of BGA into clinical applications is still hampered by the lack of reproducibility and reliability of results between different research groups. Several challenges affect the accuracy of exhaled VOCs and lead to inter- and intra-variabilities. A major challenge limiting this approach is the lack of standards in sampling procedures, pre-concentration, analytical methods, and robust statistical analyses (Lourenço and Turner 2014). Factors unrelated to disease but capable of affecting exhaled metabolites levels can be broadly classified (but not restricted) into:

1. Individual-related factors, i.e., their physiological conditions
2. Technical-related factors, i.e., sampling procedures and analytical methods
3. Environmental considerations
4. Statistical uncertainty
5. Physical and chemical properties of VOCs

Intra- and inter-variabilities due to these factors should be thoroughly investigated and understood to develop robust breath tests for clinical applications. In this section the most well-known impact factors are summarized.

Environmental and cigarette smoking exposure. — Ambient air compounds, both indoor and outdoor pollutants may influence the composition and levels of EB compounds. Some exhaled VOCs, such as nitric oxide and benzene, could originate from both exogenous and endogenous sources. Therefore, the background EVOCs should be taken in the account to accurately assess the concentration of such compounds, which may not necessarily mirror pathophysiological conditions. Different strategies have been adopted to correct the background concentrations. The first involves measuring inspired (background) and expired VOCs, and then subtracting inspiratory levels from expiratory levels, or calculating alveolar gradients as follows:

$$\Delta_{alv} = \frac{C_{alv} - C_{amb}}{C_{amb}} \tag{2.3}$$

where C_{alv} and C_{amb} represent alveolar and ambient air concentration respectively.

Calculating accurate alveolar gradients in BGA is hampered by several challenges, including technical limitations, the complex pulmonary physiology (e.g., diffusion of VOCs in the lung, variability in alveolar air), and properties of VOCs that influence their movement across the alveolar-capillary membrane. Additionally, exhaled VOCs originating from exogenous sources can further complicate the interpretation of alveolar gradients. A second strategy attempts to correct the background by breathing pure air for a specific time interval. While this approach may be more effective, it has limitations in clinical applications.

An important factor that alters the composition of exhaled breath that should be examined before interpretation of the detected VOCs is smoking behavior. Cigarette smoke contains, in addition to nicotine, thousands of chemical compounds in gas form. Several VOCs have been already detected in the breath of smokers and passive smokers, including acetonitrile, 2,5-dimethylfuran, benzene, toluene, pentane, ethane, and styrene (Gordon et al. 2002; Kushch, Schwarz, et al. 2008; Kushch, Schwarz, et al. 2008; Castellanos et al. 2018; Kwak and Preti 2011).

On the other hand, various exogenous compounds in ambient, such as biogenic VOCs and pollutants, enter the body through different pathways, and alter the breath VOCs profile. However, it is worth noting that not all VOCs with exogenous sources should be excluded from VOCs profile. Some VOCs may find application in the diagnosis and early detection of some diseases such as cancers and allergies, as previously described.

Diet habits, food intake, and medication. — Various physiological processes can cause cofounding biological background variability in the BGA. It is well-known that food intake and eating habits affect both, composition and concentrations of exhaled VOCs (Fischer et al. 2015). Indeed, several foods and beverages have specific VOCs headspace, resulting in distinct VOCs signatures detected in the exhaled breath (Løkke et al. 2012). Additionally, eating habits and frequency play a critical role in BGA. For example, several VOCs including 2-butanol, octane, 2-propyl-1-pentanol, nonanal, dihydro- 4-methyl-2 (3H)-furanone, nonanoic acid, and dodecanal have been detected in people after switching from gluten-free meals to normal meals. Further, it is important to note that many VOCs are a result from microbial fermentation of non-digestible food compounds. Therefore, abstaining from eating and drinking for 30 minutes to overnight before breath sample collection is relevant in the BGA. Moreover, small drugs or their downstream VOCs can also be found in breath samples. Additionally, diet and alcohol consumption are important factors that directly influence both antioxidant level, and the production of free radicals and ROS (Yao, Reddy, and Van Kammen 2001). Caloric intake has been directly associated with OS, particularly in the brain. However, it is not clear whether the origin of the calories or their amount is responsible for the free radical production. Decreased plasma antioxidant levels due to dietary deficiency and alcohol consumption, along with increased lipide peroxidation can result in the production of a variety of breakdown products such as alkanes, aldehydes, alcohols, ethers and ketones, as mentioned before.

Breath sample contamination from mouth and nasal cavity. — BGA predominantly focuses on mouth-exhaled breath. However, in addition to VOCs of systemic origin, breath samples can be contaminated with VOCs originating from mucus and saliva in the mouth. Moreover, certain VOCs produced in the nasal cavity as by-products of oral bacteria or infection can enter the oral expiratory air via the posterior nasopharynx, impacting the composition or/and level of exhaled VOCs (Sagnik Das and Pal 2020; Khoubnasabjafari et al. 2022). Hence, it is crucial to assess the salivary and nasal contaminations before interpreting altered VOCs profile, and differentiate between blood-born VOCs and those originating from saliva or nasal cavity. One of the foremost strategies to minimize these contaminations is measuring and comparing the biomarkers: (1) inspiring and expiring without a nose-clip, (2) inhaling and exhaling with a nose-clip, and (3) exhaling against a resistance to minimize nasal contamination (Khoubnasabjafari et al. 2022). Previous investigations have compared the exhaled breath from mouth, nose and air in the mouth cavity, revealing distinct origins of various VOCs. Acetone and isoprene were found to be entirely systemic in origin. In contrast, VOCs like ammonia, hydrogen sulfide, and ethanol were found to be predominantly mouth-generated. Methanol, propanol, and other VOCs exhibit partly systemic origins (T. Wang et al. 2008).

Exhaled breath sampling procedure. — The choice of the expired breath phase to be collected is a crucial factor in BGA. The expired air breath can be broadly divided into three fractions: dead space air, ascending respiratory phase, and end tidal air. Healthy human exhale around 500 ml of breath out, of which 150 ml is dead space air originating from the upper respiratory tract, including nose and mount air, as shown in Figure 2-5. Dead space air represents the volume of ventilated air that does not participate in gas exchange with the blood and therefore acts as diluent only. On the contrary, VOCs in end-tidal breath are expected to be in equilibrium with alveolar VOCs, offering potential information insight into metabolic processes occurring in the body. Therefore, it is desirable to collect this breath fraction. Various collection modes and sampling systems have been described by different research groups (Lawal et al. 2017; Miekisch et al. 2008; Westphal et al. 2023). In principle, there are three basic approaches to collect breath gas:

1. Mixed expiratory sampling (MES) that collects all phases of the expiratory air.
2. End-Tidal sampling (ETS) that collects only the end tidal air.
3. Late expiratory sampling (LES) that collect the end tidal and ascending expiratory phase.

MES , as it collects all phases of EB. However, the concentration of VOCs is lower compared to end-tidal air, due to dilution of the substance in dead space air (Miekisch, Schubert, and Noeldge-Schomburg 2004). Despite this limitation, MES can generally provide insights into overall trends of the exhaled VOCs. However, MES samples are prone to contamination from environmental conditions, as well as VOCs from the mouth and nose. Therefore, correction of the VOCs profile in MES is necessary to accurately estimate the concentration of potential VOCs.

End-tidal samples contain high concentrations of systemic VOCs and lower levels of contaminants from environment and airways. Typically, the initial 150 mL of breath, representing dead space air, is excluded from breath collection in ETS. Most studies that collect end tidal air involve flow-controlled sampling or CO_2 monitoring via a capnometer during the expiratory phase to visualize the different parts of the expiratory phase, and remove the air when the alveolar phase has been reached (Miekisch et al. 2010; Miekisch et al. 2008; Gilio et al. 2020a). As illustrated in Figure 2.5, during dead space ventilation, the CO_2 level is very low and rises rapidly during the ascending expiratory phase. CO_2 reaches the alveolar peak, known as the alveolar plateau, at the end of phase II, signaling the start of the end tidal phase. However, this sampling approach is not suitable for upper airway tract disorders, as it is expected to exclude airway VOCs. As an alternative to the offline ETS methods, IONICON has developed the Buffered End-Tidal (BET) sampling inlet system, which can be connected to PTR-MS for real-time BGA (Herbig et al. 2008).

Similar to ETS, LES involves minimizing dead space air and capturing of the late expiratory air. However, a standard practice for collecting this breath fraction has not been developed. Various strategies have been employed in breathomics studies to collect this part of the expiratory air. The simplest method involves escaping the first few seconds, before the sample is collected (Castellanos et al. 2016; Sanchez and Sacks 2006). However, time-controlled breath sampling has been shown to be unreliable due to several physiological properties of subjects, such as pulmonary ventilation (Miekisch et al. 2008). In response to this challenge, other sampling devices, such as BioVOCs, have been developed to collect the last 100 ml or 150 ml of the exhaled breath (Kwak et al. 2014).

Additionally, various breath maneuvers, such as deep inhalation and slow exhalation, forced exhalation or several exhalations, or deeply breathing through bags are employed to collect the MES. Similarly, ETS requires also subjects to take a deep breath before filling the sampling bag. These procedures could introduce additional variability in the BGA leading to a lake of reproducibility. Accumulating evidence suggests that breathing maneuvers significantly influence the level of exhaled VOCs. Increased levels of pentane, isoprene, 2-propanol,

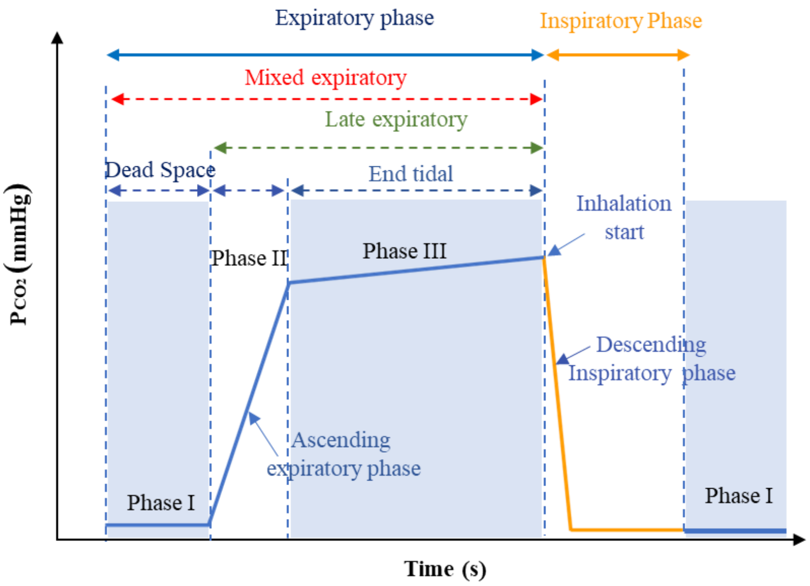

Figure 2-5| Schematic presentation of a single expiratory phase of health subjects by capnograph: *The expiratory wave consists of three parts: (1) Dead space phase represents the start of the expiration, contains of very low amount of carbon dioxide and remains relatively flat, (2) Ascending expiratory phase that presents a transition phase where dead space begins to mix with alveolar gas and the level of CO2 rises rapidly and reaches the alveolar plateau (peak), and (3) End tidal phase represents the elimination of CO2 from alveoli.*

acetaldehyde, acetone, methanol, and dimethyl sulfide were measured in breath samples taken from patients who held their breath before exhalation (Westphal et al. 2023).

Influence of storage conditions on exhaled VOCs level. — Currently, a wide range of breath sampling methods are utilized for exhaled volatomics analysis, broadly classified into online or offline sampling approaches. Offline breath sampling involves storing and transferring of breath metabolites for subsequent analysis by an analytical instrument, which may not necessarily be in close proximity to the subject. Conversely, online BGA utilizes on-site analytical devices for real time analysis of the breath. Online analysis is preferred over storing breath samples because it offers the advantage of continuous and repeated measurements in short succession, minimizing the loss of VOCs. However, offline sampling is currently the most widely implemented sampling procedure due to its simplicity. Additionally, not all breath analysis techniques support direct analysis, including GC-MS, which requires preconcentration of breath samples before analysis. Storage represents one of the crucial issues in BGA, leading to significant variability between breathomics studies. Offline sampling collection should be done with utmost care in order to prevent (1) loss of exhaled metabolites due to diffusion through the bag, (2) contamination with background pollutants, (3) influx of VOCs from the storage container materials, and (4) degradation of the sample due to chemical reactions of the sample container with the breath VOCs. Accumulating evidence suggests that water vapor content diffuses through the bag wall by extended storage, and adsorbs water-soluble compounds leading to compounds loss. Currently, there is a broad range of containers which can be used in the offline breath sampling, such as bags, glass and metal canisters, BioVOC, and ReCIVA (Harshman et al. 2020; Lawal et al. 2017; Kwak et al. 2014; Gilio et al. 2020b). Among them, Tedlar bags are the most popular way of storing breath (Marco M.L. Steeghs, Cristescu, and Harren 2007; M. M.L. Steeghs et al. 2007).

Airway versus alveolar gas exchange. — Physical-chemical properties of VOCs play an important role in their diffusion from blood to air. Gas exchange occurs either in the airways of the lungs or in the alveoli, depending on the solubility of the gas, described by the blood-air partition coefficient $\lambda_{b:a}$ (Anderson and Hlastala 2007). Highly soluble VOCs ($\lambda_{b:a} > 100$) such as acetone, isoprene, and ethanol ($\lambda_{b:a} = 1756$) exchange in the airways, whereas low-soluble gases ($\lambda_{b:a} < 10$) like oxygen ($\lambda_{b:a} = 0.7$) and carbon dioxide ($\lambda_{b:a} = 0.3$) exchange in the alveoli. VOCs with intermediate solubility, ranging between 10 and 100 undergo pulmonary gas exchange in both the alveolar and airways regions (Wilson 2015; Anderson, Babb, and Hlastala 2003; Kaloumenou et al. 2022). The measurement of less soluble VOCs in exhaled breath is easier. During expiration, soluble gases are redissolved in the mucus layer coating the airway, and diluted along the respiratory tract. Consequently, the concentration of these gases is reduced in the exhaled breath. Therefore, it is suggested to hold the breath for 10 seconds before exhalation. However, the measurement of highly soluble VOCs present challenges, as an anatomic dead-space cannot be defined for such compounds. On the other hand, during inspiration soluble gases are absorbed by the inhaled air in the airway. By the time the air reaches the alveoli, it is already saturated with soluble gases, resulting in no exchange occurring in the alveoli. As a result, for accurate quantification of the VOCs levels, airways exchange may play a significant role in the pulmonary gas exchange, and should be considered in the BGA for accurate quantification of VOCs levels.

Physiological and biological parameters. — The biological variance, encompassing physiological factors such as blood pressure, heart rate, alveolar ventilation, and physiological mechanism of pulmonary gas exchange, is a significant source of bias in the breathomics data interpretation (Lourenço and Turner 2014). Minimizing the inter-subject variability can be achieved by ensuring the homogeneity in characteristics among selected groups or subgroups, taking into account factors such as age, BMI, and gender. Intra-subject variability factors include the effect of respiration on tidal volumes, breathing frequency, airflow rates, age-dependent effects, and circadian influence. For example, several studies reported significantly elevated level of exhaled isoprene in the breath of adults as compared to children and during puberty, as well as in males compared to females (Lourenço and Turner 2014; Kushch et al. 2014). The circadian rhythm also plays an important role in the intra-variability. Indeed, previous studies reported a change of isoprene level over the day with a maximum at around 6 a.m. and a minimum at about 6 p.m. (Kushch, Arendacká, et al. 2008). Moreover, studies reported that ovulation affects the volatomics of urine headspace (Smith et al. 2006) . The acetone was significantly increased over the normal. This finding may be caused by metabolic changes occurring during the ovulation and menstrual cycle. However, to our knowledge, no alteration of exhaled VOCs profile during ovulation, menstrual cycle, or pregnancy has yet been reported.

Analytical instrumentation-specific parameters. — In addition to biological variance, technical errors pose significant challenges in large-scale breathomics studies. These errors primarily stem from devises drift, or the use of different analytical instruments, which generally offer varying sensitivity, specificity, resolution, analytical mass range, limit of detection (LOD) and ionization processes, as detailed in section 2.2.6. MS-devises are widely used in metabolomics studies. However, missing values caused by technical and biological factors are widely occurrent in MS, ranging from 10% to 40%, which can affect up to 80% of all metabolites (Wei et al. 2018), (B. Li et al. 2016), (Xia et al. 2009). Missing values that occur unexpectedly due to random errors and stochastics fluctuations during data acquisition processes, such as incomplete derivatization or ionization, are called missing completely at random (MCAR). Another source of missed value is due to LOD of the used instrumentation. This type of missing value is called missing not at random (MNAR) error source, and is widely produced by MS. Missing values can reduce the statistical power, decreasing the ability to detect significant biological differences between the groups. They can also distort data patterns, affecting multivariate analysis, and introduce bias in data interpretation. Various imputation strategies have been proposed to correct MNAR missing values. When the occurrence rate of missing values below 20% of the samples, these values can be replaced with small values, zero, half of the minimum value, or the mean. The impact of missing values on data quality, interpretation, and downstream statistical analyses depends on their nature and the handling methods used.

Statistical uncertainty. — Additional sources of variability in the BGA can be introduced by suboptimal statistical analysis of breathomics data. The uncertainty analysis becomes particularly critical due to the high heterogeneity of metabolomics data, impacting the degree of confidence in the results and precision of prediction models (Moseley 2013). Thus, the accuracy of BGA study can be influenced by various analysis procedures at different phases of the study, including spectral processing, feature extraction and validation. Inadequate sample size selection in the population sample, inaccurate raw data pretreatment, data preprocessing, excessive false discovery rates (FDR) due to the multiple hypothesis testing, inappropriate choice of computational methods, and failure to perform adequate validation and cross-validation, as well as inadequate population stratification, can all impact the confidence level of the results. Ensuring correct statistical power is crucial, particularly in breathomics studies characterized by numerous variables that greatly exceed the number of collected samples. It is important to consider that the power by through-put measurements is different from single measurements. Missed values by data acquisition can additionally be introduced because of inaccurate data preprocessing such as inaccurate peaks detection and deconvolution. Various statistical tools such as fillPeaks or XCMS have been proposed to correct the missed value. However, these methods may not necessarily correct the concentration of the compounds accurately. Further, adequately chosen pretreatment methods including centering, scaling, and non-linear transformation of spectral data can drastically improve the biological content of the large breathomics data (van den Berg et al. 2006). Additionally, the choice of robust statistical methods, such as supervised machine learning classification models or deep learning approaches, play a critical role in the accuracy of the breathprints.

2.2.6 Analytical technologies for exhaled VOCs analysis

Technological advancement has significantly contributed to the progress of BGA. A wide range of analytical instrumentations with varying sensitivity, precision, resolution, and operating modes (e.g., online or offline) are now available for investigating human breath gases (Souvik Das, Pal, and Mitra 2016; Moura, Raposo, and Vassilenko 2023; Mazzatenta, Giulio, and Pokorski 2013b; Mule, Patil, and Kaur 2021). Currently, GC coupled to mass spectrometry (MS) is an offline method that provides accurate quantification and identification of breath VOCs. GC-MS has emerged as a gold standard analytical method widely used for breath biomarkers research and screening purposes. Alongside offline GC-MS, online analytical techniques provide an alternative class of approaches for real-time detection and analysis of breath VOCs, allowing for a more rapid detection of VOCs than GC-MS. These online methods are based on electrochemistry, chemical sensors, optical spectrometry, or mass spectrometry methods (Sharma, Kumar, and Varadwaj 2023). The direct MS instrumentations include the highly sensitive proton transfer reaction-mass spectrometry (PTR-MS) (Pleil, Hansel, and Beauchamp 2019; Hewitt, Hayward, and Tani 2003), proton transfer reaction time of flight–mass spectrometry (PTR-TOF-MS) (Portillo-estrada 2014; Jordan et al. 2009), selected ion flow tube-mass spectrometry (SIFT-MS) (Smith and Španěl 2005; Španěl and Smith 2016). These methods involve the direct introduction of breath samples into analytical instruments, negating breath sample storage and pre-concentration prior to the analysis, exhibiting a limit of detection ranging from ppbv to pptv. These devices can also operate in offline mode. However, the sample must be captured in bags of varying volumes, stored, and transported to the analytical devices. Despite the clear advantage over offline techniques, online technologies have limitations. These include the detection limits that cannot be enhanced by pre-concentration of breath samples, the inability to identify compounds with high fidelity due to the absence of chromatographic separation, the high costs of the instrumentations, and the need for highly skilled operating personnel.

To reduce production costs, novel portable sensors integrated into electronic noses (E-Nose) for BGA in medical point-of-care diagnosis have become an emerging field. Portable MS instrumentations such as field asymmetric ion mobility spectrometry (FAIMS), and linear IMS (Fernández Maestre 2012) offer high sensitivity. While no single analyzer is optimized to detect all exhaled VOCs, each technique has specific strengths and weaknesses, as summarized in Table 2.1. The choice of the suitable analyzer for BGA depends on various factors such as the analytical requirements, target compounds, and experimental conditions of the application.

Therefore, it is important to understand the different principles, key features and characteristics of the analyzers and choose the one suitable for our research application. The present section provides a short overview of the most utilized analytical devices in BGA, with a special focus on the PTR-MS used in the present study.

Gas chromatography-Mass spectrometry. — GC-MS is currently the most commonly applied offline analytical technique to detect and identify trace VOCs in the exhaled breath. The GC unit separates the individual molecules in a breath sample by heating a mixture of molecules, as illustrated in Figure 2-6. The vaporized molecules are carried by a carrier gas (e.g., helium or nitrogen) through a long and thin column that separate them based on the strength of the interaction between the molecules and the column. Depending on their chemical properties, the molecules will take different amounts of time to pass through the column, known as retention times. In a subsequent step, the molecules are transferred to the mass spectrometer, which consists of a source, an analyzer, and a detector. The source promotes the ionization of the incoming neutral molecules by emitting a stream of electrons, and breaking them into fragments. The analyzer then separates all metabolites and identifies each one by its mass-to-charge (*m/z*) ratio, and a detector registers the relative number of counts per hit. The pattern of resulting fragments acts as a highly specific “fingerprint” that can be used to identify the chemical. The GC-MS device identifies individual VOCs according to their retention times, and total mass spectrum by comparing their accurate masses in a spectral chemical database such as HMDB (*https://hmdb.ca*), or NIST (*https://webbook.nist.gov/chemistry*).

Despite being highly sensitive and producible, GC-MS has limited application in clinical settings due to its expense, complexity, and the need for highly experienced analysts to operate the equipment and interpret the results. Additionally, the requirement for pre-concentration before analysis, which prolongs analysis time, represents an important

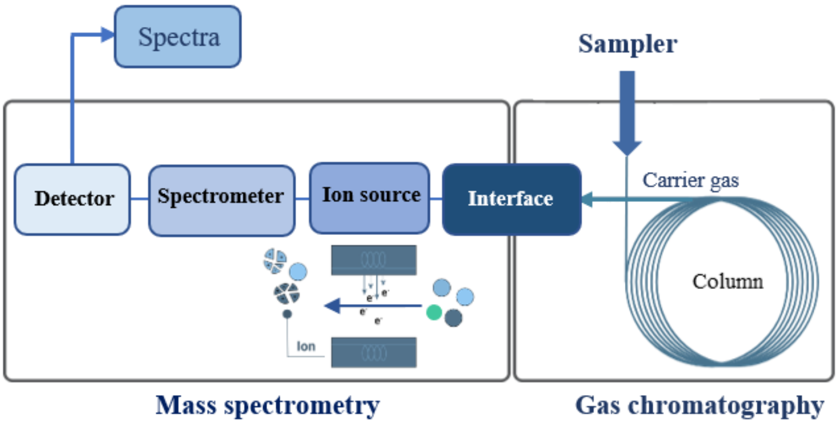

Figure 2-6| Schematic illustration of the principle of the gas chromatography-mass spectrometry: *GC-MS separates the ions in a breath sample by heating mixture of molecules. The vaporized molecules are carried by a long and thin column that separate them based on the strength of the interaction between the molecules and the column. Depending on their chemical properties, the molecules will take different amounts of time to pass through the column, known as retention times. In a subsequent step, the molecules are transferred to the mass spectrometer, which consists of a source, an analyzer, and a detector. The ionization is based on high energy electron ionization that typically led to the fragmentation of the molecule of interest.*

disadvantage of chromatographic techniques, as rapid changes in VOCs profiles cannot be monitored by means of GC-MS.

Proton transfer reactions Mass spectrometry. — PTR-MS is a direct MS technique used for online analysis of VOCs across various fields, such as atmospheric chemistry, food science, and health sciences (Pleil 2008; Ellis and Mayhew 2014; Hewitt, Hayward, and Tani 2003; Jordan et al. 2009). It provides fast response times and low detection limits down to pptv range. PTR-MS operates in both techniques, quadrupole (PTR-QMS) and time-of-flight (PTR-TOF MS), utilizing a chemical ionization process that allows a very efficient ionization for many organic compounds in gas phase, followed by high-resolution real time mass spectrometry. The ionization mechanism is based on the transfer of protons from reagent hydronium ions H_3O^+ to a trace gas molecule R, resulting in a protonated and ionized molecule RH^+ and a water molecule H_2O:

$$H_3O^+ + R \rightarrow RH^+ + H_2O \qquad \textbf{(2.4)}$$

This chemical ionization is known as soft ionization technique arising from ion-molecule reactions rather than electron impact or photoionization. This mechanism produces minimal excess energy, resulting primarily in parent ions RH^+ with significantly less fragmentation of molecules compared to GC-MS. In contrary to PTR-MS, in GC-MS high energy electron ionization typically led to extensive fragmentation of the molecule of interest. However, ion fragmentation is still observed for certain classes of compounds, complicating spectral analysis as well as absolute quantification of VOCs. Furthermore, it is important to note that only VOCs with a higher proton affinity (PA) than water (691 kJ/mol) can be ionized by proton transfer using H_3O^+. To detect VOCs with lower PA than water, alternative reagent ions, such as the nitrosonium ion NO^+, the dioxygenyl ion O_2^+, and NH_4^+, have been successfully introduced. Additionally, PTR-MS can detect a wide range of VOCs, including alcohols, aldehydes, ketones, sulfur compounds, nitriles, esters, and acids. Most exhaled VOCs have a PA higher than water, and can therefore be ionized by the proton-transfer reaction (PTR) using H_3O^+. Furthermore, the air compounds occurring in high concentration in exhaled breath, such as N_2, CO_2, O_2, H_2O do not interfere with measurements. This is because they have lower PA (~176–594 kJ/mol) than water and cannot be ionized using H_3O^+. Additionally, samples containing low analyte concentrations are not diluted with a carrier gas by PTR-MS between ion source and drift tube, preventing loss of reagent ions. This makes PTR-MS very sensitive to trace gases.

The quadrupole MS contains four parallel cylindrical metal rods inside a vacuum chamber, as illustrated in Figure 2-7. A direct current and a high frequency alternating current are applied to the quadrupole, so that only the ion with the target m/z successfully pass through the quadrupole into the detector. PTR-QMS separates ions based on the stability of their trajectories in the oscillating electric field (Sudakov and Apatskaya 2012; Brabeck and Reilly 2016). Non-resonant ions are discarded, and resonant ions are transmitted into the detector. The oscillation of ions within the quadrupole occurs according to the Mathieu equation parameters:

$$\frac{m}{z} = K\frac{v}{r^2\omega^2} \qquad \textbf{(2.5)}$$

where m, v, r, and ω are the mass of the ion, voltage applied, effective distance between quadrupole electrodes, and oscillation frequency respectively, and K is a constant. The stability of ion oscillations in quadrupole is determined by solving the Mathieu equation.

PTR-MS is highly sensitivity down to concentrations about ppt level, and capable of measuring compounds in the mass range between 21 and 200, without the need for molecules pre-concentration. PTR-MS is considered as one of the fastest analytical techniques with a time resolution lower than 100 ms, and a full analysis is achieved in time scales from 100 ms to a few seconds depending on the number of compounds in targeted or untargeted mode. Therefore, PTR-MS is well suited for real-time metabolites profiling. It is particularly suited for monitoring of rapid changes in VOCs profile induced by physiological conditions, or highly reactive compounds that could be lost in the sampling and chromatographic step by GC-MS. However, the linear quadrupole mass spectrometer characterizes VOCs according to their mass-to-charge ratio (*m/z*) without chromatographic separation. Hence, isomers[3] and even isobars[4] could not be distinguished at all, requiring additional methods or software for VOCs identification. In order to improve the detectability of isobaric compounds, PTR-TOF-MS was developed, utilizing high-resolution mass analysis (Jordan et al. 2009; Pleil, Hansel, and Beauchamp 2019)**.** Unlike PTR-QMS, which measures only one *m/z* at a time, PTR-TOF MS provides a continuous mass spectra by measuring the time that the molecules take to fly a known

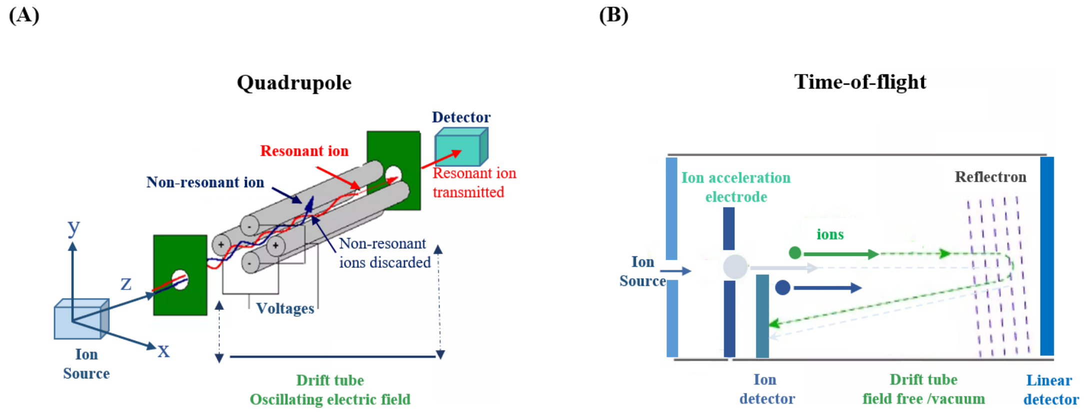

Figure 2-7| Schematic comparison between ion separation by mass spectrometers of PTR-QMS and PTR-TOF MS: *(A) The Quadrupole PTR-MS (PTR-QMS) contains four parallel cylindrical metal rods inside a vacuum chamber. A direct current and a high frequency alternating current are applied to the quadrupole, so that only the ion with the target m/z successfully pass through the quadrupole into the detector. PTR-QMS separates ions based on the stability of their trajectories in the oscillating electric field. Non-resonant ions are discarded, and resonant ions are transmitted into the detector, (B) PTR-MS Time-of-flight mass spectrometer (PTR-TOF MS) consists of an accelerator, a field free region, a reflectron and detector inside a high vacuum chamber called a flight tube. Linear TOF-MS separates and detects ions of different m/z ratios by measuring the time taken for the ions to travel through a field-free flying tube.*

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[3] Isomers are molecules with the same formula but different chemical structures

[4] Isobars are molecules with the same nominal mass

distance accelerated by a constant voltage, as well as a constant kinetic energy (Portillo-estrada 2014). Therefore, compounds with different molecular masses reach the detector at different times, enabling the simultaneous measurement of all compounds in the drift tube. Ions of different masses within a cycle arrive at the detector at different time according to their kinetic energy:

$$KE = m\frac{\vartheta^2}{2} = z.e.V \qquad \textbf{(2.6)}$$

where z, e, ϑ, V are the charge of the ion, elementary charge, velocity of the ion, and acceleration voltage applied to the ions respectively.

For a fixed flight distance L, the time-of-flight T_f of the ion can be directly converted into its m/z ratio:

$$T_f = \frac{L}{\vartheta} = \sqrt{\frac{m}{z}}\frac{L}{\sqrt{2eV}} \qquad \textbf{(2.7)}$$

allowing for precise determination of the exact mass of individuals ions within 0.001 Da. Accordingly, ions with smaller *m/z* reach the detector sooner than those with larger *m/z*. However, the separation of isomers remains challenging. To overcome this limitation, fast-GC has been developed by Ionicon Analytic for a pre-processing step prior to the PTR-MS analysis (Pallozzi et al. 2016).

Ion mobility spectrometry (IMS). — IMS is another analytical technique that separates ionized molecules in gas phase based on their mobility (W. Jiang and Robinson 2013; Dodds and Baker 2019). The principle of IMS involves the injection of the ions into a drift tube filled with an inert buffer gas at either vacuum or atmospheric pressure. Under the influence of the applied weak electrical field *E*, the ions move through the drift tube with different velocities *v* correlated to their mobility *K= v/E* in the buffer gas. The ions are then separated by their difference in gas-phase mobility through either space or time, depending on the IMS approach used. Smaller ions, being more mobile, travel faster with higher velocity in a specific electric field strength than larger, less mobile ions. Further, IMS is relatively fast, with ions typically travelling through the drift tube in the order of milliseconds (Fernández Maestre 2012).

IMS can be utilized for selective detection of ions after a chromatographic separation, for pre-separation of ions before mass spectrometry, or as a stand-alone analytical device. Combinations of IMS and MS (IMS-MS) have been considered powerful hybrid analytical devices in a diversity of biological, pharmaceutical, and environmental applications. Various IMS-MS configurations are available, such as high FAIMS. The Lonestar recently developed by Owlstone Medical Ltd (Cambridge, UK), is a FAIMS used for profiling and identification of VOCs collected from exhaled breath (Arasaradnam et al., 2013). IMS has the potential for miniaturization and provides complementary separation of isomers by utilizing differences in mobility to resolve these analytes.

Selected ion flow tube mass spectrometry (SIFT-MS). — Similar to PTR-MS, SIFT-MS is an analytical technique for real-time analytical of volatile trace gases (Smith and Španěl 2005), and is used in medical application. SIFT-MS is based on a chemical ionization of trace gas molecules using positive precursors including H_3O^+, NO^+, or O_2^+ generated in an ion source. The ion-molecule reactions generate characteristic ions corresponding to the target VOCs in a sample, which are subsequently mass-sorted and counted by a downstream detector. Further, SIFT-MS can recognize a wider range of compounds than PTR-MS, and separate isomers by switching between three precursors ions. Additionally, compared to PTR-MS, SIFT-MS does not use an electrical field to accelerate the velocity of the ions along the flow tube, the ions are injected into a fast-flowing inert carrier gas, usually pure helium at a pressure of approximately 100 Pa. SIFT-MS allow the detection of trace ions with an absolute concentration down to ppbv levels. Further, SIFT-MS has a lower sensitivity than PTR-MS due to the existence of a mass filter, which selects the precursor ions to be used according to their mass-to-charge ratio.

Electronic nose technology. — The electronic nose (eNose) technologies applied in the BGA are based on the principle of identifying smellprint, similar to how the human and animal olfactory systems work. The mammalian olfactory system utilizes various olfactory receptors, which are then processed by an automated pattern recognition system incorporated in the olfactory bulb and brain. No single receptor can identify a specific odor. Similarly, the identification of pathophysiological conditions by eNose occurs through the recognition of smellprint using pattern recognition algorithms, rather than the identification of single VOCs (Huang, Doh, and Bae 2021; Keller et al. 1995). The eNose device generally consists of a chemical sensing system (array of sensors or spectrometer) programmed to recognize different odors or gases, and a pattern recognition system using a database with pre-programmed signatures. The sensing system responds to specific characteristics of odorant molecules, and produce a characteristic pattern or signature that is specific to each breath sample. The database

Table 2.1| *Summary of analytical techniques used currently for breath gas analysis and their characteristics*

Method	**Detection Limit**	**Operation Mode**	**Advantages**	**Disadvantages**
GC-MS	ppt - ppb	Sample preparation	Highly separation efficiency Ability to identify complex mixture Widely used for VOC analysis	Time-consuming Complex operation Poor portability
PTR-QMS	Low ppb	Direct/ Real-time	High sensitivity Medium sensitivity	High cost Complex operation No identification of isobaric
PTR-TOF MS	ppt - ppb	Direct Real-time	High selectivity Fast response time Continuous monitoring	High cost Complex operation Water/background gases interference Limited ability to differentiate isomers
SIFT-MS	ppt - ppb	Direct/ Real-time	No sample preparation No interference in humid air Quantitative analysis	Limited availability High cost Lower resolution
IMS	ppm - ppb	Real-time	Portable Rapid analysis with fast response	Limited resolution and specificity Lower sensitivity Water/background gas interference
E-Noses	ppm - ppb	Real-time	Analysis of complex mixture without gas separation. Easy to use and portable. Cost-effective fast operational times	Limited specificity No identification of individual VOCs Lower sensitivity compared Sensor drift and frequent calibration

of labeled signatures is built by training the sensors using different chemicals allowing for automated pattern identification (Keller et al., 1995). ENose devices have been applied to a wide range of applications, including food quality assessment, environmental exposures monitoring, and medical diagnostics. These techniques successfully discriminate between the smellprint of healthy subjects and those of patients with respiratory tract diseases and lung cancer (Farraia et al. 2019). ENose devices are easy to use, fully automated, and portable, with relatively quick operational times and relatively low technical costs. However, these analytical approaches face several technical challenges that have hindered their introduction into the clinical setting. These challenges mainly include loss of sensitivity in presence of water and temperature, sensor drift, baseline variation, as well as the high impact of environmental factors on the breath signature. Further, the inability to identify individual compounds in complex mixtures make the identification of the altered metabolic processes and mechanistic pathways difficult.

2.3 Major Depressive Disorder

Providing accurate and early screening, diagnosis, early treatment and follow-up care is essential for achieving better outcomes in individuals with MDD. Unfortunately, many individuals suffering from MDD often wait several years before seeking help. Subjective screening and diagnosis can result in either over- or underestimation of MDD. Underdiagnosing of MDD may lead to delayed treatment, while overdiagnosis can result in unnecessary medication use. In this section, an overview on the epidemiology, etiology, pathophysiology, diagnosis, and treatment strategies of MDD is provided.

2.3.1 Diagnosis and management of major depression

Diagnosis of major depression. — Nowadays, it is still challenging to diagnose MDD with an objective and quantitative method. The diagnosis is currently based on the subjective evaluation of depressive symptoms and behavior of patients. The most commonly used manuals by psychiatrist and health care professionals are the International Classification of Diseases (ICD), and the Diagnostic and Statistical Manual of Mental Disorders (DSM) that are based on a set of criteria for diagnosing depression. Both manuals have been revised several times. Currently, the ICD is in its eleventh edition ICD-11 (Harrison et al. 2021), while the DSM is in the fifth edition DSM-5 (M. Marty and D. Segal 2017). This section emphasizes the DSM-5 criteria for diagnosing MDD. According to the DSM-5, MDD is defined by the occurrence of at least one discrete depressive episode lasting a minimum of two weeks, characterized by significant changes in mood, interests, pleasure, cognition, and physiological functions. A diagnosis of MDD is established when a patient exhibits at least five of the following core symptoms nearly every day for at least two weeks:

- Depressed mood
- Diminished interest or pleasure (anhedonia)
- Significant unexplained weight loss or gain, or change in appetite
- Insomnia or hypersomnia
- Recurrent thoughts of death (suicidal ideation or attempt)
- Psychomotor agitation or retardation
- Fatigue or loss of energy
- Feelings of worthlessness or guilt
- Impaired ability to think, concentrate or make decisions

At least one of these five symptoms must be either depressed mood or anhedonia. Furthermore, the physician must ensure that the episode is not attributed to another medical condition or physiological effects of substance use. Therefore, a differential diagnosis MDD should be considered to rule out neurological and psychological conditions with symptoms overlapping with those of MDD. Clinicians should also pay attention to organic conditions that can coexist or masquerade as MDD and should exclude these as potential causes (Otte et al. 2016). For example, thyroid dysfunction may manifest low mood. MDD is a heterogenous disorder and could accordingly be considered as a group of diseases with distinct causes, pathophysiology and symptologies. Therefore, no classification of depression subtypes has been universally accepted. However, atypical and melancholic MDD are the most accepted subtypes.

Assessment of the severity of major depression. — The severity of depression significantly impacts therapy management, including decisions about medication, psychotherapy, or the frequency of follow-up visits. Depression severity assessment is conducted by various psychometric tests. The self-rating Beck Depression Inventory-II (BDI-II) (Hailu Gebrie 2018), and the observer-rating scale 17-items Hamilton Rating Scale for Depression (HAMD-17) , (Endicott et al. 1981) are used in hospitals to assess the severity of this disorder.

BDI-II is one of the most commonly used scales to assess the severity of depression experienced during the preceding two weeks. It consists of 21 questions that measure cognitive, affective, and somatic components of depression. The test is designed for individuals aged thirteen and older. The total score of the BDI-II range between 0 to a maximum of 63 points. Interpretation of the total score varies according to different recommendations; e.g., BDI-II values below 13 points are regarded as no or minimal depressive symptoms. Values between 14 and 19 points indicate a mild expression of depressive symptoms, values between 20 and 28 points indicate a moderate severity, and scores between 29 and 63 are regarded as evidence of severe depressive disorder.

HAMD-17 is the most widely used clinician-administered depression assessment scale. It is often used alongside the BDI to ensure an accurate assessment of the severity of depression. HAMD-17 is a multiple-choice questionnaire with 21 questions aimed at detecting core symptoms of depression, including depressed mood, loss of interest, feelings of guilt, psychomotor retardation, insomnia, weight change, suicidal tentation, and impairment of personal functioning. Only 17 questions are used for scoring in HAMD-17. The total score can range from 0 to 54. Interpretation of the total score varies according to different recommendations; e.g., scores below 7 are regarded as normal or indicative of remission, values between 8 and 13 indicate mild depression, scores between 14 and 18 indicate moderate depression, and values greater than 19 are regarded as severe depression.

Management of major depression. — Several clinical guidelines are available to aid the physician in managing patients with MDD. Their recommendations aim to achieve and maintain remission of symptoms. Management of MDD includes treatment options such as psychotherapy, pharmacotherapy, social treatment, and lifestyle changes, including identification and dealing with source of stressors (Ng, How and Ng, 2017; Otte et al., 2016; Remes, Francisco, and Templeton 2021). Guidelines recommend that for patients with mild to moderate depression psychotherapy is the first-line treatment, while for patients with moderate-to-severe depression, a combination of medication and psychotherapy is recommended.

2.3.2 Mechanisms and pathophysiology of major depression

MDD is the result of a complex interaction between biological, psychological, and socio-environmental factors. Despite extensive biological research, no conclusive evidence for any specific etiological theory of this disorder has yet been provided. Additionally, the interaction between socio-environmental and biological factors is far from complete. In this section, the well-established socio-environmental, biological, and psychological determinants of MDD, as well as the well-known interactions between them, are summarized. We focus on the pathophysiological determinants of MDD that are supported by the finding of clinical studies, giving preference to aspects that have been confirmed by meta-analyses.

2.3.2.1 Environmental and social factors

The outcome and risk of developing MDD are strongly associated with socio-environmental factors that can be broadly classified as: (1) demographic factors (e.g., age, gender and ethnicity), (2) socio-economic status (e.g., financial insecurity, loss of employment, and low education), (3) neighborhood factors (e.g., inadequate and overcrowded housing, or neighborhood violence), (4) socio-environmental events like wars, natural and catastrophic disasters, migration, and (5) lifestyle factors such as alcohol consumption, smoking, physical inactivity and a high-sugar or high-fat diet (Remes, Francisco, and Templeton 2021), (Otte et al. 2016). The association between MDD and the determinants preceding its onset has been ascertained by several epidemiological studies. Various studies have shown an inverse association between the prevalence of MDD and the level of education. A study based on data from the European Health Interview Survey for 22 OECD/EU countries, Iceland, Norway and Turkey reported that the higher the educational attainment, the lower the self-reported depression (OECD 2017; OECD 2018). Additionally, gender-related factors have been recognized to play a critical role in the predisposition to MDD. Women are at a higher risk of developing depression than men. There is a body of evidence supporting the fact that this susceptibility to depression is affected by diverse hereditary, epigenetic, environmental, and endocrine risk factors (Zhao et al. 2020; Duman et al. 2016).

In addition to the determinants preceding the onset of MDD occurring in adulthood, recent research has focused on the association between MDD and stressful life events in childhood as antecedent of MDD later in life. These events typically comprise domestic violence and physical, sexual, or emotional abuse. Individuals who experience these stressful events during childhood are at a higher risk of developing depression in adulthood (Shapero et al. 2014; LeMoult 2020). Interestingly, environmental factors occurring early during gestation have been found to play critical roles in the development of various neuropsychiatric disorders, including MDD (Saavedra et al. 2016). Intrauterine conditions during this phase can have long-term effects in terms of risk of neurological or psychiatric disorders, which may be mediated through epigenetic modifications such as DNA methylation.

Large-scale epidemiological studies have shown that stressors activate immune-inflammatory, oxidative and nitrosative stress pathways that play a key role in the onset and progression of MDD, as well as its co-occurrence with other disorders (Maes, Noto and Brietzke, 2015; Calcia et al. 2016). The immune system responds to stressors and communicates with CNS through several mechanisms, including cytokine signaling, vagal innervation, and the lymphatic system (Otte et al., 2016). Previous research indicated that stressful life experiences are associated with elevated levels of proinflammatory cytokines, and depressed individuals who experienced stressful events in their childhood exhibit a stronger inflammatory response (Nobis, Zalewski, and Waszkiewicz 2020). Additionally, there is consistent evidence that mental stress can lead to neuroinflammation, especially elevated microglial activity in the hippocampus and other regions of the brain (Calcia et al. 2016). Furthermore, epidemiology research consistently reported gender difference immune response to psychological stress (Maes et al. 2009; Prather et al. 2009).

Recently, environmental, electrical and noise pollutants in combination with individual states (e.g., genetics, diseases, diets, etc.) have become susceptible factors in increasing the risk of depression (Iyer and Khan 2012), (Borroni et al. 2022). Growing evidence showed that pesticides and synthetic chemicals added to our foods (e.g., preservatives, additives, and hormones), as well as air and water pollutants, are closely linked to depression (L. Zheng et al. 2020). Nano-size particulate matter and gaseous pollutants have been epidemiologically associated with neurotoxicity, inflammation, and oxidative stress in the brain, which can significantly affect mental health and lead to depression manifestation (Ali and Khoja 2019; MohanKumar et al. 2008; Elder et al. 2006). Meta-analyses reported an increased risk of depression associated with long-term exposure to different type of pollutants, including nano-size particulate matter with different diameter and gaseous pollutants such as nitrogen dioxide, ozone, carbon monoxide, and sulfur dioxide (Borroni et al. 2022). Additionally, electrical noise including radio waves have been found to induce depression and aggression, although the causes remain poorly understood. Moreover both acute and chronic exposure to a high level of ambient noise stressors, such as workplace noise, contribute to sleep disorders, aggression, panic attacks, forgetfulness, health problems, and can increase susceptibility to emotional difficulties in children, anxiety, depression and even suicidal ideation (Iyer and Khan 2012; Beutel et al. 2016; Yoon et al. 2014). Other research reported that exposure to noise pollution causes emotional stress, neurodegeneration, cognitive impairment, and oxidative stress in the brain (Jafari et al. 2019). In the light of this evidence, the mechanisms underlying the association between the different type of pollutants and depression are not fully understood and necessitate further investigation.

2.3.2.2 Psychological factors

There is evidence suggesting that psychological factors may predispose individuals to depression and play an important role as possible causes for depression. Several studies and reviews indicated that cognitive processes, dysfunctional thinking style and personality traits increased the risk to develop MDD (Carter and Garber 2011; Fu et al. 2021). A wide range of psychological factors have been linked to depression in a large number of studies and reviews, such as mastery, self-esteem, pessimism, negative self-image, neuroticism, sensitivity to rejection, negative thinking, insight, cognitive fusion, emotional clarity, rumination, dysfunctional attitudes, low emotional clarity, interpretation bias, maladaptive perfectionism, and body image issues (Remes, Francisco, and Templeton 2021; Struijs et al. 2021). Nevertheless, no consistent evidence supported etiological theories of psychological determinant for onset of MDD. A systematic review and meta-analysis of prospective studies investigated the psychological factors derived from the different psychological theories that explain the onset of MDD (e.g., behavioral, cognitive, personal-based, psychodynamic theories, and diathesis–stress) showed that personality traits and the cognitive processes predict the onset of MDD (Fu et al., 2021).

2.3.2.3 Biological Factors

Several biological aspects have been hypothesized to play a potential role both in the etiology and progression of MDD. As summarized in Figure 2-8, these mechanisms include genetic and epigenetics factors, physical health conditions, alteration in the gut microbiome diversity and , activation of inflammatory pathways, induction of oxidative and nitrosative damage, dysregulation of HPA axis, activation of the kynurenine pathway, neurotoxicity, neurodegeneration, impairment of neurogenesis and neuroplasticity and imbalance of neurotransmitters, (Brigitta 2002; Verduijn et al. 2015). However, several studies reported that the association between these mechanisms and the clinical progression of the disorder is still inconsistent and unclear. The clinical staging may not necessarily be characterized by more advanced pathophysiological dysregulation (Verduijn et al. 2015), or may only be involved in

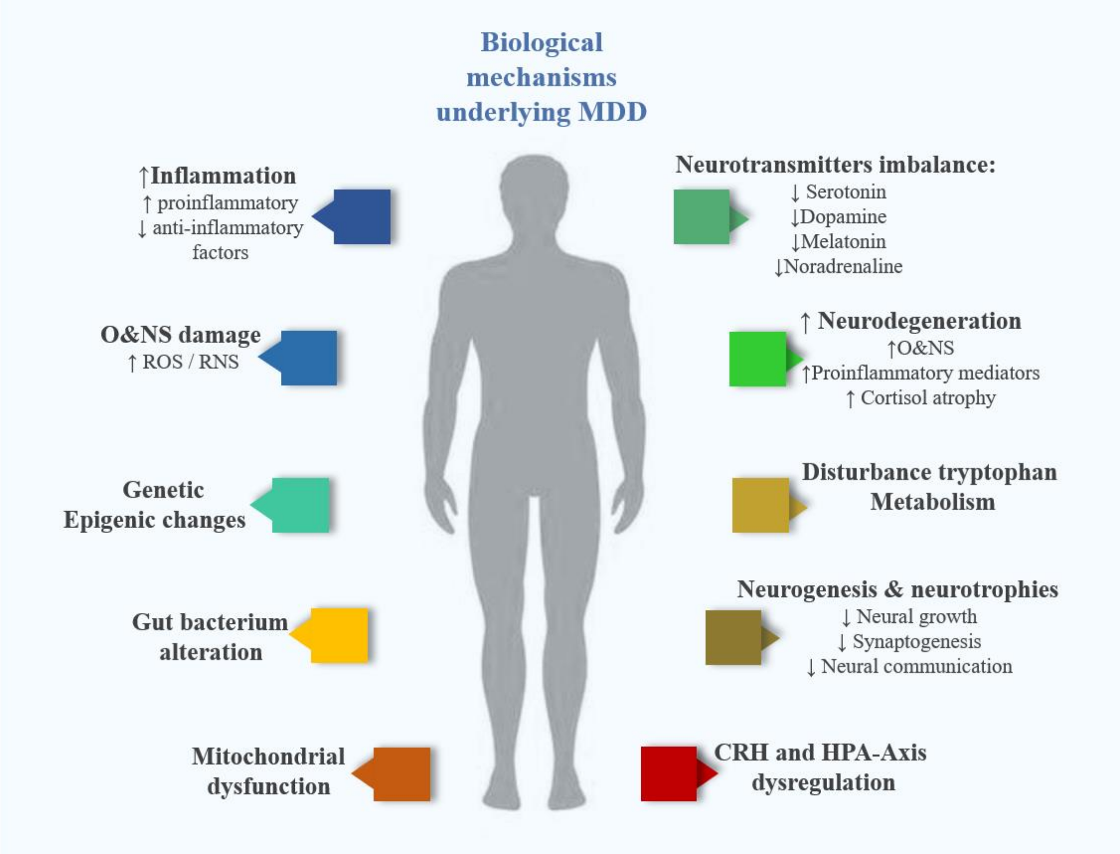

Figure 2-8| Biological factors and mechanisms underlying the pathophysiology of major depressive disorder: *Several biological mechanisms have been hypothesized to play an important role both in the etiology and progression of major depressive disorder (MDD). These include the contribution of the genetic factors and epigenetic changes as result of environmental-genetic interactions, imbalance in neurotransmitters (e.g., deficiency in serotonin, dopamine, melatonin, noradrenaline, etc.,), increased inflammation, elevated oxidative and nitrosative damage, alteration of gut bacterium and their breakdown products, impairment of neurogenesis, increased neurodegeneration, increased production of tryptophan catabolites along the tryptophan/Kynurenine pathways that are toxic for the brain, activation of corticotrophin-releasing hormone and hyperactivity of the HPA-axis, low levels of brain-derived neurotrophic factor as an indicator of reduced neurotropic growth, mitochondrial damage (e.g., size, structure, function, etc.), and decreased brain mitochondrial energy generation that influence the neurogenesis and cell survival. Abbreviations: O&NS: Oxidative and nitrosative stress; ROS: Reactive oxygen species; RNS: Reactive nitrogen* species; *HPA: hypothalamic-pituitary-adrenal, CRH: Corticotrophin-releasing hormone.*

certain subtypes or in patients with specific characteristics (Moylan et al. 2013). On the other hand, it is well-established that the presence of certain chronic conditions or mental disorders can increase the risk of developing depression, as previously noted (Otte et al. 2016).

Genetic and epigenetic factors. — Epidemiological studies have found that MDD tends to cluster within families. A meta-analysis of five twin studies, including more than 21,000 participants, suggested that genetic contributions to MDD account for 37% (95% CI: 31% - 42%) of the susceptibility to MDD relative to the general population. Additionally, evidence from family studies indicated that first-degree relatives are at a 2.84-fold (95% CI: 2.31 - 3.49) increased risk of developing depression during their lifetime (Sullivan, Neale, and Kendler 2000). Furthermore, the heritability of depression has been found to increase up to 70% when considering the disease severity (Saavedra et al. 2016). Several potential candidate genes have been associated with MDD (Hodgson and Mcguffin 2012; Saavedra et al. 2016). However, in Genome-Wide Association Study, no specific genetic variants have been identified as robust contributors to MDD despite the large population sizes. The lack of consistency and reproducibility of this study findings has been supported by the theory that genetics variants confer an increased risk of developing depression only in the presence of environmental stress stimuli, the so called environmental-genetic factors interactions (Klengel and Binder 2015).

Furthermore, previous investigations reported that environmental-genetic interactions may involve epigenetic changes, which consist of modifications of gene expression without alteration of the DNA sequence. Epigenetic changes occur through DNA methylation and histone modifications. Previous studies reported consistent epigenetic changes in animal models and post-mortem brain samples of patients suffering from MDD, particularly in individuals who experienced early-life stress and trauma (Klengel and Binder 2015; McGowan et al. 2009). On the other hand, it has been recognized that epigenetic modifications due to prenatal or early life events may predispose individuals to depression by altering the stress response of the HPA axis for a lifetime and increase the risk of suicide (McGowan et al. 2009).

Deficiency of monoamine neurotransmitters theory. — Neurotransmitter imbalance plays an important role in the pathogenesis of several mental disorders, including MDD. Alterations in brain monoamine neurotransmitters such as dopamine, serotonin (also known as 5-HT), and norepinephrine are considered direct causes of depression, and symptoms of depression have been linked to imbalanced levels of these neurotransmitters (Brigitta 2002; Elhwuegi 2004; Remes, Francisco, and Templeton 2021). Indeed, serotonin deficiency can lead to depression, phobias, and anxiety. Glutamate is the primary excitatory neurotransmitter and contributes to synaptic plasticity, cognitive activities, and motivational and emotional behavior in the brain. The monoaminergic neurotransmitter theory is one of the most extensively studied etiological theories of depression, evidenced by antidepressant effects of drugs that aim to restore neurotransmitters levels (Elhwuegi 2004; Otte et al. 2016; Nobis, Zalewski, and Waszkiewicz 2020; Badawy, Dawood, and Bano 2023). Serotonin and its receptors are major targets for depression therapeutic drugs such as tricyclics antidepressants (TCAs), selective serotine reuptake inhibitors (SSRIs), and serotonin and noradrenaline reuptake inhibitors (SNRIs) (L. Yang et al. 2015). However, meta-analysis have shown that only one-third to half of patients suffering from MDD respond to these agents, which often produce side effects, indicating the involvement of other mechanisms in depression (Liang et al. 2018). Animal and human studies have highlighted that signaling by other neurotransmitters is also altered in depression. Gamma-aminobutyric acid (GABA), glutamatergic and acetylcholine systems have been found to play a role in the etiology of depression as well (Lener 2016; Pytka et al. 2016; Zhen Wu et al. 2021).

GABA has been found inhibited in plasma, CSF, and the brain of depressed individuals, while acetylcholine was activated compared to healthy subjects (Pan et al. 2018; Sanacora et al. 2004; Zhen Wu et al. 2021). Elevated levels of glutamate in the blood, cerebrospinal fluid (CSF), and brains of patients with depression have been found in several researches (H. Tian et al. 2022).

Although considerable progress has been made in the investigation of monoamine neurotransmitters, the underlying mechanisms behind this theory remain unclear and treatments are increasingly controversial. Monoamine-research is hampered by the fact that the monoamines levels cannot be directly measured in the brain. Researchers investigate only peripheral monoamine metabolites in different bio-matrices like plasma and urine, which may not necessarily accurately reflect their central levels in the brain. CSF is thought to reflect the central concentration more accurately. However, the measurements require invasive acquisition, and the post-mortem studies of the brain of MDD patients have yielded inconsistent results.

Inflammatory and neurodegenerative hypothesis. — A large body of evidence supports the activation of both peripheral and central immune-related pathways in depression (Maes 2011; Beurel, Toups, and Nemeroff 2020; Irwin and Miller 2007; Maes 2011; Maes, Noto, and Brietzke 2015; Maes, Noto, and Brietzke 2015; Maes et al. 2009). MDD is accompanied by the activation of the inflammatory response system (IRS), immune dysregulation (Dowlati et al. 2010), activation of tryptophan catabolites pathway, induction of oxidative and nitrosative stress, increased translocation of gram-negative bacteria, and neuroprogression. These findings have been ascertained by several animal models (H. J. Park, Rhie, and Shim 2022; Otte et al. 2016). The activation of IRS in MDD patients is associated with a mild inflammatory reaction, characterized by significantly increased proinflammatory cytokines (e.g., interleukin (IL)-6, IL-1β, IL-2), tumor necrosis factor alpha (TNF-α), positive acute phase proteins, and complement factors (Dowlati et al. 2010). Additionally, growing evidence suggests that the compensatory immune-regulatory system (CIRS) plays an important role in MDD. CIRS consists of negative immune-regulatory and anti-inflammatory mechanisms that reduce the IRS and prevent excessive immune activity (Dowlati et al. 2010; Maes 2011). The reported inflammatory cytokine signatures associated with MDD show strong variation between individual studies. Meta-analyses showed that among all pro-inflammatory cytokines, IL-6 and TNF-α were the most consistently significant in depression (Dowlati et al. 2010; Carvalho et al. 2020). Elevated IL-6 could serve as an early marker for cognitive declination in depression. Previous studies reported that various stages and subtypes of MDD are characterized by different inflammatory biomarker signatures. The melancholic subtype of MDD was characterized by increased levels of acute phase proteins, expression of T cell activation markers, and increased resistance of IL-1ß and sIL-2R production compared to simple MDD. Atypical depression is accompanied by increased level of IL-2 but decreased IL-4 (Sowa-Kućma et al. 2018). Treatment-resistant depression subtype is characterized by specific immune biomarkers, including elevated levels of IL-6, sIL-1RA, and TNF-α. Additionally, increased level of TNF-α were observed in depressed individuals who experienced more than three episodes. Thus, despite the evidence which supports that MDD is an inflammatory disease, there is a difference in immune-inflammatory signatures between MDD patients, subtypes, and staging. Moreover, various factors, including smoking and obesity, which are common in depression, can influence the profile of the inflammatory markers. Furthermore, individuals exposed to physical or mental stressors in childhood have been found to exhibit stronger inflammatory response.

Inflammation is not a direct cause of depression; however, inflammatory mediators have been found to impact various factors underlying the pathophysiology of MDD, including monoamines, glutamate neurotransmitters, GR resistance, hippocampal neurogenesis, and neural functions (Arteaga-Henriquez et al. 2021; Maes et al. 2009). Upregulation of systemic inflammation might contribute to the development of depression by affecting neurotransmitters production and function, suppressing neurogenesis via decreasing brain-derived neurotrophic factor (BDNF)[5] activity, or increasing HPA axis activity. Pro-inflammatory cytokines stimulate indolamine 2,3 dioxygenase (IDO) in glial cells, leading to increased tryptophan catabolites along the kynurenine pathway, such as the neurotoxic quinolinic acid. Disruption of the tryptophan-kynurenine pathway has been linked to the inflammatory state of MDD patients.

Furthermore, plasma levels of the proinflammatory cytokine TNF-α has been closely correlated with kynurenine and the kynurenine/tryptophan ration, as well as with levels of kynurenine, kynurenic acid, and quinolinic acid in CSF (Badawy, Dawood, and Bano 2023). Additionally, the proinflammatory state reflected by elevated proinflammatory cytokines is likely responsible for impairments in cognitive function observed in MDD, as well as other symptoms such as anhedonia, anorexia, memory dysfunction, and impaired social interaction (Maes et al. 2009). Moreover, patients receiving cytokines as treatment for cancer or hepatitis, as well as patients with inflammation-associated diseases, are often observed to be at a high risk of developing depression (Otte et al., 2016). A substantial body of evidence from animal studies supports the theory that peripheral inflammation plays a pivotal role in the neuroinflammatory mechanisms of MDD. These animal models have offered new insight into both the direct and indirect pathways through which peripheral pro inflammatory mediators impact brain circuits, behavior, and mood. Peripheral cytokines have the capacity to diffuse through areas of compromised blood-brain-barrier integrity, or be transported by endothelial cell transporters, thereby affecting the brain function at the cellular level, including interaction with astrocytes, microglia, and neurons. The inflammatory signals can propagate from the periphery to the brain via the afferent vagus nerve, informing brain areas of systemic inflammation (Maes, Kubera, et al. 2011). Additionally, neuroinflammation is often accompanied by suppression of neurogenesis and a reduced production of neurotrophic molecules, such as BDNF (Maes et al. 2009; Huehnchen et al. 2011; Beurel, Toups, and Nemeroff 2020). Neuroinflammation may contribute to neurodegeneration through the neurotoxic effects of cytokines and tryptophan catabolites (Badawy, Dawood, and Bano 2023; Ogyu et al. 2018; Correia and Vale 2022; Michal et al. 2023), induced damage by ROS, activation of the DNA damage response pathway, as well as apoptosis and necrosis of neurons (Maes et al. 2009). The interaction between inflammation and other mechanisms underlying MDD is illustrated in Figure 2-9.

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[5] BDNF play important roles in the regulation of neurogenesis, synaptic and structural plasticity, which are involved in the pathology of MDD. BDNF is related to the growth and function of 5-HT neurons in the brains of adults.

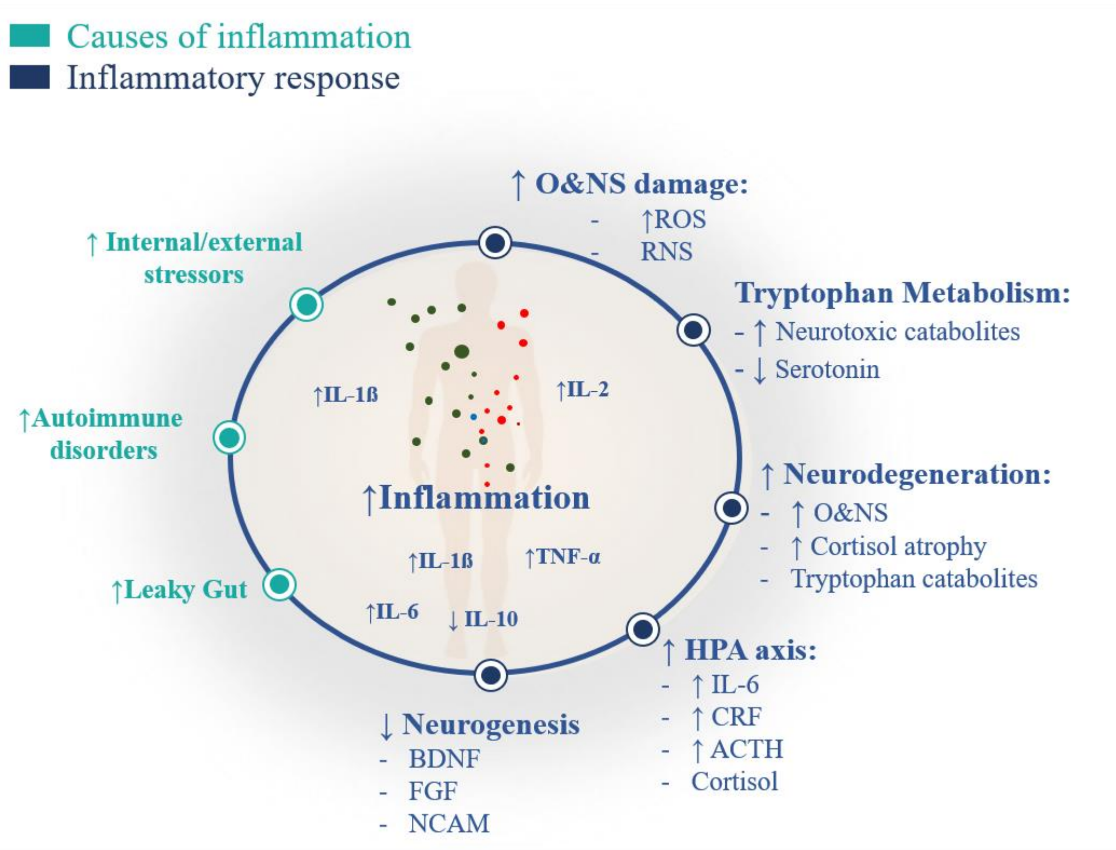

Figure 2-9| Inflammatory and neurodegeneration pathways involved in major depressive disorder: *Major depressive disorder (MDD) is associated with increased level of pro-inflammatory cytokines and relative decreased level of anti-inflammatory cytokines. External and internal stressors, as well as by an increased translocation of the lipopolysaccharide from gram negative bacteria (leaky gut) are among the main causes for the pro-inflammatory response in MDD. The inflammatory response in depression is accompanied by (1) lowered levels of zinc and a lowered ω3 PUFA status, (2) increased oxidative and nitrosative (O&NS) damage to peptides and membrane fatty acids, (3) induction of the hypothalamic-pituitary-adrenal (HPA)-axis via stimulated release/production of corticotropin releasing hormone (CRF), adrenocorticotropic hormone (ACTH) and cortisol, (4) the induction of indoleamine-2,3-dioxygenase (IDO) with decreased levels of tryptophan and serotonin, and the consequent formation of tryptophan catabolites along the Kynurenine-pathway. Inflammation induces also decreased neurogenesis in depression, which is characterized by decreased brain derived neurotrophic factor (BDNF), neural cell adhesion molecule (NCAM), and fibroblast growth factor (FGF).*

Oxidative and nitrosative stress. — It is well established that inflammation is accompanied by elevated production of oxygen radicals, which may react with the macromolecules of the cell, such as fatty acids, DNA, and proteins, thereby causing damage (Salim 2014; T. Liu et al. 2015b; Joshi and Praticò 2014). Increased evidence suggests that MDD is characterized by increased levels of oxidative stress biomarkers, such as malondialdehyde (MDA), a byproduct of polyunsaturated fatty acid peroxidation, and arachidonic acid, as well as 8-hydroxy-2-deoxyguanosine, indicating DNA damage by oxygen radicals (Maes et al. 2009). Moreover, due to its extensive oxygen consumption, lipid-rich environment, and high metabolite rate, the brain is considered highly susceptible to oxidative stress or redox unbalance. Therefore, it is not surprising that oxidative stress is implicated in several neurodegenerative and mental disorders (Maes, Kubera, et al. 2011; Joshi and Praticò 2014). Several lines of evidence indicate that MDD is accompanied by altered antioxidant defenses. Postmortem studies have demonstrated changes in glutathione levels in the brain of MDD patients. Additionally, lowered plasma antioxidant levels (e.g., zinc, vitamin E and D, coenzyme Q10), and decreased antioxidant enzymes activity have been found in depressed individuals. Additionally, activation of oxidative and nitrosative stress pathways have also been observed in MDD patients, as evidenced by increased lipid peroxidation and oxidative damage to DNA and membranes.

Although, there is a linkage between MDD and chronic low-grade inflammation, this is not specific to MDD. The activation of inflammatory pathways has also been observed in other mental disorders such as bipolar depression and schizophrenia, indicating common underlying pathogenetic pathways involving immune dysfunctions in all these disorders. However, no significant differences in inflammatory markers among these three disorders have been observed.

Neuroendocrinology theory. — Patients with MDD often show altered levels of cortisol and growth hormone (GH), indicating endocrine dysfunction, particularly dysregulation of the main endocrine stress system, the hypothalamus-pituitary-adrenal (HPA) axis (Brigitta 2002). The neuroendocrinology theory has been extensively studied as a causal factor and remains one of the most consistent biological findings in MDD. However, the mechanisms underlying this alteration are still unclear, and the clinical studies have conflicting views regarding the up- and downregulation of HPA axis in MDD patients. Furthermore, the HPA axis is a crucial part of the stress response system, which can be activated with both psychological and physiological stress. This activation results in the release of corticotrophin-releasing factor (CRF) and vasopressin by the hypothalamus. The hyperactivity of the HPA axis is presumably caused by dysfunction of glucocorticoids receptors, impairing the negative feedback circuit of the HPA-axis. Glucocorticoid receptor malfunction might cause depression via impaired neurogenesis and reduced hippocampus volumes. Additionally, several studies reported that cortisol might play a critical role in depression recurrence, suggesting that HPA axis may be involved in MDD progression.

Gut microbiota alteration. — Currently, there is growing evidence indicating that gut microbiota (GM) and microbiota-derived substances play a key role in the pathophysiology of several neuropsychiatric disorders, including depression (Capuco et al. 2020; De Angelis et al. 2013; Radjabzadeh et al. 2022). Several clinical studies and animal model research have confirmed the linkage between depression and the alteration of the diversity and richness of GM (Radjabzadeh et al. 2022), as well as the disruption of its metabolism by-products (Skonieczna-żydecka et al. 2018; Amirkhanzadeh Barandouzi et al. 2020; Simeng Liu et al. 2019; P. Zheng et al. 2016; Jianguo et al. 2019; Kelly et al. 2016; Nanthakumaran et al. 2020; Radjabzadeh et al. 2022; Zalar, Haslberger, and Peterlin 2018). In a recent gut microbiome-wide study involving 2,593 depressed participants, researchers found that thirteen microbial taxa have been linked to depression (Radjabzadeh et al. 2022). Among them, several bacteria are known to be involved in the synthesis of key neurotransmitters, such as glutamate, serotonin, butyrate and GABA. Moreover, various studies based on animal models of depression have provided evidence that GM play critical role in the neurobiological features of depression (A. J. Park et al. 2013). This includes activation of immune pathways (Galley et al. 2014), dysregulation of the HPA axis, alteration of tryptophan metabolism (Yano et al. 2015), modulation of neurotrophic factors (Lu et al. 2018), and dysregulation of neurogenesis (Ogbonnaya et al. 2015). The autonomic nervous system (ANS), the HPA-axis, and the enteric nervous system (ENS), all interconnect the brain and the GM, as highlighted in Figure 2-10.

This interconnection allows the brain to influence intestinal activity and immune cell functions, and the GM to influence mood, cognition, and trigger changes in neurotransmission, neuroinflammation, and behavior (Appleton, 2018). Several studies highlighted that GM may modulate various central processes via regulation of the gut-brain axis (GBA), the communication network that connects CNS and ENS. There is accumulating evidence suggesting that several pathways are involved in this bidirectional communication between ENS lining in the gastrointestinal tract (GI) and the CNS, namely endocrine, inflammatory, metabolic, and neural pathways, as illustrated in Figure 2-10 (Suganya and Koo 2020; Flux and Lowry 2020; Vadim Osadchiy, Clair R. Martin 2020). Current studies have provided molecular insights into how the GM influences the CNS and ENS functions, implicating the microbiota-derived products in brain hemostasis, and neuropsychological disorders. GM communicate with each other and regulate the communication between CNS and ENS through the production of several metabolites, gut hormones, neurotransmitters with neuromodulator properties, and inflammatory factors. Among them, short-chain fatty acids (SCFA), tryptophan breakdown products, serotonin, GABA, glutamine, histamine, branched-chain amino acids (BCFAs), lipopolysaccharide (LPS), bile acids, and catecholamines are the important GM derived substances that modulate important processes for neurogenesis, glial cell functions, synaptic pruning, and blood-brain barrier function (Vadim Osadchiy, Clair R. Martin 2020). Indeed, SCFAs are the most important GM metabolites produced from the fermentation of dietary fibers in the intestine tract. SCFAs can cross the blood-brain barrier (BBB) and regulate microglia function and communication, which play a key role in behavior modulation and brain development, albeit a large proportion of SCFAs is excreted within the feces (M. Wu et al. 2020). Additionally, SCFAs have been found to regulate the release of gut peptides from enteroendocrine cells and synthesis of serotonin, both of which impact the endocrine gut-brain pathway. Some peptides, such as galanin, are involved in several neurobiological functions, including sleep/wake cycle regulation, feeding, mood, and neurotrophic functions. Furthermore, peptides can stimulate the activity of the HPA axis, enhancing the glucocorticoid secretion from the adrenal cortex. Moreover, the GM is involved in the modulation of afferent nerves, and can produce neurologically active metabolites that can act as neurotransmitters, such as GABA and serotonin. Additionally, various studies focused on stress-induced altered intestinal permeability, which may play a key role in depression pathophysiology. Particularly, increased translocation of bacterial endotoxins due to a compromised gut barrier is linked to dysregulation of the HPA axis and activation of the immune system, both of which are well established mechanisms in the pathophysiology of MDD. While a causal relationship is yet to be fully established, bacterial translocation products may lead to an increased production of inflammatory mediators. Conversely, the CNS transmits information to the enteric neurons via the ANS and the HPA axis. The CNS can impact the GM directly through the secretion of endocrine mediators that interact with microbial receptors, and indirectly through the modulation of the gut environment (Vadim Osadchiy, Clair R. Martin 2020). Under stress stimuli, the HPA axis activates the release of the hormone cortisol, which eventually affects the intestinal integrity, motility, and mucus production. This may introduce changes in the composition and diversity of GM in the intestinal tract and production of microbial metabolites. Despite advanced understanding of brain-gut axis, the results are inconsistent, and no causal connection between gut microbiota and depression has yet been proven. Moreover, most research in the brain-gut linkage is based on animal models of depression.

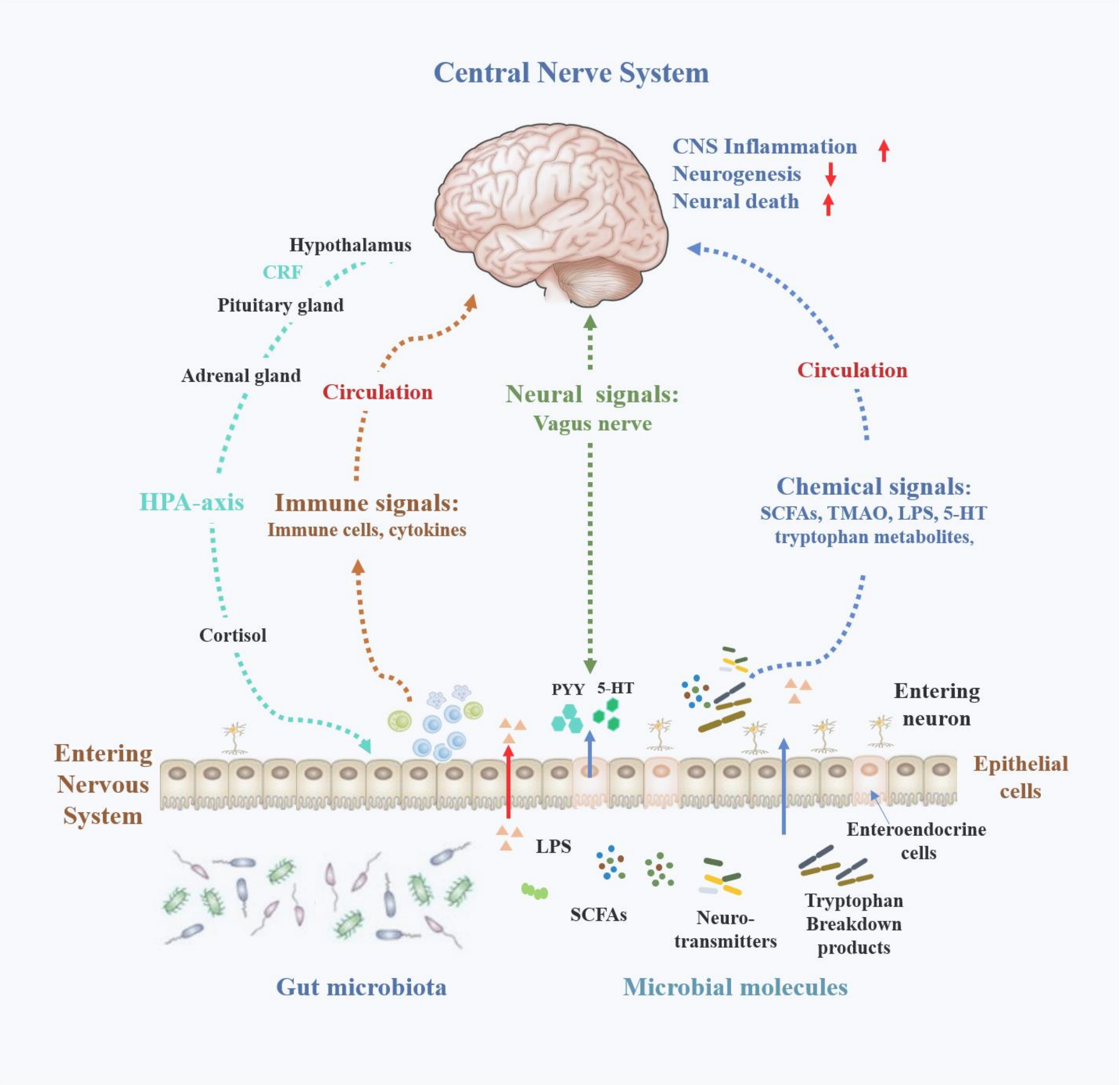

Figure 2-10| Schematic presentation of the pathways involved in the bidirectional gut-brain axis communication: *Several direct (vagus nerve) and indirect (via systemic circulation) pathways are involved in the bidirectional communication between the brain and the gut microbiota. These include endocrine (e.g., cortisol), metabolic (chemical substances), immune (cytokines and immune cells), and neural (vagus nerve, entering nervous system (ENS) and spinal cord nerve) pathways. The gut microbiota (GM) affects central nerve system (CNS) metabolism and functions by different mechanisms, including (1) releasing of immune factors such as inflammatory mediators , (2) metabolites such as short chain fatty acids (SCFAs), trimethylamine N-oxidase (TMAO), breakdown metabolites involved in tryptophan metabolism, and microbial neural substrates (neurotransmitters such as serotine (also known as 5-hydroxtryptamine (5-HT)) that can act on intestinal epithelial cells and stimulate intestinal nerves, which in turn stimulate CNS through the vagus nerve. Some microbial metabolites such SCFAs and peptides YY (PYY) can cross the blood-brain barrier and act directly on the CNS. Additionally, the gut microbiota can alter levels of circulating cytokines that in turn can markedly impact the brain functions. The lipopolysaccharide (LPS) is a proinflammatory endotoxin of gram-negative bacteria that can activate an inflammatory response and the kynurenine pathway. Inversely, CNS transmits information to the entering neurons via the nervous system and HPA axis, which under stress stimulus activates the release of corticotropin-releasing hormone (CRH) in the hypothalamus, promotes the release of ACTH by the pituitary gland, and results in the release of the hormone cortisol by the adrenal gland. Cortisol eventually affects intestinal integrity, motility, and mucus production, including changes in the composition and diversity of gut microbiota in intestinal tract and microbial metabolites.*

2.4 Suggested Markers for MDD

Despite the advanced understanding of the pathophysiology of MDD, to date, no single marker has been introduced for everyday clinical use. This is due to the lack of sensitivity and specificity of these markers and their reproducibility, the high incidence of comorbidities of MDD with several physical and psychical disorders, and the existence of different subtypes. To date, several immunological, neurotransmitter, neuroendocrine, neurotrophic, gastrointestinal (gut-related), metabolic, and O&NS markers have been suggested to be differentially expressed in MDD (Kennis, Gerritsen, and Dalen 2020). These MDD markers can be assessed at various omics levels, starting with genomics and epigenomics, followed transcriptomics and proteomics, and extending to metabolomics (Hacimusalar and Eşel 2018; Nobis, Zalewski, and Waszkiewicz 2020; Carvalho et al. 2020). Apart from omics markers, it is abundantly evident that microbiome markers are also differentially expressed in MDD. The proteome markers include growth factors (e.g., BDNF, vascular growth factor, insulin-like growth factor-1), inflammatory proteins, and oxidative stress-related enzymes such as superoxide dismutase (SOD), catalase (CAT) and myeloperoxidase (MPO). The inflammatory markers encompass pro-inflammatory (e.g., IL-1, IL-6, and TNF-α) and anti-inflammatory cytokines (e.g., IL-4,

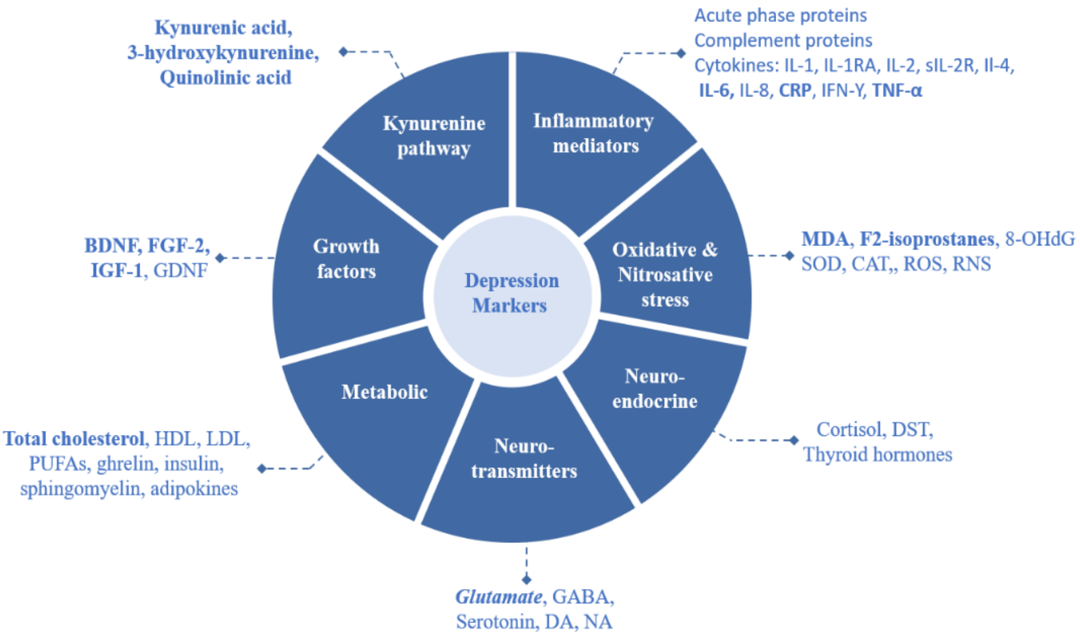

*Figure 2-11| **Summary of the potential markers of major depressive disorder:** Several markers in the inflammatory, neurotransmitter, neuroendocrine, neurotrophic, and metabolic systems have been suggested as being differentially expressed in MDD. However, the most consistent markers for MDD confirmed by the umbrella meta-analysis are the pro-inflammatory mediators IL-6, CRP, TNF- α, the oxidative stress markers MDA and F2-isoprostanes, growth factors BDNF, FGF-2 and IGF-1, the neurotransmitter glutamate, total cholesterol, as well as kynurenic acid and its breakdown products quinolinic acid and 3-hydroxikynurenine (Carvalho et al.). The most consistent markers are bold. IL: interleukin, GABA: gamma-aminobutyric acid; GDNF: glial cell line derived neurotrophic; IGF-1: insulin-like growth factor-1; DST: dexamethasone suppression test; MDA: Malonylo-dialdehyde; ROS: reactive oxygen species; NOS: nitrosative oxygen species; CAT: catalase; CRP: C-reactive protein; SOD: superoxide dismutase; FGF-2: fibroblast; 8-OHdG: 8-hydroxy-2-deoxiguanosine; HDL: high-density lipoprotein; LDL: low density lipoprotein; NA: noradrenaline; DA: dopamine; PUFAs: polyunsaturated fatty acids. FGF-2: Fibroblast growth factor-2; NA: noradrenaline.*

IL-10, and IL-13), as well as complement and acute phase proteins. Among them, IL-6, CRP, TNF-α and sIL-2R are the most consistent inflammatory mediators in MDD. The O&NS in MDD is manifested by higher levels of ROS, elevated lipid peroxidation products (e.g., MDA and F2-isoprostanes), DNA damage markers such as 8-hydroxy-2-deoxiguanosine (8-OHdG) and 8-oxo-7,8-dihydroguanosine (8-oxo-Gua), as well as protein damage products. Among all O&NS markers, the lipid peroxidation markers appear to have the greatest potential to serve as markers for depression. MDD is also accompanied by decreased levels of antioxidants such uric acid, albumin, coenzyme Q, Zinc and vitamin C. Endocrine markers include energy balance hormones (e.g., leptin and ghrelin), and the regulatory compounds involved in the HPA axis such as cortisol and corticotropin-releasing factor (CRH). The suggested metabolomics markers of MDD include mainly amino acids and their derivatives, amines, breakdown-products of lipids and neurotransmitters, as well as metabolites involved in energy metabolism. The metabolomics markers are detailed in the next section.

To date, no consistent markers have been confirmed by meta-analyses. The most recent umbrella meta-analysis by Carvalho *et al*. demonstrated that depression is accompanied by increased levels of CRP, IL-6, TNF-, sIL-2R, IGF-1, FGF-2, glutamate, lipid peroxidation markers, and decreased levels of BDNF, total cholesterol, kynurenic acid (KYNA), ration of KYNA to 3-hydroxykynurenine (3HK), and the ratio of KYNA to quinolinic acid (QA) (Carvalho et al. 2020). The potential MDD markers that have been found differentially expressed in depression are summarized in Figure 2-11.

2.5 Metabolomic Changes in Major Depression

Metabolomics has recently emerged as a particularly promising quantitative tool that provides multi-parametric biomarker signatures, reflecting alteration in various biochemical processes linked to MDD (Bot et al. 2020), or mirroring the effect of drug therapies on those biological processes (Thorn and Greenman 2012). Metabolomics, as the end point of the omics-science cascade, has the potential to directly sample downstream products from changes that occur at the genetic, transcriptional, and translational levels, as well as perturbation in the gut microbiome metabolic and global metabolic response to environmental influence and stressful events. The metabolomic profiling of various biological matrices, such as blood, plasma, urine, cerebrospinal fluid, or feces may be useful in differentiating MDD from bipolar disorder or healthy controls (HCs) (S.-W. Gui et al. 2018; Pan et al. 2018). Former pharmacometabolomic studies reported perturbation in by-products of tricarboxylic acid cycle, urea cycle, amino acids, and lipids in depressed patients exposed to sertraline (Siamak Mahmoudian Dehkordi et al. 2020), (H. Zhu et al. 2013). A large body of literature reported that MDD is associated with metabolomics changes, including altered levels of amino acids and amines, peripheral downstream metabolites originating from lipids and neurotransmitters, GM-derived metabolites, as well as metabolites involved in energy metabolism.

Amino acids. — Mounting metabolomics studies have reported alteration in amino acids and their derivatives in patients suffering from depression. Amino acids are involved in many metabolic pathways, and some of them have been suggested as potential markers for MDD. Significantly lower levels of branched chain amino acids (BCAAs) including valine, leucine, and isoleucine have been observed in MDD patients compared to HCs (F. Guerreiro Costa et al. 2022; Baranyi et al. 2016; Gu et al. 2021a). A large scale meta-analysis with 5283 participants with depression and 10,145 controls indicated that isoleucine and tyrosine were associated with depression (Bot et al. 2020). L-aspartic acid, L-glutamine, taurine, γ-amino-n-butyric acid, arginine, and L-α-amino-adipic acid have been reported as possible potential markers for the future diagnosis of depression and the development of antidepressants in both animal and human studies (P. Yang et al. 2014), (Du et al. 2021). Another rat model study suggested that biogenic amino acids were significantly reduced in the hippocampus of stressed rats compared to non-stressed ones (P. Yang et al. 2014). Specifically, lower levels of L-alanine, L-glutamic acid, glycine, L-methionine, L-phenylalanine, L-valine, L-isoleucine, and L-norleucine were significantly changed in the chronic unpredictable mild stress (CUMS) rats in tissue samples of serum, heart, liver, brain, and kidney (Geng et al. 2020). Significant increased concentrations of glutamic acid, aspartic acid, and glycine, along with low levels of 3-hydroxykynurenine were quantified by liquid chromatograph-mass spectrometry in the serum and plasma of MDD patients (Du et al. 2021). Additionally, levels of glutamic acid and phenylalanine have been found to correlate with the severity of depression (Ho et al. 2023). Decreased levels of gamma-glutamyl leucine have been also linked to MDD, suggesting potential abnormalities in the function of the antioxidant glutathione. Low levels of arginine have been observed in MDD patients, while arginine levels have been found significantly increased in remitted patients. Furthermore, several amino acids, including asparagine, glutamine, histidine, phenylalanine, threonine, and alanine, were significantly negatively correlated with the melancholia dimension score (Brydges et al. 2022).

As previously explained, tryptophan (TRP) metabolism plays a crucial role in the pathophysiology of neuropsychological conditions, including depression (Badawy, Dawood, and Bano 2023; Correia and Vale 2022; Michal et al. 2023; Correia and Vale 2022; Ogyu et al. 2018). TRP is metabolized through the kynurenine, serotonin, and microbial pathways. The kynurenine pathway (KP) is the major pathway of TRP degradation, accounting for 95% of dietary TRP metabolism. This pathway gives rise to several TRP downstream metabolites, which are mostly neuroactive and can cross the blood brain barrier (Correia and Vale 2022). Accumulating evidence reports significant alterations of tryptophan catabolite levels in MDD patients. Meta-analyses on the KYN pathway reported decreased levels of kynurenic acid and kynurenine levels in patients with depression in comparison to controls (Ogyu et al. 2018). A systematic review by Guerreiro Costa *et al* (2022) reported elevated levels of indoles in MDD patients (F. Guerreiro Costa et al. 2022). Interestingly, the TRP catabolites in the KYN pathway have been found to play a crucial role in MDD diagnosis, subtyping, and treatment response prediction (Gostner et al. 2020). A negative correlation between serum kynurenine levels and the severity of MDD has been reported (F. Guerreiro Costa et al. 2022). Moreover, ratios such as kynurenine/melatonin and 3-hydroxyquinurenine/melatonin, along with the metabolite 3-hydroxyquinurenine have been used to distinguish MDD patients responding to antidepressants from non-responder ones. On the other hand, TRP is important as precursor for the synthesis of melatonin and serotonin, which play vital roles in the physiological regulation of various behavioral and neuroendocrine functions.

Amines. — Lower levels of phosphoethanolamine, the precursor of phospholipids, have been suggested as a possible biomarker for depression. Higher baseline levels of melatonin have been observed in MDD patients responding to treatment.

Metabolites involved in the energy metabolism. — Accumulating evidence has shown that the energy metabolism is impaired in patients with depression (Gu et al. 2021a; Głombik et al. 2020; Zuccoli, Saia-cereda, and Nascimento 2017; Rezin et al. 2009). The body's energy comes from the tricarboxylic acid cycle (TCA), oxidative phosphorylation, and glycolysis. Many metabolites involved in glycolysis and the TCA cycle, such as pantothenic acid, dihydroxyacetone phosphate, creatinine, and 3-hydroxybutyric acid, have been found to be lowered in MDD subjects, indicating a state of energy deficiency (Gu et al. 2021a). Additionally, studies have indicated downregulated acylcarnitine detected in the plasma of MDD patients. This may result from decreased BCAAs, which could affect the phosphorylation levels and lead to low energy metabolism. Furthermore, studies have reported alterations in several metabolites involved in the pyruvate metabolism, such as N-acetyl-L-aspartic acid, beta-alanine, palmitic acid, steric acid, and creatine compared to HCs. During glycolysis, glucose is broken down into pyruvate, which is subsequently metabolized through various pathways. One such pathway involves the conversion of pyruvate to acetyl-CoA, which enters the TCA cycle to produce energy in the form of ATP. Therefore, alterations in metabolites involved in pyruvate metabolism can impact both glycolysis and subsequent energy production.

Monoamine neurotransmitters. — A body of evidence supports the important role of monoamine transmitters in the pathophysiology and treatments of depression. Elevated level of GABA and dopamine, as well as reduced levels of histamine and leucine-enkephalin have been found in plasma and serum of MDD patients (F. Guerreiro Costa et al. 2022). A previous study investigated the concentrations of 19 plasma neurotransmitters in individuals with MDD, bipolar disorder, and HCs. The study suggested that nine metabolites, mainly involved in GABAergic, catecholaminergic and serotonergic systems, are significantly altered in MDD subjects. Among them, dopamine, GABA, tyramine and kynurenine have been identified as potential neurotransmitters that could discriminate between individuals with MDD and bipolar disorder individuals, as well as between MDD subjects and HCs with high accuracy (Pan et al. 2018). A recent study investigated changes in neurotransmitter levels and their metabolites in GABAergic, serotonergic and catecholaminergic pathways in the nucleus accumbens of a rat model. The study reported changes in the levels of glutamine, kynurenic acid, histamine, L-phenylalanine, and tyramine after induction of stress (Y. Li et al. 2022). Despite the considerable advances in this area, the research results remain inconsistent, the underlying mechanisms are unclear, and treatments based on monoamine neurotransmitters are increasingly controversial.

Gut microbiota metabolites and neurotransmitters. — A wide range of mediators are involved in the gut microbiota-host interaction, including microbial metabolites produced from the fermentation of dietary compounds (e.g., SCFAs: acetate, butyrate, and propionate), bacterially-modified host molecules such as bile acids, or compounds directly produced by certain gut microorganisms, such as lipopolysaccharides (LPS). Disruption of the microbial metabolome due to alteration in the composition and level of GM is well known to play a critical role in the pathogenesis of MDD (Simeng Liu et al. 2019; P. Zheng et al. 2016; Liang et al. 2018). As previously described in section 2.3.2.3, several microbial metabolites are involved in the regulation of gut-brain communication (Silva, Bernardi, and Frozza 2020). These mediators include neurotransmitters with neuromodulator properties (e.g., dopamine, glutamate, serotonin, GABA), inflammatory factors, SCFAs, tryptophan breakdown products, glutamine, histamine, D-amino acids, LPS, bile acids, trimethylamine N-oxide (TMAO), and catecholamines. SCFAs are metabolic by-products of non-digestible fiber fermentation. Branched chain fatty acids (BCFAs) including isobutyric, 2-methylbutyric and isovaleric acid are bacterial down-products synthetized from the BCAAs valine, isoleucine and leucine respectively (Pan et al. 2018). Gut microbiota activate the production of monoamines such as serotonin, dopamine, and norepinephrine via stimulating the enteroendocrine cells. The largest part of serotonin in the body is stored in the endocrine cells (EC) of the gut epithelium. The release of serotonin from the EC cells into the lumen is stimulated mechanically by acids, bases, SCFAs and glucose (Nanthakumaran et al. 2020). Microbiome-linked metabolites, such as propane-1.3-diol, indole derivates of tryptophan metabolism such as indole-3-lactic acid, have also been linked to depression (Brydges et al. 2022).

Lipids. — Lipids play essential roles in several cellular processes, such as molecular signaling, energy storage, and cell membrane function. Neuropsychiatric disorders including MDD are generally associated with alteration in lipid metabolism since the brain is an organ with high lipid content (Homorogan et al. 2021; Walther et al. 2018). Nowadays, the progress of the MS opens new insight into the lipidome in MDD (M. Liu et al. 2023). An LC-MS based lipidomics study identified a signature of 20 lipids in the plasma of females suffering from MDD and HCs that could discriminate between the cohort groups with AUC of 0.897, 13 lipids that distinguish MDD from bipolar depression with AUC of 0.86 (Zhang et al. 2022). A meta-analysis showed that depression is associated with elevated blood levels of total cholesterol, triglycerides, and decreased low density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and w-3 polyunsaturated fatty acids (Bot et al. 2020). A metabolome-wide profile of depression with 2770 at baseline (1101 current MDD patients, 868 remitted MDD) and 1805 at 6-year follow up (801 HCs and 327 current MDD, 1045 remitted and 433 HCs) indicated altered lipid metabolism with lowered levels of long chain fatty acids (monosaturated and saturated), and upregulated lysophospholipids (Jansen et al. 2023). An increased ratio of LDL/HDL has been indicated in MDD patients along with a reduction in omega-3 fatty acids levels. Additionally, a positive correlation between the Montgomery-Asberg Depression Rating Scale score and serum levels of LDL, triglycerides, cholesterol, free cholesterol, and phospholipids have been observed before treatment. Disturbance of phospholipid metabolism in plasma of MDD has been reported in several metabolomics human studies and animal models (S.-W. Gui et al. 2018; M. Liu et al. 2023). However, considerable inconsistence between the lipid studies in depression was noted. This is partly due to the different methodologies used in the lipid analysis. Furthermore, alterations in circulating lipid levels have been linked to pathophysiological pathways related to depression, such as chronic activation of the HPA-axis or chronic low-grade inflammation (Bot et al. 2020). An activation of inflammatory response led to reduced levels of HDL cholesterol and phospholipids, as well as elevated levels of triglycerides. Alteration of circulating lipids was considered to be a consequence of depression.

2.6 Alterations of Breath Volatomics in other Psychiatric Disorders

To our knowledge, this study represents the first investigation of breathomics alteration in MDD compared to HCs. Therefore, in this section, we additionally summarize the state of the art of breathomics VOCs involved in psychiatric disorders that overlap with MDD symptoms and pathophysiology. The application of BGA in psychiatric disorders is still in its infancy. Most breath volatomics studies were mainly focused on schizophrenia (SCH) patients compared to HCs, or to a control group with other psychiatric disorders. Additionally, the sample size of study populations was relatively small. Consequently, the literature in this area is quite scarce, making it difficult to draw reliable conclusions. A GC-MS based breathomics study investigated the exhaled VOCs to discriminate between 25 patients suffering from SCH, 37 HCs and 26 individuals with other psychiatric disorders, serving as heterogenous psychiatric

Table 2.2*| Summary of the exhaled VOCs found to be altered in other mental disorders*

VOCs Signature	Participant groups	Stat. method	Performance	Analytical Method	Ref.
m/z 60, *m/z* 69, *m/z* 74, *m/z* 88 and *m/z* 90	SCZ:34 HCs:34 MDD:36	Bart	**SCH/HCs:** *Acc*:83.6%, *AUC*: 0.84 **SCH/MDD:** *Acc*:80.9%, *AUC*: 0.81	PTR-MS	(Henning et al. 2023)
m/z 60, *m/z* 85, *m/z* 90	SCZ:28 HCs:29	LG Bart	*Acc*:82%, *AUC*: 0.91 *Acc*:82%, *AUC*: 0.96	PTR-MS	(C. Jiang et al. 2022)
Pentane and ethane	SCZ:28 HCs:15	T-test	-	GC-MS	(B. M. Ross, Shah, and Peet 2011)
Pentane, 2-Pentanol, carbon disulphide, benzene, 2-methylbutane, 2,3,3-trimethypentane, tetrachloroethene 2,2 dimethylbutane, 1-chloro-2-methylbutane, trichlorofluoromethane	SCZ:25 PCs: 26 HCs:37	SIMCA HCA PCA	*Sen*:68%, *Spe*: 84.1%, - *Sen*: 80%, *Spe*: 61.9%	GC-MS	(M. Phillips et al. 1995)
Pentane, Carbon disulfide	SCZ:25 HCs: 37 PCs:26	T-test	-	GC-MS	(M. Phillips, Sabas, and Greenberg 1993)

SCZ: Schizophrenia; HCs: Healthy controls, PCs: Psychiatric controls; MDD: Major depressive disorder; Sen: Sensitivity; Spe: Specificity; ACC: Accuracy; AUC: Area under the curve; PCA: Principal component analysis; HCA: Hierarchical component analysis; SVM: Support vector machine; LG: Logistic regression; NB: Naïve Bayes; RFA: Random Forest analysis; PA: Pathway analysis; m/z: Mass-to-charge ratio; PTR-MS: Proton transfer reaction – Mass spectrometry; GC-MS: Gas chromatography-Mass spectrometry

controls (PCs), including MDD, bipolar disorder, dysthymic, and alcohol abuse (M. Phillips, Sabas, and Greenberg 1993). The microanalysis of breath samples showed significantly increased levels of exhaled pentane ($p < 0005$), carbon disulfide ($p < 0.001$), benzene, 2-methylbutane, and tetrachloroethene ($p < 0.05$) in the breath of SCZ patients compared to HCs. Only the level of exhaled carbon disulfide was found significantly increased in the breath of SCH patients compared to PCs. In a subsequent GC-MS study, Phillips *et al.* (1995) investigated the exhaled VOCs to discriminate between 25 SCH patients, 37 HCs and 26 PCs. The microanalysis showed that 2-Methylbutane, trichlorofluoromethane, dichloromethane, Trichloroethene, 2-Pentanol, pentane, I-Chloro-2-methylbutane, 2,3,3-Trimethylpentane, benzene, 2,2-Dimethylbutane, and tetrachloroethene represented the best breath VOCs signature, distinguishing SCH patients from controls. Using PCA, this signature achieved a sensitivity of 61.9% and sensitivity of 80%, and using SIMCA models, it achieved a sensitivity of 68.0% and specificity of 84.1% (M. Phillips et al. 1995). Ross *et al.* (2011) reported elevated levels of exhaled ethane and pentane in the breath of 28 unmedicated SCH patients compared to 15 HCs (B. M. Ross, Shah, and Peet 2011). The elevated level of these alkanes was linked in this study to the oxidative stress, which is well- known to underlie the pathophysiology of SCH. The source of ethane and pentane was associated with oxidative damage to omega-3 and omega-6 PUFA respectively (Yao, Reddy, and Van Kammen 2001), (Dietrich-Muszalska and Kontek 2010). Additionally, Ross and Glen (2002) reported that levels of butane could be used as indicators of the severity of SCH (M. A. Ross and Glen 2002). A recent publication from our research group investigated the exhaled VOCs in the exhaled breath of 28 SCH and 29 HCs patients using PTR-MS. Three exhaled VOCs with *m/z* 60, *m/z* 85, and *m/z* 90, tentatively identified as trimethylamine, hexane isomer, and ester of butyric acid, were suggested as potential VOCs for discriminating between cohort groups with an accuracy 82.4% and AUC 0.9 using logistic regression model, and with an accuracy of 91.2% and AUC of 0.96 using Bart machine analysis (C. Jiang et al. 2022). An additional study from our research group reported that five exhaled VOCs with mass-to-charge ratios *m/z* 60, *m/z* 69, *m/z* 74, *m/z* 88 and *m/z* 90 significantly differed among diagnostic groups using bootstrapped mixed-model and BART analysis. The classification models showed an *AUC* of 0.84 with an accuracy of 83.6% for differentiating between SCH patients and HCs, and an AUC of 0.81 with 80.9% classification accuracy for distinguishing between MDD and schizophrenia (Henning et al., 2023).

3 MATERIALS & METHODS

3.1 Study Population

For this study, a total of 26 patients with a DSM-V diagnosis of MDD were recruited from the Clinic for Psychiatry and Psychology, Medical Faculty, Otto-von-Guericke University in Magdeburg, Germany. Exclusion criteria for MDD patients included a known alcohol or drug dependency, neurological disorders and diseases affecting brain function. The control group included twenty-five age, sex and BMI matched healthy subjects between 20 and 65 years old, without psychiatric or endocrinologic diagnoses. They were normotensive, non-diabetic, non-smokers, and showed no evidence of any acute or chronic infection. HCs were recruited through local advertisements. The demographical and clinical characteristics of MDD and HCs are listed in Table 3.1.

Table 3.1 | Demographical and clinical characteristics of patients with MDD and HCs.

Characteristics	**HCs** (n=25)	**MDD** (n=26)	***Sig.***
Gender (w/m)	13/12	16/10	*p* = 0.58
Age	34.40 ± 8.15	38.04 ± 12.90	*p* = 0.24
BMI	24.67 ± 3.96	27.36 ± 7.99	*p* =0.21
Smoking	0	8	*p* = 0.003
Alcohol drinking	5	5	1
Marital status			*Chi* = 9.3 *P* = 0.09
Single	5	9	
Relationship	14	6	
Married	6	6	
Divorced	n.a.	5	
BDI-II	1.72 ± 3.80	32.56 ± 10.79	1.27 x 10^{-19}
HAMD-17	0.12 ± 0.44	17.2 ± 4.9	5.66 x 10^{-25}
Medication	n.a		n.a.
None		7	
SSRI		7	
SNRI		4	
NASSA		5	
Others		3	

Depicted values are represented in mean ± standard deviation. HAMD: Hamilton Depression Rating Scale; BDI: Beck Depression Inventory; SSRI: serotonin reuptake inhibitors; SNRI: serotonin and noradrenaline reuptake inhibitors; NASSA: noradrenaline and selective serotonergic antidepressants; others: valdoxane, nortiptiline, and bupropione

The study protocol was approved by the Institutional Ethics Review Board of the Otto-von-Guericke University, Magdeburg. All procedures used in the study were conducted under international ethical standards. Written informed consent was obtained from all participants after they had received a full explanation of the study procedures. Differences in study population characteristics were determined using the independent sample t-test and Fisher's exact test. A p-value less than 0.05 was considered statistically significant.

3.2 Clinical Data Assessment

To ensure the diagnosis of MDD, all subjects were clinically assessed according to DSM-V criteria by professional and independent clinicians. The diagnosis was further confirmed using SCID diagnostic interview by an additional investigator. Depression severity was assessed using the self-rated BDI-II and the observer-rated HAMD-17 scales.

3.3 Breathomics Pipeline

The breathomics analysis workflow used in this research was similar to untargeted metabolomics-based procedures applied to other bio-matrices (M. Alonso, Castellanos, and Sanchez 2010). As illustrated in Figure 3-1, the breathomics workflow for biomarker discovery involved several steps including: (1) breath sample collection, (2) Data acquisition by the analytical platform PTR-MS, followed by metabolites identification according to their mass-to-ratio (*m/z*), (3) spectral data processing, including noise reduction, dealing with missed values and imputation of missing values, scaling, normalization, and nonlinear transformations, in order to improve the biological information content of the extracted chemical data, (4) univariate and bivariate analysis of the preprocessed metabolites to rank the significant metabolites, (5) multivariate statistical analysis, and selection of the molecular features, which could be serve as potential markers for distinguishing MDD from HCs, (6) annotation and identification of potential molecular markers (7) biological prevalence and functional interpretation trough clustering and pathway- based analysis.

3.3.1 Breath samples collection

After an overnight fasting, three mixed breath samples were collected from each volunteer using 3L-Tedlar bags (SKC Inc. Eighty-Four, PA) immediately after awakening T_0, and then again at 30 minutes T_{30} and 60 minutes T_{60} post-awakening. Awakening was selected as a natural stress stimulus in this research due to its significant role in modulating and activating stress-responsive systems such as the HPA-axis, the ANS, and the immune system, as discussed previously in Chapter 2. These time intervals were selected to explore the physiological dynamic changes in relation to the cortisol awakening response, which has been found blunted in patients with MDD (Dedovic and Ngiam 2015a). The sampling device and its accuracy are detailed in previous studies (Marco M.L. Steeghs, Cristescu, and Harren 2007; M. M.L. Steeghs et al. 2007). Subjects were carefully instructed to refrain from drinking, consuming of coffee or alcohol, brushing their teeth, or smoking before breath sampling to minimize the impact of exogenous VOCs on the concentration of the exhaled gas compounds. Seated volunteers exhaled normally to fill 80% of the Tedlar bags. Additionally, reusable sampling bags were not used in this study to prevent contamination with residual VOCs. The breath samples were processed at the Hospital Clinic for Psychiatry and Psychotherapy, Otto-

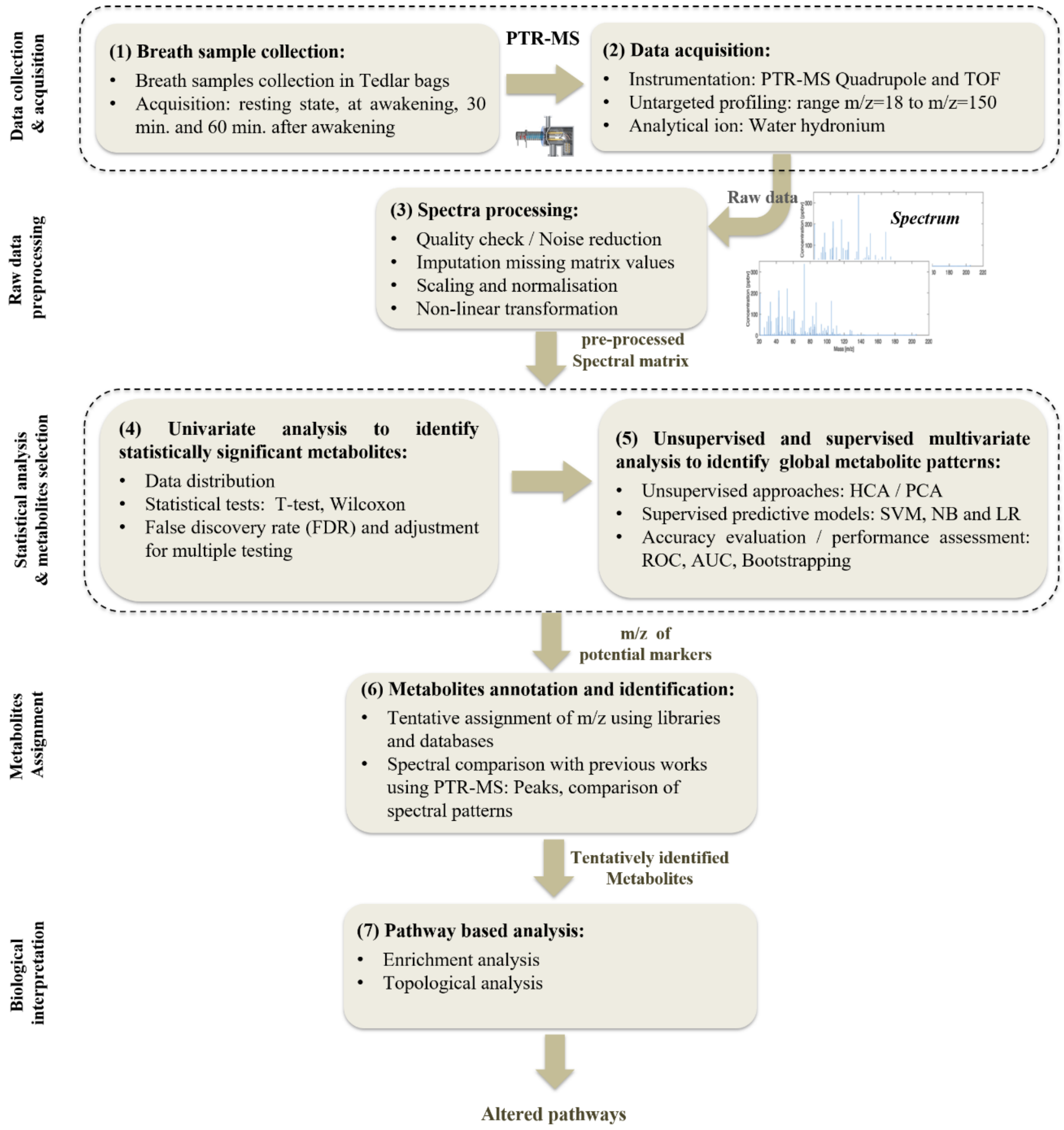

Figure 3-1| Breathomics workflow used in the present study: *the untargeted breathomics workflow for biomarker discovery involved several steps including: (1) breath samples collection in Tedlar bags in resting state immediately after awakening, 30 and 60 minutes post-awakening, (2) metabolites separation by the analytical platform PTR-MS quadrupole, and identification of analytes according to their mass-to-ratio (m/z). The PTR-MS TOF is used only to determine the compounds overlapped in similar mass, (3) Raw data processing includes reduction of noise, nonlinear transformation of data, dealing with missed values, and imputation of missing values, in order to improve the biological information content of the raw data, (4) univariate, and bivariate analysis of preprocessed spectral data in order to roughly rank the altered VOCs in MDD patients relative to HCs, (5) supervised and unsupervised multivariate data analysis to rank potential metabolites that could be used as MDD biomarkers, (6) Tentative assignment of m/z of the significant VOCs using databases and chemical libraries, and comparison of the spectral pattern identified in previous works using PTR-MS, and (6) explanation of the biological prevalence and functional interpretation by the pathway-based analysis.*

Abbreviations: TOF: Time-of-flight; HCA: Hierarchical clustering analysis; PCA: Principal component analysis; SVM: Support vector machine; LR: Logistic regression; NB: Naive Bayes; ROC: Receiver operating characteristic; AUC: area under curve von-Guericke University, Magdeburg within 5 hours after collection to avoid the loss of gas compounds. VOCs can generally remain relatively stable in Tedlar bags for up to 10 hours (Jonathan Beauchamp et al. 2008).

3.3.2 Breathomics profiling by PTR-MS

Since this study represents the first breathomics investigation of biochemical changes in MDD using PTR-MS, no prior knowledge exists regarding the breath metabolites that differentiate patient with MDD from HCs. Therefore, an untargeted breathomics profiling by PTR-MS was performed to identify a wide range of biomarkers potentially associated with various biological mechanisms underlying the pathophysiology of MDD. The microanalysis focused exclusively on VOCs in the breath of the participants. No additional investigation of Non-volatile Organic Compounds in the EBC was included in the present study.

Data acquisition was performed using the high-sensitivity PTR-QMS apparatus produced by IONICON Analytic GmbH, Austria. The hydronium ion H_3O^+ was selected as the reagent ion in all measurements. Metabolites with *m/z* between 21 and 150 were analyzed using the quadrupole-based unit. The description and working principle of this analytical devices have been detailed in previous works (Pleil, Hansel, and Beauchamp 2019; Ellis and Mayhew 2014; Hansel et al. 1999), and Ionicon (www.PTRMS.com). Briefly, PTR-QMS is a low-energy chemical ionization MS technique based on proton transfer reactions from reagent ion H_3O^+ to gaseous organic compounds R according: $H_3O^+ + R \rightarrow RH^+ + H_2O$, as previously described in last chapter. As illustrated in Figure 3-2, H_3O^+ are generated at a high density (~ 2.5 - 3.5 x 10^6/s) from pure water vapor within a hollow cathode, called the ion source, and subsequently injected into the flow drift tube, where the ionization of the volatile analytes R takes place. The analytes are introduced into this reaction room through an air inlet at a flow rate of approximately 11 mL/ min. The ionization process of trace molecules R in the drift tube can be derived as:

$$[RH^+] = [H_3O^+]_0(1 - e^{-k[R]t}) \qquad \textbf{(3.1)}$$

For an abundant supply of H_3O^+ ions, ensuring that $[RH^+] << [H_3O^+] \approx [H_3O^+]_0 =$ const, the ionization process can be simplified in a good approximation[6] to:

$$[RH^+] = [H_3O^+]_0 k[R]t \qquad \textbf{(3.2)}$$

where $[H_3O^+]_0$, $[RH^+]$, k and t correspond to the density of the primary reagent ion, the density of the protonated trace gas, the reaction rate coefficient, and the average time the ions spend in the drift tube respectively (Hansel et al. 1999). The ions RH^+ produced in drift tube are subsequently mass analyzed in a quadrupole mass spectrometer and detected by a secondary

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[6] This approximation can be justified, because only molecules with a PA higher than the PA of water undergo an ionization using H_3O^+ ion as reagent ion. Therefore, only VOCs that are present in very small density get ionized. All common constituents of the ambient cannot be ionized in the drift tube due to their lower PA than water.

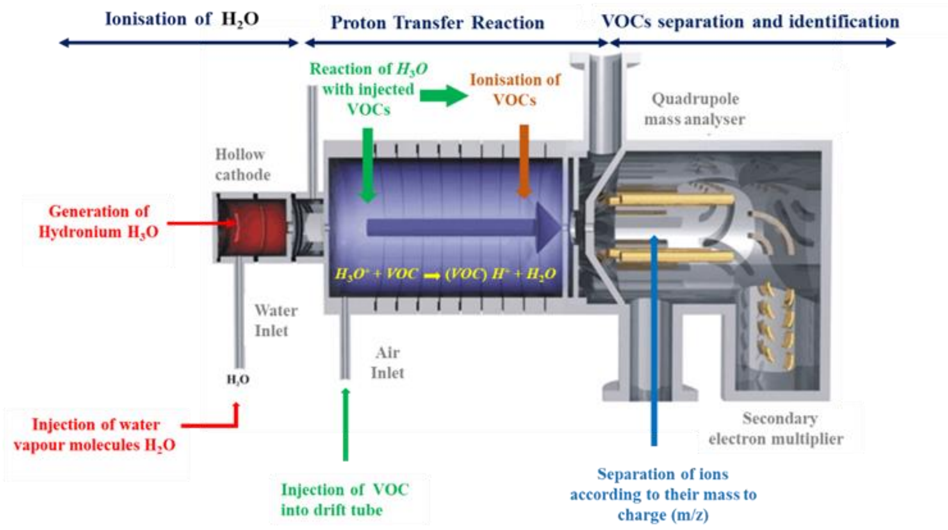

Figure 3-2| Schematic view of PTR-QMS apparatus and working principle: *The PTR-QMS consists of three parts: (1) hollow cathode for generation of reagent hydronium ions* H_3O^+ *, (2) drift tube for ionization of the VOCs using proton transfer reaction from the reagent ions according the chemical reaction* $H_3O^+ + VOC \rightarrow (VOC)H^+ + H_2O$*, and (3) the analyzing system consists of a quadrupole mass analyzer in conjunction with a secondary electron multiplier, which allow separation and identification of the VOCs according to their mas-to-charge ratio (m/z), respectively. The VOCs are introduced into drift tube through an air inlet. Adapted from Ionicon*

electron multiplier/pulse counting system, which measures count rates proportional to $[RH^+]$ and $[H_3O^+]$. The PTR-MS software calculates all parameters in (3.2), allowing the measurement of the concentration of the analyte R in the breath sample in real time in ppb or ppt:

$$[R]_{ppb} = C\frac{[RH^+]}{[H_3O^+]} \qquad \textbf{(3.3)}$$

where C is proportional to k, t, and transition factor of ions (www.PTRMS.com).

For all measurements in this study, PTR-QMS operated under standard conditions: drift tube voltage of 600V, drift tube pressure of approximately 2.2 mbar, drift tube temperature of 60◦C, and the E/N ratio of approximately 120Td. PTR-QMS cannot differentiate between substances contributing to the same nominal mass and is therefore a low selective method that requires further complementary analysis for compound identification. For this purpose, PTR-TOF-MS was used parallel to PTR-QMS in six MDD patients and two HCs to identify possible isobaric compounds. PTR-TOF-MS operated at standard condition. A more in-depth review can be found elsewhere (Jordan et al. 2009), (Watson 2016), (Portillo-estrada 2014).

3.3.3 Spectral processing

The concentration of the raw data was first averaged over at least 10 spectra (cycles) belonging to the same sample to reduce the background noise, then standardized using Log-transformation. This non-linear transformation adjusts for heteroscedasticity and converts skewed breathomics data to a more symmetric distribution. Missing values present in more than

20% of samples were removed, while those existing in less than 80% of samples were corrected with a small determined value, such as 2 (Wei et al. 2018), as explained in section 2.2.5. The processed spectra matrices of MDD patients and HCs served as input data for subsequent statistical and chemometric analysis.

3.3.4 Statistical analysis and potential metabolites selection

All data in the present study were evaluated using the Statistical and Machine Learning Toolbox as well as the Bioinformatic Toolbox in MATLAB (R2019b). Given the high complexity, multidimensionality, and collinearity of the metabolome, coupled with the small sample size particularly in the untargeted design, we employed a combination of univariate, bivariate and multivariate statistical tools to identify the potential exhaled metabolites that differentiate patients with MDD from HCs, and to investigate the chemical changes in MDD patients.

Univariate and bivariate analysis. — Due to the small study population, the univariate distribution of the data was investigated using several normality tests, including empirical distribution function tests including Kolmogorov-Smirnov test (Lilliefors test, limiting form, Stephen's method, Marsaglia method), Moment tests (Jarque-Bera test, D'Agostino and Person test), and correlation and regression-based tests (Shapiro-Wilk test, Shapiro-Francia test). Hypothesis testing was first carried out to obtain an overview and a rough ranking of potentially altered VOCs in MDD patients relative to HCs and to establish features applicable to multivariate approaches in subsequent analysis. Student's t-test on the log-transformed data and non-parametric Wilcoxon test were carried out to discriminate between the cohorts' groups. VOCs showing a p-value below 0.05 were considered statistically significant. As with other omics data, breathomics data involve a large number of simultaneous tests of thousands of hypotheses. Therefore, to account for multiple testing, *p*-values were adjusted according to the Benjamini Hochberg approach to control the false discovery rate (FDR).

Exploratory data analysis of breath metabolome alterations in MDD by principal component analysis. — A preliminary exploratory analysis of the breathomics data was conducted using the principal component analysis (PCA). This analysis included all statistically significant VOCs at all response times from baseline to T_{60} to derive and visualize clusters separating the patients with MDD patients and HCs. PCA is an unsupervised machine learning approach extensively used in the exploratory analysis and dimension reduction in metabolomics studies. The main idea behind PCA is to find a linear transformation of the original correlated data into a set of linearly uncorrelated variables, known as principal components (PCs), which capture most of the variability in the dataset. This decomposition method maximizes the variance explained by the first component while the subsequent components explain increasingly reduced amounts of variance (Tharwat 2016). Mathematically, PCA represents an eigenvalue decomposition of a semi-definite matrix of the data points. The PCs refer to the directions given by eigenvectors of the data's covariance matrix. After applying the PCA method, a set of loading vectors and score vectors are obtained. The loading vectors represent the PCs, and each vector coefficient corresponds to the individual contribution of each variable to the PCs. The score vectors represent the projection of each sample onto the new orthogonal basis. Plotting these sample scores over the first principal components is a convenient way of summarizing the global dataset, since normally these first PCs capture most of the variability in the dataset.

Analysis of the VOCs response to awakening stress by hierarchical clustering analysis. — The metabolites play a crucial role in the pathophysiology of diseases via interactions with each other within a metabolic pathway or biological network. Thus, in order to consider these interactions and understand the redundancies of the chemical compounds, we additionally focused on identifying groups of VOCs that showed similar patterns of change in MDD patients from baseline to T_{60} after awakening in response to the awakening stress. In a first step, a correlation network with changes in VOCs levels of MDD patients in logarithmic scale from baseline to T_{60} was created by Spearman correlation. In a second step, the hierarchical clustering analysis (HCA) using the agglomerative algorithm was applied to the Spearman's coefficients matrix (Nielsen 2019). The HCA is an unsupervised machine learning approach useful in the exploratory analysis, which provides a powerful and intuitive method for discovering structure in the data. It is particularly suitable for detecting non-linear trends in the data that are not conveniently detected by PCA. HCA is widely used in metabolomics to identify clusters of metabolites that show similar patterns of change in response to various stimulus (e.g., conditions, stress, therapy, etc.) or changes at specific time intervals.

Breath metabolite selection for binary classification. — To develop a cost-effective and reproducible clinical breath test for MDD, the supervised multivariate analysis should be performed with a concise, filtered list of VOCs. This approach aims to produce robust suitable predictive outcome while minimizing computational cost. Therefore, selecting the most relevant breath metabolites for binary classification is essential. This process involves identifying an optimal subset of significantly altered VOCs in MDD patients that offer maximal discriminating power between MDD and HCs. Before selecting multiple potential breath markers, non-informative breath VOCs that do not contribute to the VOCs fingerprint were initially filtered out, and redundancy in the data set was minimized. As previously described, non-informative VOCs include VOCs with very low values close to the LOD, or those detected in only small number of samples. Thus, a preliminary (putative) list of predictors was selected from the most significant VOCs identified through univariate hypothesis testing. These VOCs were available in more than 80 % of the samples and survived the adjustment for FDR. Next, VOCs with similar information content, such as VOCS with very high collinearity (e.g., isotopes) or those clustered with similar responses to the awakening stress stimulus, were considered to minimize redundancy among the informative metabolites. Subsequently, the potential VOCs were ranked based on variable importance scores calculated using neighborhood component analysis (NCA) applied to the filtered of putative predictors. Feature selection involved ranking the VOCs used in predictive models in order of importance, and then repeating the modelling process using the top metabolites. Generally, a higher absolute score or importance weight indicates a more influential metabolite. However, it is no guarantee that the top N variables from the full model will yield the optimal subset model. Therefore, feature selection was performed using forward selection to further refine the set of predictors. In this approach, at each step, one breath metabolite from the putative list is tested for inclusion in the predictive model. The metabolite that most significantly improves the model's prediction is added to the model. This process is repeated until no further significant improvement is observed. For optimal selection, the bootstrap averaged (with 1000 iterations) ROC curves for all single potential VOCs with high scores was initially performed to evaluate their performance (Xu et al. 2014). The single VOCs exhibiting the highest AUC, and optimal accuracy were combined in subsets to identify potential multiple VOCs that are correlated with the outcome.

Supervised machine learning approaches for potential breath test selection. — In order to achieve a comprehensive and robust description of the breathomics data and its predictive potential, the supervised machine learning analysis was performed using linear predictive models, including linear support vector machines (SVMs) (Awad and Khanna 2015; Cervantes et al. 2020; Nayak, Naik, and Behera 2015), logistic regression (LR) and Naive Bias (NB), as well as the non-linear kernel SVM predictive model using the Gaussian kernel radial basis function (T. Jiang, Gradus, and Rosellini 2020). In contrast to unsupervised learning, supervised learning utilizes labeled data sets to supervise (or to train) algorithms, enabling for accurate classification or prediction of outcomes. Multivariate predictive models are powerful tools for the discovery and development of biomarkers. In contrast, unsupervised learning focuses on identifying inherent patterns and structures within unlabeled data, making it useful for discovering novel biological processes.

SVMs are binary classifiers that seek to find an appropriate hyperplane or function $g(x) = w^T x + b$ that correctly separates two classes (e.g., cases and controls) with a maximum margin by training data samples in a multidimensional space (i.e., across many variables). The hyperplane may be linear in the origin space of the metabolite space or in a higher dimensional space. Figure 3-3 shows a separating hyperplane corresponding to a linear SVM. A decision hyperplane for a binary classification can be written as a set of points x satisfying:

$$w^T x + b = 0 \tag{3.4}$$

Where w is the normal vector to the hyperplane, b/||w|| determines the offset of the hyperplane from the origin along the normal vector w, as illustrated in Figure 3-3. For given training samples $\{\boldsymbol{x}_i, 1 \leq i \leq n\}$ that belong to two linearly separable classes C_1 and C_2, the distance from any point $\boldsymbol{x}_i$ to the hyperplane is:

$$\varepsilon_i = |wx_i + b| = y_i(wx_i + b) \tag{3.5}$$

where $y_i = 1$ for C_1 and $y_i = -1$ for C_2. The geometric distance is the normalized distance:

$$\delta_i = \frac{\varepsilon_i}{||w||} = \frac{|w^T x_i + b|}{||w||} \tag{3.6}$$

SVM aims to find w and b, such as $w^T x_i + b = 1$ for the nearest data points belonging to C_1 and $w^T x_i + b = -1$ for the nearest ones to C_2. Geometrically, this can be viewed as having a margin of $\frac{1}{||w||} + \frac{1}{||w||} = \frac{2}{||w||}$ between the two hyperplanes $w^T x + b = 1$ and $w^T x + b = -1$. Thus, to maximize this margin, the distance ||w|| should be minimized. The training data can be accurately divided into two classes, and the constraints that each data point belongs to the correct class assigned to the same side of the hyperplane can be written as:

$$\begin{cases} wx_i + b \geq +1,\ for\ y_i = +1 \\ wx_i + b \leq -1,\ for\ y_i = -1 \end{cases} \quad \text{for all } 1 \leq i \leq n \qquad \textbf{(3.7)}$$

These constraints can be rewritten as follows:

$$y_i(wx_i + b) = |wx_i + b| \geq 1 \qquad \text{for all } 1 \leq i \leq n \qquad \textbf{(3.8)}$$

Thus, the optimization problem of the linear SVM classifier can be written as:

$$\begin{cases} min\ \frac{||w||^2}{2} \\ y_i(wx_i + b) \geq 1, for\ i = 1,2,\ldots,n \end{cases} \qquad \textbf{(3.9)}$$

The values of w and b that solve this optimization problem determine the maximal margin and linear SVM classifier defined as: f(x) = sgn($w^T x\ + b$), where sgn(.) the sign function. These optimization parameters are determined by the closest data points to the separating hyperplane.

For non-linear separable data in input space, the linear SVM cannot find a robust separating hyperplane that minimizes the number of misclassified data points. For that, SVM algorithms perform a nonlinear mapping of the data from the input space into a high-dimensional feature space φ: $R^d \rightarrow H$. The classification vector *w* in *H* satisfies:

$$w = \sum_{i=1}^{n} c_i\, y_i \varphi(x_i) \qquad \textbf{(3.10)}$$

To reduce the complexity and computation of the problem in the high dimensional space *H*, SVM algorithm uses specific functions called kernel functions *K* that satisfy:

$$K(x_i, x_j) = \varphi(x_i)\varphi(x_j) \qquad \textbf{(3.11)}$$

Kernel functions map the nonlinear problem into a linear problem in a higher-dimensional space, and approximate the nonlinear separating decision boundaries in the original space (T. Jiang, Gradus, and Rosellini 2020; Noble and Street 2006). The SVM algorithm tries to find some index i such that $\varphi(x_i)$ lies on the boundary of the margin in the high dimensional space $y_i = w^T\varphi(x_i) + b = \sum_{j=1}^{n} c_j y_j\ k(x_i, y_j) + b$. The non-linear SVM optimization problem can be written as:

$$\begin{cases} \max\ (\sum_{i=1}^{N} c_i - \frac{1}{2}\sum_{i=1}^{n}\sum_{j=1}^{n} y_i y_j\ c_i c_j\ k(x_i, y_j) \\ \sum_{i=1}^{n} c_i y_i = 0, and\ \ 0 \leq c_i \leq C \end{cases} \qquad \textbf{(3.12)}$$

The value of the coefficients $\{c_{i,}\ 1\leq i\leq n\}$ can be obtained from the optimization problem. The optimal classification function is:

$$f(z) = sgn\left[\sum_{i=1}^{n} c_i\, y_i K(x_i, z)\right] + b \qquad \textbf{(3.13)}$$

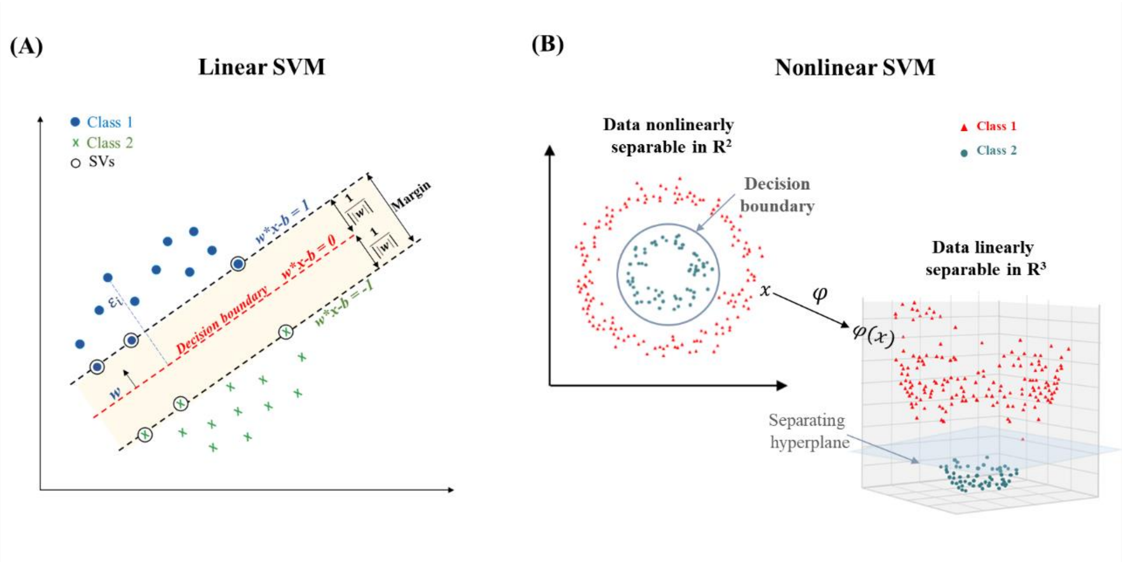

Figure 3-3| Illustration of classification principle of the support vector machine classifier: *(A) For linearly separable data (class1 and class 2), the support vector machine (SVM) classifier tries to find a hyperplane {x, w x-b=0} in the feature space that distinctly classifies the data points. For this purpose, SVM maximizes the margin, i.e., the maximum distance between data points of both classes corresponding to 2/||w||. To determine the position and orientation of the hyperplane, and maximize the margin, the SVM algorithm use the support vectors (SVs) (circle), which belong to the hyperplane* $w^T x_i + b = 1$ *for the nearest data points belonging to* C_1 *and* $w^T x_i + b = -1$ *for the nearest ones to* C_2*. Hyperplane parameters w and b are results of the optimization problem of the margin corresponding to the minimization of ||w||, (B) example of nonlinearly separable data in* R^2*. SVM algorithms map the data from feature space into a higher dimensional space using the function* $\varphi\colon x \rightarrow \varphi(x)$*. In order to minimize the computation and complexity of the optimization problem, SVM algorithms use kernel function defined as* $k(x,y)= \varphi(x).\varphi(y)$ *that allows implicit mapping and approximate the nonlinear separating decision boundaries in the original space. In this example the date is not separable in the feature space* R^2*. However, the transformed data in* R^3 *can be distinctly classified using a linear decision boundary, the two-dimensional hyperplane (light blue).*

SVM classifiers are widely used in various BGA and metabolomics applications (A. Smolinska et al. 2014). SVMs are powerful statistical approaches, even in studies with small sample sizes, and are very insensitive to perturbation and outliers.

LR is a supervised classifier that tries to find the best hyperplane to separate two classes. In contrary to SVM algorithms that are based on statistical approaches, LR is based on probabilistic approaches. Using a set of input features, the LR model aims to predict the probability that a given instance belongs to a particular class (e.g., diseased or non-diseased group). The LR model consists of a linear function of the input features, which is transformed using the logistic function, also called sigmoid function $g(z) = \frac{1}{1+e^{-z}}$, into probabilistic values between 0 and 1. Using a threshold, the probabilistic values of the sigmoid function can be assigned to a binary output {0,1} (e.g., 0 for diseased or 1 for controls) corresponding to two classes linearly or nonlinearly separable with the LR decision boundary. The LR hypothesis can be derived from the sigmoid function as follows:

$$h_w(x) = g(w^T x)\ , for\ 0 \leq h_w \leq 1 \tag{3.14}$$

The LR parameters w can be estimated by solving a LR optimization problem. The LR cost function for two classes is defined as:

$$Cost(h_w(x), y) = \begin{cases} -\log\,(h_w(x)) & if\ y = 1 \\ -\log\,(1 - h_w(x)) & if\ y = 0 \end{cases} \tag{3.15}$$

The cost function using these constraints can be rewritten as:

$$J(w) = -[\mathrm{y} \log\,(h_w(x) - \log\,(1 - h_w(x))] \tag{3.16}$$

The cost function is convex. Thus, the LR optimization problem consists in the minimization of cost function $\min_{w} J(w)$ with respect to the parameter w.

The NB is a linear classifier based on the so-called Bayes' Theorem, which assumes that the occurrence of a feature is completely uncorrelated with the occurrence of another feature within the class. The term "Naïve" comes from the fact that we assume that the features are statistically independent when in fact, we know that they are not. Given the assumption that a set of exhaled metabolites F: $\{\boldsymbol{F}_1, F_2,\ldots, F_n\}$ are independent, NB states the probability of the output Y from the features F_i for i=1,..,n:

$$P(Y|F) = P(Y|F_1)P(Y|F_2)P(Y|F_3) \ldots P(Y|F_n) = \prod_{i=1}^{n} P\,(Y|F_i) \tag{3.17}$$

Bayes' theorem sates the probability of the output Y from the feature F_i:

$$P(Y|F_i\,) = \frac{P(F_i|Y)P(Y)}{P(F_i)} \tag{3.18}$$

Model Validation and performance assessment. — The performance assessment of the classifier is a crucial part of the data analysis, as it measures how well the outcome predicted by the used model matches the real outcome. Several complementary measures were attained to assess the classifier's performance, including the sensitivity, the specificity, receiver operating characteristic (ROC) curve, and the area under curve (AUC) of the ROC. The ROC curves for SVM, LR, and NB predictive models were generated to verify which breath VOCs signature had the optimal sensitivity and specificity for potentially discriminating between MDD patients and HCs. The bootstrap technique with 1,000 iterations was used to calculate the bootstrap-corrected *AUC* and 95% confidence interval (95% CI) of the *AUC*.

The sensitivity represents the percentage of true positives that are correctly classified, also called true positive rate (TPR). It is defined as:

$$Sen = \frac{TP}{TP + FN} \tag{3.18}$$

The specificity indicates the percentage of true negatives that are correctly classified. It is defined as:

$$Sp = \frac{TN}{TN + FP} \tag{3.19}$$

where:

TP: the number of diseased individuals that are correctly identified as diseased

TN: the number of HCs that are correctly identified as healthy

FP: the number of HCs that are incorrectly identified as diseased

FN: the number of diseased individuals that are incorrectly identified as healthy.

ROC analysis is used in clinical epidemiology to assess the diagnostic ability of biomarkers, or to quantify how accurately medical tests can discriminate between two patient states, typically referred to as “diseased” and “non-diseased” (Hajian-Tilaki 2013), (Çorbacıoğlu and Aksel 2023). The ROC curve is a non-parametric procedure that depicts the balance of true positive rate and false positive rate, and is obtained by plotting TPR against FPR

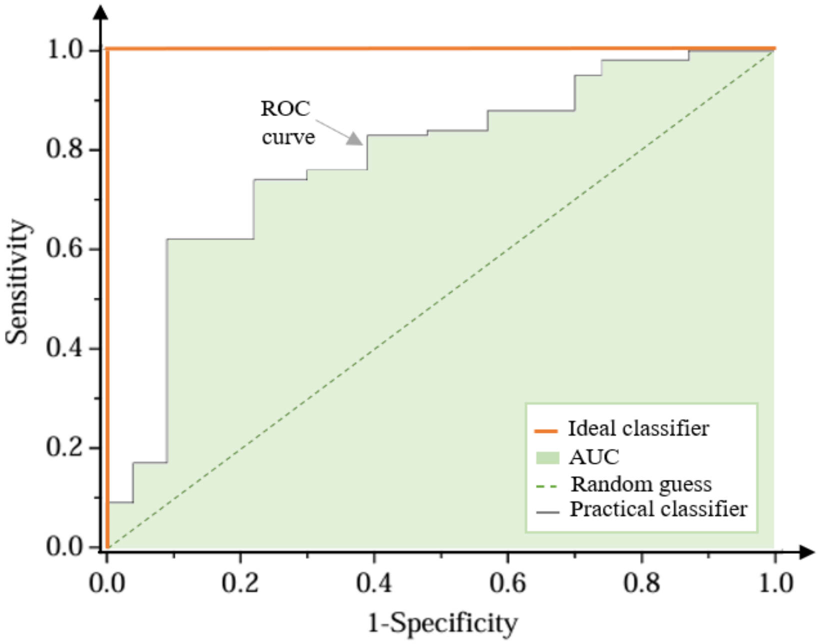

Figure 3-4| Example of the receiver operating characteristic (ROC) curves and the area under curve (AUC) of ROC*: The empirical ROC curve is obtained through the connection of (sensitivity, 1-sensitivity) values points from all possible cut-offs. The ideal ROC curve (orange line) goes through TPR=1 and FPR=0, which means that the classifier can achieve 100% accuracy under some threshold. A classifier based purely on a random guess is a straight line, while a practical classifier is somewhere in-between.*

under different thresholds. The ROC curve analysis is generally considered the standard method for evaluating the performance of biomarker classifiers (Xia et al. 2013). As illustrated in Figure 3-4, an ideal ROC evaluation curve passes through TPR = 1 and FPR = 0 indicating the that classifier can achieve 100% accuracy at some threshold. A classifier based purely on a random guess is a straight line, while a practical classifier falls somewhere in-between. The *AUC* serves as another important metric for evaluating classifier performance across all thresholds, as illustrated in Figure 3.4. *AUC* values range from 0.5 to 1, with *AUC* = 1 for an ideal classifier, *AUC* = 0.5 for random guess, and *AUC* values between 0.5 and 1 indicate the performance of a practical classifier. Various *AUC* labeling systems are substantial. In diagnostic studies, A rough guide for assessing the utility of a biomarker based on its *AUC* is as follows: *AUC* values above 0.90 are interpreted as very good diagnostic performances of the test, values between 0.80 and 0.90 are considered good, values between 0.70 and 0.80 are fair and values below 0.60 are interpreted as limited clinical usability of the test (Xia et al. 2013).

3.3.5 Breath volatomics data annotation and identification

VOCs annotation and identification. — The tentative identification of the detected exogenous and endogenous VOCs with PTR-QMS and their properties was performed based on previous studies utilizing PTR-MS, as well as the chemical library created by (Pagonis, Sekimoto, and de Gouw 2019). This library summarizes different trace gases detected by PTR-MS and SIFT-MS using soft ionization with hydronium ions (Pagonis, Sekimoto and de Gouw, 2019). Additional chemical libraries and database, such as PubChem (https://pubchem.ncbi.nlm.nih.gov) and Human Metabolome Database (*https://hmdb.ca*) were also used for metabolite annotation and property determination. The tentative identification of SCFAs and their fragments was additionally based on a previous study that investigated carboxylic acids using PTR-MS (Hartungen et al. 2004). Additionally, the identification of exogenous VOCs related to smoking habits was performed using T-test and clustering analysis with PCA. For this purpose, the participants were regrouped into three groups, namely smoker MDD (S-MDD) patients, non-smoker MDD (NS-MDD) patients and HCs (all non-smoker). The statistical difference between the levels of smoking-related VOCs among the three groups was investigated using T-Test at a significance level $p \leq 0.05$. Furthermore, VOCs of all S-MDD patients and those of all non-smoker participants, including NS-MDD patients and HCs, were clustered using PCA. Additionally, mass calibration, compound identification, and isotope correction with PTR-MS TOF were performed using the interface of the software PTR-MS Viewer (version 3.3, IONICON Analytic GmbH, Innsbruck).

Identification of VOCs fragments. — The contribution of the fragments in the main compounds was quantified at awakening, T_{30} and T_{60} using the regression and correlation analyses. The Pearson's correlation coefficients measure the strength of the linear correlation between the variables ***X*** and ***Y*** as follows:

$$\rho_{X,Y} = \frac{\sum_{i=1}^{n}(X_i - \bar{X})\,(Y_i - \bar{Y})}{\sqrt{\sum_{i=1}^{n}(X_i - \bar{X})^2}\,\sqrt{\sum_{i=1}^{n}(Y - \bar{Y})^2}} \qquad \textbf{(3.20)}$$

Where X_i and Y_i correspond to the ith components of the markers X and Y respectively, $\bar{X}$ and $\bar{Y}$ are their arithmetical means, and n is the sample size. The coefficient $\rho_{X,Y}$ ranges between −1 and 1. The strongest linear relationship is indicated by a $\rho_{X,Y}$ of -1 or 1, while the weakest relationship is indicated by a $\rho_{X,Y}$ of 0. A Pearson correlation coefficient of between 0 and 0.3 (or -0.3 and 0) indicates a weak relationship between the two variables, coefficient values between 0.4 and 0.6 (or -0.4 and -0.6) indicate a moderate strength relationship, and values between 0.7 and 1 (or between - 0.7 and 1) indicate a strong relationship. Correlations with $p \leq 0.05$ were identified as significant relationships between the breath VOCs.

3.4 Pathway-Based Analysis of Breath Metabolites

Pathway-based analysis of omics profiles has shown greater stability of biological activities and better performance (Aittokallio and Schwikowski 2006), (Draghici et al. 2007). Recent advancements in graph neural networks (GNNs) have opened new horizons and expanded the capacities to capture hidden topological information in pathways. Therefore, to comprehensively examine metabolomic changes manifested in MDD patients, we conducted an analysis of chemical changes occurring in metabolic pathways using the metabolic pathway enrichment analysis (MPEA) and metabolic pathway topological analysis (MPTA). Significant volatile breath metabolites were mapped into metabolic pathways using the homo sapiens Kyoto Encyclopedia of Genes and Genomes (KEGG) and homo sapiens SMPDB pathways database using the MetPa tool (Xia, Wishart, and Valencia 2011). MPEA analyzes a list of differentially expressed metabolites by comparing the number of metabolites detected in each pathway of interest with the number of metabolites expected to be found in the given pathway. The tool calculates *p*-values for each pathway based on the hypergeometric distribution (Goeman, Geer, and Kort 2004). To estimate the importance of each metabolite, MPTA was conducted using two topology measures: relative-betweenness centrality and out-degree centrality. In this analysis, each metabolite is represented as a node within a specific pathway. The degree of centrality is defined by the number of connections associated with this node. Generally, for a directed graph, there are two types of degree centrality: in-degree, which counts the links coming into the nod from other nodes, and out-degree, which counts the links initiated from the current node to the others. MetPa tool use the out-degree centrality as measure of node importance, assuming that upstream nodes play regulatory roles over downstream nodes. The betweenness centrality measures the number of shortest paths passing through a node, indicating its role as a bridge within the metabolic pathway or network. Since the metabolic network is directed, relative betweenness centrality is used to assess the importance of a metabolite within the pathway or network. Degree centrality measure focuses more on local connectivity, while the betweenness centrality emphasizes the global network topology. For more details on various graph-based methods, refer to the article by Tero Aittokallio et al. (Aittokallio and Schwikowski 2006).

The pathway impact is determined by the cumulative centrality values of matched metabolites normalized by the sum of centrality for all metabolites within that pathway. Given that multiple pathways are simultaneously tested in the metabolic pathway analysis, the statistical *p* values from the MPEA are further adjusted for multiple testing using Holm-Bonferroni and False Discovery Rate methods.

3.5 Association between VOCs and Depression Severity

The linear association between potential metabolites and depression severity, as measured by the HAM-D17 and BDI-II total scores, was evaluated using Peason correlation coefficients.

To assess the monotonic relationship between the candidate VOCs and the depression severity scores, Spearman's correlation coefficients were used. Spearman's correlation coefficients between 0 and 0.3 (or between -0.3 and 0) indicate a weak monotonic relationship between two variables. Coefficients between 0.4 and 0.6 (or between -0.6 and -0.4) indicate a moderate strength monotonic relationship, while coefficients between 0.7 and 1 (or between -1 and -0.7) suggest a strong monotonic relationship. In the first method, all three measurements from baseline to T_{60} were included as predictors in the correlation analysis. This approach accounts for the entire trajectory awakening response of the breath marker over time. In the second method, the measurement at the most significant response time was chosen as the single best breath marker to represent the predictor.

3.6 Impact of Confounding Factors on VOCs Profile

The impact of critical confounding factors on the accuracy of the breathomics analysis was evaluated by re-running the classification analysis on MDD subgroups after reorganizing the patients based on the confounding factors, including age, gender, BMI, smoking-related EVOCs, environmental exposure, and medication. The subgroups for smoking habits were defined as smoker and non-smoker subpopulations. No information on Ex-smokers or passive smokers was available, so these subgroups were not considered. Two approaches were used to assess the bias introduced by smoking. First, the impact of smoking on the exhaled markers at the most significant response time was evaluated in S-MDD, NS-MDD and HCs. Second, all three measurements from baseline to T_{60} of the exhaled markers were included in the analysis.

4 RESULTS

4.1 Baseline Characteristics of Study Population

In total, 26 MDD participants (38% men, 62% women), with a mean age of 38.04 ± 12.90 years and BMI of 27.36 ± 7.99 kg/m^2 were included in the breathomics study. The prevalence of mild, moderate, and severe depression according to HAMD-17 was 42%, 50% and 4% respectively and 4% in remission. The prevalence of minimal, mild, moderate, severe according to BDI-II was 4%, 4%, 23%, and 65%, respectively. One participant (4%) was excluded from the BDI-II depression severity statistics but remained included in the study due to the availability of HAMD-17 scale data for this individual. No significant differences in age, gender and BMI were identified between cohort groups. Patients with MDD show significant higher depression severity compared to HCs ($p < 0.001$). The smoking status was significantly different between the cohort groups ($p = 0.003$). Demographic, lifestyle, and clinical information of the study population is summarized in Table 3.1.

4.2 Breathomics Metabolites Identification

Identification of significant VOCs. — A total of 132 breath metabolites were identified in 153 samples of a cohort of 51 subjects in the range between *m/z* 18 and *m/z* 150. Out of which, 26 VOCs were statistically different between patients with MDD and HCs at level $p < 0.05$. The altered metabolites in the exhaled breath of the MDD patients belong to various chemical classes, namely alcohols, aldehydes, amino acids (AAs), biogenic amines (BA), benzoic acids, hydrocarbons compounds, pyridines, unsaturated hydrocarbon, short chain fatty acids (SCFAs), and short chain fatty acids esters (SCFAEs). However, the composition of altered metabolites in MDD breath was clearly dominated by volatile AAs and microbial metabolites, such SCFAs and acetaldehyde, as well as VOCs involved in the metabolism of AAs by intestinal tract gut microbiota, including intermediate metabolites of the essential amino acid tryptophan and biogenic amines. Table 4.1 summarizes all significant breath metabolites in MDD patients, their corresponding response times to the awakening stimulus, chemical classes and regulations.

Considering the awakening response trajectory from baseline T_0 to T_{60}, the compound at *m/z* 70 showed a significantly high linear correlation with isoprene at *m/z* 69 in HCs ($R = 0.83$, $p < 0.001$) and MDD patients (T_0-T_{60}: $R = 0.74$, $p < 0.001$; T_0: $R = 0.91$, $p < 0.001$), as illustrated in Figure 4-1 and Figure 4-2 A-B. Thus, *m/z* 70 was tentatively identified as isoprene fragment. The compound at *m/z* 89, tentatively identified as butyric acid (with a possible interference with pyruvic acid), showed significantly a high linear trend with the metabolite at *m/z* 88 in both, HCs ($R = 0.996$, $p < 0001$) and MDD patients ($R = 0.991$, $p < 0.0001$), as well as with the compound at m/z 90 in both HCs ($R = 0.93$, $p < 0.0001$) and MDD patients ($R = 0.95$, $p < 0.0001$). These VOCs were tentatively identified as derivates of butyric acid (*m/z* 88 was tentatively identified as an anion of the butyric acid with possible interference with the Tedlar bag compound, and *m/z* 90 was tentatively identified as an ester or salt of butyric acid). The VOCs at *m/z* 47 and *m/z* 65 showed a highly significant linear relationship $R = 0.96$ ($p < 0.0001$) in MDD patients,

Table 4.1| *List of all significant VOCs discriminating between patients with major depression and HCs*

m/z	Tentative Identification	T_{resp}	Chemical Class	*p*-values	p_{FDR}	Trend
42	Acetonitrile	T_0, T_{30}, T_{60}	Aromatic hydrocarbons	**0.009** 0.032 **0.0035**	0.035 0.039 **0.012**	up
44	Ethenamine	T_{60}	Amine	0.026	0.037	down
45	Acetaldehyde	T_{60}	Aldehyde	0.036	0.040	up
46	Ethylamine / dimethylamine	T_{30} T_{60}	Amine	**0.0031** **0.0041**	0.016 **0.012**	down
47^{t}	Ethanol	T_0	Alcohol	0.040	0.047	down
60	Trimethylamine / acetic acid ester	T_{30}	Amine	0.047	0.048	down
69^{t}	Isoprene	T_{30}	unsaturated hydrocarbons	0.0389	0.045	down
65^{*}	Ethanol cluster	T_{60}	Alcohol	**0.007**	0.017	up
70	Isoprene isotope	T_0 T_{30}	unsaturated hydrocarbons	0.029 0.026	0.038	down
74	n-Butanamine	T_{30} T_{60}	Amine	**0.0046** **0.0005**	0.0162 **0.0006**	down
79^{*} ($61^{p,int}$)	Benzene / Acetic acid^{int}	T_{30}	SCFAs	**0.0066**	0.039	up
85^{a} (103^{p}) **85^{a} (147^{p})**	Valeric acid / Lysine isotope^{int}	T_0 T_{30}	SCFAs Amino acid	0.0195 **0.0051**	0.035 0.016	down
87	Ethenamine derivative 2,2-Dimethylbutane 2,3-Butandione^{int}	T_{30} T_{60}	Alkane Alcohol	0.015 0.0389	0.038 0.040	down
88	(Iso)Butyric acid anion Pyruvic acid anion	T_0	SCFAs	0.041	0.041	down
89	(Iso)Butyric^{i} Pyruvic acid	T_0	SCFAs Keto-acid	0.029	0.035	down
90	(Iso)Butyric acid Pyruvic acid Derivate	T_0 T_{30} T_{60}	Amino acid	0.0238 0.0302 0.0253	0.035 0.039 0.037	down
91	Lactic acid / 2,3-Butandiol	T_{30}	Alcohol SCFAs	0.0297	0.048	down
93	Toluene / Propionic acid^{int}	T_{30}	Aromatic hydrocarbons	**0.0093**	**0.0048**	up
94	3-Methylpyridine	T_{60}	Pyridine	**0.0002**	**0.001**	down
102	2-Methylbutyrate 1-Butanamine, N,N- dimethyl	T_{60}	SCFAEs Amine	0.0232	0.040	down
106 (88^{a})	Serine	T_0	Amino Acid	0.040	0.041	down
116^{w}	1-Propane-amine,N,N-dimethyl	T_0	Amine	0.037	0.044	down
123^{t}	Nicotinamide	T_{30}	Pyridine	0.0269	0.0336	down
132 /$86^{t,a}$	(Iso)leucine^{i}	T_{30}	Amino Acid	0.0384 0.033	0.0477 0.039	down
138^{t}	Anthranilic acid	T_0	Benzoic acid	0.038	0.047	up

^{a}: abundant; SCFAs: Short chain fatty acids; SCFAEs: Short chain fatty acid esters, ^{p}: Precursor; ^{int}: Interference; t: significant compound using with t-test directly applied to concentration without transformation; ^{f}: fragment; T_{resp}: Response time to awakening; ^{W}: non-parametric Wilcoxon test; ^{i}:structural isomers that cannot be distinguished using PTR-QMS alone

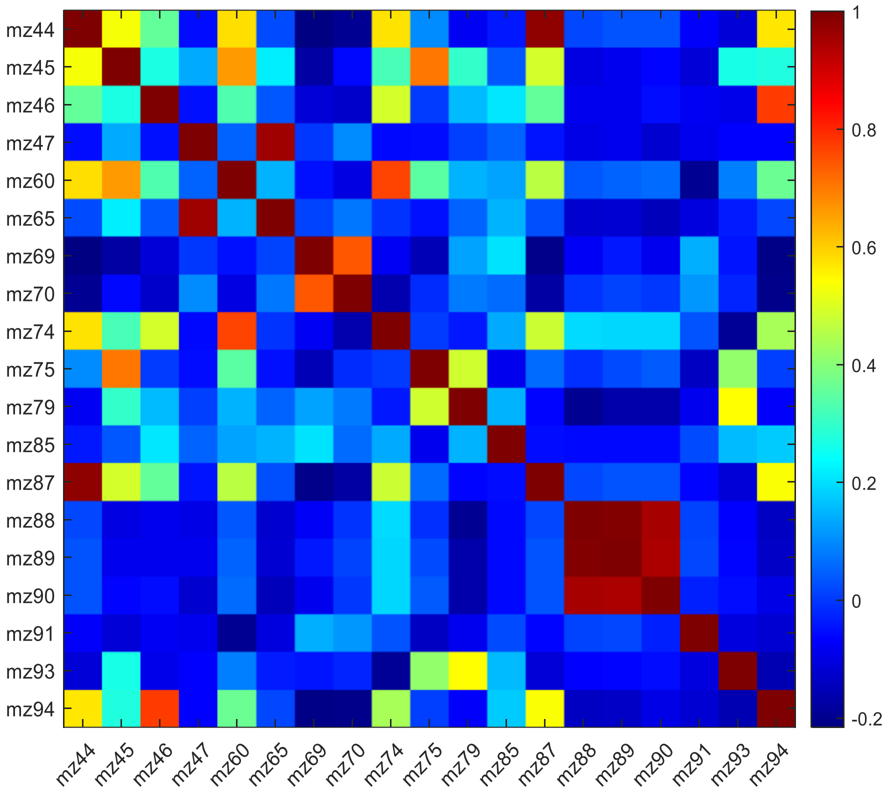

Figure 4-1| Visualization of the collinearity between the significant altered VOCs in patients with major depression by Pearson's correlation heat map: *The Pearson's correlation analysis shows strong collinearities between several significantly (p < 0.05) altered VOCs in MDD patients: A high significant Pearson's coefficient of R = 0.99 (p < 0001) is depicted between the VOCs at m/z 88 and m/z 89, and R = 0.94 (p < 0.0001) between m/z 89 and m/z 90, a coefficient R = 0.96 (p <0.0001) between m/z 65 and m/z 47, as well as a strong correlation R = 0.98 (p <0.0001) between m/z 44 and m/z 87. Additionally, a good correlation of R = 0.74 (p < 0.001) is depicted between the VOCs at m/z 69 and m/z 70. Furthermore, good linear relationships with Pearson's coefficient between 0.7 and 0.8 is illustrated between m/z 94 and m/z 46, and between m/z 75 and m/z 45, as well as between m/z 74 and m/z 60.*

and were tentatively identified as ethanol's main fragment and its water cluster. However, a weak association between these VOCs was detected in HCs, possibly due to the interferences with other metabolites. The VOCs at *m/z* 44 and *m/z* 87 also showed a highly significant linear correlation with each other in both HCs (R = 0.98, p <0.0001) and MDD patients (R = 0.99, p < 0.0001). The metabolites at *m/z* 44 was tentatively identified as ethenamine, and the compound at *m/z* 87 could be a fragment or derivative of ethenamine. Additionally, a good linear relationship (0.70 < r < 0.80) was observed between the metabolites at *m/z* 94 and *m/z* 46, between the VOCs at *m/z* 75 and *m/z* 45, and between VOCs at *m/z* 74 and *m/z* 60, as illustrated in Figure 4-1. These associations may be due to the involvement of these compounds in similar pathways or networks, as explained in the following sections.

Moreover, regression analysis showed a strong linear trend between *m/z* 69 and *m/z* 70. The contribution of the isoprene isotope at *m/z* 70 to the main compound at *m/z* 69 was 5% in MDD patients and 4.6% in HCs, as shown in Figure 4-2 A-B. This is in accordance with previous studies, which reported a contribution of the isoprene fragment at *m/z* 70 to the main compound of approximately 5.9% (Kushch, Schwarz, et al. 2008). Additionally, strong

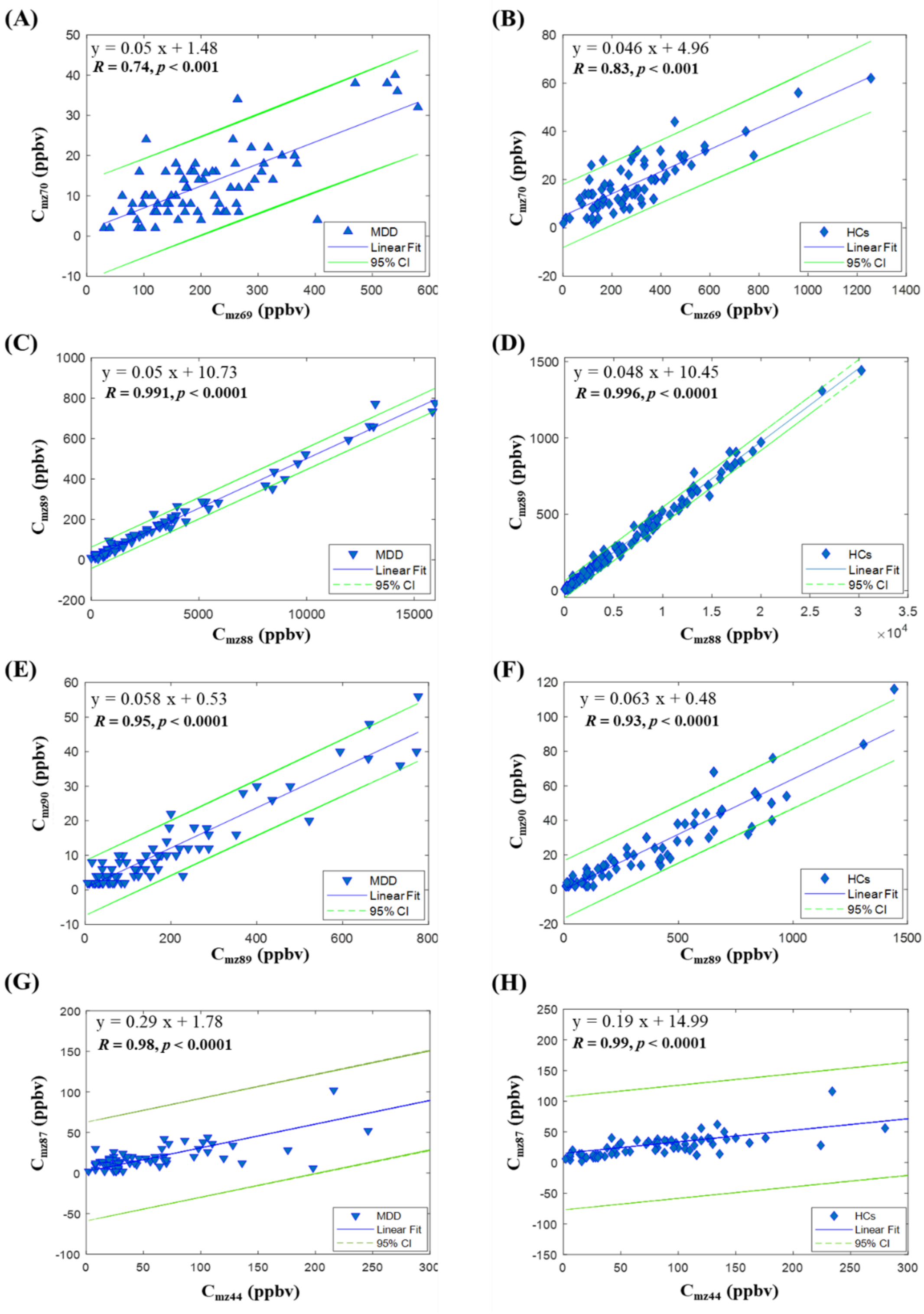

Figure 4-2| Identification of the collinearity between the breathomics data of patients with major depressive disorder using the regression analysis: *The regression analysis shows linear relationship between the VOCs at m/z 69 and m/z 70 in (A) MDD patients, and (B) healthy controls (HCs), as well as strong linear relationships between the VOCs at m/z 89 and m/z 88 in MDD patients (C) and HCs(D), between m/z 90 and m/z 89 in MDD patients (E) and HCs (F), and between m/z 44 and m/z 87 in MDD patients (G) and HCs (H). The VOCs at m/z 70, m/z 90, and m/z 87 in MDD patients contribute with 5%, 5,8% and 29% in the main compounds at m/z69, m/z 89 and m/z 44 respectively, and in HCs with 4.6%,6.3% and 19% respectively. The VOCs at m/z 70, m/z 88, m/z 90 and m/z 87 were tentatively identified as isoprene fragment, anion, and ester of butyric acid and ethenamine derivates respectively.*

linear relationships between m/z 88, m/z 89 and m/z 90 were observed, as seen in Figure 4-2 C-F. Thecompound at m/z 90 contributes with 5% to m/z 89 in MDD patients and 6.3% in HCs. The contribution of the ethanol water cluster at m/z 65 to ethanol main fragment was very low, corresponding to 1.3% in MDD patients. The compound at *m/z* 87 contributed 29% in *m/z* 44 in MDD patients and 19% in HCs.

The significant metabolites showed lower responsiveness to the awakening stress and were decreased in the breath of patients with MDD compared with HCs, except for the ethanol cluster at *m/z* 65, acetaldehyde and anthranilic acid. The level of the exhaled propionic acid from the baseline to T_{60} was found to be significantly elevated in MDD non-smokers ($p = 0.48$) compared to HCs, and significantly decreased in smokers at T_{30} ($p = 0.031$) relative to HCs. Additionally, the propionic fragment at *m/z* 57 was ranked among the top twenty key features for all MDD patients using the Bioinformatic Tool in MATLAB. Therefore, propionic acid was added to the putative list of ranked VOCs for MDD.

Identifcation of EVOCs related to smoking and enviromental exposure. — The concentrations of the VOCs at *m/z* 42 and *m/z* 93 were significantly increased ($p < 0.001$) in smoker MDD (S-MDD) patients compared to HCs (all non-smokers) and non-smoker patients with MDD (NS-MDD). However, these VOCs did not have a discriminatory effect between HCs and NS-MDD patients as illustrated in Figure 4-3A. Therefore, we primarily attributed the increased levels of these EVOCs to smoking. In contrast, the metabolite at *m/z* 79 was significantly increased in the breath of both S-MDD ($p < 0.001$) and NS-MDD patients ($p = 0.034$) relative to HCs. Additionally, no difference between S-MDD and NS-MDD subgroups was detected. This indicates that m/z 79 significantly distinguished betwenn all MDD patients and HCs, and could play an important role in MDD pathophysiology. The significantly increased level of this compound in all MDD patients is not only associated with active smoking but also results from outdoor and/or indoor exposure to this compound, including passive smoking. Therefore, this volatile metabolite was added to the putative VOCs-list for MDD.

Additionally, PCA clustering of smokers and non-smokers using *m/z* 42, *m/z* 79 and *m/z* 93 showed that *m/z* 42 and *m/z* 93 clearly discriminated between the S-MDD patients and all non-smokers (HCs and NS-MDD) with a sensitivity of 0.75 and a specificity of 0.95, as depicted in Figure 4.3-B. The metabolite at *m/z* 79 did not impact the PCA clustering of smoker and non-smoker subgroups. The metabolites at *m/z* 42, *m/z* 79 and m/z 93, which were associated in previous PTR-MS works and in chemical libraries with smoking and environmental exposure, were identified as acetonitrile, benzene, and toluene, respectively (Kushch, Schwarz, et al. 2008). Additionally, the PTR-MS TOF analysis of a subgroup of the study population showed that the acetic acid fragment contributed to the peak at *m/z* 79 with a very low relative abundance. This is consistent with a previous study investigating SCFAs using PTR-MS (Hartungen et al. 2004). Additionally, the PTR-MS TOF analysis also showed that a fragment of propionic acid contributes in very low amounts to the peak at *m/z* 93 (Hartungen et al. 2004). Among these EVOCs, the level of acetonitrile was significantly higher in S-MDD compared to benzene and toluene, despite the overnight fasting. This is consistent with previous reseach. Indeed, acetonitrile is considered a hallmark of smoking due to its prolonged accumulation in the body, remaining elevated for several days before reaching non-smoker levels, even after smoking cessation (Kwak and Preti 2011). On the other hand, it is important to note that growing evidence suggests psychological disorders, including depression, are associated with smoking (Zhaoping Wu et al. 2023). However, the relationship between

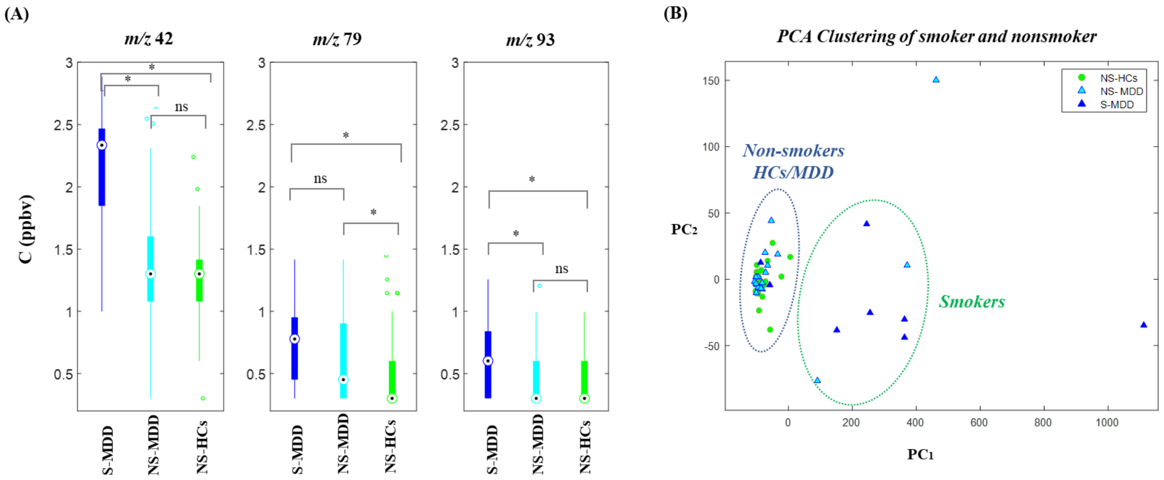

*Figure 4-3| **Identification of exogenous volatile organic compounds related to smoking and environmental exposure:*** *(A) The exhaled acetonitrile at m/z 42 and toluene at m/z 93 were significantly increased in the breath of smoker patients with major depressive disorder (S-MDD) compared with the non-smoker group ones, including non-smoker with MDD (NS-MDD) and HCs (all non-smoker. Benzene at m/z=79 was significantly elevated in S-MDD compared with HCs, but hat discriminatory effect on NS-MDD and HCs. This may indicate that the elevated level of benzene in all MDD is not restricted to smoking, but it is a result of the environmental exposure to this compound. (B) The exploratory analysis using the principal components analysis (PCA) shows that acetonitrile and toluene can clearly discriminate between all nonsmoker (HCs and NS-MDD within blue cluster) and S-MDD (green cluster) with a high sensitivity of 0.93.*
*ns: not significant; * significant at level p<0.05. The boxes contain 50% of the data distributed around the median, upper and lower quartile of values. Outliers in boxplot presentation are depicted with crosses.*

different aspect of smoking behavior and depression is controversial (Fluharty et al. 2017). Therefore, these EVOCs cannot be excluded from the candidate VOCs discriminating between HCs and MDD, especially benzene that was significantly altered in boh S-MDD and NS-MDD compared to HCs. A more in-depth investigation into the relationship between these VOCs and depression is necessary.

Putative list[7] of the most important VOCs associated with MDD. — After adjusting the *p*-values using Benjamini Hochberg method, identifying exogenous compounds related to smoking and environmental exposure, and excluding VOCs present in fewer than 80% of samples, the putative list of the most important breath metabolites includes ethenamine at *m/z* 44 and its derivate at *m/z* 87, ethylamine at *m/z* 46, acetaldehyde at *m/z* 45, the ethanol at *m/z* 47 and *m/z* 65, trimethylamine at *m/z* 60, isoprene at *m/z* 69 and *m/z* 70, butylamine at *m/z* 74, butyric acid at *m/z* 89 and its derivatives (*m/z* 88, and *m/z* 90), lactic acid at *m/z* 91, and methylpyridine at *m/z* 94, as depicted in Figure 4-4. Meanwhile, the most significant VOCs ($p < 0.01$) identified through univariate analysis were butylamine at *m/z* 74 ($p = 0.0005$, $p_{\mathrm{FDR}} = 0.0006$), methylpyridine at *m/z* 94 ($p = 0.0002$, $p_{\mathrm{FDR}} = 0.0012$), ethanol isotope at m/z 65 ($p =$

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[7] The putative list of VOCs comprises the filtered list of VOCs that could discriminate between patients with MDD and HCs, and can be used to rank the candidate VOCs for further binary classification.

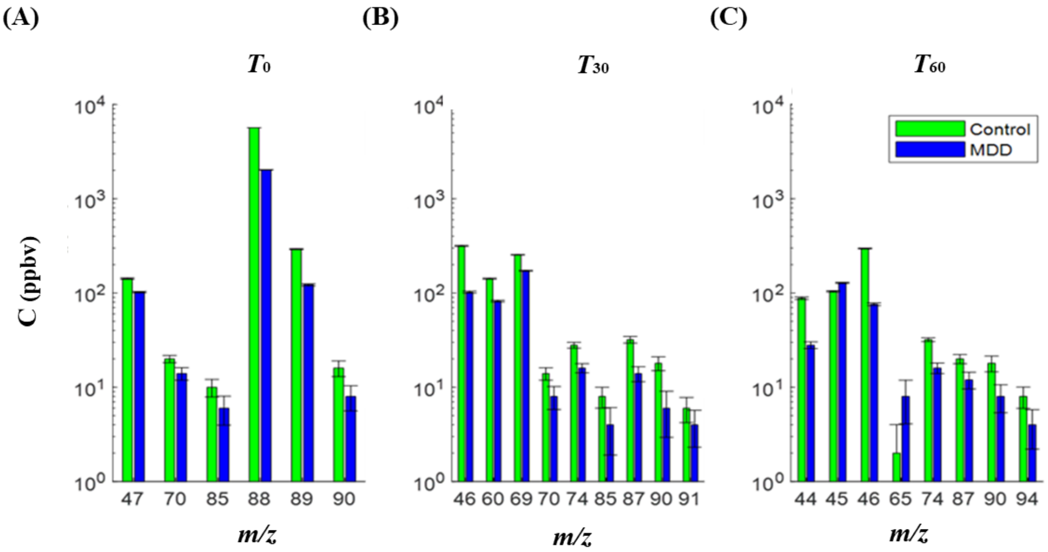

Figure 4-4| Putative list of the most important VOCs altered in MDD patients relative to healthy controls: *significant exhaled VOCs discriminating between patients with major depression (blue) and healthy controls (green) after exclusion of exogenous VOCs related to smoking and environmental exposures, VOCs available in lower than 70% of samples: (A) at awakening (T_0), (B) 30 minutes after awakening (T_{30}), and (C) 60 minutes after awakening (T_{60}). All exhaled VOCs are decreased in the breath of MDD patients relative to the healthy controls, except VOCs at acetaldehyde at m/z 45 and ethanol fragment at m/z 65. All concentrations of the VOCs are plotted in median and geometric standard deviation.*

0.0073, $p_{\text{FDR}} = 0.0174$) and valeric acid (interference with lysine) at *m/z* 85 ($p = 0.005$, $p_{\text{FDR}} = 0.0162$), where p_{FDR} represents the adjusted *p*-value for multiple testing.

Benzene discriminated between all MDD patients and HCs, while propionic acid at m/z 75 was found to be significantly increased in NS-MDD and significantly decreased in S-MDD at T_{30} ($p = 0.031$) relative to HCs, as discussed in section 4.6. Therefore, these VOCs were added to the putative list for further multivariate analysis. Toluene and acetonitrile cannot be excluded from the list, and their discriminatory effect and predictive value should be investigated in the multivariate analysis. Taking in account the collinearity between the VOCs, the tentatively identified isotopes and water cluster can also be excluded from the putative VOCs-list.

Exploratory analysis of volatomics alteration in MDD by PCA. — The exploratory analysis of the exhaled volatomics from MDD patients and HCs using PCA revealed that isoprene at the significant response time of T_{30} and butylamine at T_{60} are the most promising VOCs for achieving linear separation of the cohort groups, with a sensitivity 0.85 and specificity of 0.76. PCA clearly highlighted the separation of cohort groups into two distinct clusters. However, some overlap between the cohort's clusters is observed, as depicted in Figure 4-5A. This overlap could be associated with various factors, including the severity of depression and smoking. As shown in Figure 4-5B, some MDD patients with mild depression are clustered within or near the HCs cluster. Additionally, the smoker patients are also positioned close to the HCs cluster. This may be due to the impact of smoking habits on the levels of VOCs at *m/z* 69 and *m/z* 74, as explained in section 4.7. Active smoking is well-known to influence the level of VOCs. Previous studies reported that smoking increases the level of exhaled isoprene compared to non-smokers (Jia et al. 2024) . However, to our knowledge, there is no information available on the impact of smoking on the concentration of butylamine.

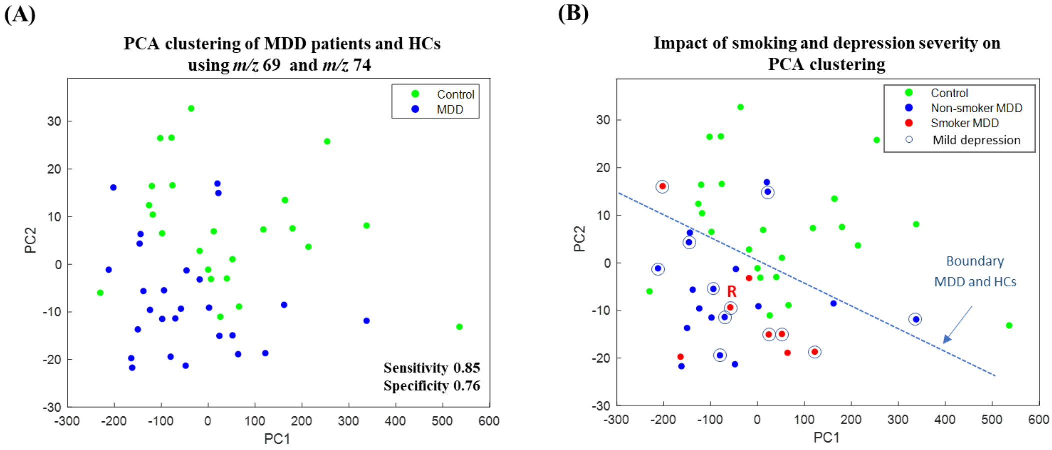

Figure 4-5| Exploratory analysis of volatomics changes in major depression using the principal component analysis: *(A) Butylamine and isoprene represent the most potential VOCs allowing a linear separation of patients with major depressive disorder (MDD) (blue cluster) and healthy controls (HCs) (green cluster) with a sensitivity of 0.76 and a specificity of 0.85. (B) The overlapping of the cohort's clusters could be associated with various factors, including the severity of depression and smoking. MDD patients with mild depression or MDD smokers are clustered within or close to the cluster of HCs.*

4.3 Investigation of Awakening Response of VOCs using Hierarchical Cluster Analysis

HCA revealed the presence of seven breath metabolite modules, where the member metabolites showed statistically significant correlations ($p < 0.05$) with each other in their perturbation patterns from baseline to T_{60}, as illustrated in Figure 4.6. The metabolites within each cluster primarily belong to the same biochemical class, are involved in a specific metabolic network or pathway, and have functional relationships with one another. The first cluster (C_1) mainly included microbial byproducts. The first subcluster of C_1 consisted of biogenic amines produced by microbial decarboxylation of amino acids. Ethylamine and butylamine are decarboxylase products of the non-essential alanine and norvaline, respectively, along with their derivatives, including 1-Butanamine,N-N-dimethyl and 1-Propane-amine,N,N-dimethyl. These derivatives are derived from propanamine, which is a decarboxylate product of the amino acid alpha-aminobutyrate (Thorn and Greenman 2012). The second cluster (C_2) mainly contained essential volatile amino acids, including (Iso)leucine, lysine and serine, as well as intermediate metabolites involved in the metabolism of the essential amino acid tryptophan via the tryptophan-nicotinate pathway. The third cluster (C_3) encompassed the main isoprene compound at *m/z* 69 and its fragment at *m/z* 70. The association of the compound at *m/z* 91 with the isoprene cluster is not clear. The fourth cluster (C_4) primarily included the aromatic smoking VOCs acetonitrile, benzene, and toluene, which have minor interferences with the fragments of acetic acid and propionic acid at *m/z* 79 and *m/z* 93, respectively. The fifth cluster (C_5) included volatiles involved in pyruvate metabolism, while the sixth cluster (C_6) included SCFAs such as propionic, acetic and valeric acids, along with their isotopes. The presence of (iso)butyrate at *m/z* 89 in C_1 and not in C_6 may indicate that this SCFA was not produced by anaerobic fermentation of fiber, but rather by bacterial fermentation of proteinaceous material such as amino acids, or it could be due to interference with pyruvate acid at the nominal mass of m/z 89. The results of the inter-correlation between the metabolites and the hierarchical clustering are visualized in Figure 4-6 and cluster details are presented in Table 4.2.

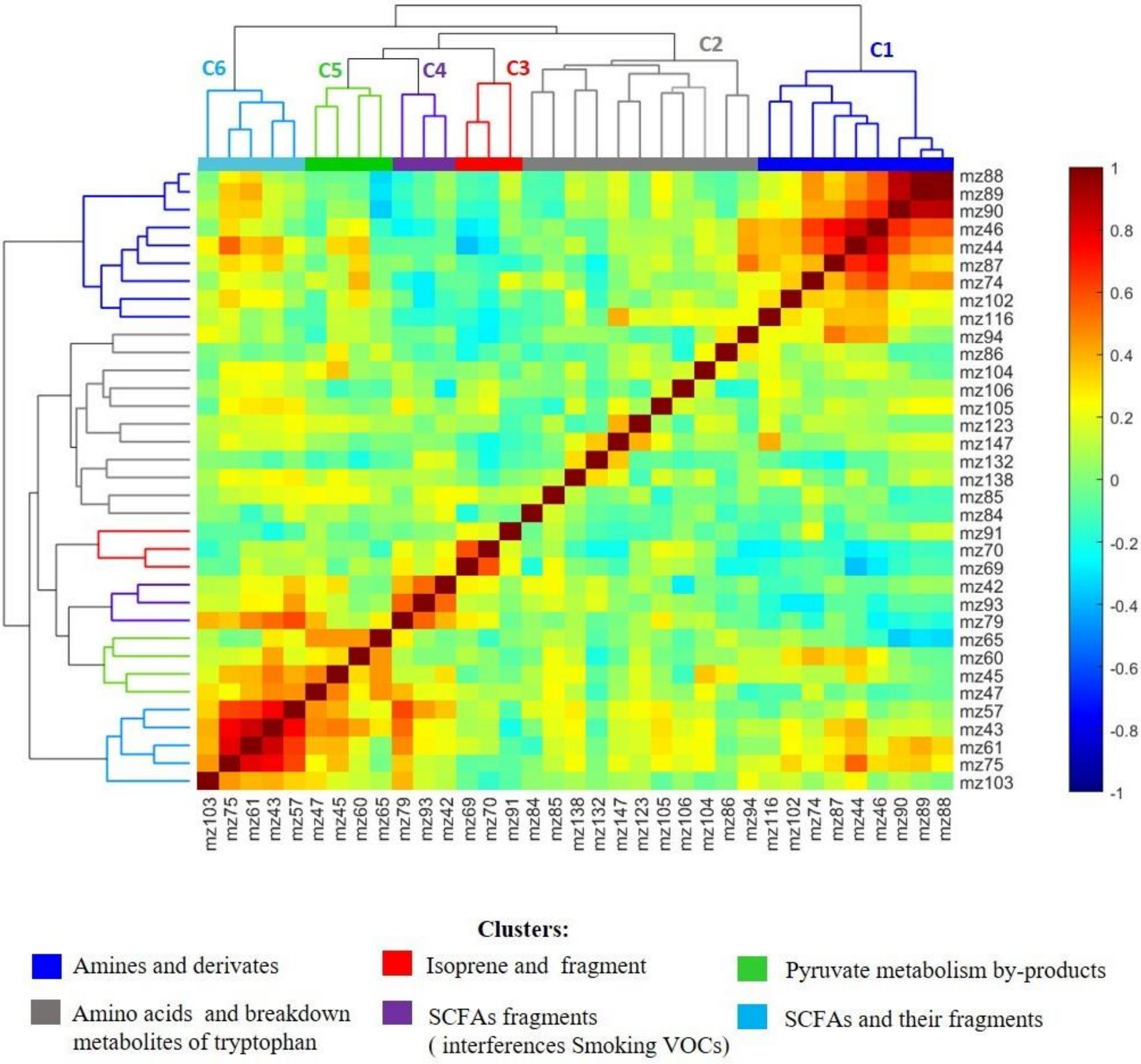

Figure 4-6| Heat map visualization and hierarchical clustering analysis of Spearman's distance between significant altered breath metabolites of MDD patients compared to healthy controls (p<0.05) from awakening to 60 minutes after awakening: *The HCA shows the presence of six clusters of breath metabolites in which the member VOCs show statistically significant correlation among each other in their perturbation from baseline to 60 minutes after awakening. The clusters are primarily predominated with the same chemical class or metabolites involved in the same pathway/network. The cluster* C_1 *(dark blue) contains mainly amines,* C_2 *(grey) comprises amino acids and breakdown metabolites of amino acid tryptophan,* C_3 *(red) includes isoprene and its fragment at m/z 70. C4 (violet) includes fragments of SCFA that are overlapped with aromatic smoking VOCs. C5 (green) metabolites involved in pyruvate metabolism and* C_6 *(light blue) includes SCFAs and their fragments. The metabolites in* C_2 *showed lower correlation with each other due to the intra- and inter-variability of amino acids in exhaled bread using PTR-MS quadrupole. It is important to note that for the significant VOCs fragments, the main compounds were added in the HCA in order to detect the contribution of these VOCs in their main compound. Abbreviations: C: Cluster; m/z: Masse-to-ratio; SCFAs: Short chain fatty acids; VOCs: Volatile organic compounds*

Table 4.2 | Clustering of differential breath metabolites with similar responsiveness to awakening stress from baseline to 60 minutes after awakening using the hierarchical clustering analysis, and their corresponding dominant chemical class or probable pathway

Cluster	***m/z***	**Metabolites**	**Class / pathway / Network**
Cluster #1	44 46 74 87 102 116	Ethenamine Ethylamine 1-Butanamine Ethenamine derivative/ 2,2-Dimethylbutane/ 1-Butanamine, N, N-dimethyl 1-Propane-amine, N,N-dimethyl	Biogenic amines: Microbial decarboxylation products of amino acids and derivatives
	88 89 90	(Iso)Butyric acid anion (Iso)Butyric acid/pyruvic acid^{int} (Iso)Butyrate esters	Branched chain SCFAs and derivates
Cluster #2	147^{p}, 84^{f},85$^{f, a}$ 106^{p}, 88^{f} 132^{p}, 86^{a}	Lysine Serine Isoleucine/Leucine	Amino acids
	123 138 94	Nicotinamide Anthranilic acid 3-Methylpyridine (precursor of nicotinamide)	Breakdown metabolites of tryptophan metabolism in Tryptophan-Nicotinic pathway
Cluster #3	69^{m}/70^{f} 91	Isoprene lactic acid / 2,3-Butandiol	Mevalonate pathway
Cluster #4	42 79 93	Acetonitrile Benzene (Acetic acid $^{int, L}$) Toluene (Propionic acid $^{int, L}$)	Aromatic smoking VOCs with low amount of SCFAs fragments interferences
Cluster #5	47^{m}/65^{c} 45 60	Ethanol Acetaldehyde Trimethylamine	Pyruvate metabolism Glycolysis
Cluster #6	61^{m}, 43^{f} 75^{m}, 57^{f} 103^{m}	Acetic acid Propionic acid Valeric acid	Straight chain SCFAs

m/z: mass-to-charge ration; SCFAs: Short Chain Fatty acids; VOCs: Volatiles organic compounds, ^{int}: interference; ^{a}: abundant; ^{m}: main compound; ^{f}: fragment; ^{L}: low amount, ^{c}: Water cluster.

4.4 Ranking of Breath Markers for Binary Classification

Feature selection for binary classification using NCA. — The NCA applied to the filtered list of significantly altered VOCs in MDD patients at the most significant response times indicated that the VOCs with the highest importance weights are methylpyridine at *m/z* 94, butylamine at *m/z* 74, and isoprene at *m/z* 69, as illustrated in Figure 4-7. The breath metabolites in the cluster C_5 with similar awakening response, including those at *m/z* 45, *m/z* 47, *m/z* 60 and *m/z* 65, have nearly similar weights. The metabolite with redundant information, including the fragment of isoprene at *m/z* 70, the isotope of butyric acid at *m/z* 90, the isotope of valeric acid at m/z 85, and the isotope of ethylamine at *m/z* 87 were ranked as VOCs with the lowest weights close to zero. Additionally, ethylamine at *m/z* 44 and lactic acid at *m/z* 91, which have similar awakening response to the top-ranked VOCs butylamine and isoprene, respectively, have also the lowest importance for binary classification. Ethylamine at *m/z* 46 and butyric acid at *m/z* 89 in C_1 have also similar weight around 0.25. Benzene at m/z 79 showed also low importance.

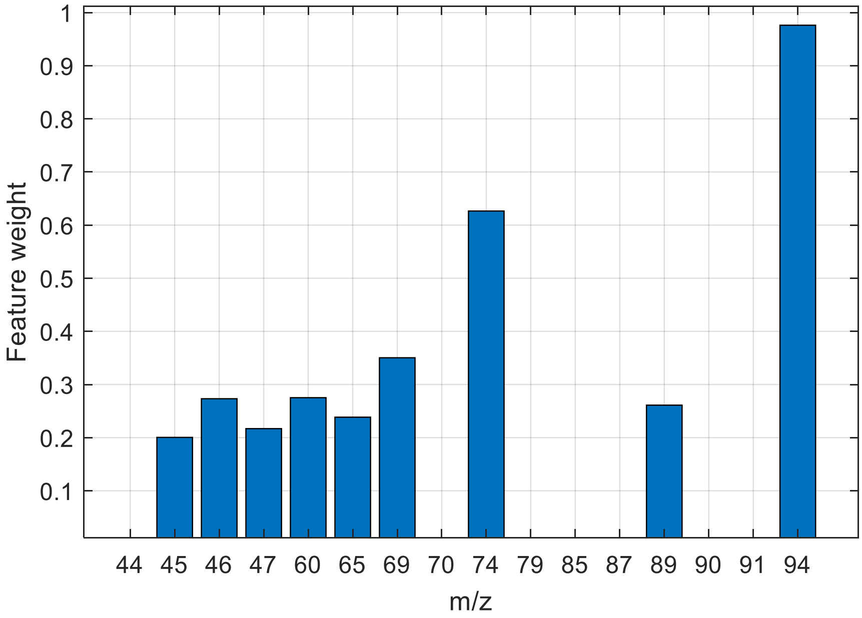

Figure 4-7| Feature selection for binary classification using neighborhood component analysis: *the neighborhood analysis shows that the VOCs with highest importance weights are the metabolites at m/z 69, m/z 74 and m/z 94. The VOCs with analytically redundant information, including m/z 70, m/z 85, m/z 87 have the lowest weights close to zero. The VOCs at m/z 44, m/z 85, m/z 91 with similar awakening response such as m/z 74, m/z 94 and m/z 69 respectively, which have highest ranking. The VOCs m/z 45, m/z 47, m/z 60, m/z 65 involved in cluster 4, as well m/z 89, and m/z 46 have nearly similar weight around 0.25.*

Predictive strength for single preselected markers. — In addition to feature selection with NCA, we also ranked the VOCs with the strongest predictive values within the different HCA clusters. In cluster C_1, butylamine at T_{60} demonstrated the highest predictive value for discriminating patients with MDD from HCs, with a sensitivity of 0.81, a specificity of 0.70 using all models, an *AUC* of 0.81 (95% CI: 0.67- 0.91) using SVM, LR and NB, and an *AUC* of 0.82 (95% CI: 0.67 - 0.92) using K-SVM. Ethylamine at T_{30} distinguished the cohort groups with a fairly good predictive value, similar to butylamine, with an *AUC* of 0.75 (95% CI: 0.60 - 0.86) using K-SVM, SVM and LR. It exhibited a lower sensitivity of 0.70 but higher specificity of 0.80. The other VOCs within C_1 generally had a sensitivity higher than 0.70 and a specificity between 0.60 and 0.70. In cluster C_2, the isotope of valeric acid (m/z 85) at T_{30} and methylpyridine (*m/z* 94) at T_{60} demonstrated strongest predictive values, with approximately similar performance: a sensitivity of 0.62, a specificity of 0.80, and an *AUC* of 0.82 using all predictive models except NB. All VOCs within C_3 exhibited nearly similar performance: *m/z* 69 had an *AUC* of 0.68, a sensitivity of 0.62 and a specificity of 0.65; *m/z* 70 had an *AUC* ranging from 0.68 to 0.70, a sensitivity of 0.62, and a specificity of 0.75; *m/z* 91 had an *AUC* between 0.63 and 0.67, a sensitivity of 0.62 and a specificity of 0.60, using all predictive models. In cluster C_4, the exhaled acetaldehyde at T_{60} (sensitivity 0.73, specificity 0.65) and trimethylamine at T_{30} (sensitivity of 0.73 and specificity of 0.6) demonstrated the strongest classification performances. Taking into account the tobacco and environmental-related VOCs in C_5, benzene at T_{30} showed the best performance using all models (*AUC* of 0.70, sensitivity of 0.60 and specificity of 0.75). The propionic acid had a fairly good predictive value using NB (*AUC* = 0.77, sensitivity of 0.73 and specificity of 0.80). Therefore, this volatile metabolite was also considered a potential candidate for breath markers in MDD.

Selection of Breathprint for MDD using forward feature selection. — To develop an optimal diagnostic breath test for MDD using a concise breathprint, all four predictive models were applied to assess the performance of the selected breath markers at each step of the forward selection method. Initially, the predictive models were built using the most promising marker, butylamine at *m/z* 74, measured at T_{60}. The Bootstrap-averaged (1000 iterations) ROC of this compound alone already demonstrated good predictive accuracy, with an *AUC* between 0.81 and 0.82, a sensitivity of 0.81 and a specificity of 0.70 across all four predictive models, as depicted in Figure 4-8A. Subsequently, at each step, one volatile compound with a strong predictive value from the other HCA-clusters and/or from the ranked VOCs with NCA was added to the predictive models. According to the bootstrapped (n = 1000) ROC analysis, the combination isoprene (*m/z* 69) at T_{30}, butylamine (*m/z* 74) at T_{60} and methylpyridine (*m/z* 94) at T_{60} are the most promising markers for MDD. This breathprint demonstrated clinically acceptable performance across all linear models (SVM, LR and NB), with AUC ≥ 90, sensitivity ≥ 80 and specificity ≥ 80), as illustrated in Figure 4-8C. The nonlinear K-SVM model achieved even higher performance, although this may reflect overfitting, a known risk with K-SVM in small datasets and in the presence of correlated or redundant features.

To further refine the breathprint, additional VOCs from other HCA-derived clusters were tested. The inclusion of trimethylamine (m/z 60) modestly enhanced sensitivity in both the LR model (from 0.92 to 0.96) and SVM model (from 0.85 to 0.88), without comprising the specificity. However, adding a fifth VOC did not substantially improve the overall AUCs, as illustrated in Figure 4-8 E-F. When propionic acid was added as a fifth marker, NB model showed notable gains in classification performance (AUC: from 0.87 to 0.94, Sensitivity from 0.73 to 0.81, Specificity from 085 to 0.95). Similarly, SVM model showed improved specificity (from 0.85 to 0.95), albeit with a reduction in sensitivity (from 0.88 to 0.77). A comparable pattern was observed when benzene was added as a fifth marker, both NB accuracy and SVM specificity (0.90 vs. 0.85) improved slightly, while SVM sensitivity decreased modestly (from 0.88 to 0.81). These findings suggest that propionic acid and benzene enhance specificity at the expense of sensitivity, likely due to their association with specific MDD subtypes, propionic acid being more relevant to NS-MDD subgroup, while benzene is more closely linked to environmental exposome factors.

Interestingly, several alternative breathprints were identified by substituting core VOCs (Butylamine, 3-Methylpyridine, isoprene, trimethylamine) with biologically related metabolites, exhibiting similar post-awakening dynamics, as shown in Table 4-3. These alternatives maintained comparable classification performance, suggesting functional redundancy due to shared metabolic pathways or overlapping roles. Importantly, they met clinically accepted diagnostic thresholds (AUC ≥ 85, Sensitivity and specificity ≥ 80) across all linear models, underscoring their clinical utility for flexible, application-specific breath test development. Among the most consistent alternatives were ethylamine (*m/z* 46), (iso)butyric acid (*m/z* 89), while ethanol (*m/z* 47) met these criteria only in the SVM model. Acetaldehyde (*m/z* 45), showed more balanced sensitivity and specificity, although its association with very high AUC values suggests potential overfitting, may due to redundancy or interference with other VOCs.

The combination of methylpyridine, isoprene, butylamine at the response times represents the most promising 3-variable breathprint with all models. The K-SVM model demonstrated the highest performance and accuracy for most 3-variable, 4-variable, and 5-variable signatures. K-SVM generally achieves higher accuracy than the linear SVM model and the other linear classifiers when the data is complex and not linearly separable. Transforming the data into higher dimensions using the kernel trick allows a linear separation of the data in these high dimensional spaces, making K-SVM powerful for nonlinear classification. However, K-SVM provided very high *AUC* values, though with potential overfitting. K-SVM typically demonstrate high *AUC* values in cases where non-linear boundaries are necessary. However, overfitting in K-SVM typically occurs due to feature redundancy or insufficient data points, which prevent capturing the true underlying patterns.

The SVM model demonstrated strong overall performance with robustness against overfitting, while LR provided a good balance between sensitivity and specificity, making the predictive models robust for the breathomics data. The NB predictive model also demonstrated good classification performance but underperformed compared to the linear LR and SVM models. This was expected because NB model is based on the assumption of independence between the markers, which is generally violated in medical diagnosis, and particularly in the metabolomics data. Additionally, NB showed relative high sensitivities but lower specificities across different combinations of candidate VOCs, a known issue when features are highly correlated.

Importantly, candidate VOCs demonstrated their strongest best discriminatory performance at the most significant responsiveness time during the post-awakening interval (T_0 – T_{60}), a critical window associated with physiological stress. Several metabolites returned to baseline levels beyond this period, indicating transient, state-dependent physiological responses. Similar to the cortisol awakening response, these transient patterns may offer diagnostic value when breath sampling is standardized to this specific timeframe. This approach allows for detection of altered stress reactivity and impaired metabolic adaptation in MDD patients, physiological disruptions that may remain undetectable under resting conditions.

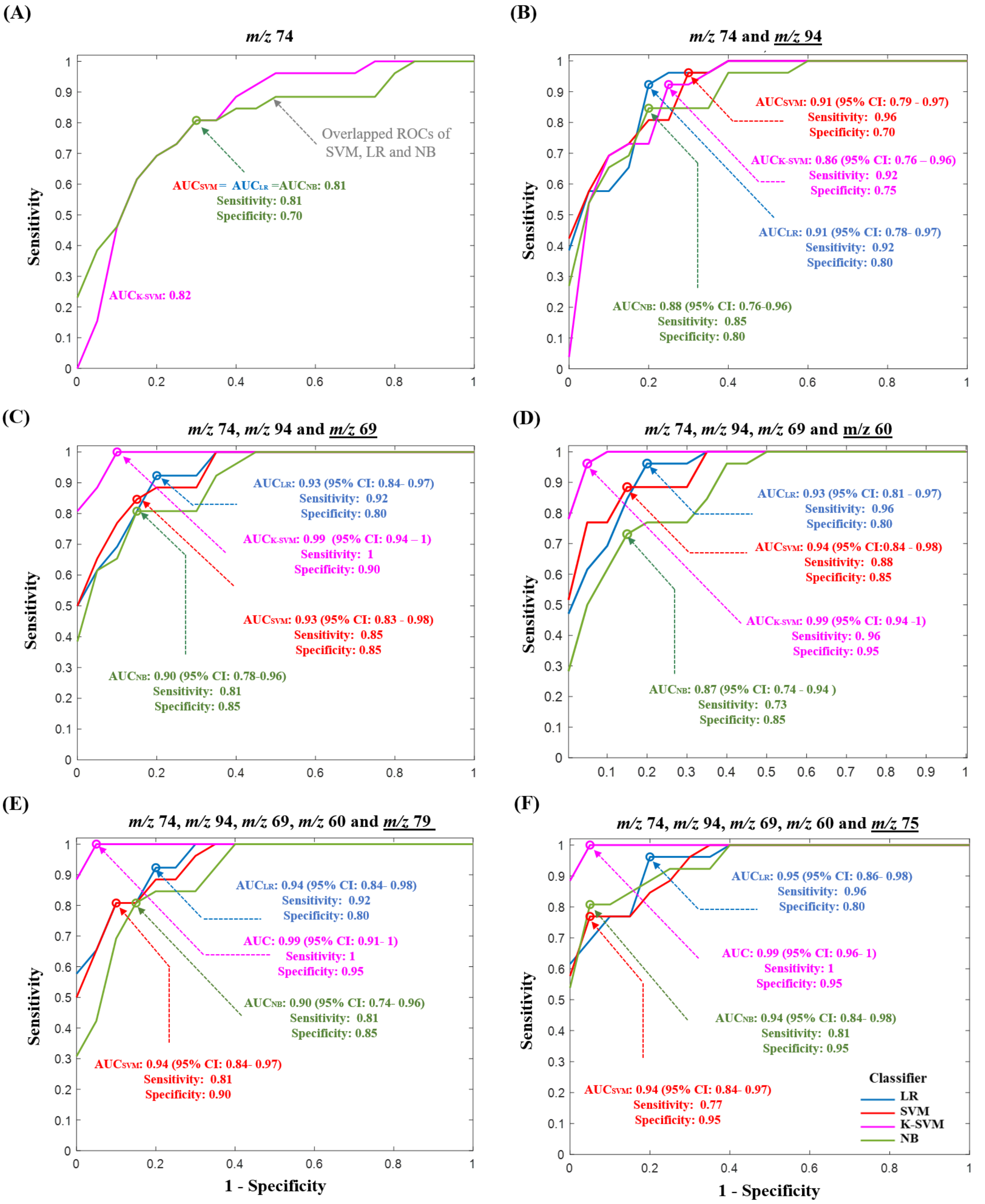

Figure 4-8*| *Feature selection for binary classification of MDD patients and healthy controls using forward selection: *(A) First, the predictive models, including support vector machine (SVM) (red), kernel SVM (K-SVM) (pink), logistic regression (LR)(blue) and Naïve Bayes (NB), were built with the most potential marker m/z 74 discriminating between MDD patients and HCs , and their separation performance were evaluated using the Bootstrap averaged receiver operating characteristic (ROC) curves. Then subsequently all four models were built with the other important markers with one marker more in a step: (B) best two volatile breath metabolites m/z 74 and m/z 69, (C) three variables m/z 74, m/z 69 and m/z 94, (E) four VOCs m/z 74, m/z 69 and m/z 94, and m/z 60, and (F) five VOCs at m/z 74, m/z 69 m/z 94, m/z 60 and m/z 79, and (G) m/z 74, m/z 69 m/z 94, m/z 60 and m/z 79. The inclusion of trimethylamine (m/z 60) modestly enhanced sensitivity in both the LR model and SVM model without comprising the specificity. Adding VOC at m/z 75 or m/z 79 as a fifth VOC did not substantially improve the overall AUCs. Both VOCs improved specificity but reduced the sensitivity. The Bootstrap averaged ROC were calculated with 1000 iterations. The arrows in the panels indicates the point on the ROC curve with the optimal sensitivity and specificity.*

Table 4.3| Summary of the candidate 3-variable, 4-variable, and 5-variable breathprints for major depressive disorder and their diagnostic performance with the linear predictive models support vector machine (SVM), logistic regression (LR) and Naïve Bayes (NB).

VOCs Signature (m/z)	**Predictive Model**	**Sensitivity**	**Specificity**	**AUC**	**95% CI**
			3-Variables breathprint		
74, 94, **69**	SVM	0.85	0.85	0.93	0.83 – 0.98
	LR	0.92	0.80	0.93	0.84 - 0.97
	NB	0.81	0.85	0.90	0.78 - 0.96
74, 94, **91**	SVM	0.96	0.85	0.93	0.81 – 0.98
	LR	0.92	0.85	0.93	0.82 - 0.97
	NB	0.96	0.70^{L}	0.91	0.78 - 0.96
46, 94, 69	SVM	0.88	0.85	0.88	0.76 – 0.96
	LR	0.85	0.85	0.88	0.73 – 0.96
	NB	0.85	0.80	0.86	0.73 – 0.94
			4-Variables breathprint		
74, 94, 69, **45**	SVM	0.92	0.90	0.97^{H}	0.90 - 0.99
	LR	0.92	0.90	0.97^{H}	0.88 - 0.99
	NB	0.84	0.89	0.91	0.80 - 0.96
74, 94, 69, **60**	SVM	0.88	0.85	0.94	0.85 - 0.98
	LR	0.96	0.80	0.93	0.82 - 0.97
	NB	0.73^{L}	0.85	0.87	0.74 - 0.94
74, 94, 69, **47**	SVM	0.88	0.85	0.94	0.86 - 0.98
	LR	1^{H}	0.70^{L}	0.93	0.82 - 0.97
	NB	0.77^{L}	0.85	0.88	0.76 - 0.95
			5-Variables breathprint		
74, 94, 69, 60, **75**	SVM	0.88	0.85	0.94	0.84 – 0.98
	LR	0.96	0.80	0.93	0.81 – 0.97
	NB	0.73^{L}	0.85	0.87	0.74 - 0.94
46, 94,69, 45, 75	SVM	0.92	0.85	0.92	0.80 - 0.98
	LR	0.96	0.80	0.94	0.85 - 0.99
	NB	0.92	0.80	0.92	0.82 - 0.97
89, 94,69, 45, 75	SVM	0.85	0.95	0.95	0.85 - 0.99
	LR	0.85	0.95	0.96	0.87 - 0.99
	NB	0.88	0.85	0.93	0.84 - 0.98

The most significant awakening-stress response times used in the predictive models for the volatile organic compounds at *m/z* 94, *m/z* 91, *m/z* 75, *m/z* 74, *m/z* 69, *m/z* 60, *m/z* 47, *m/z* 46, *m/z* 45 are depicted in Table 4.1; ^{L}: lower values compared to the minimal recommended diagnostic performance thresholds; ^{H}:very high, suggesting overfitting due to interferences with other VOC or due to collinearity

4.5 Metabolomic Pathway Analysis

The metabolic pathway analysis using the KEGG library, MPEA and MPTA revealed that 16 pathways may be affected in MDD patients compared to HCs, as shown in Figure 4-9. However, the MPEA using Fishers' Exact test and the Hypergeometric test showed that most significant ($p < 0.05$) altered metabolic pathways in patients suffering from MDD compared to HCs were (1) valine, leucine and isoleucine leucine biosynthesis, (2) pyruvate metabolism, (3) glycolysis and gluconeogenesis; (4) nicotinate and nicotinamide metabolism, and (5) valine, leucine and isoleucine degradation at significant level p of $5.50 \cdot 10^{-4}$, $7.97 \cdot 10^{-4}$, 0.0015, 0.005 and 0.035 respectively. Similar results were calculated with both tests. However, the pathway impact changed depending on the centrality measure used for estimating node importance by MPTA. Among the statistically significant pathways, (1) valine, leucine and isoleucine leucine biosynthesis, (2) pyruvate metabolism and (3) glycolysis and gluconeogenesis demonstrated the highest impact (0.25, 0.11 and 0.09 respectively) using out degree centrality. In contrast, the MPTA using the relative-betweenness centrality showed that the pathways nicotinate/nicotinamide metabolism, pyruvate metabolism, and glycolysis and gluconeogenesis had the highest pathway impacts, with values of 0.03, 0.19 and 0.015 respectively, as

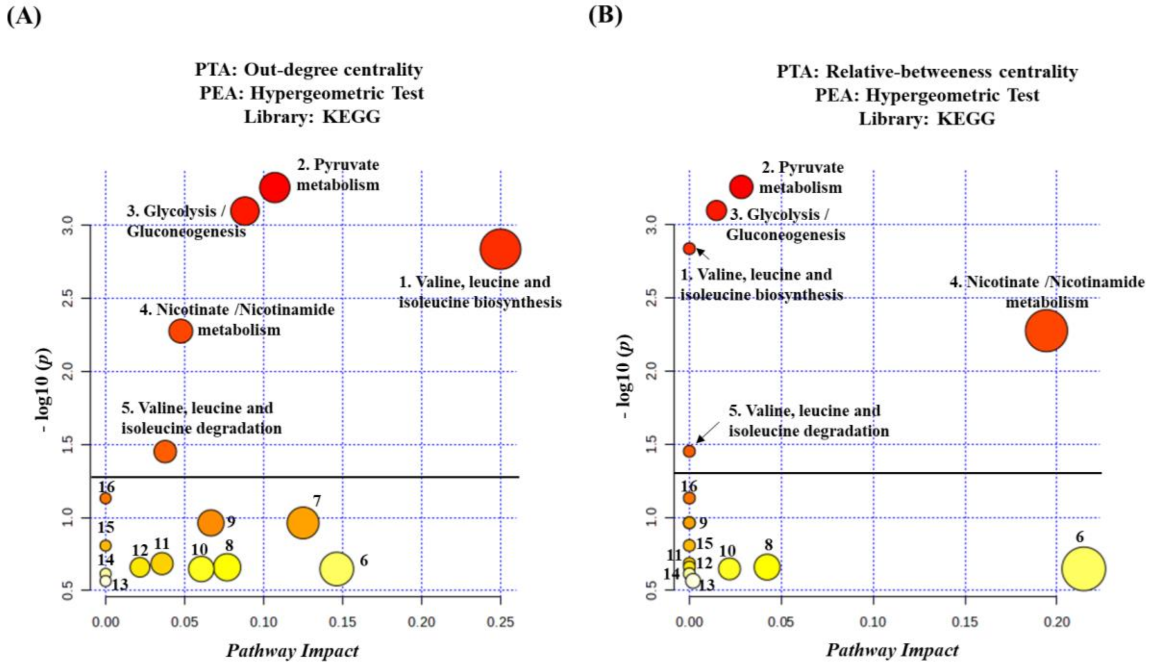

Figure 4-9| The metabolome view map of altered metabolic pathways identified in breath samples from MDD patients compared with healthy controls conducted by Metabo Analyst tool: *(A) Out-degree and (B) relative betweenness centralities. The significant pathways with the enrichment analysis using the different centrality measures are (1) Valine, leucine, and isoleucine biosynthesis, (2) pyruvate metabolism, (3) Glycolysis and gluconeogenesis, (4) Nicotinate and nicotinamide and (5) Valine, leucine and isoleucine degradation. The pathway topological analysis shows different impact factors of the significant altered pathways using: (A) out-degree centrality and (B) relative betweenness centrality. The matched nodes show varied heat map colors and is based on p-value while the node radius is determined based on the pathway impact values. The bold lines indicate the significance level of a p-value = 0.05. The pathways under the bold line are probable altered pathways but not significant (6) Glycine, serine and threonine metabolism, (7) D-amino acid metabolism, (8) Glyoxylate and dicarboxylate metabolism, (9) butanoate metabolism, (10) Cysteine and methionine metabolism, (11) Lysine degradation, (12) Sphingolipid metabolism, (13) Tryptophan metabolism, (14) Glycerophospholipid metabolism, (15) propanoate metabolism and (16) biotin metabolism* graphically map ped in Figure 4.9. Additionally, after adjusting the *p*-value with FDR for multi-testing only pyruvate metabolism, glycolysis and gluconeogenesis and BCAAs valine, leucine and isoleucine biosynthesis were significant with p_{FDR} of 0.032, 0.032 and 0.039 respectively. A detailed information about the most relevant pathways is summarized in Table 4.4. On the other hand, the results showed that expected pathways with metabolic pathway analysis are strong dependent on the used library. Changing the KEGG library with the homo sapiens SMPDB library led to the identification of only one significant pathway, the ethanol degradation pathway (*p* = 0.007 with a pathway impact 0.25). Furthermore, the Metabolic pathway analysis was mainly based on the KEGG library that is originally for genome analysis. There are some significant VOCs reported in VOCs analysis, which were not taken into account or not identified in KEGG Pathway library and worth further discussion for their associations with MDD status. Isoprene is thought to be a by-product of cholesterol biosynthesis along the mevalonate acid pathway (Pereira et al. 2015).

Table 4.4| *Significant altered metabolic pathways in patients with MDD compared to healthy subjects (p <0.05) conducted by MetaboAnalyst tool using pathway enrichment analysis (PEA) and pathway topological analysis (PTA). The pathway analysis was based on the breathomics altered metabolites. PTA was performed using out-of-degree (OD) and relative betweenness (RB) centrality measures to calculate the impact factor of the pathway.*

Pathway Name	**Match Status**	**Detected Metabolites**	**MPEA**		**MPTA**	
			***p*-value**	**- $\mathbf{Log_{10}(p)}$**	**OD**	**RB**
Pyruvate metabolism	3/23	Lactate, acetaldehyde, ethanol	$5.50 \cdot 10^{-4}$	3.26	0.11	0.03
Glycolysis/ Gluconeogenesis	3/26	Acetaldehyde, Ethanol, lactate	$7.97 \cdot 10^{-4}$	3.099	0.09	0.015
Valine, leucine and isoleucine biosynthesis	2/8	Leucine, isoleucine	0.0015	2.83	0.25	0
Nicotinate /nicotinamide Metabolism	2/15	Nicotinamide, nicotinate	0.005	2.28	0.048	0.19
Valine, leucine and isoleucine degradation	2/40	Leucine, isoleucine	0.035	1.45	0.038	0

4.6 Subgroups Analysis and Confounding Factors

In this section the impact of the confounding factors on the profile of candidate VOCs that discriminate between MDD patients and HCs is investigated. These factors include exposure to EVOCs from cigarette smoking, environment exposure, and medication, as well as MDD patients' demographic data such as gender, BMI, and age.

Environmental and smoking exposures. — The analysis of S-MDD, NS-MDD, and HCs subgroups at single response times from baseline to T_{60} revealed that S-MDD and NS-MDD have distinct VOCs profiles, and differing response times to the awakening stress. Notably, several additional exogenous chemical substances associated with smoking were found to be significantly increased in the breath of S-MDD compared to HCs. In addition to acetonitrile, benzene and toluene, the levels of the VOCs at *m/z* 31 at T_{60} ($p = 0.33$), *m/z* 58 at T_{30} ($p = 0.018$), m/z 81 at T30 (p = 0.0078), tentatively identified as formaldehyde, hydroxy acetonitrile, and pyrazine, were significantly increased in the breath of S-MDD compared to HCs. These EVOCs

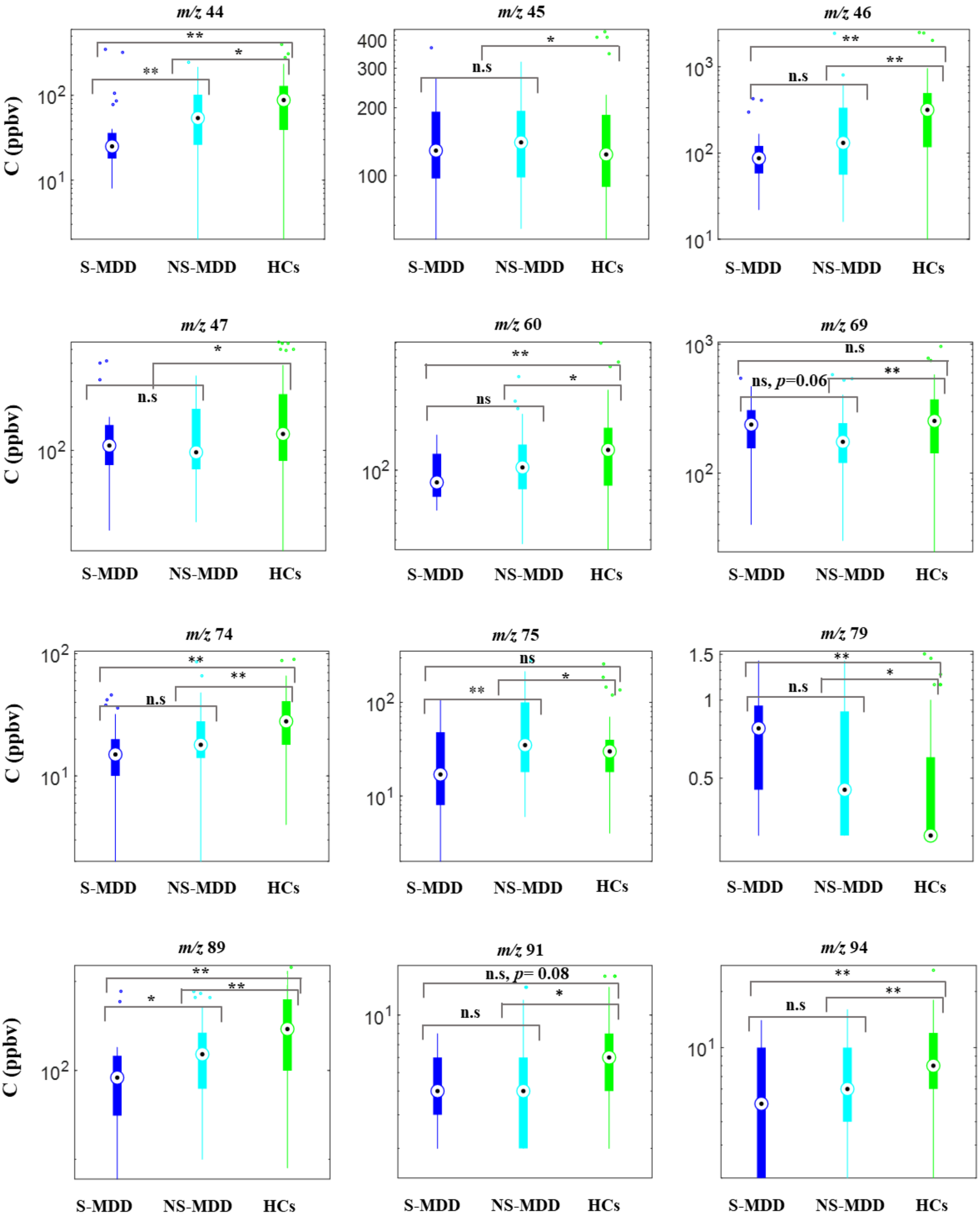

Figure 4-10| Boxplot representation of the impact of smoking on the levels on the candidate exhaled VOCs for major depression: *smoking influence significantly (p < 0.05) the levels of the VOCs at m/z 44, propionic m/z 75 and butyric acid at m/z 89 in S-MDD compared with NS-MDD. The concentration of isoprene at m/z 69 was significantly decreased (p<0.05) in the breath of NS-MDD compared with healthy controls (HCs). In contrary, smoking significantly elevated the isoprene level in S-MDD relatively to NS-MDD (p=0.06), so that no difference between HCs and S-MDD is detected. Propionic was significantly decreased in S-MDD compared to NS-MDD and lowered against HCs but not significantly. On the other hand, the levels of the VOCs at m/ 46, butylamine at m/z 74, and methylpyridine at m/z 94 are significantly lowered in S-MDD, but not significantly. Smoking had no discriminatory effects on the concentrations of ethanol m/z 47, acetaldehyde and trimethylamine m/z 60 between S-MDD and NS-MDD subgroups. and NS-MDD relative to HCs. However, no significant difference between S-MDD and NS-MDD was detected.*

have been previously detected in the breath of smokers (Kushch, Schwarz, et al. 2008), and were excluded from the list of the ranked VOCs for MDD due to their very low concentration. However, we cannot rule out their neurotoxicity in S-MDD. Additionally, smoking significantly affects the level of various compounds in S-MDD compared to NS-MDD and HCs, such as isoprene and microbial byproducts such as amines and SCFAs, as illustrated in Figure 4.10. Considering the best single response time for each metabolite, the levels of the propionic acid at *m/z* 75 at baseline, butyric acid at *m/z* 89 at baseline, ethenamine at T_{60}, and trimethylamine at T_{30} were significantly ($p < 0.05$) decreased in S-MDD relative to HCs. To increase the accuracy of the subgroup analysis, the response trajectories of candidate VOCs from baseline to T_{60} in S-MDD were additionally compared to those in NS-MDD and HCs. The levels of candidate VOCs for MDD were influenced by smoking, as depicted in Figure 4.10. The concentrations of the candidate VOCs at *m/z* 44, *m/z* 46, *m/z* 60, *m/z* 74, *m/z* 89 and *m/z* 94 were significantly lower in both NS-MDD and S-MDD groups compared to HCs. However, significant differences between S-MDD and NS-MDD were observed only in *m/z* 44 ($p = 0.008$) and *m/z* 89 ($p = 0.023$). Propionic acid was significantly ($p = 0.048$) elevated in NS-MDD compared to HCs and S-MDD ($p = 0.009$). Smoking lowered the propionic acid level in S-MDD relative to HCs, resulting in no significant difference between S-MDD and HCs. The compensation of elevated levels of propionic acid in NS-MDD and lowered levels in S-MDD led to no overall difference between all MDD patients and HCs. Isoprene (*m/z* 69) was significantly decreased in NS-MDD ($p = 0.005$) compared to HCs and relatively lowered ($p = 0.06$) compared to S-MDD. Smoking elevated the level of isoprene in S-MDD resulting in no difference between S-MDD and HCs, aligning with previous studies that reported elevated isoprene levels in smokers (Mochalski et al. 2023). Additionally, smoking did not have a discriminatory effect on the concentrations of ethanol (*m/z* 47), acetaldehyde (*m/z* 45) and trimethylamine (*m/z* 60) between S-MDD and NS-MDD subgroups.

Impact of patient's data on the candidate VOCs concentrations. — The investigation into the influence of the patient data on the candidate VOCs for MDD highlighted that age, BMI, and gender are significant factors determining the levels of certain exhaled metabolites. As substantiated by the correlation analyses, BMI showed a positive significant linear association with the log-transformed levels of the exhaled (iso)butyric acid (*m/z* 89: $r = 0.23$, $p = 0.048$), and its derivatives (*m/z* 88: $r = 0.25$, $p = 0.032$; *m/z* 90: $r = 0.23$, $p = 0.48$). Conversely, there were negative linear trends with the log-transformed *m/z* 85 ($r = 0.26$, $p = 0.026$) and propionic acid (*m/z* 75: $r = -\ 0.22$, $p = 0.06$). Additionally, BMI was positive correlated in a monotonic manner with isoprene (*m/z* 69: $\rho = 0.28$, $p = 0.02$) and toluene (*m/z* 93: $\rho = 0.22$, $p = 0.05$), as illustrated in Figure 4-17 A-B. This suggests that BMI could be a confounding factor with significant impact on the levels of these VOCs. Additionally, the correlation analysis within the S-MDD and NS-MDD subgroups revealed a strong negative monotonic association between BMI and butylamine at *m/z* 74 in S-MDD ($\rho = -\ 0.85$, $p = 0.02$). However, no significant BMI effect on butylamine levels was observed in the NS-MDD subgroup.

The correlation analysis supported the influence of age on certain candidate exhaled metabolites for MDD. Age showed negative linear correlations with the log-transformed levels of ethenamine (*m/z* 4: $r = -\ 0.36$, $p = 0.0015$), ethylamine *m/z* 46 ($r = -\ 0.30$, $p = 0.02$), trimethylamine (*m/z* 60: $r = -\ 0.23$, $p = 0.04$) and butylamine *m/z* 74 ($r = -\ 0.22$, $p = 0.06$). Conversely, positive linear correlations were observed with the log-transformed levels of isoprene (*m/z* 69: $r = -\ 0.30$, $p = 0.014$), ethanol (*m/z* 47: $r = 0.30$, $p = 0.01$), acetonitrile (*m/z* 42: $r = 0.34$, $p = 0.0026$), benzene (*m/z* 79: $r = 0.39$, $p = 0.006$), and toluene (*m/z* 93: $r = 0.33$,

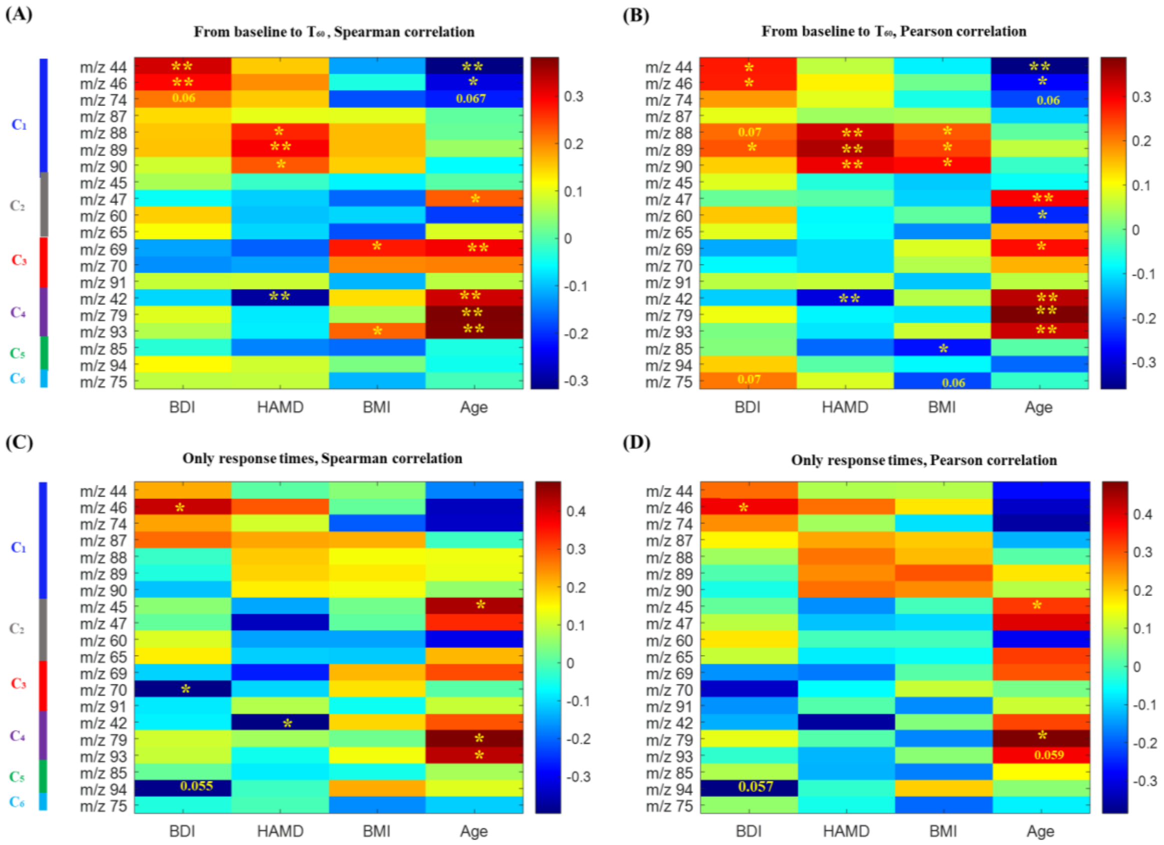

Figure 4-11| Heat map displaying the association between the candidate breathomics markers for major depressive disorder with the patient's BMI and age, and with the depression severity total scores of BDI-II and HAMD-17 tests: *Investigate of the monotonic and linear relationships between the log-transformed levels of the candidate the VOCs from the awakening to 60 minutes after awakening and confounding factors using (A) Spearman and, (B) Person's correlation analysis respectively, as well as the investigation of the monotonic and linear trends between the Log-transformed candidate VOCs at the most significant response time with the confounding factors using (C) Spearman and (D) Pearsons correlation analyzes.*
**: significant level p <0.05; **: significant level p <0.01;* T_{60}*: 60 minutes after awakening; HAMD: Hamilton rating scale for depression; BDI: Beck Depression Inventory.*

p = 0.0037), as illustrate in Figure 4-11. Therefore, the age could be a potential factor influencing the levels of these exhaled VOCs.

Moreover, several breath metabolites were found to be discriminatory between men and woman with major depression in this breathomics study, as illustrated in Figure 4-12. Woman consistently exhibited higher median levels of exhaled ethylamine (*p* < 0.0001), ethenamine (*p* = 0.002), methylpyridine (*p* = 0.042), propionic acid (*p* = 0.016) compared to men. The level of butylamine was also increased in the breath of the women, though not at the significant level *p* = 0.066. In contrast, the averaged levels of exhaled isoprene (*p* = 0.061) and lactic acid were lower in the breath of the woman with MDD compared to men, but these differences were not significant. Additionally, no gender-based differences were observed for exhaled ethanol, acetaldehyde, and trimethylamine. To obtain more accurate results for isoprene and butylamine, the impact of sex on their levels should be investigated in a larger population.

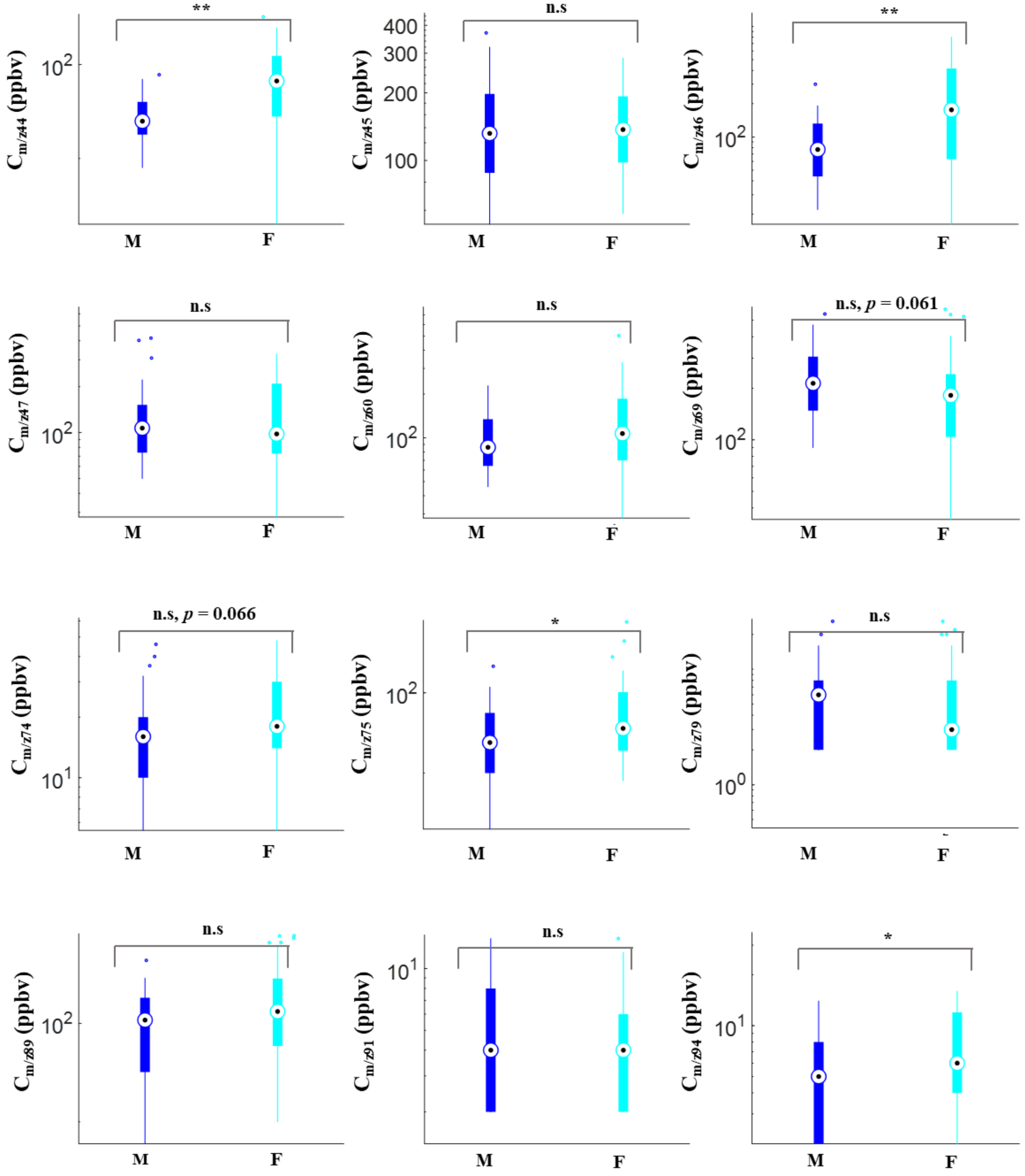

Figure 4-12| Boxplot illustration showing the gender differences of the exhaled candidate VOCs for major depression: *The median concentrations of ethylamine at m/z 44, ethenamine at m/z 46, propionic acid at m/z 75, butyric acid at m/z 89, and methylpyridine at m/z 94 were significantly increased in the breath of women with MDD compared to men. Butylamine was also elevated in butylamine at m/z 74 but at the level p= 0.066. In contrast, the averaged isoprene concentration was lowered in women (p = 0.06) relative to men. No discriminatory effects of the biological sex were observed on the concentrations of the exhaled ethanol at m/z 47, acetaldehyde at m/z 45, trimethylamine at m/z 60, and lactic acid at m/z 91.* *: significant at the level p <0.05; **: significant at the level p <0.01; M: Men; F: Female; $C_{m/z}$: Concentration of the volatile compounds at the masse- to- ration (*m/z*).

Medication. — The candidate breath markers did not show any significant association with medication. However, the small sample size limited the ability to accurate investigation of the impact of different types of medication (SSRI, SNRI, and NASSA) on the composition and levels of the exhaled candidate VOCs for MDD.

Impact of error analysis on the VOCs profile. — Accurate detection of VOCs in exhaled breath is generally a crucial task, but PTR-QMS faces a significant challenge due to the interference of VOCs with similar nominal mass-to-charge ratios, which can affect the detected metabolite levels.

In summary, considering the preliminary results from this breathomics data clearly indicate that smoking has a strong impact on the composition and levels of the exhaled VOCs in S-MDD patients compared to NS-MDD and HCs. Furthermore, emerging evidence suggests that metabolomic responses to the awakening stress stimulus and regulation differ between man and woman. This aligns with fundamental biological differences between men and woman, and existing evidence that the gender influences metabolomic data in other matrices. The association between BMI and the candidate VOCs was weak and should be investigated in a larger population for more accurate results. The association between the levels of the candidate metabolites and the patient data underscores the importance of considering differential stimulus response in MDD patients. Therefore, smoking and patient data should be incorporated into the analysis of breathomics data as a first step toward developing an accurate breath test for MDD.

Association between Candidate Metabolites and Depression Severity

The correlation and regression analyses indicated a tendency for a significant monotonic association between increased depression severity and elevated levels of the microbial VOCs, including the exhaled ethylamine (*m/z* 44), ethenamine (*m/z* 46), the SCFAs butyric acid (*m/z* 89, *m/z* 88, *m/z* 90) and propionic acid at *m/z* 75. In contrary, a negative monotonic association was detected between the isoprene fragment at *m/z* 70, methylpyridine (*m/z* 94) and acetonitrile *m/z* 42 in all MDD patients, with a negative monotonic trend observed for *m/z* 91 specially in S-MDD. As illustrated in Figure 4.11-B and Figure 4.14, Pearson's correlation analysis revealed a significant linear relationship between BDI-II total score and the log-transformed concentrations of ethylamine ($r = 0.30$, $p = 0.017$), ethenamine ($r = 0.30$, $p = 0.018$), (iso)butyric acid main compound at *m/z* 89 ($r = 0.30$, $p = 0.018$). These relationships were further supported by the monotonic associations between the BDI-II total score and the transformed VOCs, with Spearman coefficients of $\rho = 0.32$ ($p = 0.0048$) for ethylamine (*m/z* 44) and $\rho = 0.30$ ($p = 0.012$ for ethenamine (*m/z* 46), as illustrated in Figure 4.17 A. Additionally, the log-transformed level of butylamine (*m/z* 74) showed a weak positive monotonic relationship with a Spearman coefficient of $\rho = 0.21$ ($p = 0.06$). Stronger moderate monotonic trends between BDI-II total score and log-transform metabolites were detected at single response times. As depicted in Figure 4-17 C, ethenamine, the isoprene fragment at *m/z* 70 and methylpyridine showed moderate correlations with Sperman coefficients $\rho = 0.41$ ($p = 0.039$) at time T_{30}, $\rho = - 0.40$ ($p = 0.05$) at T_{30}, and $\rho = - 0.40$, ($p = 0.055$) at T_{60} respectively.

Furthermore, the correlation and regression analyses indicated tendencies for positive linear correlations between HAMD total score and log-transformed concentration of (iso)butyric acid at *m/z* 89 and its derivative (*m/z* 88 and *m/z* 90) with the significant Pearson' coefficients $r = 0.35$ ($p = 0.002$), $r = 0.33$ ($p = 0.004$) and $r = 0.30$ ($p = 0.008$) respectively. In contrast, the log-transformed concentration of acetonitrile from baseline to T_{60} showed a negative linear association r = - 0.30 (p = 0.01) and a stronger negative monotonic association of ρ = - 0.40 (p = 0.049) at T_{60}, as depicted in Figure 4-11 B and C respectively. No association between toluene and depression severity was detected in either S-MDD or in NS-MDD patients. However, a moderate correlation between benzene (*m/z* 79) and BDI-II total score in S-MDD, with a Pearson coefficient of r = 0.35 (p = 0.082).

On the other hand, the correlation analysis in S-MDD and NS-MDD subgroups revealed a strong strength monotonic relationship between the BDI-II total score and the lactic acid (*m/z* 91) in S-MDD with a Spearman coefficients ρ = - 0.73 (p = 0.046). Additionally, a strong monotonic relationship was observed with isoprene fragment (*m/z* 70), showing r = - 0.76 (p = 0.028) and ρ = - 0.72 (p = 0.046). The isoprene main compound at *m/z* 69, the fragment of valeric acid at *m/z* 85, benzene at m/z 79 showed moderate correlations, with r = 0.35 (p = 0.082), r = - 0.35, and r = 0.31, and r = - 0.40, respectively, through these correlations were not significant. In NS-MDD group, BDI-II total score exhibited a moderate relationship with m/z 85 (r = 0.42, p = 0.078). These finding may be attributed to the small sample size and warrant further investigation be in a large population for future validation.

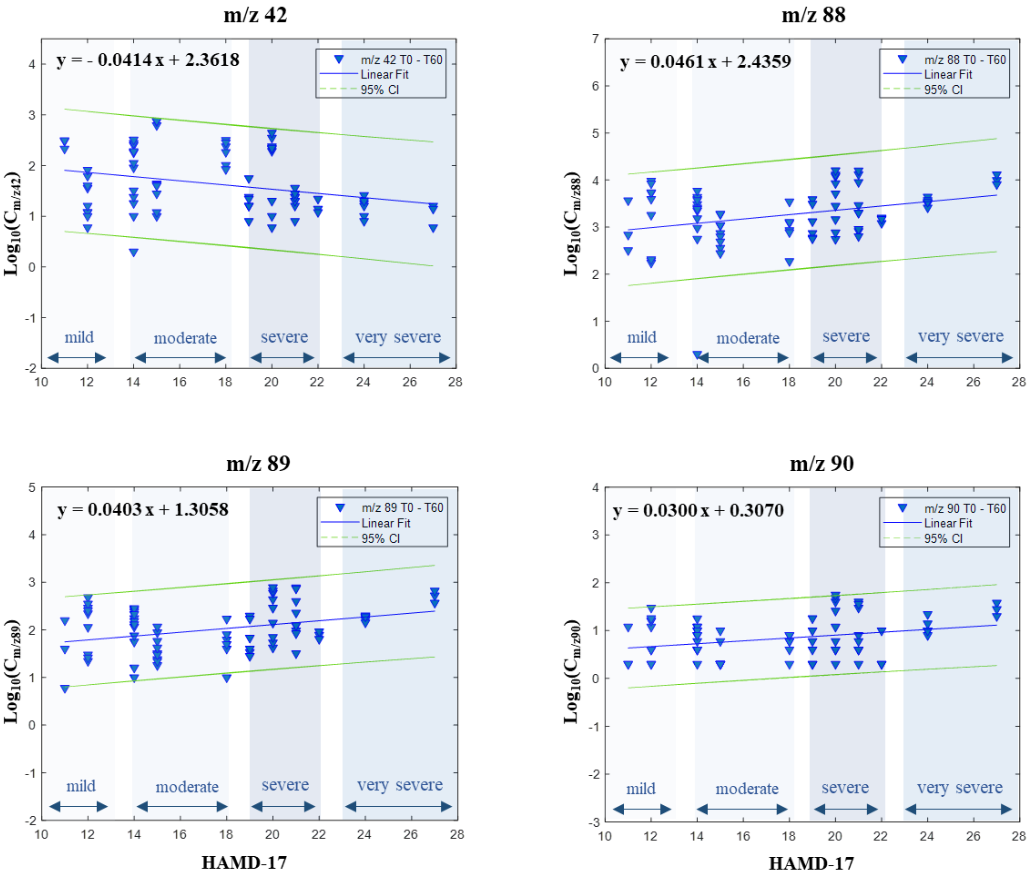

Figure 4-13| Illustration of the candidate VOCs for major depression with tendencies for association with the depression severity HAMD-17 total score: *The regression analysis indicates that the log-transformed concentration of acetonitrile at m/z 42 has a negative linear relationship with HAMD-17 total score, whereas butyric acid main compound at m/z 89 and its derivatives at m/z 88 and m/z 90 have positive linear trend with HAMD-17 total score. The interpretation of depression severity using HAMD-17 scores: values <7 no depression or remission; values 8 -13 mild depression;14 -18 moderate, 19 - 22 severe, and values >23 very severe depression.*

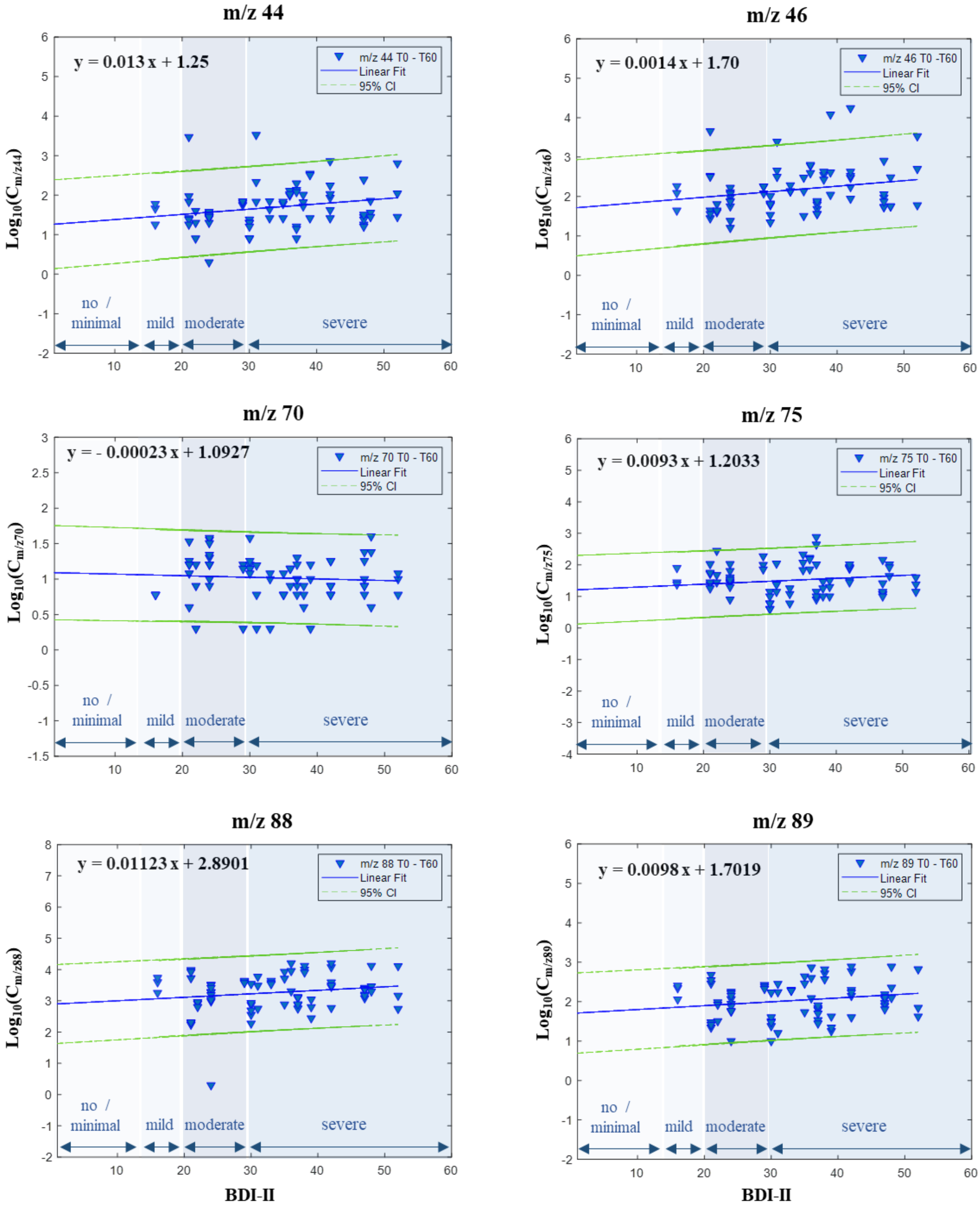

__Figure 4-14| Illustration of the candidate VOCs for major depression with tendencies for an association with the depression severity HAMD-17 total score:__ The regression analysis indicates that the log-transformed concentration of isoprene at m/z 70 has a negative linear relationship with BDI-II total score, whereas the log-transformed levels of ethenamine at m/z 44, ethylamine at m/z 46, propionic acid at m/z 75, as well as butyric acid main compound at m/z 89 and its derivative at m/z 88 have a positive linear correlation with the depression severity using BDI-II total scores. The interpretation of depression severity using BDI-II scales: values < 13 no or minimal depression; values be 14-19 mild depression; 20 - 28 moderate, 29 - 63 severe.

Further, as depicted in Figure 4.16 the regression analysis indicated that the following candidate VOCs in this breathomics study exhibited tendencies for the monotonic relationships with the depression severity, as measured by the HAMD-17 total score:

$$Log_{10}\left(C_{m/z42}\right) = -\,0.0414\ HAMD + 2.3618 \tag{4.7}$$

$$Log_{10}\left(C_{m/z88}\right) = 0.0461\ HAMD + 2.4359 \tag{4.8}$$

$$Log_{10}\left(C_{m/z89}\right) = 0.0403\ HAMD + 1.3058 \tag{4.9}$$

$$Log_{10}\left(C_{m/z90}\right) = 0.0300\ HAMD + 0.3070 \tag{4.10}$$

Additionally, as depicted in Figure 4.17 the regression analysis showed that the following candidate VOCs exhibited tendencies for monotonic relationships with depression severity, as measured by the BDI-II total score:

$$Log_{10}\left(C_{m/z44}\right) = 0.013\ BDI + 1.2500 \tag{4.1}$$

$$Log_{10}\left(C_{m/z46}\right) = 0.0140\ BDI + 1.7000 \tag{4.2}$$

$$Log_{10}\left(C_{m/z70}\right) = -\,0.023\ BDI + 1.0927 \tag{4.3}$$

$$Log_{10}\left(C_{m/z75}\right) = 0.00093\ BDI + 1.2033 \tag{4.4}$$

$$Log_{10}\left(C_{m/z88}\right) = 0.01123\ BDI + 2.8901 \tag{4.5}$$

$$Log_{10}\left(C_{m/z89}\right) = 0.0098\ BDI + 1.7019 \tag{4.6}$$

These relationships between candidate VOCs and depression severity, as measured by HAMD-17 and BDI-II, were primarily calculated using a small sample size of breathomics data. Given the limited size of this data, further investigation in a large population is warranted to validate and ensure the accuracy of these findings. This would help to confirm the potential breath markers and strengthen the reliability of breathomics as a diagnostic tool for MDD.

5 DISCUSSION & CONCLUSION

5.1 Results Discussion

Major depression is a complex and heterogeneous mental disorder in both its presentation and pathophysiology. It is associated with serious socio-economic impacts, and a broad spectrum of clinical challenges, including a high global prevalence, a high rate of misdiagnosis, symptom overlap with other disorders, delayed therapeutic onset, and a high incidence of relapse and recurrence. Additionally, it is associated with substantial disability, and mortality. Therefore, there is an urgent clinical need to deepen our understanding of the molecular pathophysiology underlying this mental disorder to identify objective markers for accurate diagnosis, disease progression monitoring, and treatment response, ultimately leading to improved outcomes for individuals with MDD. The primary hypotheses of this study were that the analysis of the exhaled breath of patients with MDD could provide a qualitative or relatively quantitative multiparametric VOCs signature that may mirror dysregulation of several biological pathways and mechanisms underlying MDD, and could yield a concise breathprint of potential markers for MDD, which may be valuable for developing a non-invasive and low-cost MDD breath test in clinical settings. For these purposes, we employed non-targeted breath-based metabolomics profiling using the high-throughput analytical instrumentation PTR-MS, combined with advanced machine learning and deep learning approaches, to rank candidate markers for MDD breath test and identify biochemical changes that occur in patients with MDD.

Our preliminary findings supported the hypotheses of this thesis. The pathway analysis revealed that at least five significant metabolic pathways were significantly affected and enriched in MDD patients, including (1) pyruvate metabolism, (2) glycolysis and gluconeogenesis, (3) valine, leucine and isoleucine biosynthesis, (4) valine, leucine and isoleucine degradation, and (5) nicotinate and nicotinamide metabolism. A total of 26 differential VOCs were partially identified within these pathways, linking them to various mechanisms underlying the pathophysiologies of MDD. Additionally, many of these altered VOCs in the breath of MDD patients have also been identified in other biological matrices as potential markers tied to key mechanisms contributing to MDD. These mechanisms include alterations in gut microbiota (Barandouzi et al. 2020), imbalances in energy metabolism (Gu et al. 2021a), dysregulation of amino acids and their breakdown products (Baranyi et al. 2016), alteration of intermediate VOCs involved in the metabolism of tryptophan, increased inflammation, elevated oxidative and nitrosative stress (Sinumvayo, Li, and Zhang 2021; Sakurai et al. 2020). Among these metabolites, isoprene, methylpyridine, and butylamine were identified by SVM, K-SVM, NB and LR predictive models as potential markers in the altered pathways and HCA clusters, discriminating between MDD patients and HCs with a high AUC (≥ 0.91), sensitivity (≥ 0.84), and specificity (≥ 0.89). This 3-variable signature demonstrated strong overall performance as a concise breathprint for MDD, supporting the development of a low-cost and non-invasive molecular breath test for MDD diagnostic. Adding trimethylamine slightly improved the performance, while expanding to five-marker signatures with propionic acid or benzene further enhanced specificity, supporting their potential for phenotype-specific diagnostics. Propionic acid may be particularly relevant for non-smokers with MDD, while benzene may serve as an exposome marker in environmentally exposed individuals. Similarly, the utility of isoprene as biomarker may be compromised in smoking populations, as smoking appears to mask or overrides the disease-related metabolites, highlighting the importance of considering behavioral confounders in breathomics studies. To ensure the reliability of theses breath signatures involving isoprene and propionic acid, their diagnostic performance should be validated in a larger population with a balanced sample size of S-MDD and NS-MDD patients.

Interestingly, substituting compounds from the core breathprint with others that exhibit similar post awakening temporal dynamics, or belong to related metabolic functional cluster yielded comparably high classification performance. This finding suggests that exploring biological redundancy within functional metabolic groups offer flexibility in tailoring breath test panels to specific clinical or operational priorities, whether to goal is to maximize sensitivity, improve specificity, or strike a balance between both for broader diagnostic applicability.

Importantly, the significantly altered VOCs in the exhaled breath of MDD patients observed in this study primarily fall into three categories: (1) volatile amino acids, (2) volatile by-products of gut microbiota produced through various fermentation pathways, such as pyruvate breakdown metabolites (e.g., ethanol, acetaldehyde, and lactate) and SCFAs such as propionic and butyric acid, (3) volatile compounds from gut microbiota involved in amino acid metabolisms, such as microbial decarboxylation products of amino acids like butylamine. The alterations in the gut microbiota metabolome and amino acids observed in depressed individuals in this study are consistent with findings from other biomatrix-based metabolomics research (Bot et al. 2020; Skonieczna-żydecka et al. 2018). A growing body of literature supports that alteration in gut microbiota profiles, and their derived metabolites plays a critical role in pathogenesis of depression (Cheung et al. 2019; Amirkhanzadeh Barandouzi et al. 2020; Dinan et al. 2015). Fecal and blood-based metabolomics studies have demonstrated a correlation between bacterial fingerprints and depression, highlighting the potential association between gut microbiota and the development of depressive symptoms (P.J. Kennedy, J.F. Cryan , T.G. Dinan 2018). The alteration of gut microbiota metabolome observed in this study could be indicative of both an altered gut microbiota profile and/or changes in dietary intake, which are closely interlinked. Microbial by-products are highly sensitive to dietary intake, which directly influences the gut microbiota profile since different types of food (fibers, fats, proteins) promote the growth of different microbial species. Decreases in the diversity and abundance of the gut microbiota have been linked to depression and psychological stress in both human and animal models exposed to mild, chronic or prolonged stressors (Jianguo et al. 2019; Bailey et al. 2010). Several bacterial metabolic by-products are often considerate key mediators of gut-brain crosstalk, as explained in Chapter 2 (Silva, Bernardi, and Frozza 2020; Kennedy et al. 2017). Previous studies reported that SCFAs were depleted in patients with MDD (P. Zheng et al. 2016; Skonieczna-żydecka et al. 2018), while their administration was found to improve intestinal permeability and HPA-axis reactivity, exhibiting antidepressant effect and presenting a novel therapeutic target for depression (L. Liu et al. 2023). SCFAs acting within intestinal endocrine cells stimulate the production of neuroactive molecules such as serotonin and γ-aminobutyric acid (Oleskin and Shenderov 2016). Accumulating evidence supported by several studies in humans and animals reported that alteration of SCFAs is linked to behavioral and neurological pathologies, such as depression (P.J. Kennedy, J.F. Cryan , T.G. Dinan 2018), Alzheimer’s, Parkinson’s diseases and autism spectrum disorders (Silva, Bernardi, and Frozza 2020), as well as metabolic disorders such as type 2 diabetes and obesity (Sanna et al. 2019).

Moreover, SCFAs play a very important role in regulating energy metabolism and supply, maintaining the homeostasis of the intestinal tract (He et al. 2020), regulating mitochondrial function (Schönfeld and Wojtczak 2016), regulation of inflammatory processes, emotional state and cognition through the gut-brain axis (Vinolo et al. 2011; Skonieczna-żydecka et al. 2018). SCFAs can cross the gut epithelial barrier and enter the systemic circulation. However, a significant proportion remains within the gut ecosystem and is excreted in the feces. Only a fraction of colon-derived acetate, propionate and butyrate reaches systemic circulation and other tissues. In this context, it is important to note that SCFAs detected through breathomics are reflective of blood SCFAs concentration and cannot be used as a direct proxy for their production in the colon. Despite the small fraction of free SCFAs in systemic circulation, several studies have reported significant impacts of these metabolites on health (Pluznick 2017; Deng et al. 2019; Lee and Zhu 2021).

SCFAs are the main metabolites produced by the bacterial fermentation of dietary fibers, whereas acetate, propionate, and butyrate are the main produced SCFAs. Levels of exhaled butyric acid were found significantly lowered in the breath of both S-MDD and NS-MDD patients in this breathomics study in comparison to HCs, with no significant gender difference. The lowered levels of butyric acid are in accordance with previous metabolite studies based on other bio-matrices (Skonieczna-żydecka et al. 2018) and animal models (Deng et al. 2019), and could be an indicator for imbalance in various processes, potentially contributing to MDD pathophysiology. Butyric acid plays a crucial role in the oxidative generation of ATP and supplies anabolic pathways such as gluconeogenesis with carbon-containing precursor molecules, as well as the regulation of serotonin, a neurotransmitter essential for mood regulation. Thus, the downregulation of butyric acid observed in this study in MDD patients, may indicate a disturbance in energy metabolism, which aligns with the symptomatic profile of major depression. Moreover, butyric acid exhibits anti-inflammatory effects, notably through the inhibition of the production of pro-inflammatory cytokines. Lowered butyric acid level observed in MDD patients may be linked to depressive behaviors caused by neuroinflammation, possibly due to increased microglial activation (Capuco et al. 2020). Furthermore, butyric acid has been found to stabilize HPA axis, which regulate stress responses. This modulation can help reducing the excessive stress reactivity often seen in depressive patients. On the other hand, animal studies have shown that butyrate supplementation can have antidepressant-like effects, improving both mood and behavior (Yamawaki et al. 2012).

Propionic acid levels were found to be lower in all MDD patients compared to HCs, though this difference was not statistically significant. This is primarily due to a reduction in propionic acid among S-MDD patients (significantly altered only at T_0) and a significant increase in NS-MDD relative to HCs when considering all concentration from baseline to 60 minutes post awakening, resulting in a compensatory effect in the overall level in all MDD. The alteration of propionic acid concentration in MDD have been confirmed in several researches. However, the results concerning the regulation were conflicting (Skonieczna-żydecka et al. 2018). Propionic acid plays a crucial role in the gut-brain axis communication, acting like other SCFAs as a signaling metabolite between gut microbiota and CNS, and affecting the neurotransmitter balance, which are crucial for mood regulation. Reduced propionic production have been associated with development of depressive symptoms and neurodegenerative conditions. However, elevated levels have been linked to neurotoxicity effects, neuroinflammation and behavioral changes in neurodegenerative disorders, particularly in autism spectrum disorder. Previous studies an animal models reported that elevated levels of propionic can increase oxidative stress, disrupt energy metabolism, and elevated inflammatory markers like IL-6 and TNFα, leading to neuroinflammation and neurotoxicity (El-Ansary, Bacha, and Kotb 2012; Choi et al. 2018). Therefore, increased levels of propionic acid in NS-MDD in this study may serve as hallmarks of neuroinflammation and neurotoxicity. This is further supported by the positive correlation found between propionic levels and BDI-II total score among MDD patients, indicating a link between heightened inflammation and the severity of depressive symptoms. Moreover, propionate acid is involved in energy metabolism through its role as a substrate in gluconeogenesis and the citric acid cycle.

Exhaled acetic acid exhibited patterns similar to propionic acid, with elevated levels in NS-MDD and reduced levels in S-MDD compared to HCs. However, these changes were not statistically significant, resulting in no overall significant difference between the entire MDD group and HCs. The involvement of acetic acid is in gut-brain axis regulation, neuroinflammation, neurotransmitter synthesis, and energy metabolism suggests that it may contribute to the development or exacerbation of mental health conditions including depression (Cheng et al. 2024). Further research with a larger population is needed to determine whether exhaled acetic acid could be a relevant marker for MDD breath test.

Lactic acid is produced during anaerobic metabolism, typically rises in response to acute stress due to increased sympathetic nervous system activity. Lactate have been found to act as a protective molecule against stress in healthy individuals and in the early stages of brain disorders (Shunfeng Liu and Zhou 2024). Therefore, downregulated levels of exhaled lactic acid observed in this study in MDD patients compared to HCs may suggest a reduced metabolism of lactic acid or lowered stress responsiveness in individuals with MDD. This diminished response is likely driven by dysregulation of the HPA axis and neurotransmitter imbalances, both of which underly the pathophysiology of depression. Interestingly, research suggests that lactate, rather than glucose, is preferentially metabolized by neurons in the brain of several mammalian species, enabling the brain to maintain high levels of activity. Lactate not only serve as a critical energy source, but also promotes neurogenesis and inhibits neuroinflammation, further highlighting its role in brain health. Studies have shown that lactate has neuroprotective effects and plays a critical role in the onset and progression of mental disorders, including depression, anxiety, Parkinson’s disease, and Alzheimer (Shunfeng Liu and Zhou 2024). Furthermore, Gut microbial-derived lactate has been implicated in the development of depression (Shan et al. 2020). In fact, oral administration of lactate has demonstrated antidepressant-like effects in animal model (Shaif et al. 2018), suggesting that lactate could be an important target for understanding and treating mental health disorders like depression.

Additional dysregulated bacterial metabolites observed in this study include acetaldehyde and ethanol, which are linked to altered glycolysis and pyruvate metabolism. The dysregulation of glycolysis and gluconeogenesis revealed by metabolic PA in this pilot study is in accordance with previous researches. Mounting omics studies suggest link between pathogenesis of depression and imbalanced energy metabolism (Gu et al. 2021a; Zuccoli, Saia-cereda, and Nascimento 2017). MDD patients have generally mitochondrial energy metabolism obstacles and energy imbalance in the brain. Both acetaldehyde and ethanol water cluster at m/z 65 were significantly elevated in the breath of patients with MDD compared to non-depressed individuals, while ethanol main compound was increased, possibly due to interference with other VOCs at the *m/z* 47 like formic acid. Acetaldehyde being highly reactive and toxic, is especially noteworthy in this context. Its elevated levels may indicate heightened oxidative stress, as acetaldehyde is a by-product of lipid peroxidation, which occurs during oxidative damage to cell membranes (Ayala, Muñoz, and Argüelles 2014). This oxidative stress is a key hallmark of neuropsychiatric conditions like depression, suggesting that acetaldehyde could serve as a potential marker for oxidative stress in MDD patients in this breathomics study. Moreover, endogenous ethanol (EE) is constantly formed from acetaldehyde through metabolic processes within the human body. Under typical conditions, the concentration of EE in the blood of healthy individuals remains minimal. However, intestinal dysbiosis can lead to a significant increase in ethanol production. This can compromise the gut permeability, allowing both endotoxins and ethanol to pass through the intestinal barrier into the blood stream. Once in circulation, these compounds reach the liver, where they activate a pro-inflammatory response, contributing to systemic inflammation. Additionally, elevated levels of EE increase the activity of enzyme cytochrome P450 2E1, which catalyzes the ethanol oxidation, thereby promoting oxidative and nitrosative stress (Teresa Auguet 2021). Increased EE has been reported in several conditions and metabolic disorders such as diabetes mellitus (Simic et al. 2012), non-alcoholic fatty liver disease (Elshaghabee et al. 2016) and obesity (Cope, Risby, and Diehl 2000). The increased alveolar elimination of ethanol in the exhaled breath of patients with MDD may be due to both its increased production and impaired metabolic breakdown. Previous researchers reported unusually high concentrations of EE in blood and cerebrospinal fluid of hospitalized patients suffering from various psychiatric disorders, indicating a potential association between gut dysbiosis, EE accumulation, and mental health conditions (Logan and Jones 2000). Furthermore, increased EE production has been linked to a significant abundance of Candida albicans in antibiotic-disrupted bacterial community of the murine cecum in mice (Mason et al. 2012). On the other hand, research in plant has demonstrated that environmental stress induce the production of acetaldehyde and ethanol in plants, suggesting a substantial alteration of respiratory metabolism in stressed plants (Kimmerer and Biology 2014)). Thus, elevated levels of acetaldehyde and ethanol in MDD patients could indicate the lowered responsiveness to stress in awakening stimulus.

Additional groups of altered metabolites in this study include the volatile AAs and their breakdown products. HCA and metabolic PA revealed that several HCA-clusters and altered pathways are closely related to AAs metabolism, including degradation and biosynthesis of essential BCAAs valine, leucine, and isoleucine, as well as the tryptophan metabolism. There is growing evidence that AAs and their metabolites not only serve as essential building blocks for protein synthesis but also act as critical substrates and regulators in various metabolic processes. Accumulating research has showed that alteration of amino acids levels contributed to the pathophysiology of depression (Baranyi et al. 2016; Geng et al. 2020; Bhattacharyya et al. 2019; Siamak Mahmoudian Dehkordi et al. 2020), and are linked to both mental and physical stress in animal models (Guo et al. 2018; Geng et al. 2020). These findings are consistent with previous metabolomics reports from various biomatrices, underscoring the role of AAs metabolism in the pathophysiology of MDD. In the present breathomics study, we observed disrupted levels of the BCAAs (iso)leucine and their associated metabolic pathways in individuals with MDD, as well as the fragment of lysine. However, (iso)leucine and lysine were not abundantly detected at their main compounds, potentially due to their low volatility and the relatively low sensitivity of the PTR-QMS instrumentation. Additionally, leucine and isoleucine interfered due to their similar nominal masses. Likewise, lysine fragment interfered with valeric acid fragments at *m/z* 85, which complicated the identification and quantification of these metabolites. These limitations may have hindered the accurate detection of these less volatile amino acids, pointing to the need for more advanced analytical tools to capture a broader range of metabolites in future studies. The interferences can be investigated in future studies using more sensitive techniques like PTR-TOF, which can provide higher mass resolution and allow for better differentiation between these compounds. Moreover, García-Gómez and co-workers (2016) have reported that slightly volatile amino acids, including isoleucine, leucine, and lysine can be quantified in the human breath condensate in real time using secondary electrospray ionization coupled to high-resolution mass spectrometry. They found that the concentrations of these AAs in breath condensate correlated with their plasma concentrations, indicating the potential of breath analysis as a non-invasive approach for monitoring levels of AAs in the body (García-Gómez et al. 2016).

Overall, BCAAs play a critical role in various physiological processes that are disrupted in MDD, highlighting their significance in the disorder's pathophysiology. For instance, alterations in leucine levels can affect serotonin production, a key neurotransmitter in mood regulation. In addition, BCAAs regulate the Mammalian Target of Rapamycin (mTOR) pathway, which is essential for vital cellular functions such as growth, protein synthesis, mitochondrial activity, and energy metabolism. In this study, the observed lower levels of BCAAs and altered BCAAs metabolism pathways in depressed individuals may suggest reduced mTOR activity and decreased mitochondrial energy metabolism, both of which are common in neuropsychiatric disorders (Gu et al. 2021a; Baranyi et al. 2016). Moreover, BCAAs modulate immune responses by regulating the mTOR pathway, influencing immune cell function, controlling tryptophan metabolism, reducing oxidative stress, and impacting cytokine production. The disruptions in BCAA levels and their metabolic pathways observed in this study may indicate imbalance in these processes, potentially contributing to MDD pathophysiology. On the other hand, pharmacologic studies have shown significant changes in BCAAs levels in the depressed individuals who responded to antidepressants compared to those who did not, with these changes strongly correlated with symptom reduction after treatment (Siamak Mahmoudian Dehkordi et al. 2020; Kaddurah-Daouk et al. 2013).

The third group of disrupted exhaled VOCs in the breath of MDD patients in the present study primarily included metabolites involved in AAs metabolism by gut bacteria. Anthranilic acid (AntA), 3-methylpyridine and nicotinamide are key metabolites of metabolism of the essential amino acid tryptophan via tryptophan-nicotinic pathway. Tryptophan metabolism leads to the generation of several neuroactive agents within the CNS, including the neurotransmitter serotonin (Ruddick et al. 2006). Dysregulation of brain tryptophan concentrations has been reported in neuropsychiatric disorders (Davidson et al. 2022; Schopman et al. 2021; Sakurai et al. 2020). HPA may activate tryptophan metabolism through corticosteroids in response to awakening stress. Moreover, AntA was found in this study to be significantly upregulated in the exhaled breath of all MDD patients with severe depression and in one patient with the highest moderate depression score, as assessed by HAMD-17 and BDI-II. No differences in AntA levels were observed between HCs and MDD patients with mild depression. This finding suggests that AntA levels could mirror the severity of depression and warrant further investigation in a larger population. Increased levels of AntA have been reported in various mental disorders, including schizophrenia (Oxenkrug et al. 2016) and MDD (Sakurai et al. 2020). Additionally, elevated levels AntA have been observed in metabolic disorders such as chronic hepatitis C, rheumatoid arthritis, osteoarthritis (Igari, Shimamura, and Tsuchizawa 1987) and type 1 diabetes (Oxenkrug et al. 2015; Pawlowski et al. 2021). A clinical study has also reported that blood serum levels of AntA may predict the onset and progression of clinical depression (Sakurai et al. 2020). Interestingly, an increase in AntA concentration was associated with decreased levels of anti-inflammatory factors in high risk MDD subjects. Pawolwski and coworkers (2021) provided the first direct evidence of a role for anthranilic acid in the pathogenesis of inflammation-induced MDD (Pawlowski et al. 2021). Generally, downstream metabolites of the kynurenine pathway and the availability of tryptophan play a critical role for the functioning of efferent nervous system and CNS, and thus in the gut-brain communication. Moreover, the kynurenine pathway was identified as a likely, but not significant altered pathway. This is primarily due to the slight volatility of the metabolites involved, which require mass range up to *m/z* 265. This mass range exceeds the detection limit of the PTR-QMS instrumentation used, which operates within a mass range below 150. This limitation may have hindered the detection of these less volatile organic compounds, pointing to the need for more advanced analytical tools to capture a broader range of downstream metabolites of the kynurenine pathway. A previous study detected 20 low-volatile metabolites of this pathway in exhaled human breath condensate using secondary electrospray ionization coupled to high-resolution mass spectrometry (García-Gómez et al. 2016).

3-methylpyridine is a primary precursor to nicotinic acid (NA), also known as B3 or niacin. NA derivatives play a critical role in cellular energy metabolism and DNA repair. NA deficiency is associated with various psychiatric symptoms and neuropsychiatric disorders, including Alzheimer’s, Parkinson’s, Huntington’s diseases, cognitive impairment, schizophrenia, and depression (Periyasamy et al. 2019). Case reports have shown that NA treatment was effective in improving depressive symptoms in patients with bipolar type II disorder (Jonsson 2018) and depression (Prousky 2010). Additionally, a cross sectional study with 16,098 adults reported a U-shaped association between niacin intake and depression, suggesting that moderate daily dietary intake of 36 mg/day of B3, but not high, may help protect against depression (S. Tian et al. 2023). However, the precise role of NA in depression remains unclear.

The metabolic PA was based on the KEGG library, which was originally designed for genome studies. As a result, some metabolites may not be identified in humans and might require additional verification. There are some candidate VOCs for MDD in this study, which were not taken into account or not identified in KEGG pathway library and worth further discussion for their associations with MDD status. These included isoprene and biogenic amines. Isoprene is one of the most abundant compounds in the human breath. However, its exact origins and physiological significance are not fully understood, though it is known to have an endogenous origin and is not produced in the airways (Sukul et al. 2023). The altered levels of isoprene observed in this study could have different pathophysiological implications. While the precise role of isoprene in neuropsychiatric disorders remains unclear, several hypotheses and emerging evidence suggest its involvement in key physiological processes that could impact the development and progression of mental health conditions, including cholesterol biosynthesis, brain function, altered energy metabolism, cell membrane integrity, oxidative stress, and neuroinflammation. Indeed, isoprene is thought to be a by-product of the mevalonate acid pathway, which is crucial for cholesterol biosynthesis (Pereira et al. 2015). Cholesterol plays a key role in energy metabolisms in CNS, maintaining cell membrane integrity and functioning of neurons, particularly the formation and maintenance of synapses. Membrane dysfunction has been linked to several neuropsychiatric disorders, including schizophrenia, bipolar disorder, depression, Parkinson disease, and multiple sclerosis. This dysfunction can lead to altered neurotransmitter signaling, neural connectivity, and overall brain function (Kurup and Kurup 2003; Ding et al. 2014). Additionally, low serum total cholesterol level can disrupt serotonin metabolism, which is negative correlated with depression severity, poor control of aggressive impulses, and increased risk of suicides (Messaoud et al. 2017; Ju young, Jerry, and René 2008). Furthermore, a large number of studies have suggested that exhaled isoprene could serve as a non-invasive and rapid biomarker for detecting alteration of blood cholesterol levels (Kushch et al. 2014; Kushch, Schwarz, et al. 2008; Karl et al. 2001). The downregulation of the exhaled isoprene in NS-MDD in comparison to HCs, as observed in this breathomics study, may also serve as a marker for decreased serum cholesterol synthesis, impairment of energy metabolism, reduced serotonin function. Furthermore, some studies have suggested that isoprene could be linked to oxidative stress or inflammatory status, both of which are critical factors in the development of many neuropsychiatric conditions, including MDD. On the other hand, evidence shows that isoprene possesses antioxidant properties in plants, where it minimizes oxidative stresses induced by ozone and play a protective role against toxins and temperature fluctuations (Vickers et al. 2009; Velikova et al. 2005; Pollastri, Baccelli, and Loreto 2021). However, these mechanisms have not yet validated in humans, and further research is needed to clarify isoprene's specific mechanisms. Additionally, alterations in exhaled isoprene levels have been investigated as potential markers for various health conditions, such as cystic fibrosis (Alkhouri et al. 2015), and cancers (D. Fuchs et al. 2012). While alterations in isoprene levels may reflect underlying metabolic dysfunctions associated with these conditions, further research is needed to establish a clear connection.

The levels of the volatile biogenic amines (VBAs) ethenamine, ethanamine, butylamine and trimethylamine were found significantly disrupted in MDD patients compared to HCs in this breathomics study. These group of microbial VOCs demonstrated a particular high discriminatory performance between the cohort' groups. Trimethylamine is the precursor of the trimethylamine N-oxidase (TMAO) that has a negative association with a broad range of diseases including neurological, metabolic and brain disorders (Heaney 2020; Gątarek and Czaplińska 2021). Although the link between TMAO levels and neurological disorders has been previously hypothesized, its role in the disease etiology has not been fully explored (Cryan and Dinan 2012; Janeiro et al. 2018). There is convincing evidence suggesting an association between TMAO and inflammation that is well known to contribute to the pathophysiology of MDD. Furthermore, TMA is a microbial metabolite produced by various taxa of gut microbiota quaternary amines, mainly choline and carnitine and is subsequently transformed in the liver into TMAO, which eventually acts as a neuronal protein stabilize (Rath et al. 2017). Therefore, we assumed that lowered levels of these compounds observed in this breathomics study could indicate either alteration of gut microbiome profile or disturbed nutritional status, particularly choline and carnitine.

Butylamine, one of the most discriminatory VBAs between MDD and HCs in this breathomics study, is less commonly studied in the context of neuropsychiatric disorders. However, it may play a potential role in various physiological and pathological processes like other candidate biogenic amines. Additionally, butylamine is one of the four isomeric amines of butane, the latter of which is recognized as biomarker for lipid peroxidation (Souvik Das, Pal, and Mitra 2016). Nevertheless, no direct association between butylamine, lipid peroxidation, or neuropsychiatric disorders has been identified in the literature. Dysregulation of butylamine has been linked to liver diseases in a few studies (Al-Ani 2015). Moreover, VBAs are produced as a consequence of microbial decarboxylation of amino acids in low PH intestinal tract environment (Thorn and Greenman 2012; Özogul and Özogul 2007). The low PH decarboxylase enzymes act on AAs producing carbon dioxide and free VBAs. Decarboxylase activity was through to be protective mechanism against acid stress across a broad range of naturally occurring acidic circumstances (Thorn and Greenman 2012). Thus, the downregulation of VBAs in MDD patients observed in this study could be a potential indicator for gut microbiota alteration, low protective mechanism of gut microbiota as response to their environmental change (e.g., PH change) induced by the psychological stressor at awakening through the GBA or due to the chronic and persistent activation of HPA system in rest and in response to short-term exposure to environmental stress in major depression (Dedovic and Ngiam 2015a).

With respect to metabolomics, it is evident that smoking and environmental exposure significantly impact the metabolomic profile and must be considered in breathomics analysis. Growing evidence suggests a connection between smoking and psychological disorders, including depression (Zhaoping Wu et al. 2023). However, the relationship between various aspects of smoking behavior and depression remains controversial (Fluharty et al. 2017). In this breathomics study, smoking was found to influence the composition of the exhaled breath in S-MDD individuals. Additionally, the levels of several potential markers were found to be altered in S-MDD compared to HCs and NS-MDD. Toluene and acetonitrile were significantly elevated in S-MDD compared to HCs and NS-MDD, with no significant difference between HCs and NS-MDD. However, benzene levels were significantly elevated in both S-MDD and NS-MDD compared to HCs, with no significant difference between S-MDD and NS-MDD. Therefore, acetonitrile and toluene were considered as marker for smoking for first time, while benzene could be considered a marker of environmental exposure to this compound in all MDD patients. The significantly elevated benzene levels in NS-MDD compared to HCs could serve as a marker for exposure to this compound from the outdoor or/and indoor environment pollutants, including passive smoking. Indoor benzene levels in smokers' home are reported to be 50% to 69% higher than in nonsmokers' homes (Gordon et al. 2002). Additionally, smokers receive 89% of their benzene exposure directly from smoking, while nonsmokers derive about 10% of their exposure from environmental tobacco smoke. Exposure to toluene, widely used as an industrial feedstock, has been previously associated with cognitive dysfunction in the neuropsychological tests (Echeverria et al. 1989). Additionally, exposure to toluene has been reported to alter dopaminergic neurotransmission (Nimitvilai et al. 2016). Furthermore, many smoking compounds are classified as free radicals. In light of this evidence, chronic cigarette smoking can be associated with oxidative stress due to elevated level of free radicals (Yao, Reddy, and Van Kammen 2001). In animal models, both chronic and acute inhaled toluene have been found to induce oxidative stress in the brain, a process that was already hypothesized to be linked to the pathophysiology of depression. Particularly interesting is the significant negative moderate association between depression severity, as measured by BDI-II total score, and acetonitrile levels in S-MDD observed in this study. This finding, however, may not align with existing research. Previous studies have indicated that smoking exposure is associated with an increased risk of depressive symptoms. Yet, no direct association between blood acetonitrile and MDD has been identified in earlier research. The observed trend in this study may therefore attributed to the small sample size and warrants further investigated in a larger population to validation this potential relationship. Additionally, there was a moderate but not significant ($p = 0.082$) positive correlation between benzene and depression severity, as measured by the BDI-II total score. This finding may align with a cross-sectional study of 3,449 participants, which reported a positive correlation between blood benzene and depression (Y. Zhu et al. 2023). Therefore, significant exhaled EVOCs related to cigarette smoking and environmental exposure cannot be excluded from this breathomics study. A more in-depth investigation into the relationship between depression, and environmental factors and smoking exposure is necessary.

On the other hand, smoking impacted the levels of various candidate VOCs associated with MDD. Lowered levels of butylamine, ethylamine, ethenamine, trimethylamine, methylpyridine, butyric acid and lactic acid were detected in S-MDD individuals compared to NS-MDD. However, only levels of butyric acid and ethenamine were significantly different between S-MDD and NS-MDD. On the other Hand, isoprene and propionic acid were particularly influenced by smoking. Isoprene showed a potential discriminatory effect between NS-MDD and HCs ($p = 0.005$). However, smoking elevated its level in S-MDD, eliminating any significant difference between HCs and S-MDD. Propionic acid was elevated in NS-MDD compared to both S-MDD and HCs, resulting in no significant difference between S-MDD and HCs, as well as no overall significant distinction between HCs and all MDD patients. While isoprene and propionic acid are likely impacted by smoking, which reduces their specificity, it is important to note that these VOCs have limited utility as diagnostic markers for depression in S-MDD individuals. Furthermore, the alteration of the potential microbial by-products for MDD in smokers observed in this study could result from the significant influence of cigarette smoking on the gut microbiota composition and diversity. Previous research has shown that smoking can affect the gut microbiota by altering intestinal irrigation, impairment of mucosal immune response, and increased permeability of the mucosa (X. Gui, Yang, and Li 2021). These changes can disrupt the balance of gut bacteria, potentially impacting microbial metabolism in S-MDD.

5.2 Strengths, Limitations and Future Directions of the Study

Our preliminary results show that BGA by sensitive PTR-MS, coupled to advanced machine and deep learning approaches could be a potential tool discriminating MDD patients from HCs with high sensitivity and specificity. Additionally, breathomics provide the opportunity for a non-targeted metabolites analysis, enabling the detection of a broad range of altered metabolites and metabolic pathways. Many of these candidate metabolites have already been identified as potential (bio)markers for MDD in previous bio-fluid and fecal-based metabolomics studies. These metabolites are linked to the mechanisms underlying MDD, including dysregulation of gut microbiome, tryptophan-kynurenine pathway, imbalance of energy metabolism, increased inflammation and oxidative stress, and dysfunction of amino acids acting as inhibitory neurotransmitters. On the other hand, the altered VOCs demonstrated an impaired response to awakening stress, likely linked to dysfunctions in key biological systems involved in stress regulation, such as HPA. Additionally, selecting candidate markers is a critical step in developing diagnostic models for diseases and characterizing the features of the disorders. Most studies have relied on single strategies to select the most important metabolites, which may limit the comprehensiveness of the diagnostic model. A more integrative approach to biomarker selection could enhance the accuracy and reliability of such models. Therefore, a key strength of the analysis of breathomics data in this study lies in the implementation of various univariate and multivariate machine learning algorithms to rank candidate markers for MDD used to train the classifier models, potentially enhancing the performance of supervised machine learning. These methods include the hypothesis testing approaches (e.g., T-test, Wilcoxon test), clustering methods such PCA and HCA, ranking approaches like NCA. Furthermore, the study utilized multiple linear and nonlinear classifiers based on different principles, including statistical and probabilistic approaches, to develop a machine learning-aided diagnostic model using breathomics. This comprehensive approach increases the robustness and potential clinical utility of the diagnostic model for MDD.

Despite the comprehensive biochemical analysis, the present breathomics study has several limitations related to the study design, analytical instrumentation, statistical analysis, and impact of confounding factors:

Study Design. — The findings of the present study should be interpreted with caution due to the correlational nature of the data, derived from a cross-sectional design. As such, this methodology precludes us from drawing definitive conclusions about the underlying mechanisms or relationships between the observed metabolic alterations and MDD. The association between changes in metabolites from breath and those from blood, as well as brain function has still to be determined. Additionally, due to the complexity of the pathogenesis of MDD, multi-omics methods could enhance our understanding and treatment of the disorder, as well as improve prevention strategies, thereby advancing precision medicine. The integration of breathomics, other biomatrices-based metabolomics with other omics-discipline could characterize various aspects.

Another important limitation is the relatively small sample size of the study, which may affect the generalizability of the findings and introduce uncertainty in the predictive accuracy of the breath test. The limited population size restricted our ability to conduct a thorough analysis of how individual patient data, habits and exposure to EVOCs influence metabolite concentrations. Additionally, this small sample did not allow for categorical comparison among different MDD subgroups, particularly regarding treatment outcomes. While we did not observe any significant influence of the medication on the VOCs concentrations, we cannot rule out that antidepressant drugs may still impact he metabolomic profiles. In a follow-up study, it is essential to extend the sample size to enable a more accurate estimation of predictive performance. We aim thus to recruit 80 patients and 80 controls to investigate the impact of medication, smoking, nutrition, and hormonal status in females on breath metabolites profile. Determining appropriate sample sizes for metabolomic studies is inherently complex. This complexity arises from several factors, including the unknown nature of the expected effects, the variable number of the detected metabolites, which are highly dependent on the analytical platform, the high-dimensionality, and the strong multi-collinearity of the metabolites involved. Despite recent advancements, there are currently no standard methods for sample size estimation in metabolomic phenotyping. This underscores the need for continued methodological development in this area to enhance the robustness of future studies.

On the other hand, no background air sampling was conducted to assess the effects of environmental contaminations from the clinical setting on the profile of the exhaled metabolites. As a results, the concentrations of the analyzed VOCs may not accurately reflect the absolute levels of these potential metabolites in the exhaled breath of the study participants.

Analytical instrumentation. — PTR-QMS cannot differentiate between substances contributing to the same mass, such as leucine and isoleucine. To address this interference issue, we intend to utilize the more sensitive PTR-MS TOF instrumentation in follow-up studies. An additional limitation relates to the low mass range limit of the used PTR-MS device (<150). Several amino acids and metabolites from the tryptophan-kynurenine pathway are slightly volatile and detectable in a mass range beyond the device's limit (~150-200) in the exhaled breath condensate. Thus, more sensitive instrumentation with larger masse ranges should be employed to detect a broader array of compounds in the exhaled breath to identify additional disrupted metabolites and pathways involved in the pathophysiology of MDD. Additionally, it is important to note that PTR-QMS can only detect molecules with proton affinities higher than water, utilizing water hydronium H_3O^+ as precursor ion. Several VOCs with lower affinity than water, such as such butane and pentane, cannot be detected by PTR-QMS, despite their potential to indicate oxidative stress. Switching to alternative precursor ions, such as NO^+ and O_2^+, would allow for the detection of a wide range of compounds with varying molecular affinities, thereby improving the accuracy of VOCs identification.

Moreover, in breathomics research, various high-throughput analytical platforms are commonly used to identify metabolic signatures that differentiate between diseased and control groups. However, integrating the outcomes of different analytical technologies remains uncommon. Recently, data fusion has gained popularity in metabolomics. Thus, combining results from multiple platforms can enhance the robustness of models and improve the accuracy of breathomics studies, as each platform has distinct strengths and limitations regarding compound detection, analytical reproducibility, and sensitivity.

Statistical and pathway analysis. — The missing values were imputed with a small number in the preprocessing step. This approach may introduce biases and lead to an underestimation of the standard deviation. More robust methods, such as kernel-based methods, should be implemented for this purpose (B. Li et al. 2016). Furthermore, the breath markers for MDD and mechanisms identified in this study were based on putative and ranked lists of VOCs, relying on the tentative identification and annotation of the candidate exhaled VOCs, as well as the grouping of VOCs with similar functions, responses to awakening stress, and chemical classes. To improve high-confidence compound identification, powerful analytical processes are required. Additionally, the pathway analysis was limited by the KEGG and SMPDB libraries used in the Metabo-Analyst tool, which struggled to map some potential VOCs to specific pathways.

Confounding Factors. — Smoking, environmental exposure, nutrition, lifestyle, patient data, and medication may have affected VOC concentrations observed in this study, but the small sample size limited a detailed analysis of these potential confounding factors, as previously noted. Addressing these confounding factors, along with considering the fundamental gender differences in VOC levels, is essential for advancing the development of precision medicine for MDD. It is also a critical step toward creating an accurate and reliable breath test for diagnosing MDD. Incorporating these considerations in larger populations into future research will help to improve the validity and robustness of breath-based diagnostics for MDD.

5.3 Conclusion

In summary, the preliminary results of this breathomics study in MDD patients compared to HCs largely supported the hypothesis that the peripheral molecular changes detected through BGA, utilizing high-throughput analytical instrumentation combined with advanced computational approaches in machine and deep learning, could provide valuable insights into the molecular alterations underlying the pathophysiology of MDD. The integration of artificial intelligence and psychiatry in this way could open new avenues for precision psychiatry. Furthermore, BGA enabled the identification of concise breathprints with a limited number of potential markers for MDD, showing promise for developing a non-invasive, low-cost breath test in clinical settings. However, the breathprint displayed certain limitations, particularly in smokers. Future studies with larger sample sizes, more sensitive instrumentation, and better control of confounding variables are needed to address these limitations and refine the diagnostic utility of breathomics in MDD.

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Magdeburg, May 27, 2025

Laila Gbaoui

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