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*Coordinatore del Corso: Prof.ssa Margherita Maioli*

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# ***Assessing the Neurobiological Modulation Results of* Radioelectric Asymmetric Conveyer (*REAC) on Salivary Metabolomics in Parkinson's Disease.***

***Coordinatore:***

Prof.ssa Margherita Maioli

***Tutor***:

Dott.ssa Margherita Maioli

Dott. Salvatore Rinaldi

***Tesi di dottorato di:***

Dott.ssa Ana Rita Pinheiro Barcessat

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**Abstract**

Parkinson's disease (PD) is a multifaceted neurodegenerative disease characterized by motor and non-motor symptoms resulting from the degeneration of dopaminergic neurons in the brain. Endogenous Bioelectrical Activity (EBA) plays a crucial role in cellular physiology, intercellular communication and tissue organization, making it a focal point for neurobiological modulation therapies such as REAC technology. This raises the question of whether REAC protocols, specifically neuropostural optimization (NPO) and neuropsychophysical optimization (NPPO), can induce EBA changes measurable in saliva metabolomics in PD patients. **Purpose:** This study seeks to evaluate salivary metabolic profiles in individuals with PD undergoing NPO and NPPO with REAC technology. **Methods:** Salivary samples from 33 patients were collected and analyzed using Nuclear Magnetic Resonance (NMR) spectroscopy on a 500 MHz Bruker Biospin device (Rheinstetten, Germany). The resulting NMR spectra were then subjected to Principal Component Analysis (PCA) to identify patterns in the data. Additionally, discriminant analyses were performed using the Partial Least Squares (PLS-DA) and Sparse Partial Least Squares (sPLS-DA) methods to differentiate between pre- and post-therapy metabolomic profiles. The quality of the predictive models was evaluated using the Q2 metric (prediction quality), R2 metric (quality of adjustments), and accuracy (ACC). Univariate analyses were also conducted, adopting a 95% confidence interval to determine the significance of changes in metabolite levels. **Results** Significant changes in the metabolomic profile were observed after the NPO and NPPO-REAC cycles, affecting a panel of 27 metabolites. Multivariate analysis revealed variations in metabolite abundances before and after the therapy. Specifically, decreases were noted in acetic acid, isocaproate, saturated fatty acids, ketovaleric acid, hydroxyproline, isoleucine, butyric acid, N-caproate, lysine, lipids, and histidine. Conversely, increases were observed in sarcosine, threonine, valine, lactate,

sucrose, leucine, and propionic acid, along with other unmarked metabolites. These changes suggest that REAC therapy can significantly alter the metabolomic profile. **Conclusion:** significant changes in the salivary metabolic profile highlight biochemical changes associated with the intervention, indicating systemic effects that deserve further investigation. These findings collectively suggest promising therapeutic effects and argue for the continued exploration and use of REAC technology in the treatment of neurodegenerative diseases.

*Keywords*: Endogenous Bioelectrical Activity, Neurodegenerative diseases, salivary metabolites

# 1 INTRODUCTION

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder that primarily affects the central nervous system. It is characterized by a constellation of motor symptoms, notably including tremors, rigidity, bradykinesia (slowed movement), and postural instability[1]. These symptoms result from the gradual loss of dopaminergic neurons in the substantia nigra, a region of the brain responsible for producing dopamine, a neurotransmitter crucial for coordinating movement and regulating mood[2, 3]. Beyond motor symptoms, PD can also manifest with a range of non-motor symptoms, such as cognitive impairment, mood disturbances, sleep disturbances, autonomic dysfunction, and sensory disturbances[4]. These non-motor symptoms often significantly impact the quality of life of individuals with PD and may precede the onset of motor symptoms by several years[5]. The exact cause of PD remains elusive, but both genetic and environmental factors are believed to contribute to its development[6]. Mutations in specific genes, including SNCA, LRRK2, and Parkin, have been identified as key contributors to familial forms of Parkinson's disease (PD). Additionally, environmental factors such as lifestyle habits, exposure to pesticides, heavy metals, and various toxins have been linked to an elevated risk of developing the disease.[2, 7]. As PD progresses, individuals may experience worsening motor symptoms, fluctuations in medication response, and the development of treatment-related complications, such as dyskinesias (involuntary movements)[8]. Assessing functional mobility and monitoring disease progression are critical aspects of managing PD, as they inform treatment decisions and help optimize therapeutic interventions to improve patients' quality of life.

Endogenous Bioelectrical Activity (EBA) emerges as a fundamental aspect governing biological processes, including neuronal function and communication[9, 10]. Notably, EBA is the

target of treatments utilizing Radio Electric Asymmetric Conveyer (REAC) technology[11]. This complex network of electrical signals regulates various biological processes, including synaptic transmission, neuronal excitability, and information processing[12-18]. Disruptions in EBA have been implicated in neurodegenerative disorders like PD, where aberrant electrical activity contributes to neuronal dysfunction and degeneration[19]. Moreover, emerging evidence suggests a bidirectional relationship between EBA and epigenetic modification[20-24], adding another layer of complexity to the understanding of neurological disorders.

Epigenetic mechanisms, including DNA methylation, histone modification, and non-coding RNA regulation, modulate gene expression without altering the underlying DNA sequence[22, 25, 26]. The interplay between EBA and epigenetic processes highlights the intricate connections between cellular physiology, environmental cues and disease pathology[23].

### 1.1 Mechanisms and Physiological Significance of Endogenous Bioelectrical Activity (EBA):

Endogenous Bioelectrical Activity (EBA) can be defined as the totality of electrical activity originating from individual cells, their interactions within the extracellular matrix, the overall tissue architecture and, ultimately, the organic level, conventionally and contemporaneously referred to as the electrome [27-29].

EBA primarily originates from the selective movement of charged particles (ions) across cellular membranes. The unequal distribution of ions across the membrane establishes an electrical potential difference, which governs various cellular functions[27, 30, 31]. Sodium-potassium pumps and ion channels actively regulate this ionic flux, enabling processes like neurotransmission and muscle contraction[32]. EBA also influences cellular differentiation, proliferation, and tissue regeneration through complex signaling pathways[14, 15, 33].

This phenomenon governs and underpins all fundamental biological processes in living organisms, even before their pathological or physiological manifestation[13, 33, 34].These

electrogenic processes, coupled with the spatial organization of cells within tissues, generate complex patterns of bioelectrical activity[12]. Additionally, the extracellular matrix and tissue architecture influence the propagation and modulation of electrical signals, contributing to the overall EBA[27]. The basis of Endogenous Bioelectrical Activity lies in the intricate interplay between cellular physiology, intercellular communication, and tissue organization. At the cellular level, ion channels and pumps regulate the flow of ions across membranes, generating electrical potentials[20, 35-39]. This activity is coordinated within tissues through gap junctions and other intercellular connections, enabling synchronization of cellular behavior and propagation of signals[38-42]. Furthermore, the structural organization of tissues provides pathways for electrical conduction and spatial constraints that shape the distribution of bioelectrical activity, thereby influencing physiological processes[37]. In summary, EBA represents a fundamental aspect of biological systems, essential for orchestrating cellular functions and maintaining tissue homeostasis. This electrical activity is important for crucial physiological processes, including nerve conduction, muscle contraction, and wound healing.

### 1.2 The Relationship between EBA and Epigenetics

EBA is essential for regulating changes in gene expression that occur without altering the DNA sequence itself[26]. These changes, known as epigenetic modifications, influence gene activity and cellular function, and are crucial for development, differentiation, and disease.[14-16, 33]. At the molecular level, epigenetic modifications primarily involve three key mechanisms: DNA methylation, histone modifications and non-coding RNA-mediated regulation[43]. However, it's important to note that these are the most well-known epigenetic alterations, but they are not the only ones. The relationship between EBA and epigenetic is multifaceted, reciprocal and dynamic, with EBA influencing epigenetic processes and, in turn, epigenetic modifications shaping EBA-mediated signaling pathways, it involves regulation of epigenetic machinery, propagation of

epigenetic signals, integration of environmental cues, maintenance of cell identity, implications for disease and regeneration[23, 44-46].

EBA, for example, influences the activity of enzymes involved in epigenetic modifications. For instance, changes in intracellular ion concentrations can affect the activity of DNA methyltransferases and histone-modifying enzymes, thereby altering the epigenetic landscape of the cell[21, 26].

Bioelectrical signals generated by EBA can also propagate across cell populations through gap junctions and other intercellular communication channels. These signals can trigger cascades of intracellular events, including the activation or repression of genes associated with specific epigenetic modifications[24, 47].

Concurrently EBA serves as a conduit through which environmental stimuli, such as electromagnetic fields or mechanical forces, are transduced into intracellular signaling pathways[14, 48]. These signals can modulate the activity of epigenetic regulators, allowing cells to adapt their gene expression profiles in response to changing environmental conditions[33, 49].

EBA contributes to the maintenance of cell identity by regulating epigenetic mechanisms involved in cellular differentiation and tissue development. By modulating the expression of key transcription factors and signaling molecules, it helps to establish and preserve cell fate decisions encoded within the epigenome[9, 13, 33].Dysregulation of EBA-mediated epigenetic processes has been implicated in various diseases, including cancer, neurodevelopmental disorders and tissue regeneration defects[16, 17, 50]. Understanding the interaction between endogenous bioelectric activity (EBA) and epigenetic modification holds promise for the development of innovative therapeutic strategies targeting these conditions. In this regard, the focus of Radioeletric assimetric conveyer (REAC) technology lies on EBA[51].

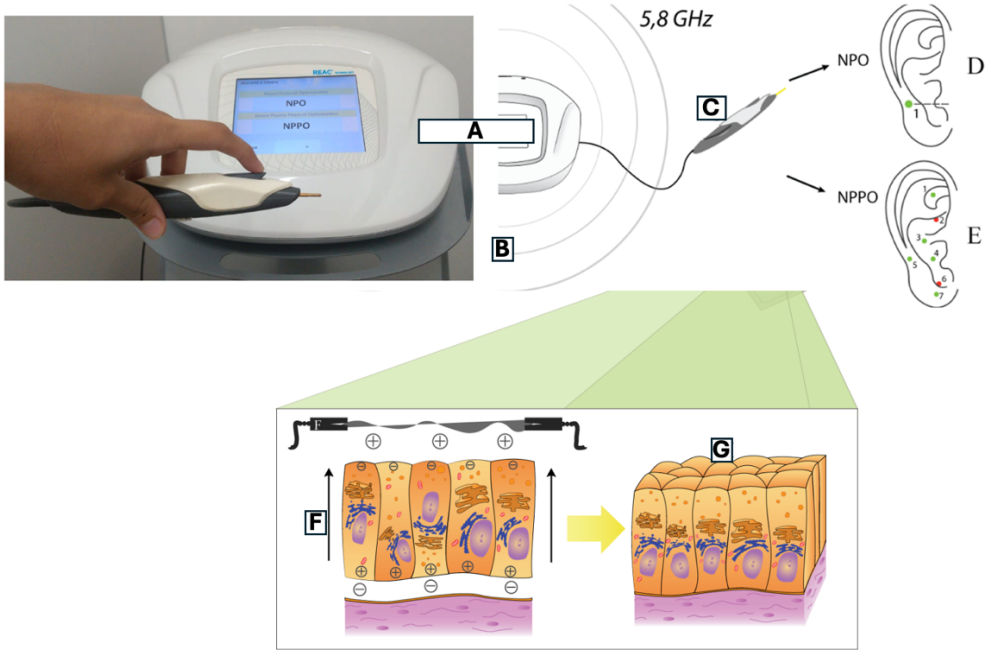
## 1.3 REAC technology

### 1.3.1 General Mechanism

REAC (Radioelectric Asymmetric Conveyer) technology represents a novel approach for modulating EBA. REAC devices employ low-intensity radio waves (2.4 GHz or 5.5 GHz) with an asymmetric waveform to interact with biological tissues [50]. Although the underlying mechanisms of this interaction are still being elucidated, it can be assumed that the radio waves influence cellular polarity and EBA via the polyelectrolyte gradient and charge relief phenomenon[12, 38, 40, 52]. Notably, this modulation targets fundamental cellular functions such as neurotransmission, conceivably leading to therapeutic benefits in a variety of clinical applications[53-57].

The REAC biotechnology equipment operates via an asymmetric conveying probe (ACP), emitting radiofrequency at 2.4 GHz for biomodulation protocols or 5.8 GHz for neuromodulation [51]. The ACP possesses only one physical pole acting as an attractor pole for induced currents within the body, thereby creating an asymmetric circuit with the organism. This interaction results in the formation of an electrical gradient, thus allowing the modulation of ionic flows from the cellular to the body level, providing therapeutic effects[13, 51, 58, 59]. By leveraging the body's natural electrical properties, REAC treatments hold promise for various therapeutic applications, including neurological disorders [11, 51, 54, 60-65] chronic pain management [51, 54, 62, 65-67] and tissue regeneration[68-72].Through the use of asymmetrically delivered low-power radio electric fields inside the body, REAC technology can improve neural communication and promote bioelectric activity within cells[10, 32, 38, 73, 74]. The release of neurotransmitters and neuromodulators may be altered as a result of this stimulation, which may improve the nervous system's overall performance[9, 75, 76].

**Figure 1 Schematic representation of REAC neurobiological modulation.**




(A) B.E.N.E 110 device equipped with the neuropostural optimization protocols (NPO) and neuro-psychophysical optimization protocol (NPPO) of the REAC technology. (B) 5.8 GHz radio wave emitted to the environment and recovered in the asymmetrical punctiform probe (ACP) (C). (D) Application point of the NPO protocol on the antitragus of the auricular pavilion. (E) Sequence of 7 application points of the NPPO protocol. (F) Graphic scheme of the optimization of endogenous bioelectrical activity (EBA) exemplified by the adjustment of current flow and consequent improvement in the asymmetric distribution of cellular constituents, cellular polarity (G). Source: Author, 2024[77].

### 1.3.2 REAC technology General Applications:

REAC technology's ability to target EBA holds significant potential for both general applications across various biological processes and specific therapeutic interventions for neurodegenerative disorders. By modulating EBA, REAC therapeutic protocols can offer a novel approach to promoting a wide range of responses such as enhanced cellular communication. Once optimizing bioelectrical signaling pathways, it may promote more efficient intercellular communication, leading to improved tissue function and overall physiological health[53, 78, 79].

By targeting EBA, REAC technology could stimulate regenerative processes in damaged or diseased tissues[80, 81]. By promoting the activation of specific cellular pathways involved in

tissue repair, REAC may facilitate faster healing and regeneration following injury or disease[80, 82].

Considering neurodegenerative disorders the responses of EBA optimization could include modulation of neuronal activity, symptom management and adjunctive therapy with existing treatments.

In neurodegenerative disorders, aberrant EBA may contribute to neuronal dysfunction and degeneration. REAC technology could be utilized to modulate neuronal activity, restoring proper bioelectrical signaling patterns and potentially slowing the progression of neurodegeneration[9, 66, 83]. REAC protocols may promote neuroprotection and enhance neuroplasticity in individuals with neurodegenerative diseases. This could involve stimulating the production of neurotrophic factors, promoting synaptic remodeling, and supporting the survival of vulnerable neuronal populations[68, 83, 84].

In Parkinson's disease context motor symptoms such as tremors, rigidity, and bradykinesia result from the loss of dopaminergic neurons in the substantia nigra. REAC technology could potentially alleviate these symptoms by modulating EBA to improve dopaminergic neurotransmission and restore motor function[11, 60, 62] .

REAC protocols could also complement existing therapeutic approaches for neurodegenerative disorders, such as medication or different devices for brain stimulation[85, 86]. By targeting EBA, it may enhance the efficacy of these treatments and potentially reduce the dosage or frequency of medication needed to manage symptoms.

### 1.3.3 Neuro Postural Optimization (NPO) and Neuro Psycho-Physical Optimization (NPPO) REAC Protocols:

REAC Neuro Postural Optimization (NPO) treatment was developed to induce initial and stable electro metabolic and functional reorganization in the brain, potentially altered by dysfunctional adaptive processes, including those with an epigenetic basis[53, 59]. This treatment

involves a single administration lasting a few milliseconds, capable of modifying gradients of endogenous bioelectric activity, leading to a soliton effect that explains the long-term stability even after a single administration[87]. One of the significant clinical outcomes observed with the REAC NPO treatment is the consistent reduction in functional dysmetria (FD) phenomenon[87, 88].

Fluctuating asymmetry (FA), a measure of deviations from perfect bilateral symmetry and a potential indicator of stress, has been linked to functional dysmetria (FD) by Rinaldi and Fontani. They propose that FD arises from adaptive motor responses driven by environmental stress-induced changes in the cerebellum and neural circuits, not structural lesions. Building on this concept, they developed Radio Electric Asymmetric Conveyer (REAC) technology protocols, such as Neuro postural optimization (NPO) and Neuro Psychophysical Optimization (NPPO), a neuromodulation approach designed to optimize neuro-psycho-motor strategies for environmental interaction [87].

This outcome could possibly be linked to the treatment's capacity to adjust the bioelectric activity of neurons and promote better communication between different areas of the brain[83, 89-93]. As a result, there may be enhancements in neural plasticity and functional reorganization, contributing to this effect[51, 61].

Otherwise, Neuro Psycho-Physical Optimization (NPPO) is a REAC neuromodulation intervention aimed at enhancing neurological, psychological, and physiological functions. Demonstrated efficacy extends to individuals afflicted with emotional and behavioral disturbances, stress-related ailments, and somatization disorders[79, 94-98]. It serves as a therapeutic modality for targeting the central nervous system's regulatory mechanisms, encompassing the autonomic, endocrine, immune, and motor systems susceptible to maladaptive responses[94, 95]. Administered via precise auricular stimulation, NPPO is characterized by its painless, non-invasive nature and expeditious application, comprising therapeutic cycles 18 sessions each. A maximum of four sessions per day, separated by a minimum interval of one

hour, with a maximum inter-session gap of 15 days, is recommended by the manufacturer. Treatment cycles should be tailored based on individual clinical presentations, necessitating periodic reiterations every few months, under professional evaluation[81]. Broad NPPO indications encompass mood and behavioral disorders[99], while specific applications include acute and chronic depression, anxiety, stress-related conditions, developmental emotional and cognitive disorders such as autism and attention deficit hyperactivity disorder (ADHD), somatization syndromes, post-traumatic stress disorder, adjustment disorders, and enhancement of neuropsychological functioning in healthy cohorts[79, 95, 97, 100, 101].

NPO and NPPO represent two of REAC neuromodulation interventions. Each treatment modality addresses distinct facets of neurological and physiological function, collectively contributing to the comprehensive enhancement of individual health and functioning[62, 91, 93].

REAC protocols offer potential benefits in managing Parkinson's disease by modulating endogenous bioelectric activity, optimizing neurotransmission, and enhancing neuronal function[60, 83, 102]. These treatments have demonstrated favorable effects on diverse neurological conditions, potentially ameliorating motor symptoms, mitigating inflammation, and bolstering neuroprotection[76, 103, 104]. Furthermore, REAC protocols may play a role in addressing mood and behavioral disorders linked with Parkinson's disease, providing a comprehensive strategy for symptom alleviation[96, 97, 105, 106].

## 1.4 Parkinson's Disease and Psychopathological Symptoms

Parkinson's Disease (PD) stands as the second most prevalent neurodegenerative disorder affecting individuals over the age of 60, manifesting in a chronic and progressive form often associated with autonomic dysregulation across various regulatory mechanisms, with an epidemiological forecast indicating exponential growth in the coming decades[4].The

pathophysiology of PD, while not entirely elucidated, is characterized by the progressive loss of dopaminergic neurons in the brain's substantia nigra. Inclusion of Lewy bodies within nerve cells, comprising aggregates of alpha-synuclein proteins, constitutes a distinguishing feature of the disease, leading to degeneration and death of dopamine-producing neurons crucial for movement regulation[107]; The deficiency of this neurotransmitter results in dysfunctional neural circuits such as the nigrostriatal and cortico-basal-ganglionic circuits, pivotal for movement regulation, leading to imbalances in basal nuclei activity and subsequent motor symptoms including tremors, muscular rigidity, and bradykinesia[6, 108]. Non-motor symptoms such as pain, mood alterations, autonomic nervous system dysfunctions, sleep disturbances, fatigue, apathy, hypotensive instability, anorexia, cognitive dysfunction, and depression further compound the clinical picture[109], profoundly impacting quality of life and motivating ongoing exploration of novel technologies aimed at slowing disease progression and alleviating associated symptoms[110].

Stress, particularly, emerges as a non-motor symptom exacerbating the overall symptomatology and negatively affecting quality of life, thereby perpetuating a pathological feedback loop[86, 110]. Both acute and chronic stress play significant roles in PD, with acute stress triggering physiological responses to perceived threats, exacerbating motor symptoms such as gait freezing, dyskinesias, and tremors, and diminishing the efficacy of dopaminergic medication[86, 111, 112]. Conversely, chronic stress, marked by sustained activation of the stress system, disrupts homeostasis, increasing the risk of developing depressive and anxious disorders [86, 113, 114] thereby contributing to accelerated disease progression, as evidenced by rodent model studies demonstrating faster dopaminergic neuronal loss[111]. Stress can induce maladaptive alterations in neural networks, precipitating a cascade of various behavioral and mood disorders and eventual degeneration of these neurons, culminating in the development of a neurotoxic environment[115].

Consequently, not only does the brain suffer from stressful stimuli and toxic insults, but also its capacity for recovery and neural circuit reconnection, i.e., neuroplasticity, becomes compromised, initiating a vicious cycle of toxic damage and stress, resulting in further neuronal degeneration and depressive symptoms, thereby intensifying stress levels and perpetuating the cycle[113]. The treatment of stress and depression in individuals with PD poses challenges, with research indicating that even following a depression diagnosis, conventional antidepressant therapy may not be effective. This resistance may be partial or complete, leading to further reductions in cognition and quality of life[116].

### 1.5 Salivary Metabolomics and physiological expression

In the context of possible measures for physiological outcomes, saliva emerges as a biological fluid primarily produced by major salivary glands regulated by the autonomic nervous system[117-119]. Comprising 99% water along with electrolytes, nucleic acids, and proteins, saliva's metabolite concentrations reflect overall health, including the nervous system's function, enabling detection of early pathological changes in organisms[119-122]. Metabolites, low-molecular-weight organic compounds expressed in both pathological and physiological processes, aid in monitoring disease progression and early detection, underscoring their utility in disease management[119, 123, 124]. These metabolic compounds are not directly produced from gene expression but rather result from the interplay between the genotype, EBA, epigenetic mechanisms, and environmental factors[117, 118, 125]. Studying metabolic alterations aims to elucidate how the metabolic chain responds to various environmental factors, microbial changes, genetic dysregulations, and pathological conditions, considering in this way, the metabolome as an epigenetic expression[119, 126-128]. Metabolomics functionality in understanding pathologies or even molecular physiology enables its application in precision medicine, diagnostic use, evolutionary studies, and biomarker detection[124].

Additionally, nuclear magnetic resonance (NMR) spectroscopy, an analytical technique utilizing magnetic fields and radio waves to study molecular structure and dynamics, offers a comprehensive view of an individual's metabolic profile[129, 130]. NMR spectra, predominantly one-dimensional, serve as a "fingerprint" of the sample's metabolome, distinguishing specific groups of individuals, such as control and disease groups[131].

Metabolites play significant roles in the context of Parkinson's disease. Increased levels of acetic acid were observed in plasma using the NMR technique; this is associated with cognitive decline and neuroprotective effects [132] Decreased levels of sarcosine were detected in cerebrospinal fluid and plasma using LC/MS and NMR techniques, linked to cognitive decline and neuroprotective effects[133, 134]. Decreased levels of threonine were identified in plasma using light transmission aggregation; this is correlated with sleep quality and neurotransmission[135]. Reduced levels of valine were detected in plasma using the NMR technique, which are associated with neurotransmission disorders and oxidative stress[131, 136].

Increased lactate levels were observed in the hippocampal electrochemical system, indicating its involvement in neuronal energy homeostasis[137]. Elevated levels of ketovaleric acid were detected in plasma and cerebrospinal fluid, associated with alterations in leucine and tyrosine metabolism, as well as mitochondrial functional disorders[138]. Decreased levels of leucine in cerebrospinal fluid are linked to cognitive impairment[139]. While increased hydroxyproline levels in urine are associated with oxidative stress[140].

Isoleucine levels were found to be increased in plasma, cerebrospinal fluid, and urine, correlating with mitochondrial functional disorders[124, 141-143]. Elevated butyric acid levels in plasma are linked to decreased motor effects [7]. Increased lysine levels in plasma, cerebrospinal fluid, and urine are associated with the stimulation of alpha-synuclein aggregation and reduced glutamate metabolism [7, 138, 142] . Higher histidine levels in plasma and blood are connected to antioxidant mechanisms and improvements in dopaminergic neurotransmission[131, 136, 143]. Conversely,

decreased propionic acid levels in feces, plasma, and cerebrospinal fluid are linked to cognitive impairment, depression, inhibition of neuroinflammatory activity, and attenuation of blood-brain barrier damage in Parkinson's disease [138, 144].

Salivary metabolomics can provide valuable insights into the effects of a treatment in various areas, including treatment efficacy, toxicity and side effects[145]. It can help identify these effects by monitoring changes in metabolites associated with cellular damage, oxidative stress, or inflammation[128, 146, 147]. However, the greatest importance of salivary metabolomics lies in individual response, especially because it has a physiological target[128, 146, 148]. Analysis of salivary metabolites can reveal individual variations in response to treatment[147]. Some patients may exhibit more pronounced metabolic changes than others, which can help clinicians personalize treatment plans to maximize outcomes.

In light of the intricate interplay among Parkinson's Disease (PD), psychopathological symptoms, and stress, there arises a compelling necessity for innovative therapeutic strategies capable of addressing the multifaceted nature of the condition, encompassing both its motor and non-motor manifestations. The integration of advanced methodologies such as Radio Electric Asymmetric Conveyer (REAC) technology and salivary metabolomics emerges as a promising avenue for devising personalized and comprehensive interventions. REAC technology's capacity to modulate endogenous bioelectric activity represents a pivotal mechanism with potential implications for ameliorating neuronal dysfunction and attenuating neurodegenerative processes in PD. Concurrently, leveraging salivary metabolomics enables non-invasive assessment of biochemical alterations associated with PD progression and stress reactivity, thereby facilitating timely detection and tailored therapeutic interventions. By synergistically harnessing the capabilities of REAC technology and salivary metabolomics, a comprehensive approach to understanding the multifactorial etiology of PD and its psychopathological correlates can be

achieved, thereby envisaging improved clinical outcomes and heightened quality of life for individuals grappling with this debilitating ailment.

The primary objective of this research is to assess whether neuropostural optimization (NPO) and neuro psychophysical optimization (NPPO) protocols using REAC technology can induce measurable changes in the salivary metabolomic profiles of patients with Parkinson's disease (PD). Specifically, the study aims to utilize sophisticated statistical tools such as Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), and Sparse PLS-DA (sPLS-DA) to identify patterns and differences in metabolomic data before and after the therapy. Another objective is to evaluate the quality of predictive models using metrics like R2 (fit quality), Q2 (prediction quality), and accuracy (ACC) to determine the reliability of the observed metabolomic changes. The study seeks to identify specific metabolites that show significant changes in abundance levels between the pre- and post-therapy groups, using Variable Importance in Projection (VIP) scores and univariate analysis.

Furthermore, the study aims to explore the utility of salivary metabolomics as a non-invasive biomarker tool for monitoring treatment efficacy and individual response in the management of Parkinson's disease. Finally, the study aims to discuss the clinical significance of the observed metabolomic changes, which could potentially contribute to understanding the effects of REAC technology on PD and its therapeutic implications.

# 2 OBJECTIVES

## 2.1 General

Evaluate the Impact of REAC Technology on Salivary Metabolomics in Parkinson’s disease Patients.

## 2.2 Specific objectives

Analyze Metabolomic Data Using Advanced Statistical Methods

Determine the Predictive Quality of Metabolomic Changes

Identify Key Metabolites Responsible for Observed Differences

Assess the Clinical Relevance of Metabolomic Changes

# 3 METHODS

## 3.1 Study Design

This study examined the impact of REAC technology on saliva metabolite profiles and functional capacity in persons diagnosed with Parkinson's disease (PD). It was a single-arm, longitudinal, before-and-after design study. The investigation was carried out at the Federal University of Amapá (UNIFAP) in Macapá, Brazil, in the laboratory of the health department. The UNIFAP Ethics Committee granted ethical approval (Opinion No. 5.232.551). The acquisition of spectra and analysis using Nuclear Magnetic Resonance (NMR) spectroscopy was conducted at the National Center for Magnetic Resonance of the Federal University of Rio de Janeiro (UFRJ) with the support of the State University of Rio de Janeiro (UERJ) Rio de Janeiro, Brazil.

## 3.2 Participants

### 3.2.1 Inclusion Criteria

Adults (aged 18 years and above) diagnosed with PD by a neurologist at least six months prior to enrollment.

Willingness and ability to understand and comply with study requirements (over 23 points in Mini Mental State Examination – MMSE) [149]24-30 points: Generally considered normal for healthy adults 18-23 points: Indicates possible mild cognitive impairment (MCI) or mild dementia.

### 3.2.2 Exclusion Criteria

Participants were excluded if they exhibited cognitive or behavioral impairments that affected their ability to comprehend or express themselves. Additionally, individuals with debilitating physical conditions that hindered their participation in the study were excluded. This included individuals who were bedridden, immobile, or otherwise unable to engage in the therapy. Furthermore, participants who did not provide a sufficient amount of saliva were excluded from the metabolomic study.

### 3.2.3 Sample Size

A sample size of 33 participants was calculated using G*Power software [18, 19] (version 3.1.9.4) with the following parameters: effect size (0.50), error α (0.05), power ( 0.95) and Wilcoxon sign classification test. To account for possible dropouts and losses to follow-up, the final sample size was increased to 55 participants. 50 patients reached the end of the 18 NPPO sessions, but only 34 were considered for salivary analysis, the exclusion occurred mainly due to insufficient quantity of saliva for analysis < 600 µL.

## 3.3 Procedures

### 3.3.1 Saliva Collection and Processing

Participants abstained from food, beverages (except water), and oral hygiene for at least 1 hour before saliva collection. Unstimulated saliva was collected using a cotton roll placed in the mouth, then transferred to a sterile syringe (600 µL) and stored in Eppendorf tubes. Samples were refrigerated and subsequently stored at -80°C. Lyophilization was performed for safe transport. Samples were placed in a freeze dryer (-100°C) for 24 hours to remove water by sublimation, preserving the samples without refrigeration during transport.

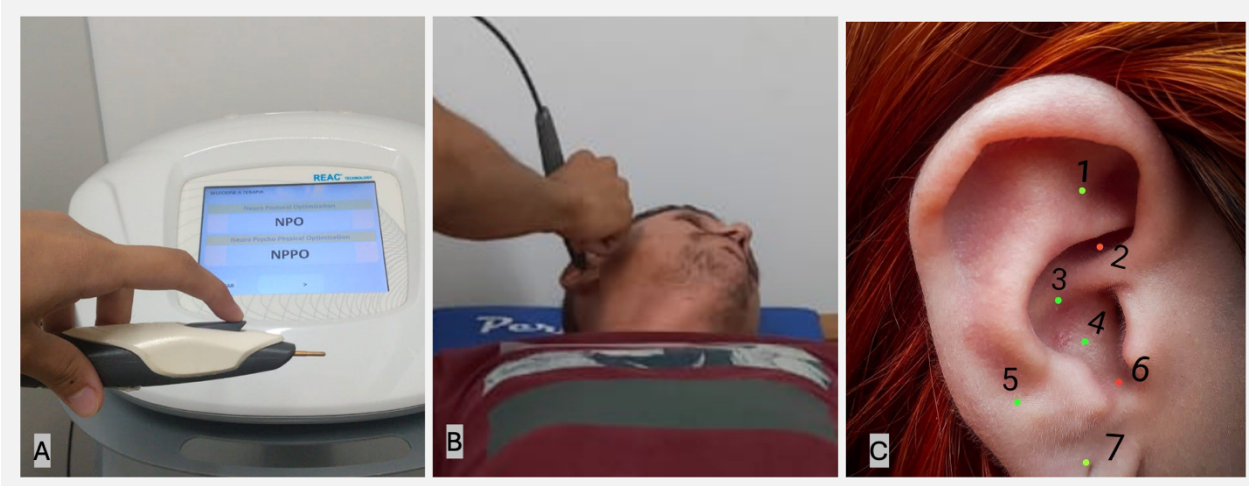
### 3.3.2 Therapeutic Intervention

Following saliva collection, participants received the REAC Neuro-Postural Optimization (NPO) protocol, followed by the REAC Neuropsychophysiological Optimization (NPPO) protocol.

3.3.2.1 **REAC NPO:** A single session applied to the ear to neuromodulate the central nervous system, targeting postural control and stability ( **Error! Reference source not found.**).

3.3.2.2 **REAC NPPO:** Eighteen applications with a probe on specific points of the right auricle, with one-hour intervals between applications and limited to three sessions per day. Target points corresponded to somatotopic references in cortical and cerebellar regions involved in neuroendocrine regulation and stress response ( Figure 2).

**Figure 2- Schematic representation of NPPO application.**




A) Handle, Cable and asymmetric point probe (B) Application of the protocol with the patient lying down (C) highlighting the 7 application points of the NPPO protocol. 1: Anterior branch of the helix (scaphoid fossa); 2: Posterior region of the antihelix branch; 3: Anti-helix; 4: Shell; 5: Anti-helix; 6: Antitragus; 7: Ear lobe.

## 3.4 Data Analysis Procedures

### 3.4.1 Salivary Metabolites

Data obtained from NMR spectroscopy were stored using TopSpin software (version 4.0.3; Bruker Biospin, Karlsruhe, Germany). Spectral data extraction was performed using the AMIX program (Bruker Biospin, Rheinstetten, Germany), obtaining buckets of 0.03 ppm and removing the water region, Triton peaks, and DSS for analysis. The resulting list of peak areas for each metabolite was converted into a .csv file for exportation to the MetaboAnalyst 5.0 software (www.metaboanalyst.ca) (accessed on February 9, 2023) for multivariate analysis. Before analysis, spectral peak values were normalized by dividing them by the sum of signal intensities and subjected to Pareto scaling.

Multivariate analysis was conducted using the online software MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Data were submitted to Principal Component Analysis (PCA), an unsupervised analysis allowing observation of the trend of separation/clustering of metabolites in the analyzed samples, as well as identification of inconsistent samples (outliers); Discriminant Analysis with Partial Least Squares method (PLS-DA), a supervised linear regression method enabling classification or clustering of samples and determining the variables responsible for predicting classes, thereby explaining the variability between data, in addition to generating Variable Importance in Projection (VIP) scores determining the relative amount of metabolites contributing to the separation before and after; and Discriminant Analysis with Sparse Partial Least Squares method (sPLS-DA), obtaining predictive performance parameters of metabolites through cross-validated Q2, R2, and accuracy (ACC) validation.

Based on the Variable Importance in Projection scores, metabolites were identified using 2D spectra and the Human Metabolome Database (HMDB, http://www.hmdb.ca, accessed on October 9, 2023), as well as the assignment established in the work of SILWOOD et al. (2002)[130].

Statistical analysis was performed using SPSS 20.0 software (IBM, Chicago, IL, USA), applying normality tests and standardizing the statistical significance level at 95%. Univariate analysis was conducted using the Wilcoxon test. A confidence interval of 95% was adopted for all statistical analyses.

## 3.5 **Risks and Benefits**

The NPO and NPPO protocols of REAC therapy employ low-intensity radio waves (5.8 GHz) to generate an electric gradient through specific points in the ear. The absorption rate of these waves is only 7μW/kg, resulting in no stimulation, making it a non-invasive and painless application, with the only sensation being the touch of the probe on the application points. Participants had access to water, restroom facilities, chairs, and could at any time express discomfort or embarrassment, allowing them to wait a few minutes, reschedule for another time, or even suspend the applications altogether.

In conducting risk analyses for the research, the risk of discomfort/embarrassment during questionnaire administration, which could take a long time, was observed. To mitigate or avoid this risk, REAC scale application occurred in an appropriate room (private, climate-controlled, well-lit) with two applicators and the participant on separate days depending on participant convenience. Fluid collection took place in a laboratory (climate-controlled, well-lit) in the presence of two specialized researchers and technicians.

## 3.6 **Ethical Considerations**

The research adheres to Resolution 466/12 of the National Health Council, which establishes guidelines and regulatory norms regarding the ethical aspects of research involving human subjects, emphasizing "respect for human dignity and special protection due to research participants involving human beings," and "all progress and advancement must always respect the dignity, freedom, and autonomy of the human being." Participant consent was ensured through the signing of the Informed Consent Form (ICF), and confidentiality and anonymity were respected using numerical codes to identify participants.

Participants were given the opportunity to interrupt the application at any time upon request, as well as the freedom not to answer any item on the scale and to withdraw from

technology application, with no detriment to the participant. Protocols and regulations related to the pandemic situation were adhered to by researchers and participants.

The research received approval from the Research Ethics Committee of UNIFAP with protocol number 5.468.146.

# 4 RESULTS

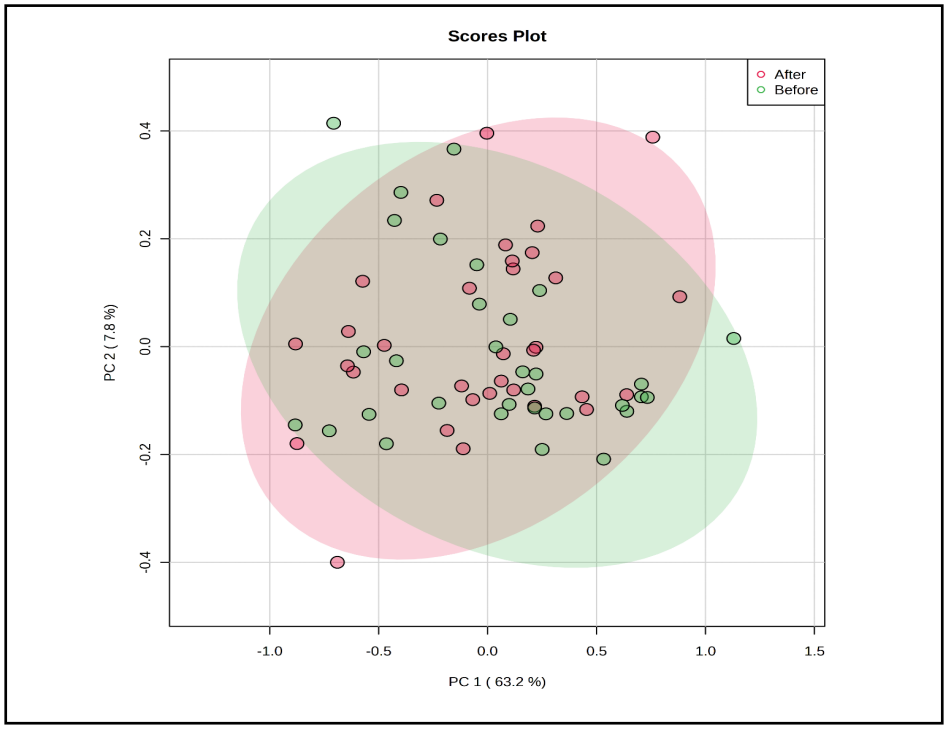
## 4.1 Multivariate analyses

The multivariate analyses of the metabolomic data from the 33 participants were conducted using MetaboAnalyst 5.0 software due to its capacity to process a variety of data and express the differences or similarities among the metabolites subjected to analysis[150]

### 4.1.1 Principal Component Analysis (PCA)

The Principal Component Analysis (PCA) plot (Figure 3) revealed a variability of 71% when comparing the "Before" and "After" groups. However, the PCA did not show a clear separation between these groups, indicating that the overall metabolomic profiles of the participants before and after the therapy were not distinctly different.

**Figure 3 – Principal Component Analysis (PCA) plot**

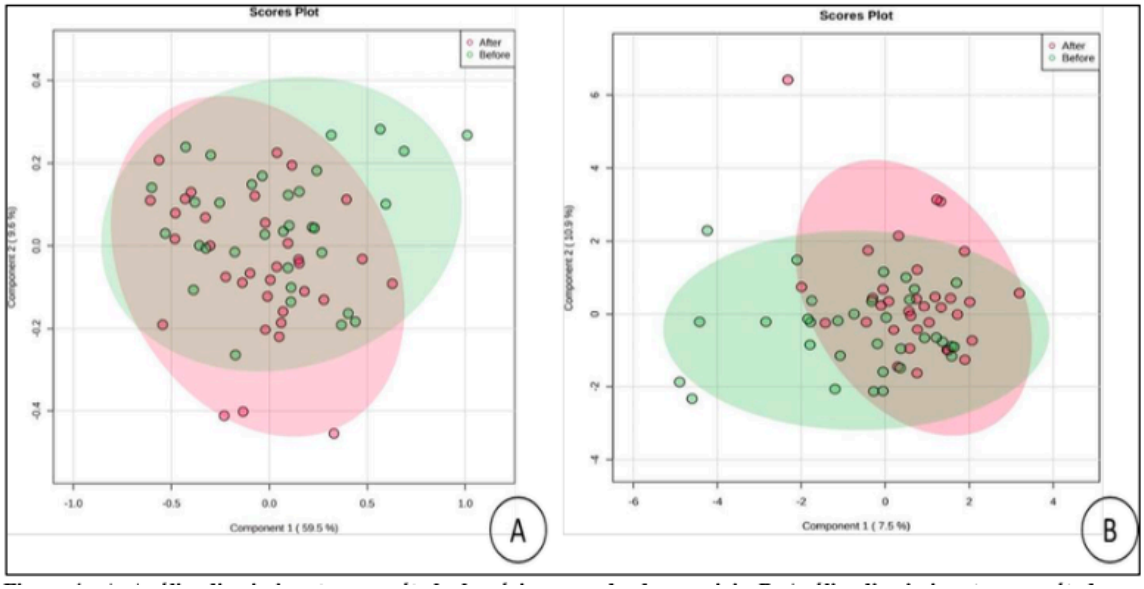



Source: Results (2024)

### 4.1.2 Partial Least Squares Discriminant Analysis (PLS-DA) and Sparse PLS-DA (sPLS-DA)

Sequentially, the PLS-DA (Figure 4A) showed a variability of 69.10%, and in the s-PLS-DA (Figure 4B) a variability of 18.40% was observed. These analyses suggested a slight difference between the metabolomic profiles before and after, with a greater distinction between the groups in the s-PLS-DA.

**Figure 4 – A: Discriminant Analysis with Partial Least Squares Method; B: Discriminant Analysis with Sparse Partial Least Squares Method**

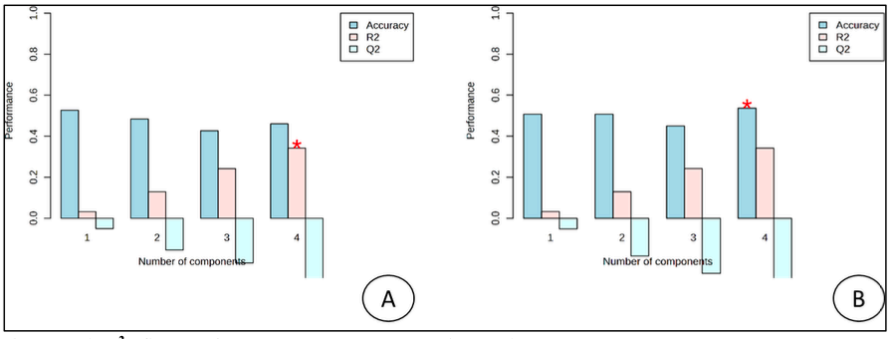



Source: Results (2024)

### 4.1.3 Predictive Model Quality

The quality of the predictive models was assessed using the R2 (fit quality), Q2 (prediction quality), and accuracy (ACC) metrics. The results indicated a low prediction quality of metabolites in the groups, as shown in Figure 5A and 5B.

**Figure 5– A: R2 - Sum of Squares of the Sample; B: Sample Accuracy.**




Source: Results (2024)

### 4.1.4 Cross-Validation Analysis

**Table 1** presents the results of the cross-validation analysis, showing the accuracy, R2, and Q2 values for different numbers of components. The accuracy values ranged from 0.46333 to 0.54667, R2 values ranged from 0.031136 to 0.34134, and Q2 values were negative, indicating poor predictive performance.

***Table 1- Cross-Validation Analysis***

| Measure | 1 comps | 2 comps | 3 comps | 4 comps* |
|---|---|---|---|---|
| Accuracy | 0.54333 | 0.46333 | 0.47333 | 0.54667 |
| R2 | 0.031136 | 0.12846 | 0.24203 | 0.34134 |
| Q2 | -0042964 | -0.16308 | -0.31858 | -0.55908 |

*Components signaled by accuracy test. Source: The author (2024)

Source: Results (2024)

## 4.2 Univariate analysis

### 4.2.1 Metabolite Differences

The metabolomic results generated by the MetaboAnalyst analysis indicate differences between before and after, as shown in Table 2. The metabolites are listed according to their abundance levels expressed in the Variable Importance in Projection scores generated by the multivariate analysis, followed by the univariate analysis, ranking the metabolites responsible for the observed difference between the groups in descending order.

**Table 2** Displays the main concentration of salivary metabolites shown with Chemical Shift, Median, Minimum, Maximum of Samples Before and After, Significance Level, Multivariate Analysis, and Univariate Analysis.

***Table 2-Main Concentration of Salivary Metabolites***

| Metabolites | Chemical shift (δ) | State Atomic | Median Before (Min/Max) | Median After (Min/Max) | *p*-value |
|---|---|---|---|---|---|
| Acid Acetic[I] HMDB00042 | 1.90 | Singlet | 0,40 x$10^{-3}$ (0,08 x$10^{-3}$ – 0,94 x$10^{-3}$) | 0,35 x$10^{-3}$ (0,10 x$10^{-3}$ – 0,67 x$10^{-3}$) | 0,271 |
| Sarcosine[I] HMDB00271 | 3.61 | Singlet | 1,06x$10^{-3}$ (0,23 x$10^{-3}$ – 1,70 x$10^{-3}$) | 1,10x$10^{-3}$ (0,44 x$10^{-3}$ – 1,71 x$10^{-3}$) | 0,985 |
| Threonine[I] HMDB00167 | 3.58 | Dublet | 0,46x$10^{-3}$ (0,12 x$10^{-3}$ – 8,7 x$10^{-2}$) | 0,50x$10^{-3}$ (0,14 x$10^{-3}$ – 1,22 x$10^{-3}$) | 0,777 |
| Unassigned[I] | 0.67 | | 0,55 x$10^{-3}$ | 0,58 x$10^{-3}$ | 0,971 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | (0,02 x10$^{-3}$ – 10,2 x10$^{-3}$) | (0,17 x10$^{-3}$ – 1,01 x10$^{-3}$) | | |
| 3 | Valine[I] HMDB0088 | 3.64 | Dublet | 0,93x10$^{-3}$ (0,31 x10$^{-3}$ – 1,43 x10$^{-3}$) | 0,97x10$^{-3}$ (0,49 x10$^{-3}$ – 1,43 x10$^{-3}$) | 43 | 0,9 |
| 190 | Lactate[I] HMDB0000 | 1.33 | Dublet | 0,10 x10$^{-3}$ (0,05 x10$^{-3}$ – 0,16 x10$^{-3}$) | 0,11 x10$^{-3}$ (0,05 x10$^{-3}$ – 0,62 x10$^{-3}$) | 0,490 | |
| 8 | Sucrose[I] HMDB0025 | 3.76 | Multiplet | 0,14x10$^{-3}$ (0,10x10$^{-3}$ - 0,19x10$^{-3}$) | 0,15x10$^{-3}$ (0,10x10$^{-3}$ - 0,25x10$^{-3}$) | 57 | 0,0 |
| [I] | Unassigned | 1.66 | | 0,06 x10$^{-3}$ (0,02 x10$^{-3}$ – 0,12 x10$^{-3}$) | 0,05 x10$^{-3}$ (0,03 x10$^{-3}$ – 0,09 x10$^{-3}$) | 0,059 | |
| 465 | Isocaprate[I] HMDB0302 | 0.91 | Dublet | 0,06 x10$^{-3}$ (0,03 x10$^{-3}$ – 0,19 x10$^{-3}$) | 0,05 x10$^{-3}$ (0,02 x10$^{-3}$ – 0,07 x10$^{-3}$) | 0,220 | |
| fatty acid[I] | Saturated | 0.88 | Triplet | 0,05 x10$^{-3}$ (0,02 x10$^{-3}$ – 0,19 x10$^{-3}$) | 0,04 x10$^{-3}$ (0,02 x10$^{-3}$ – 0,07 x10$^{-3}$) | 88 | 0,1 |
| 1 | 3-Methyl-2-kethovaleric acid[I] HMDB0049 | 0.85 | Triplet | 0,04 x10$^{-3}$ (0,01 x10$^{-3}$ – 0,13 x10$^{-3}$) | 0,03 x10$^{-3}$ (0,01 x10$^{-3}$ – 0,06 x10$^{-3}$) | 21 | 0,1 |
| 7 | Leucine[I] HMDB0068 | 3.73 | Triplet | 0,11x10$^{-3}$ (0,06x10$^{-3}$ - 0,15x10$^{-3}$) | 0,12x10$^{-3}$ (0,05x10$^{-3}$ - 0,19x10$^{-3}$) | 0,357 | |
| 5 | Hidroxyproline[I] HMDB0072 | 2.23 | Multiplet | 0,06 x10$^{-3}$ (0,02 x10$^{-3}$ – 0,14 x10$^{-3}$) | 0,06 x10$^{-3}$ (0,02 x10$^{-3}$ – 0,11 x10$^{-3}$) | 0,116 | |
| Isoleucine[I] HMDB00172 | | 0.94 | Triplet | 0,04 x10$^{-3}$ | 0,04 x10$^{-3}$ | 0,366 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | (0,02 x10$^{-3}$ – 0,15 x10$^{-3}$) | (0,01 x10$^{-3}$ – 0,06 x10$^{-3}$) | | |
| Sucrose[I] HMDB00258 | 3.46 | Triplet | 0,05x10$^{-3}$ (0,03 x10$^{-3}$ – 0,10 x10$^{-3}$) | 0,06x10$^{-3}$ (0,03 x10$^{-3}$ – 0,10 x10$^{-3}$) | 0,139 | |
| Lactate[I] HMDB0000190 | 1.30 | Dublet | 0,10 x10$^{-3}$ (0,05 x10$^{-3}$ – 0,31 x10$^{-3}$) | 0,11 x10$^{-3}$ (0,06 x10$^{-3}$ – 0,51 x10$^{-3}$) | 0,672 | |
| Butyric acid[I,II] HMDB0000039 | 1.15 | Dublet | 0,01 x10$^{-3}$ (0,006 x10$^{-3}$ – 0,03 x10$^{-3}$) | 0,01 x10$^{-3}$ (0,004 x10$^{-3}$ – 0,01 x10$^{-3}$) | 01 | 0,0 |
| N-Caprate[I] HMDB00535 | 0.82 | Triplet | 0,01 x10$^{-3}$ (0,009 x10$^{-3}$ – 0,3 x10$^{-3}$) | 0,01 x10$^{-3}$ (0,007 x10$^{-3}$ – 0,02 x10$^{-3}$) | 03 | 0,0 |
| Lysine[I] HMDB00182 | 1.75 | Multiplet | 0,03 x10$^{-3}$ (0,009 x10$^{-3}$ – 0,07 x10$^{-3}$) | 0,02 x10$^{-3}$ (0,009 x10$^{-3}$ – 0,06 x10$^{-3}$) | 0,067 | |
| Leucine[I] HMDB00687 | 3.73 | Triplet | 0,11x10$^{-3}$ (0,06x10$^{-3}$ - 0,15x10$^{-3}$) | 0,12x10$^{-3}$ (0,05x10$^{-3}$ - 0,19x10$^{-3}$) | 0,357 | |
| Butyric acid[I] HMDB00039 | 2.32 | Dublet | 0,1x10$^{-3}$ (0,03 x10$^{-3}$ – 0,17 x10$^{-3}$) | 0,09x10$^{-3}$ (0,03 x10$^{-3}$ – 0,16 x10$^{-3}$) | 95 | 0,3 |
| Unassigned[I] | 2.20 | | 0,04 x10$^{-3}$ (0,02 x10$^{-3}$ – 0,14 x10$^{-3}$) | 0,04 x10$^{-3}$ (0,02 x10$^{-3}$ – 0,11 x10$^{-3}$) | 0,154 | |
| Lipid[I,II] | 0.79 | | 0,01 x10$^{-3}$ (0,006 x10$^{-3}$ – 0,02 x10$^{-3}$) | 0,009 x10$^{-3}$ (0,005 x10$^{-3}$ – 0,01 x10$^{-3}$) | 001 | <0, |
| Histidine[I,II] HMDB00177 | 3.28 | Dublet | 0,02 x10$^{-3}$ (0,009 x10$^{-3}$ – 0,04 x10$^{-3}$) | 0,01 x10$^{-3}$ (0,008 x10$^{-3}$ – 0,05 x10$^{-3}$) | 42 | 0,0 |
| Unassigned[I,II] | 1.78 | | 0,02 x10$^{-3}$ (0,008 x10$^{-3}$ – 0,04 x10$^{-3}$) | 0,01 x10$^{-3}$ (0,008 x10$^{-3}$ – 0,03 x10$^{-3}$) | 0,038 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Unassigned[,II] | 1.09 | | 0,01 x10$^{-3}$ (0,007 x10$^{-3}$ – 0,02 x10$^{-3}$) | 0,01 x10$^{-3}$ (0,005 x10$^{-3}$ – 0,01 x10$^{-3}$) | 08 | 0,0 |
| Proprionic acid[,II] HMDB0000237 | 1.06 | Triplet | 0,0062x10$^{-3}$ (0,006 x10$^{-3}$ – 0,01 x10$^{-3}$) | 0,001x10$^{-3}$ (0,008 x10$^{-3}$ – 0,0003 x10$^{-3}$) | 0,029 | |
| Unassigned[II] | 0.76 | | 0,005 x10$^{-3}$ (0,0003 x10$^{-3}$ – 0,1 x10$^{-3}$) | 0,004 x10$^{-3}$ (0,001 x10$^{-3}$ – 0,1 x10$^{-3}$) | 29 | 0,0 |

*δ = chemical shift; [I]Indicates which metabolites showed statistical difference (p ≤ 0.05) in the multivariate analysis (VIP score of the PLS-DA analysis); [II]Indicates which metabolites showed statistical difference in the univariate analysis; The statistically significant metabolites were presented above according to multivariate analysis (VIP score of the PLS-DA analysis) median, minimum and maximum between before (B) and after (A) REAC protocols, their respective chemical shifts, State atomic and the significance value obtained in the univariate analysis*

The analysis of salivary metabolites revealed subtle but noteworthy changes between the "Before" and "After" groups following Neurobiological modulation REAC therapy. The metabolites were ranked according to their Variable Importance in Projection (VIP) scores from multivariate analysis and further examined through univariate analysis.

### 4.2.2 Key findings:

1. Leucine (HMDB00687) showed a slight increase after therapy, with median concentrations rising from 0.11 × 10^-3 to 0.12 × 10^-3, though this change was not statistically significant (p = 0.357).
2. Butyric acid (HMDB00039) exhibited a minor decrease, with median concentrations changing from 0.1 × 10^-3 to 0.09 × 10^-3 (p = 0.395).
3. An unassigned metabolite at δ 2.20 remained stable with a median concentration of 0.04 × 10^-3 in both groups (p = 0.154).

4. Lipid levels showed a statistically significant decrease (p = 0.001), with median concentrations dropping from 0.01 × 10^-3 to 0.009 × 10^-3.
5. Histidine (HMDB00177) demonstrated a significant decrease (p = 0.042), with median concentrations falling from 0.02 × 10^-3 to 0.01 × 10^-3.
6. Two unassigned metabolites (δ 1.78 and δ 1.09) showed significant decreases (p = 0.038 and p = 0.008, respectively).
7. Propionic acid (HMDB0000237) exhibited a significant decrease (p = 0.029), with median concentrations changing from 0.0062 × 10^-3 to 0.001 × 10^-3.
8. Another unassigned metabolite (δ 0.76) showed a significant decrease (p = 0.029), with median concentrations dropping from 0.005 × 10^-3 to 0.004 × 10^-3.

These results indicate that while some metabolites remained relatively stable, others, particularly lipids, histidine, propionic acid, and certain unassigned compounds, showed statistically significant decreases following REAC therapy. These changes, although subtle, suggest that the therapy may have induced measurable alterations in the salivary metabolomic profile of Parkinson's disease patients.

## 5 DISCUSSION

The observed changes in metabolite abundance following NPO and NPPO protocols suggest a systemic response to REAC technology, potentially reflecting changes in metabolic pathways, cell signaling, and homeostatic regulation[59, 117, 118, 151]. Furthermore, due to changes in the general behavior of the central nervous system (CNS), resulting in improved neurotransmission and impacting the metabolic profile[76, 89, 118].

By optimizing the mechanisms of neuromodulation, REAC therapy enhances the adaptive response of the CNS, effectively mitigating the progression of neurological disorders. It acts as a forceful intervention, stifling the disease's progression by bolstering endogenous defense mechanisms[76, 79, 98].Moreover, REAC therapy augments the capacity for environmental interaction and modifies how environmental factors influence epigenetic processes. This comprehensive approach not only improves neuromodulators mechanisms but also reshapes the response to environmental stimuli, thereby altering epigenetic regulation[43, 81].

The observed alterations in salivary metabolites post-REAC therapy may be linked to improvements in clinical parameters. Previous study with the same individuals of the present trial showed significant improvements in functional dysmetria, postural stability, and quality of life (QLF) following the REAC NPO and NPPOs treatments. Notably, the treatments demonstrated a positive impact on physical function, as evidenced by enhanced performance in the five times sit to stand test (FTSST), indicating improvements in strength, balance, and overall physical function. Furthermore, the assessment of physical and mental health using the 12-item Short-Form Health Survey (SF-12) revealed significant enhancements post-treatment, underscoring the efficacy of REAC interventions in improving both physical and mental well-being[60].

Decreases in metabolites such as acetic acid, isocaproate, saturated fatty acids, hydroxyproline and histidine could signal changes in metabolic pathways associated with Parkinson's disease pathophysiology. For instance, reductions in acetic acid, butyric acid, and lipids may reflect alterations in energy metabolism, while decreases in lysine and isoleucine levels might correspond to disruptions in protein metabolism observed in Parkinson's disease[152, 153].

Conversely, increases in metabolites such as sarcosine, threonine, valine, lactate, and sucrose could indicate metabolic adaptations or compensatory mechanisms following REAC therapy[136, 154, 155]. Elevated levels of lactate may suggest enhanced glycolysis, whereas higher levels of amino acids like threonine and valine could be indicative of shifts in protein metabolism. These findings underscore the intricate metabolic changes occurring in response to REAC treatment, potentially contributing to the amelioration of Parkinson's disease symptoms[156-159].

Assessing the predictive potential of these salivary metabolomic profiles for disease progression and treatment response calls for longitudinal studies. These would help to link changes in metabolites with disease progression over time and gauge responses to different treatments, including REAC therapy. Additionally, further inquiries are necessary to grasp the specific mechanisms driving these metabolic shifts and their relevance to Parkinson's disease development and treatment strategies. Despite the need for more research, some initial insights will be explored.

### 5.1 Unveiling Neurobiological Insights: The Salivary Expression Perspective

Glandular reactions, owing to their direct linkage with the autonomic nervous system, serve as indicators of a neurovegetative state, with mental health disorders frequently accompanying Parkinson's Disease (PD)[123]. The neurobiological stress associated with

oxidative stress perpetuates individual dysfunctions of endogenous bioelectric activity (EBA), epigenetic processes, and exposomic adaptive responses, phenomena that inherently influence the salivary metabolic profile, regardless of the presence of PD or other conditions. These disturbances in EBA can impact epigenetic mechanisms, thereby altering cellular function and physiology [5, 24, 43, 151, 160-164].

Saliva primarily comprises locally produced glandular components and passive diffusion in the exposome, wherein only free molecules can permeate, indicating that its constituents predominantly exist in biologically active concentrations. Therefore, all salivary metabolites detected across different experimental time points in this study reflect the physiological expression of the individual. The responsiveness of salivary glands as effector organs, along with their neural command origin, constitutes manifestations of bioelectric cellular communications, which contribute to epigenetic and transcriptional cascades, thereby eliciting changes in cellular activity[13, 23, 37, 165].

Targeted by REAC technology protocols, the EBA undergoes modification after a single cycle of the REAC ONP and ONPF protocols, two therapies designed to treat postural and behavioral disorders[11, 62, 80, 101]. The modification in the metabolic response among individuals with PD could be detected through salivary metabolomics tests based on nuclear magnetic resonance. These results reinforce the premise that the salivary metabolite profile can serve as one of the most reliable and individualized indicators of an individual's biological status. Thus, machine learning algorithms were employed to predict the presence of metabolites in saliva samples, revealing significant variation in a set of 27 metabolites among individuals with PD after a single cycle of REAC neuromodulation protocols. These results are in agreement in terms of number and type of metabolites with studies carried out in other biological fluids, such as plasma and blood.[7, 166, 167].

## 5.2 Branched-chain amino acids (BCAAs) modulation and potential neuroprotective mechanisms in Parkinson's disease

Following the cycle of REAC neurobiological modulation, levels of branched-chain amino acids (BCAAs), including threonine, sarcosine, sucrose, and lactate, exhibited an increase, while histidine, lysine, lipids, and organic acids such as acetic acid, propionic acid, isocaproate, butyric acid, and N-caproate showed a decrease. BCAAs, namely isoleucine, leucine, and valine, play a pivotal role beyond protein genesis, extending to immune function and energy metabolism[166, 168]. Notably, these BCAAs are involved in modulating neurobiological stress by stimulating the BDNF/TRKB (brain-derived neurotrophic factor and tropomyosin receptor kinase B) signaling in the hippocampus[169]. Furthermore, isoleucine and valine have been linked to the regulation of nervous behavior, highlighting the significance of BCAAs in brain protection[43]. Studies in animal models have demonstrated that reduced levels of leucine correlate with hippocampal neuronal count, exacerbating cognitive impairment[137]. Conversely, leucine supplementation has shown positive effects on motor function, neuronal protection, and dopaminergic cell survival[114, 170]. Low levels of isoleucine and leucine have been associated with alterations in neurotransmission and induction of oxidative stress in the hippocampus of rodents [131, 136]

The observed increase in these amino acids following REAC intervention, counteracting their known decrease in PD [171, 172], may indicate a restoration of balance in neurobiological and oxidative stress mechanisms. This restoration could potentially contribute to improved neuronal function and protection against neurodegeneration, given the crucial roles of BCAAs in protein synthesis, neurotransmitter regulation, and mitochondrial function. Moreover, the alterations in BCAA levels post-REAC therapy underscore the therapeutic potential of modulating endogenous bioelectric activity in neuroprotective interventions for PD.

In contrast, hydroxyproline, also implicated in oxidative stress processes, was found to be increased in the biofluids of PD patients compared to the control group, attributed to intensified collagen degradation through matrix metalloproteinase activity associated with PD pathophysiology[140]. In the present study, decreased levels of hydroxyproline may indicate modulation of the stress process, resulting in less degradation of matrix proteins in PD. This reduction suggests a potential attenuation of collagen breakdown, indicating a protective effect on connective tissue integrity. Furthermore, it highlights the complex interplay between oxidative stress, protein degradation, and neurodegenerative processes in PD pathology.

### 5.3 Sarcosine, Lactate, and Modulation of Stress Response and Depressive Behavior:

Research highlights the multifaceted properties of sarcosine, an amino acid derivative, encompassing anti-inflammatory, antioxidant, and neuroprotective attributes [154, 173] et al., Sarcosine's capacity to modulate stress response via N-methyl-D-aspartate receptor (NMDAR) activity underscores its potential role in cortisol regulation[155, 174]. Notably, elevated sarcosine levels have shown promise in mitigating cognitive decline, particularly evident in studies involving dementia patients[133]. Moreover, augmented neuroprotective effects associated with increased sarcosine levels further accentuate its therapeutic potential[134].

In the realm of Parkinson's disease (PD), the rise in sarcosine levels post-REAC protocols may stem from the therapy's influence on neurobiological pathways. REAC therapy has demonstrated efficacy in modulating neurophysiological processes[54, 96, 97].Thus, the observed elevation in sarcosine levels possibly signifies the restoration or enhancement of neuroprotective mechanisms in PD patients undergoing REAC treatment.

Additionally, the increase in lactate levels post-REAC therapy could be intricately linked to the modulation of stress response and amelioration in depressive indices established by REAC

neurobiological modulation[96, 97]. Despite its traditional association with exercise-induced stress, lactate is increasingly recognized for its pivotal role beyond energy metabolism, serving as a signaling molecule in diverse biological processes[175-177]. Including cell differentiation[157], inflammation and angiogenesis[176, 178]. The homeostasis of lactate metabolism is related to neuronal energy demands contributing to neuron survival and long-term memory formation[137, 156, 157].

Emerging evidence suggests lactate's potential as a treatment for conditions like major depressive disorder (MDD), with its ability to counteract corticosterone-induced depressive-like behavior[159, 179]. Furthermore, lactate's involvement in synaptic plasticity, energy production, and antioxidant enzyme regulation underscores its therapeutic promise in neurodegenerative diseases and aging[176, 180].

The observed metabolic adjustments, notably the increase in lactate levels post-REAC therapy, may signify enhanced neuroprotective mechanisms and metabolic adaptation in PD patients. This elevation in lactate levels potentially reflects an upsurge in energy metabolism to support neuronal function and synaptic plasticity, thereby contributing to improved cognitive and motor function. Moreover, the rise in lactate may signify a reduction in oxidative stress and inflammation, further bolstering neuronal resilience and overall brain health in Parkinson's disease.

### 5.4 Enhancing Neuroprotection: Histidine, Threonine, and REAC Therapy in Parkinson's Disease

Histidine, a notable amino acid, has consistently shown elevated levels in the saliva of individuals with Parkinson's disease (PD) compared to controls[142, 181]. However, intriguingly, following REAC therapy, histidine levels exhibited a decrease in the study group. Histidine serves

as a precursor for histamine, a neurotransmitter pivotal for various brain functions, including stress response modulation, alertness, and sleep regulation[182]. The reduction in histidine levels post-REAC therapy suggests a potential modulation of neurotransmitter pathways, particularly those involving histamine, which could significantly influence the overall neurological response.

Furthermore, the observed decrease in histidine/histamine levels may entail a positive impact on dopaminergic neuron transmission within the striatal system as evidenced in rodent models[8]. This potential link between histaminergic neuronal activity and dopaminergic function holds significance for motor behavior, notably in the context of PD. The modulation of histamine levels through REAC therapy could contribute to improvements in motor symptoms commonly associated with PD, as indicated by incremental enhancements in motor function measured by the FTSST test in previous study [60] . Moreover, the reduction in histidine levels following REAC therapy may be linked to its neuroprotective and anti-inflammatory effects, supported by various studies demonstrating REAC's ability to modulate inflammatory responses and enhance neuroprotection[183-185]. These findings imply that the observed improvements in motor function and quality of life parameters [60] could be attributed, at least in part, to the neuroprotective and anti-inflammatory effects of REAC therapy mediated by histamine and histidine modulation.

In addition to histidine, the increase in threonine levels observed after REAC therapy further underscores the therapeutic effects of the treatment. Threonine, known for its role in promoting sleep and closely linked to the GABAergic control of sleep regulation, aligns with the observed improvements in sleep quality indices associated with REAC therapy[77, 135, 186]. This association suggests that threonine modulation may contribute to the observed clinical benefits, offering insights into the multifaceted mechanisms underlying REAC therapy in PD management.

## 5.5 Organic acids and sugars and alteration of microbial activity

Following REAC neuromodulation therapy, a notable decrease in the levels of organic acids was observed. These compounds, essential for maintaining metabolic and physiological homeostasis, are intricately involved in lipid (N-caproate), energy, and amino acid metabolism (acetic acid, propionic acid, and butyric acid)[121]. However, their reduction post-therapy suggests a potential alteration in microbial activity, as organic acids are predominantly associated with microbial metabolic pathways.

Microbial degradation processes play a crucial role in converting sugars into organic acids, including acetic acid, and other short-chain fatty acids. Proteolytic bacteria also contribute to the degradation of amino acids, leading to the production of organic acids. Notably, microbial activity within the oral cavity, including species associated with oral diseases such as *Streptococcus mutans, Prevotella intermedia,* or *Porphyromonas gingivalis*, as well as those commonly found in the intestinal microbiome of individuals with Parkinson's disease (PD), may influence the levels of these metabolites.

Furthermore, the elevated level of acetic acid has been associated with enhanced cognitive function, implying that prolonged deficiency may predispose individuals to cognitive decline. Conversely, lysine, which is derived from bacterial peptide lysis, exhibited a decrease in concentration in the sample. Salivary free amino acids are products of oral cells, proteases, and microbiota activity. Notably, lipids and saturated fatty acids present in saliva are linked to bacterial adhesion [187, 188] and were found to be elevated in PD patients[172, 189], suggesting a potential clinical correlation.

Considering the reduction of these metabolites associated with increased availability of sucrose and other sugars (unattributed), a reduction in microbial activity, especially proteolytic activity, after therapy can be presumed[187, 188].

In light of the diminished levels of organic acids observed in the sample, it is pertinent to highlight the prevalent pattern of these metabolites in the saliva of individuals with PD[172], particularly butyric and propionic acids[142, 172]. Furthermore, recent studies have underscored a positive correlation between elevated valeric acid levels and both cortisol levels and depressive symptoms[190]. Notably, propionate has been identified as capable of inhibiting pathways linked to microbial infections and safeguarding the blood-brain barrier against oxidative stress[144], while also being implicated in cognitive decline and depression among PD patients[191, 192]. Moreover, plasma concentrations of butyrate and valerate have been associated with cognitive impairment, with levels found to be elevated in PD patients compared to the control group[142, 193]. Remarkably, our metabolomic analysis revealed a decrease in these metabolites following treatment with REAC therapy, suggesting a potential effectiveness of REAC in modulating these metabolic pathways. This adds to the growing body of evidence supporting the therapeutic efficacy of REAC in managing PD and its associated symptoms.

In summary, the increase in branched-chain amino acids and lactate suggests an improvement in both the stress response and neuroprotective behavioral responses, potentially accompanied by modulation of the inflammatory response and reduction in proteolytic microbial activity. These effects may also be associated with the availability of sugars and lipids, as well as the reduction of organic acids, histidine, and lysine.

Moreover, it is essential to emphasize the need for further comprehensive investigation, as the metabolic alterations identified in this study hold significant implications. Utilizing a

proposed clinical device to reprogram endogenous bioelectric activity, already employed in managing psychiatric disorders and neurodegenerative conditions, not only enhances metabolic conditions but also offers relief from psychopathological symptoms in Parkinson's disease (PD). This improvement has the potential to mitigate epigenetic entropy and adaptive dysfunctions triggered by exposome factors in PD. Furthermore, it is paramount to highlight that the present study analyzed salivary metabolomics based on a single therapeutic cycle for all patients indiscriminately. The underlying mechanisms of the REAC technology, which are non-stimulatory and involve the alteration of currents towards the organism, suggest precision medicine targeting physiological rather than morphological aspects. The observed pattern of modification in the metabolic profile of this sample after a single therapy cycle confirms this trend.

## 6 CONCLUSION

The study's findings suggest that REAC therapy, through NPO and NPPO protocols, induces measurable changes in the salivary metabolomic profiles of Parkinson's disease patients. These changes, although subtle, indicate a potential modulation of metabolic pathways associated with the disease. Significant decreases in metabolites such as lipids, histidine, and propionic acid, along with observed improvements in clinical parameters, highlight the therapeutic potential of REAC technology in managing Parkinson's disease.

However, the predictive models showed low accuracy and poor fit, suggesting that the changes in metabolite levels were not substantial enough to be clearly distinguished by the applied analytical methods. This underscores the need for further research with larger sample sizes and more sensitive analytical techniques to fully elucidate the impact of the therapy on the metabolomic profiles of Parkinson's disease patients.

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**Future Directions**

In summary, while the study provides promising insights into the potential benefits of REAC therapy, it also highlights the complexity of metabolomic changes and the challenges in accurately predicting these changes. Future studies should aim to address these challenges and further explore the therapeutic mechanisms of REAC technology in neurodegenerative diseases.

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