


## RESEARCH ARTICLE

# Detection of a Parkinson's Disease–Specific MicroRNA Signature in Nasal and Oral Swabs

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**ABSTRACT: Background:** Biomaterials from oral and nasal swabs provide, in theory, a potential resource for biomarker development. However, their diagnostic value has not yet been investigated in the context of Parkinson's disease (PD) and associated conditions.

**Objective:** We have previously identified a PD-specific microRNA (miRNA) signature in gut biopsies. In this work, we aimed to investigate the expression of miRNAs in routine buccal (oral) and nasal swabs obtained from cases with idiopathic PD and isolated rapid eye movement sleep behavior disorder (iRBD), a prodromal symptom that often precedes  $\alpha$ -synucleinopathies. We aimed to address their value as a diagnostic biomarker for PD and their mechanistic contribution to PD onset and progression.

**Methods:** Healthy control cases ( $n = 28$ ), cases with PD ( $n = 29$ ), and cases with iRBD ( $n = 8$ ) were prospectively recruited to undergo routine buccal and nasal swabs. Total RNA was extracted from the swab material, and the

expression of a predefined set of miRNAs was quantified by quantitative real-time polymerase chain reaction.

**Results:** Statistical analysis revealed a significantly increased expression of hsa-miR-1260a in cases who had PD. Interestingly, hsa-miR-1260a expression levels correlated with diseases severity, as well as olfactory function, in the PD and iRBD cohorts. Mechanistically, hsa-miR-1260a segregated to Golgi-associated cellular processes with a potential role in mucosal plasma cells. Predicted hsa-miR-1260a target gene expression was reduced in iRBD and PD groups.

**Conclusions:** Our work demonstrates oral and nasal swabs as a valuable biomarker pool in PD and associated neurodegenerative conditions. © 2023 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

**Key Words:** Parkinson's disease; Lewy body disease; oral and nasal swab; biomarker; microRNA

## Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder characterized by the

progressive development of bradykinesia, muscle rigidity, resting tremor, and postural instability. In addition to these motor signs, several prodromal symptoms have been defined, including isolated rapid eye movement

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(REM) sleep behavior disorder (iRBD) or hyposmia.<sup>1-3</sup> These signs were collectively termed prodromal PD (pPD)<sup>2,4</sup> and often herald the appearance of typical motor signs by 10 to 20 years.<sup>5,6</sup> These prodromal symptoms have a high predictive value, with iRBD exhibiting a >80% risk of conversion to clinical PD (cPD), multiple system atrophy, or dementia with Lewy bodies (DLB) within 15 years.<sup>7,8</sup>

Neuropathological studies suggest that by the time a patient is diagnosed with cPD, a significant proportion of dopaminergic (DA) neurons is already lost<sup>9</sup> and within 4 years of diagnosis, DA terminals in the dorsal putamen have almost disappeared.<sup>10</sup> Therefore, estimates propose that DA cell loss commences 5 to 10 years before the clinical diagnosis.<sup>11,12</sup> Predicting the conversion of pPD to cPD is therefore highly relevant in the context of developing disease-modifying therapies that may no longer be effective at the time of a clinical diagnosis. Therefore, in addition to phenotyping pPD, there is a high need for diagnostic biomarkers that may help to predict disease progression in PD.

Several strategies have been developed to assess pathological changes in iRBD, such as the Real-Time Quaking-Induced Conversion assays (RT-QuIC).<sup>13-15</sup> RT-QuIC detected misfolded  $\alpha$ -synuclein ( $\alpha$ -Syn) in the cerebrospinal fluid (CSF) in iRBD with a high sensitivity and specificity, and  $\alpha$ -Syn positivity was associated with an increased risk for subsequent conversion to cPD or DLB.<sup>7,16,17</sup> Another report examined  $\alpha$ -Syn aggregates in the olfactory mucosa of a large cohort of subjects with iRBD by RT-QuIC<sup>16</sup> and found the olfactory mucosa to be  $\alpha$ -Syn positive in 44.4% of iRBD cases, 46.3% of cPD cases, but in only 10.2% of the control subjects. In addition to these assays, previous work investigated the significance of microRNAs (miRNAs) as potential markers of disease progression. For instance, a recent study found sustained deregulation of 12 miRNAs across the RBD continuum.<sup>18</sup> We ourselves investigated the expression of miRNAs in routine colonic biopsies from PD cases by miRNA sequencing (miRNA-seq) and found several differentially expressed submucosa-enriched miRNAs, with hsa-miRNA-486-5p having the highest effect size for PD.<sup>19</sup> Because colonic biopsies are less well suited for routine diagnostics, we here tested the diagnostic value of the eight most promising candidate miRNAs from our previous report in buccal (oral) and nasal swabs in PD and iRBD cases and in healthy controls. Different from colonic biopsies, we found a significantly increased expression of the miRNA hsa-miR-1260a in cases who had PD. Notably, the abundance of hsa-miR-1260a in oral swabs correlated with disease severity, as well as olfactory function, across the PD and iRBD cohorts. In summary, our results thus identified hsa-miR-1260a as a novel PD-specific miRNA in oral

and nasal swabs that correlates with disease progression.

## Subjects and Methods

### Study Subjects

Cases from the PD cohort were recruited through the Outpatient Clinic for Movement Disorders; iRBD cases were recruited through the Sleep Outpatient Clinic at the Ludwig Maximilian University Hospital. Subjects with PD were eligible if they met the Movement Disorder Society Clinical Diagnostic Criteria for PD and had no primary gastrointestinal disease. All subjects with iRBD met the International Classification of Sleep Disorders criteria for iRBD, as confirmed by polysomnography. Within a singular study visit, all PD and iRBD cases were subjected to the Movement Disorder Society Unified Parkinson's Disease Rating Scale (UPDRS), the 12-item Sniffin' Sticks assessment,<sup>20</sup> the Hoehn & Yahr Scale, the Montreal Cognitive Assessment Scale, the constipation module of ROME-III Questionnaire,<sup>21</sup> and the REM-Sleep Behavior Disorder Questionnaire (RBDSQ).<sup>22</sup> Healthy control subjects were subjected to a similar assessment, but not to the UPDRS. All participants provided written informed consent, and all procedures were conducted in accordance with a protocol that received prior ethical approval through the ethics boards at Ludwig Maximilian University (Munich, Germany).

### Nasal and Oral Swab

Swabs were performed using the OmniSwab (Qiagen), a sterile swab with a 2.2-cm-long tip. To collect samples from the oral mucosa, the swab was repeatedly moved horizontally for 10 seconds with moderate pressure on the inside of the cheek of either side. Nasal mucosa was collected from the lateral nasal wall. The tip of the swab was inserted into the nasal cavity until resistance was felt, the nasal wing was pulled laterally, and the swab pulled out once with sustained pressure. This was repeated with the same swab on the opposite side. The tip of the swab was immediately transferred into a tissue preservative (DNA/RNA Shield; Zymo Research), frozen, and stored at  $-80^{\circ}\text{C}$  until use.

### RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction and Digital Droplet Polymerase Chain Reaction

For RNA isolation, 200  $\mu\text{l}$  of the tissue preservation solution in which the tip of the swab was stored in was used as starting material. Total RNA isolation, including small RNAs, was performed using the miRNeasy Serum/Plasma Advanced Kit according to the manufacturer's instructions (Qiagen). Reverse transcription of

the isolated miRNA was performed using the miRCURY LNA RT Kit (Qiagen). The quantitative polymerase chain reaction (PCR) was performed using the miRCURY LNA SYBR Green PCR Kit and the miRCURY LNA miRNA PCR assays (both Qiagen). The Applied Biosystems ViiA 7 system from Thermo Fisher Scientific was used as a real-time cycler. hsa-miR-103a and hsa-miR-191-5p were quantified as endogenous controls for normalization. For the expression analysis of miR-1260a target genes, 25 ng RNA was depleted of genomic DNA and reverse transcribed to cDNA with the QuantiTect RT kit (Qiagen) following the manufacturer's instructions. For digital droplet PCR (ddPCR), 1  $\mu$ l corresponding to 1.25 ng of cDNA was mixed with 10  $\mu$ l of EvaGreen Supermix (Bio-Rad), 0.5  $\mu$ l of each forward and reverse primer (concentrated at 10  $\mu$ M), and 8  $\mu$ l H<sub>2</sub>O. After droplet generation on the QX200 droplet generator (Bio-Rad), endpoint PCR was carried out for 40 cycles with an annealing temperature of 60°C. Droplets were read on a QX200 droplet reader (Bio-Rad), and absolute quantification was carried out using QuantaSoft software (Bio-Rad) by manually setting a common threshold based on clear separation of positive and negative droplets. Primer sequences were retrieved from PrimerBank<sup>23</sup> and are given in Supporting Information Table S1. Nontemplate controls using water instead of sample were run for all primer pairs and verified to yield no amplifiable signal.

### Bioinformatics Analysis

Delta-Cq values were calculated by subtracting the average Cq value of the target miRNA from the Cq value of the endogenous control miRNA. For determining the log<sub>2</sub> fold-change per group and miRNA over the endogenous controls, we used the  $2^{-\Delta\Delta CQ}$  method.<sup>24</sup> Both endogenous control miRNAs showed a high intercorrelation (Pearson's  $r = 0.90$ ). We chose to continue the analysis using the control miRNA with a slightly higher expression, which was hsa-miR-103a. A Kolmogorov-Smirnov test showed a nonnormal distribution of the delta-Cq values ( $D = 0.51$ ,  $P < 2.2 \times 10^{-16}$ ); therefore, the nonparametric Wilcoxon test was used on delta-Cq values to test for group differences. Raw  $P$  values were adjusted for multiple comparisons with the Holm-Bonferroni method, and results were considered significant if adjusted  $P$  value was  $<0.05$ . To determine the miRNA expression-phenotype relationship, we based the test statistic on Pearson's product moment correlation coefficients between the delta-Cq values and the phenotypic response variable. Sex and age were included as additive factors in this linear model. Target genes for hsa-miR-1260a were identified by querying the miRDB database<sup>25</sup> and filtered for a target prediction score of  $>70$ , yielding 227 unique genes. Target

genes were functionally annotated with their Gene Ontology terms using the enrichR R package.<sup>26</sup> For the identification of putative target gene-expressing cells, we downloaded single-cell RNA sequencing data taken from the oral mucosa<sup>27</sup> as an *anndata* object, which was converted to a *Seurat* object for downstream analysis with the *Seurat* package.<sup>28</sup> Cluster assignments for cells were directly taken from the *anndata* object (identical to the publication), and for each cluster, an aggregate target gene expression score was computed by averaging the expression levels of the putative 227 hsa-miR-1260a target genes. All analysis steps were carried out using the R programming environment (R version 4.2.1).

## Results

### Patient Demographics and Clinical Scores

A total of  $n = 65$  patients completed the study. Of these,  $n = 29$  subjects were in the PD group and  $n = 8$  subjects were in the iRBD group;  $n = 28$  subjects were in the control group. The average age in the PD group was 63 years and in the iRBD group was 70 years; in the control group, the average age was 62 years ( $P = 0.093$ ). We found no significant group-specific differences in the Montreal Cognitive Assessment Scale score (two-way ANOVA,  $P = 0.6556$ ) or the ROME-III questionnaire (two-way ANOVA,  $P = 0.4488$ ) to assess PD-associated gastrointestinal symptoms. As expected, PD and iRBD cases showed a lower Sniffin' Sticks score compared with healthy control cases (two-way ANOVA,  $P = 0.002$ ), indicating hyposmia in these groups. In accord with a more severe disease phenotype, the PD group had a higher UPDRS sum score ( $t$  test,  $P = 0.0028$ ), as well as higher UPDRS Part II ( $t$  test,  $P = 0.0057$ ) and Part III ( $t$  test,  $P = 0.0009$ ) scores compared with the iRBD group (Table 1, Supplementary Fig. S1).

### Differential Expression of miRNAs in Nasal and Oral Swabs

We previously examined the diagnostic validity of miRNAs in routine colonic biopsies from PD and healthy control cases by using miRNA-seq.<sup>19</sup> Based on this previous expression profiling, we identified a panel of miRNAs shown to be differentially expressed in colonic biopsies from patients with PD (adjusted  $P < 0.05$ ) with strong effect sizes (absolute fold-changes  $>1.6$ ). We termed this panel miRNA<sub>ENTERIC</sub> (Table 2). When we quantified the expression of our miRNA<sub>ENTERIC</sub> panel in oral and nasal swabs by quantitative real-time PCR, we found a significant increase of hsa-miR-1260a in both oral and nasal swabs from PD cases as compared with control subjects (Fig. 1A). Compared with the iRBD group, we found no significant differences in the

expression of hsa-miR-1260a (Fig. 1A). Taken together, these results demonstrate specific differences in the expression of hsa-miR-1260a in oral and nasal swabs from PD.

In addition to quantifying the expression of the miRNA<sub>ENTERIC</sub> panel, we correlated the expression of hsa-miR-1260a in the oral swabs with specific clinical parameters, including the total UPDRS, the UPDRS Part III, and the Sniffin' Sticks score (Fig. 1B). Oral swabs were chosen over nasal ones because of the higher power level (Power > 0.975) regarding the differential expression of miR-1260a. When we pooled miRNA expression data, we found that the expression of hsa-miR-1260a correlated with all these clinical parameters. Specifically, we observed that higher levels of miR-1260a expression (reflected by a more negative Cq value) were associated with higher UPDRS scores and lower Sniffin' Sticks scores. Although the correlation was comparably low, it reached statistical significance. Conversely, we found no significant correlation with the levodopa (L-dopa) equivalent daily dose. These results suggest hsa-miR-1260a to predict disease severity across the iRBD and PD continuum independent from DA therapy.

### hsa-miR-1260a Expression Segregates to Golgi-Associated Structures

To delineate the cellular and molecular significance of hsa-miR-1260a, we next determined potential target genes of hsa-miR-1260a by searching the miRDB database. This led to the identification of 227 unique target genes that interact with hsa-miR-1260a with a high confidence (Supporting Information Table S1). A Gene Ontology analysis based on these target genes revealed their functional association with "supercoiled DNA binding" (Fig. 2A). From a spatial perspective, hsa-miR-1260a segregated to the "Golgi *cis* cisterna" and, to a lesser degree, to "Golgi cisterna membrane," the "Golgi cisterna," the "*cis*-Golgi network," and the "Golgi membrane," thus demonstrating a tight association of hsa-miR-1260a with the Golgi apparatus (Fig. 2B). The strongest association with a biological process was found with "spindle assembly" and "Golgi organization" (Fig. 2C). Taken together, these results suggest significant molecular relationships of hsa-miR-1260a-regulated genes to Golgi function. Next, we adopted a recently published single-cell transcriptome

**TABLE 1** Summary of the demographic and clinical characteristics of the participating study subjects

Characteristics	Total (N = 65)	PD (n = 29)	iRBD (n = 8)	Control (n = 28)	P Value
Demographic					0.093
Age (y)	63.1 ± 10.9	62.8 ± 10.3	69.8 ± 12.8	61.6 ± 10.8	
Female sex, n (%)	28 (41.8)	12 (38.7)	1 (12.5)	15 (53.6)	
Clinical scores					
UPDRS Part I		9.1 ± 7.2	4.8 ± 3.6		0.1081
UPDRS Part II		8.6 ± 7.0	1.3 ± 1.8		0.0057
UPDRS Part III		24.0 ± 12.6	7.6 ± 3.9		0.0009
UPDRS Part IV		2.5 ± 4.4			0.1162
UPDRS total		43.6 ± 26.1	13.6 ± 6.1		0.0028
ROME-III score	8.8 ± 8.1	9.9 ± 9.6	8.3 ± 7.9	7.8 ± 6.2	0.4488
RBDSQ score	4.2 ± 3.2	5.1 ± 3.4	7.6 ± 2.3	2.4 ± 1.6	
RBDSQ score ≥ 5, n (%)		14 (45.2)	7 (87.5)	3 (10.7)	
Hoehn & Yahr score		1.5 ± 0.6			
MOCA score	27.0 ± 2.5	27.4 ± 2.9	26.5 ± 2.9	26.8 ± 1.9	0.6556
MOCA score ≤ 25, n (%)		5 (16.1)	3 (37.5)	9 (32.1)	
Sniffin' Sticks score	6.9 ± 2.9	5.5 ± 2.7	6.6 ± 3.2	8.5 ± 2.1	0.002
Sniffin' Sticks score ≤ 6, n (%)		20 (64.5)	4 (50)	3 (10.7)	
Sniffin' Sticks score 7–10, n (%)		9 (29.0)	3 (37.5)	21 (75)	

Note: The clinical scores are presented as scores on their respective scales. In addition, the table provides information on the percentage and number of subjects who scored below the specific threshold for REM-Sleep Behavior Disorder Questionnaire (RBDSQ), Montreal Cognitive Assessment Scale (MOCA), and Sniffin' Stick score and the respective P values.

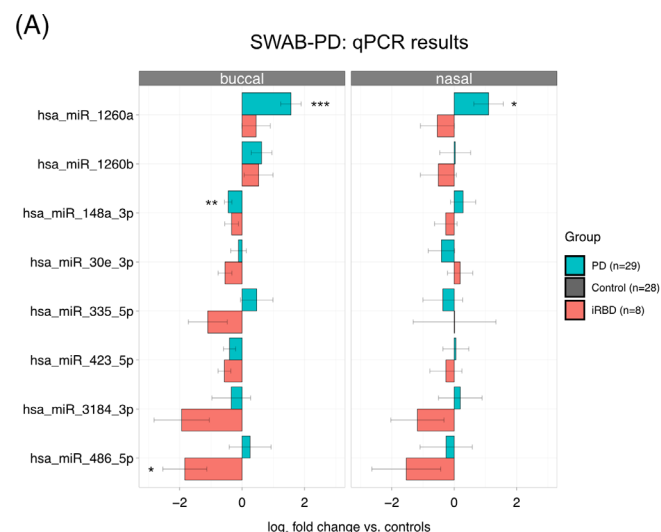
Abbreviations: PD, Parkinson's disease; iRBD, isolated rapid eye movement sleep behavior disorder; UPDRS, Unified Parkinson's Disease Rating Scale.



database that investigated gene expression in oral swabs to identify potential cell types expressing hsa-miR-1260a.<sup>27</sup> Although miRNA levels cannot be directly measured in single-cell assays, we reasoned that a cell type with decreased expression of target genes could indicate higher miRNA expression indirectly. When we tested the expression levels of hsa-miR-1260a target genes for their cluster-wise expression on this

**TABLE 2** Table depicting the specific microRNAs that were previously shown<sup>13</sup> to be differentially expressed with a high effect size in colonic biopsies from patients with Parkinson's disease and therefore termed *microRNA<sub>ENTERIC</sub>*

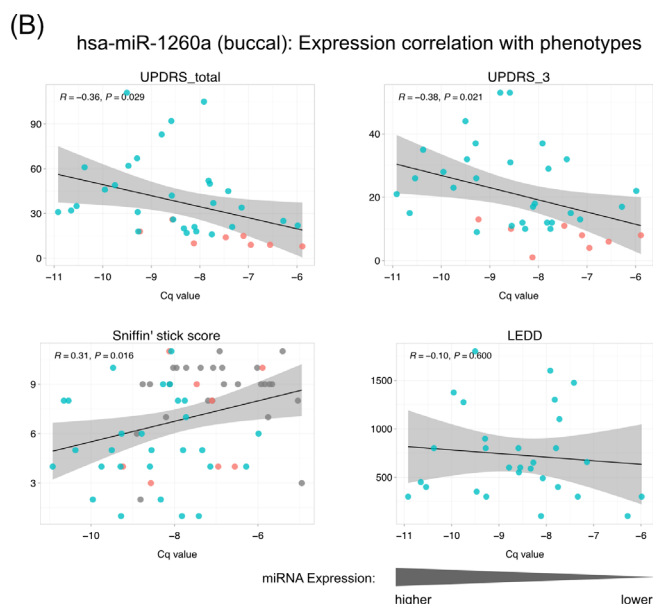
miRNA	Log2 fold-change	P Value	Adjusted P Value
hsa-miR-486-5p	1.4	5.59e-03	4.96e-02
hsa-miR-1260a	0.93	2.24e-05	7.46e-04
hsa-miR-1260b	0.88	1.82e-05	7.46e-04
hsa-miR-30e-3p	-0.88	2.41e-03	2.95e-02
hsa-miR-3184-3p	0.79	1.09e-04	2.89e-03
hsa-miR-148a-3p	-0.77	3.65e-06	2.42e-04
hsa-miR-335-5p	-0.77	2.44e-03	2.95e-02
hsa-miR-423-5p	0.77	1.85e-04	4.10e-03



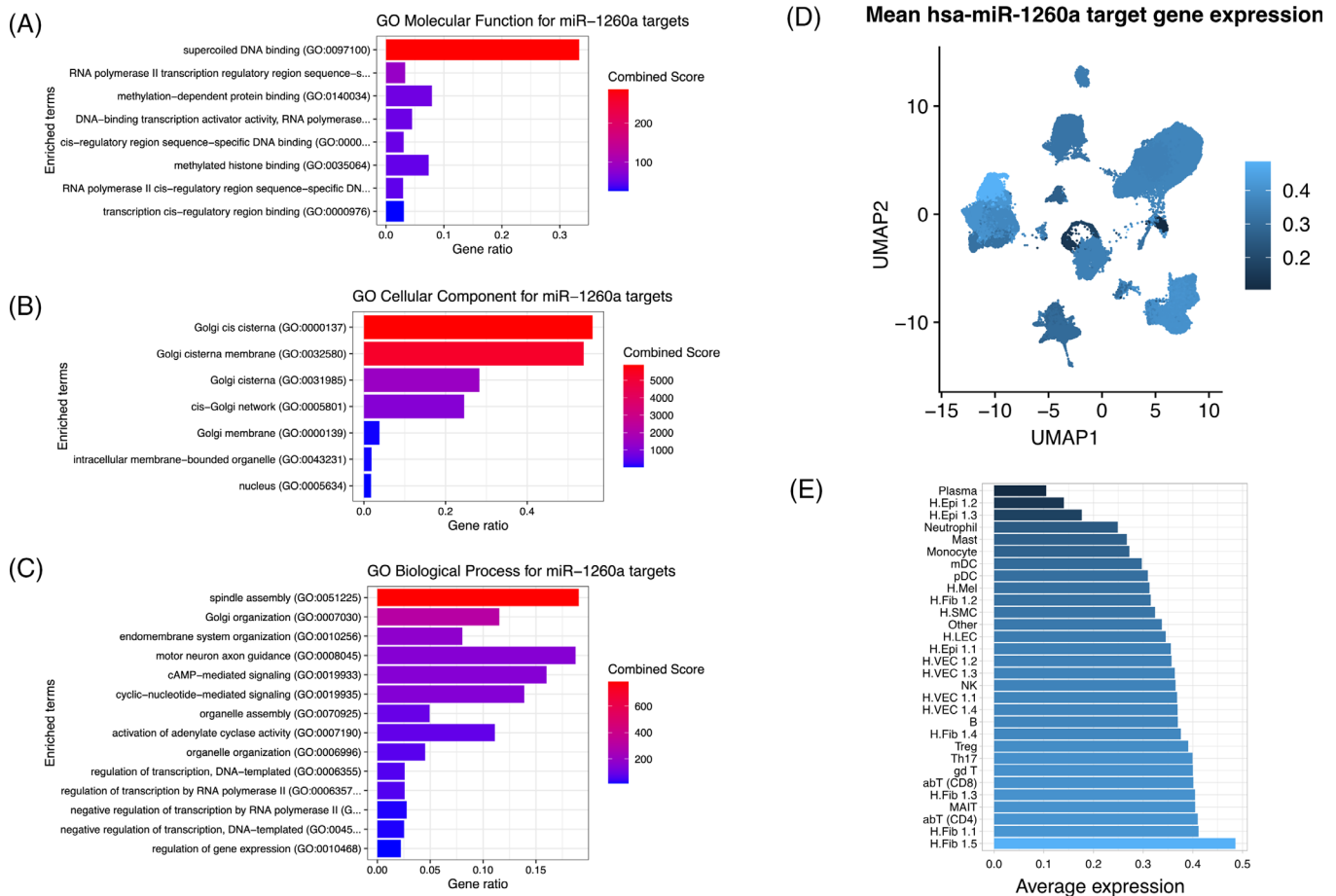
dataset, we found that plasma cells showed the lowest expression levels of the hsa-miR-1260a targets. Under the common assumption that miRNA expression levels correlate inversely with their target gene levels, this could suggest high hsa-miR-1260a expression in plasma cells (Fig. 2D,E).

### hsa-miR-1260a Target Genes Are Downregulated in Oral Mucosa of Patients with iRBD and PD

It is commonly accepted that miRNAs fine-tune gene expression by binding to the 3'-untranslated region of a transcript, leading to downregulation of the corresponding target gene. To investigate the functional relevance of an increased hsa-miR1260a expression, we performed ddPCR gene expression assays for select miRNA targets in the same oral swabs from control, iRBD, and PD cases with the highest miR-1260a abundance ( $n = 3/\text{group}$ ). Based on the single-cell gene expression data (Fig. 3), we selected four genes that exhibited high expression levels in the oral mucosa and had a high prediction score in the miRDB database (Table S2): (1) *ATF6*, a gene associated with the unfolded protein response (UPR) with reported roles in PD<sup>29</sup> and, additionally, a reported genome-wide association study locus<sup>30</sup>; (2) *CTAGE1*, a gene predicted to be involved in Golgi vesicle-mediated transport;



**FIG. 1.** hsa-miR-1260a correlates with Parkinson's disease (PD). **(A)** Bar graph illustrating the differential expression of the *miRNA<sub>ENTERIC</sub>* panel members in oral (buccal) and nasal swabs. As compared with healthy control subjects, cases who had a diagnosis of PD exhibited a significantly increased expression of hsa-miR-1260a in swabs obtained from either region (PD:  $n = 29$ ; isolated rapid eye movement sleep behavior disorder [iRBD]:  $n = 8$ ; control:  $n = 28$ ). **(B)** Graphs illustrating the results of regression analyses correlating the expression of hsa-miR-1260a with distinct clinical parameters, including the Unified Parkinson's Disease Rating Scale (UPDRS) total score, the UPDRS Part III (motor) score, the Sniffin' Sticks score, and the REM-Sleep Behavior Disorder Questionnaire (RBDSQ) score. For comparison of the means, Wilcoxon's rank sum test with Holm-Bonferroni correction was used in **(A)**. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ . Data are shown as means  $\pm$  SEM. LEDD, levodopa equivalent daily dose; qPCR, quantitative polymerase chain reaction. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



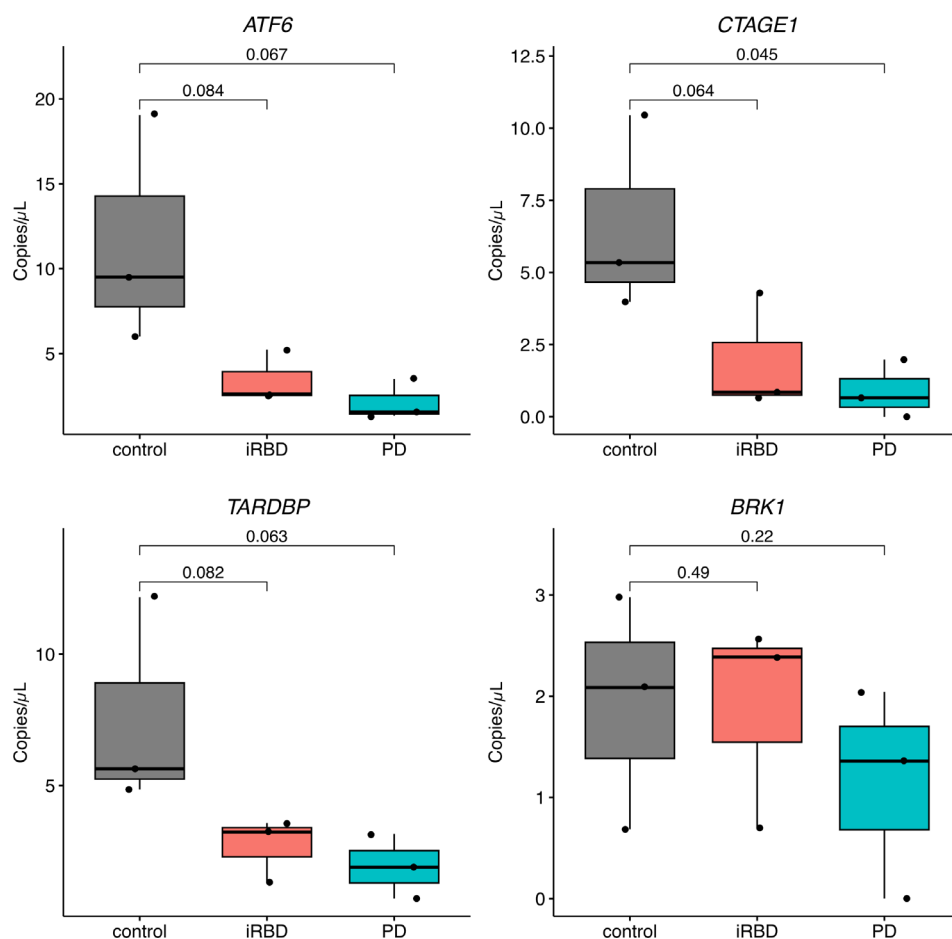
**FIG. 2.** hsa-miR-1260a target genes segregate to Golgi-associated cellular processes. **(A–C)** Bar graphs illustrating the results of a Gene Ontology (GO) analysis showing the relative enrichment over background genes of hsa-miR-1260a target genes with molecular functions **(A)**, cellular compartments **(B)**, and biological processes **(C)**. The combined score multiplies the log of the *P* value computed with Fisher's exact test by the *z* score computed by assessing the deviation from the expected rank. GO numbers correspond to GO IDs. **(D)** Uniform Manifold Approximation and Projection (UMAP) plot from a single-cell RNA sequencing experiment of the oral mucosa, where clusters are colored by average expression values of the 227 putative hsa-miR-1260a target genes. **(E)** Bar graph illustrating the relative abundance of hsa-miR-1260a target genes in distinct cell types, based on the single-cell expression data from Williams et al.<sup>27</sup> Color mapping equivalent to **(D)**. Note the lowest abundance of hsa-miR-1260a target genes in plasma cells. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

(3) *TARDBP*, also known as TDP-43, a prominent protein whose mutations cause frontotemporal dementia, amyotrophic lateral sclerosis, and, rarely, PD<sup>31</sup>; and (4) *BRK1*, a component of the SCAR/WAVE complex known to be involved in actin dynamics.<sup>32</sup> *BRK1* was expressed only at low levels and did not show group differences. However, there was a clear trend in RNA levels that aligned with the expected pattern of patients showing a reduced expression of *ATF6*, *CTAGE1*, and *TARDBP* in buccal swabs. Therefore, these findings support the hypothesis that elevated expression of miR1260a leads to the suppression of target genes in the oral mucosa.

## Discussion

An ideal biomarker should be valid and reliable, have a high discriminative and predictive value, and should

be easily obtained during routine clinical practice. Previous work, for instance, examined  $\alpha$ Syn by RT-QuIC to predict the conversion rate in RBD cases.<sup>7,16,33</sup> Our own previous work examined gut biopsies as a potential source for miRNA biomarkers.<sup>34</sup> In this study, we investigated a predefined panel of miRNAs in buccal (oral) and nasal swabs from PD and iRBD cases. We found hsa-miR-1260a to be significantly upregulated in oral and nasal swabs from PD compared with control cases (Fig. 1A). Although we found an overall correlation of hsa-miR-1260a with disease severity (Fig. 1B), this miR was not significantly changed in the iRBD group. The distinctive miR-1260a pattern observed in the oral and nasal mucosa during disease progression in certain groups could have been caused by complex, stage-specific changes in the molecular and cellular environment, including variations in miRNA expression. In addition, our iRBD cohort was considerably smaller than the PD cohort. We observed a noteworthy



**FIG. 3.** hsa-miR-1260a target genes are downregulated in the oral mucosa of isolated rapid eye movement sleep behavior disorder (iRBD) and Parkinson's disease (PD) cases. Boxplots showing the results of four digital droplet polymerase chain reaction (ddPCR) absolute quantification assays performed in control, iRBD, and PD groups ( $n = 3/\text{group}$ ). RNA was reverse transcribed from buccal samples.  $P$  values were derived using one-sided  $t$  tests. *BRK1* showed no differences in expression. There was a clear trend for reduced expression in iRBD and PD groups for *ATF6*, *CTAGE1*, and *TARDBP*, with the comparison between control and PD groups reaching significance for *CTAGE1* ( $P = 0.045$ ). [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

inverse correlation of hsa-miR-486p in the iRBD group's oral swabs, although with a lower statistical power ( $<0.8$ ) (Fig. 1A). This result differs from previous findings indicating that miR-486p is upregulated in gut biopsies of PD cases.<sup>19</sup> These seemingly conflicting results could possibly be attributed to differences in miR expression that are specific to disease stages and tissue types, as has been documented in neurodegenerative disorders.<sup>35</sup> Our work thus reinforces the usefulness of certain miRNAs from our previous miRNA-seq experiments and illustrates their applicability in a more easily accessible specimen, available from routine clinical diagnostics. In accord with miRNAs being suitable LBD biomarkers, a recent study reported a consistent deregulation of 12 miRNAs across the RBD continuum, including DaT-negative iRBD, DaT-positive iRBD, and LBD phenoconverted iRBD, both in cross-sectional and longitudinal analyses.<sup>18</sup> Using age and sex adjustment, the authors developed a predictive model based on the differential expression of these 12 miRNAs that

accurately discriminated between iRBD and PD or DLB cases and controls, with an area under the curve of 98%. These results and our own data support the investigation of miRNAs as potential disease biomarkers that allow monitoring disease progression in  $\alpha$ Syn-associated conditions.

Previous work demonstrated that more than 90% of iRBD cases develop  $\alpha$ -synucleinopathies within 15 years, including PD, multiple system atrophy, or DLB.<sup>8,36</sup> Because iRBD and PD can be regarded as a clinical and, possibly, pathological continuum, and because iRBD may exhibit olfactory dysfunction and mild motor symptoms,<sup>37-39</sup> we pooled data from PD and iRBD cases to better represent clinical progress (Fig. 1A). Because the expression of hsa-miR-1260a correlated significantly with disease severity and hyposmia, our data suggest hsa-miR-1260a as a possible additional biomarker reflecting disease progression in iRBD and PD.

Using nasal swabs and measuring (mi)RNA quantity practically mirrors a typical SARS-CoV-2 test setting.

As of December 2022, more than 1.1 billion SARS-CoV-2 PCR tests had been performed in the United States alone. Because we have demonstrated specific differences between PD and healthy control subjects, our study reveals the principal applicability of using oral and nasal swabs for biomarker testing in PD. In line, recent reports showed successful miRNA-seq from such swabs.<sup>40,41</sup> Future studies using such material may support the development of useful biomarkers for neurodegenerative diseases on a larger scale.

Among the 227 target genes of hsa-miR-1260a that we have identified, some have already been associated with PD. For example, the expression of *SPEN* is upregulated in astrocytes and neurons in PD. Silencing of *SPEN* resulted in upregulated lipid metabolism in *Drosophila* glial cells.<sup>42</sup> Likewise, *GLP1R* is more strongly expressed in microglia and astrocytes in PD. In addition, *GLP1R* was shown to play an important role in immune-mediated neurodegeneration. This was demonstrated by the *GLP1R* agonist-mediated conversion of astrocytes into proinflammatory phenotypes in PD.<sup>43</sup> Interestingly, *GLP1R* mRNA expression is significantly increased in the substantia nigra of PD cases,<sup>43</sup> and *GLP1R* is also highly expressed in the myenteric plexus throughout the gut.<sup>44</sup> *NCAPG2*, which plays a role in chromosome assembly and segregation in mitosis, was found to be differentially expressed in a transcriptome analysis of blood in *LRRK2*-associated and idiopathic PD.<sup>45</sup> Another interesting hit is the gene *ATF6*, which governs the UPR in the endoplasmic reticulum and which was found to be expressed at lower levels in buccal RNA of patients with PD and iRBD. A genome-wide association study identified a common single-nucleotide polymorphism (rs10918270) in the *ATF6* gene associated with PD.<sup>30</sup> Other studies showed that the presence of  $\alpha$ -Syn inhibits the *ATF6* signaling pathway in PD. This attenuated the UPR and disrupted the endoplasmic reticulum-Golgi transport by COPII vesicles.<sup>29</sup>

Numerous previous studies have described a dysregulation of miRNAs in plasma, CSF, and the brain of patients with PD.<sup>46,47</sup> miRNAs bind to the 3' untranslated region of target gene mRNA to mediate the degradation or translation inhibition of mRNA, usually leading to lower levels of specific target proteins.<sup>48</sup> Because we found the lowest expression levels of hsa-miR-1260a target genes in plasma cells (Fig. 2D,E) and because miRNAs are thought to decrease the abundance of their cognate target mRNAs, our bioinformatics analyses appear to suggest a high abundance and, possibly, a significant role for hsa-miR-1260a in regulating plasma cell function. In line with exerting a negative regulatory influence, we observed an inclination toward reduced mRNA levels for multiple target genes of miRNA-1260a (Fig. 3). Nevertheless, apart from this influence mediated by miRNA, it is

plausible that alternative molecular mechanisms could contribute to the decline in these targets. Consequently, our findings obtained through ddPCR emphasize the need for additional investigations in future experiments. Immune mechanisms are likely to contribute to PD onset and progression.<sup>49</sup> In accord with a role for miRNAs in immune cells in PD, a recent report found a differential expression of miRNAs in white blood cells in PD with an association to L-dopa treatment.<sup>50</sup> Furthermore, B lymphocytes have been specifically implicated in PD pathology (reviewed by Scott<sup>51</sup>): For instance, bulk IgG obtained from PD cases resulted in selective DA neuron loss in passive transfer experiments compared with IgG from control individuals.<sup>52</sup> B cell levels might be reduced in patients with PD,<sup>53,54</sup> which could be linked to alterations in the expression of B cell-related genes.<sup>55,56</sup> Moreover, the expression of *LRRK2* (encoding dardarin, also known as leucine-rich repeat serine/threonine-protein kinase 2), a gene linked to familial PD, is increased in B cells in patients with PD<sup>57</sup> and is associated with altered B cell function in mice.<sup>58</sup> Together with additional evidence, linking antibody-mediated mechanisms to PD pathology<sup>59-64</sup> and therapy,<sup>65</sup> these results suggest a role for B cells in PD. Based on the results and our own work, we believe that investigating B cells and associated antibody-mediated immunity warrants further studies of mucosal immunity in PD.

The nasal and oral mucosa are sites of first encounters with pathogens. Life-sustaining substances such as food, water, and air pass through the oral cavity, carrying along allergens, microbes, and sometimes toxins as they enter the lower gastrointestinal and respiratory tract. These areas are thought to have a particular role for initiating PD-associated pathology,<sup>4,66</sup> where a presumed environmental pathogen would initiate a pathological process that would in turn progress toward the central nervous system via autonomic nerves. The oral and nasal mucosa consist of a multilayer squamous cell epithelium with unique anatomical and functional characteristics and comprising epithelial, endothelial, fibroblast, and immune cells.<sup>67</sup> As such, mucosal immunity has been demonstrated to significantly contribute to regulating immune responses in the entire organism in various model systems, a mechanism better known as oral tolerance.<sup>68</sup> Based on our results, we believe that further investigating the specific molecular and cellular function of hsa-miR-1260a in the nasal and oral mucosa might support the understanding of mucosal immunity in PD. ■

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## Data Availability Statement

Data available in article supplementary material.



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## Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.