Cortisol and Alpha-Synuclein Stability in Saliva under Varying Storage and Handling Conditions

Mo Zheng, Sujata Srikanth, Jeremiah Carpenter, Delphine Dean

Clemson University

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by motor impairments and non-motor symptoms, significantly impacting patients' quality of life. Currently, cerebrospinal fluid (CSF) is the primary biofluid used for PD biomarker studies, notably α-synuclein, despite the invasive nature of lumbar puncture procedures. Recent work has shown that some of these PD biomarkers have been measured in saliva. As an alternative to CSF, saliva can be non-invasively self-collected by patients repeatedly over time to monitor biomarker levels. However, the stability of these biomarkers in saliva needs to be evaluated before saliva can be considered for patient self-collection studies. Therefore, the goal of this study was to determine if α-syn and cortisol levels were stable in saliva using different common sample storage conditions. Salivary samples were analyzed using enzyme-linked immunosorbent assay (ELISA) kits to measure α-synuclein and cortisol levels under various storage conditions, simulating real-world scenarios of home collection and transport. Our findings indicate that both biomarkers are quantifiable in saliva with high reliability and stability. The results showed that cortisol and α-synuclein concentrations were well-maintained at -20°C and -80°C storage conditions, with minimal degradation observed under room temperature overnight storage. Moreover, freeze-thaw cycles did not significantly affect the biomarker stability. These findings support the potential of saliva as a feasible and non-invasive biofluid for PD biomarker analysis, facilitating easier and more frequent monitoring of disease progression. Further studies are warranted to validate these preliminary findings and to establish standardized protocols for saliva collection and storage in clinical settings.

Introduction

Parkinson's disease (PD) is a progressive brain disorder caused by degeneration of the nerve cells that control movement. In the United States, PD is the second most common neurodegenerative disease and nearly one million Americans are living with PD, with around 90,000 diagnosed yearly [1]. Because it has vast clinical heterogeneity and complexity, PD is difficult to diagnose due to its subtle onset. Parkinson's disease causes high rates of disability and, currently, while there are treatment options, there is no cure for PD. The shaking, stiffness, decreased coordination and balance caused by PD eventually lead to difficulty in speech and inability to walk [2]. Moreover, the non-motor effects of PD include cognitive impairment, sleep disorders, pain, mental health complications, and sensory disturbances [3]. With such life-altering effects, it is important for early diagnosis to let individuals alter their lifestyles/routines and prepare for life with PD, as well as explore potential treatment options depending on the stage of the disease. For example, physical therapists, speech therapists, social workers, neuropsychologists, and pharmacists may be regularly required to work with patients to address the disease's progressive debilitating symptoms [4]. The most commonly investigated biofluid used in investigating the progression of PD is cerebrospinal fluid (CSF) [5]. CSF is obtained through the lumbar puncture (LP), or spinal tap, where a hollow needle is inserted into the space surrounding the spinal column (subarachnoid space) in the lower back [6]. Unfortunately, especially since PD patients are generally aged sixty or over, such a process of obtaining the CSF biofluid can be painful and may even be detrimental to their health. LP can cause post-lumbar puncture headaches (25% of people who undergo LP reported), back pain, bleeding at the needle site, and brainstem herniation (in rare cases) [7]. The cost of monitoring the progression of PD or potential diagnosis through LP may outweigh the benefits. That said, currently, there are no suitable biochemical markers for diagnosis and progression monitoring [8]. One of the most promising biomarkers for monitoring PD is currently α-synuclein (α-syn)[5], [9], [10]. In a 2023 study, researchers tested the spinal fluid of 1,000 participants, looking for the clumps due to agglomeration of the α-syn protein to determine if the participants had Parkinson's disease. Such a test resulted in an 87.7% accurate identification of participants with Parkinson's disease and those at risk [11]. Brain cells of most people with PD contain Lewy bodies constituted by clumps of α-syn making it a pathological hallmark. As a major component of Lewy bodies, α-syn has been investigated as a potential PD biomarker, since it is present in biofluids like CSF, plasma, and saliva [9], [10], [11], [12], [13]. Studies are underway to understand better the functions of α-synuclein and its relationship to other factors impacting PD progression and prognosis.

Now, due to the invasive nature of LP in obtaining CSF for α-syn, less invasive alternative biofluids are needed for the monitoring of α-syn levels in patients. Urine has been studied as it can contain α-syn, but there is a low prevalence of studies involving urine. Instead, saliva is shown to be a better candidate for study, having similar levels of α-syn compared with CSF, meaning saliva is a potential non-invasive biofluid for monitoring and diagnosing Parkinson's disease [10], [14].

Cortisol, an extensively researched hormone, has the potential to disturb mitochondrial function and elevate oxidative stress and neuroinflammation, which are both acknowledged as pivotal pathological mechanisms in PD [15]. Cortisol levels are also discriminatory in PD diagnosis with higher salivary cortisol levels also seen in PD. Such levels of cortisol are shown to be higher in individuals with depression (a symptom of PD: psychological disorder), sleep disturbances, and neurodegenerative diseases such as PD [15], [16], [17].

Saliva is an attractive biofluid due to its accessibility and homogeneity[18], [19], [20]. Both cortisol and α-syn are prevalent in saliva, and early studies indicate salivary α-synuclein and cortisol could be used together as biomarkers for PD in diagnosing and monitoring disease progression [19], [20], [21], [22], [23]. In addition, saliva is already used as a good sample type for cortisol testing in at-home settings [24], [25], [26]. Therefore, saliva is a promising alternative compared to CSF. Elderly or not, patients with Parkinson's disease will be able to have more frequent disease progression monitoring even in the comfort of their homes by sending saliva samples to nearby pharmacies, and then to saliva testing locations. Such an alternative to CSF reduces the strain on the patient's physical health by limiting intrusive medical procedures and the need to navigate themselves to the testing sites. Careful examination must be done to monitor the possible degradation and changes in concentration of both cortisol and α-syn in saliva to eliminate the possibility of unusable samples by the time the saliva reaches the lab for testing. In addition, storage conditions must be tested to determine the optimal temperatures in which the samples are stable.

In our study, we evaluated the usability of salivary α-synuclein and cortisol ELISA assays for real-world use. We want to determine if these markers are stable enough in saliva with differing standard storage and transport conditions.

Methods

In the ELISA kits for α-syn and cortisol, control samples of saliva containing each biomarker were assayed by ELISA for levels in (ng/ml) of α-synuclein and cortisol. Quantitative analysis of α-syn and cortisol levels in the saliva samples was performed using enzyme-linked immunosorbent assay (ELISA) kits from [Invitrogen™(A-syn) and Crystal Chem Inc (Cortisol)]. One important factor to note is that the Cortisol ELISA kit was a competitive ELISA, meaning the higher the concentration of analyte (cortisol in ng/ml), the lower the absorbance reading (450 nM). Standard protocols provided by the manufacturer were followed for sample preparation, assay procedures, and data analysis. All experiments were repeated in duplicates three times for a total of 6 total replicates. Quality control measures were implemented to ensure the accuracy and precision of the assay results.

Upon confirming the validity of both ELISA tests by analyzing the R^2 values of both (near 1) graphs (Cortisol absorbance and α-syn absorbance), we moved to test the stability of both biomarkers in saliva in different real-world storage conditions.

To assess the stability of samples, we tested pooled saliva (PS, Lee Biosolutions Inc.) spiked with cortisol (Crystal Chem Inc) and α-syn (Invitrogen) under storage conditions simulating situations of home collection, transport, and lab handling conditions. These include fresh saliva, overnight at room temperature (RT), at 4°C, frozen at -20°C with 1 or 2 freeze-thaw cycles, and frozen at -80°C with 1 or 2 freeze-thaw cycles.

This stability testing on both biomarkers was repeated after the first trial to solidify the results, but instead, measuring the percentages present instead of concentration (ng/ml). Ultimately, the effect of storage and transport variables will be evaluated to decide upon the best conditions to be used in real-life situations.

Results

We validated commercially available cortisol (Figure 1) and α-syn (Figure 2) ELISA kits using pooled saliva samples spiked with cortisol and α-syn. The tests showed good linear response with concentration showing that we have been able to measure the levels of the markers in sample saliva correctly.

Our results show that commercial ELISA assays for cortisol and α-synuclein can be used to quantify the markers in saliva. Figure 1 displays the inverse linear relationship (R^2= 0.9964) between the concentration in ng/ml of cortisol and the absorbance reading (450 nM), due to the nature of a competitive ELISA. Figure 2 displays the positive linear relationship between concentration in ng/ml of α-syn and absorbance (450 nM), with an R^2 value of near 1 (0.9977). Overall, both R^2 values indicate a high degree of correlation between the concentrations of these biomarkers (α-syn and cortisol) and the corresponding absorbance readings obtained from the ELISA tests. This strong correlation enhances the reliability and validity of the ELISA results, signifying that the measured absorbance values are closely associated with the actual concentrations of α-synuclein and cortisol in the saliva samples. Furthermore, the salivary matrix did not affect the ELISA results in any of the trials.

To determine the effect of storage and handling conditions on cortisol and α-syn, we ran ELISA tests on pooled saliva samples stored at different conditions (Figure 3). Normal pooled saliva (NPS, Lee Bisolutions Inc) was saliva pooled from 100 healthy patient donors. Fresh condition samples were NPS spiked with cortisol and α-syn. Room temperature (RT) samples were stored at ~23C for 24hrs. Refrigerated samples were stored at 4C for 24hrs. Frozen samples (at -20C or -80C) were stored in the freezer for 24hrs. The samples with 1 freeze-thaw cycle (1F/T) were thawed immediately prior to experimentation at 24hrs. The samples with 2 freeze-thaw cycles (2F/T) were thawed at 12 hrs then refrozen for another 12hrs and then thawed just prior to experimentation.

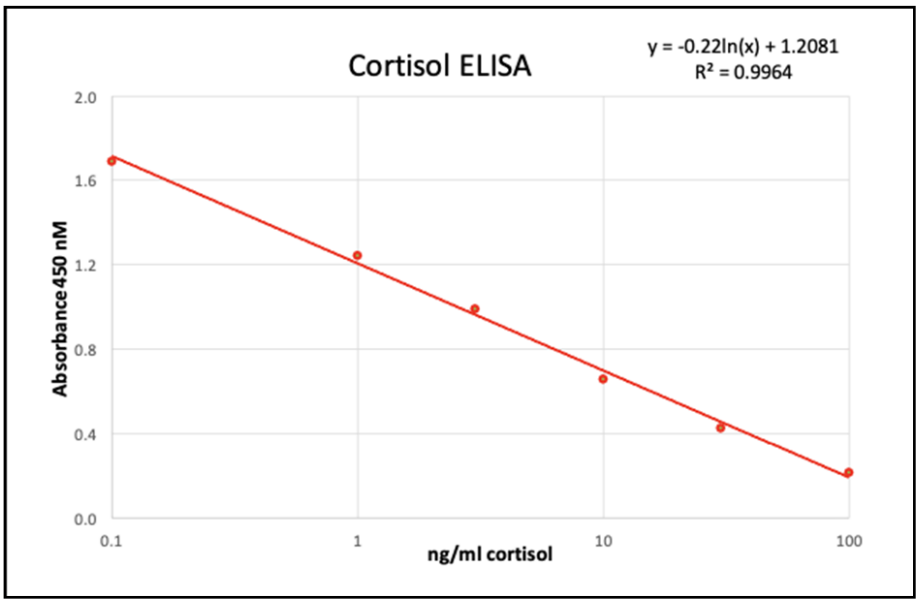
The results show that cortisol levels in saliva are stable after overnight storage at room temperature or when stored in frozen conditions with 1-2 freeze-thaw cycles (Figure 3). This is consistent with prior studies on cortisol testing in saliva. Since α-syn levels can vary by patient, for the α-syn stability testing, we will be doping pooled saliva samples with recombinant α-syn and then running the storage conditions testing.

In Figure 3, the highest concentrations of α-syn were found in the fresh spiked saliva, as expected since it is the control. Then, after comparing overnight at room temperature (RT), at 4°C, frozen at -20°C with 1-2 freeze-thaw cycles, and frozen at -80°C with 1-2 freeze-thaw cycles, saliva at -80°C under 2 free-thaw cycles had near similar concentrations of α-syn in fresh saliva. Saliva at room temperature overnight had the lowest amounts of α-syn (ng/ml) showing slight degradation of the stability of α-syn under such temperature. Working forwards, under 4°C, there was a slight increase in the concentration of α-syn, and a similar phenomenon is observed the lower the temperature the saliva was stored in, i.e. -20°C and -80°C. Furthermore, freeze-thaws appear to not affect the stability of the biomarker, and possibly even increase the stability, as higher concentrations of α-syn were seen with more than 1 freeze-thaw. However, there was less degradation of α-syn concentration percentages in the saliva under storage conditions overnight at room temperature, and the percentages of α-syn were relatively higher as storage temperatures decreased from RT to -80°C (Figure 3).

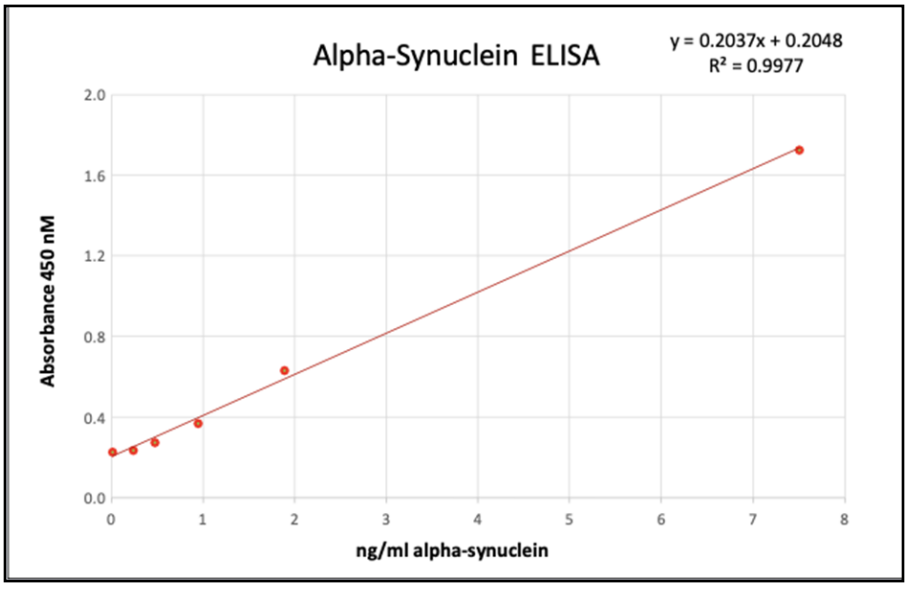
The concentration of cortisol (ng/ml) steadily decreased as storage temperatures increased: at -20°C and 4°C the concentration was similar but comparably lower to the -80°C, and at room temperature, there was a significant decrease in cortisol concentration levels in the saliva sample (Figure 3). Cortisol concentrations displayed similar patterns of higher percentages at temperatures, and freeze-thaws have little to no effect on the stability of cortisol percentages in the saliva sample (Figure 3). The cortisol under RT decreased substantially compared to lower temperatures.

Discussion

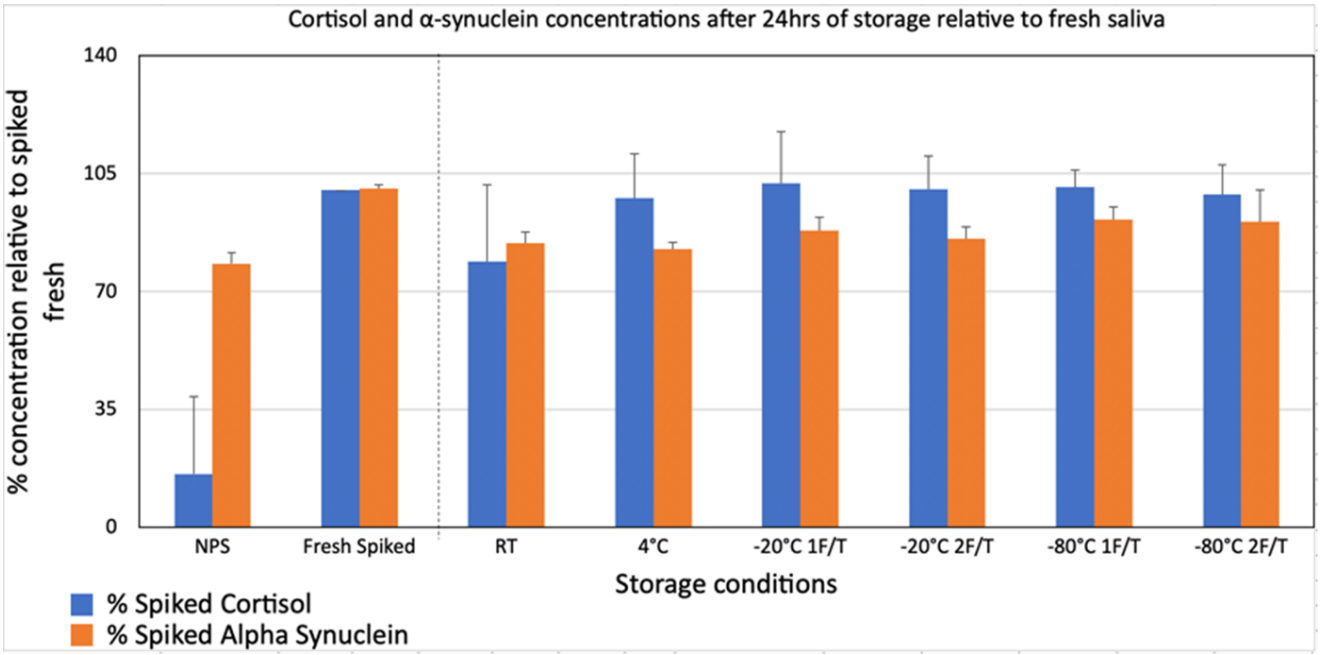
Overall, there were no significant differences (Student's t-test $p>0.01$) in either cortisol or α-syn levels in saliva after 24 hours for any of the storage conditions when compared to the initial fresh spiked concentration. Initially, we hypothesized that room temperatures overnight would cause substantial degradation to the saliva samples' biomarkers (both cortisol and α-syn). While there was some decrease in cortisol and α-syn concentrations/percentages after 24hrs of storage under RT, these differences were not found to be statistically significant (Student's t-test $p = 0.17$ for cortisol and $p= 0.47$ for α-syn between the fresh spiked control saliva and the saliva stored at room temperature for 24hrs). However, it is important to note that compared to cortisol decrease under RT, α-syn decrease may indicate a possible higher tolerance and stability in RT compared to cortisol. Although not experimented with, we hypothesize that the longer stored in RT, the more degradation of stability of both biomarkers in saliva. These results are consistent with the literature on cortisol concentration stability; several prior studies have shown that salivary cortisol levels are relatively stable after up to 72hrs at room temperature[27], [28] but that longer storage at room temperature showed significant decreases in measured cortisol levels [29].



(Figure 1): The concentration of cortisol in the control sample of saliva and its linear negative correlation with its absorbance reading (450 nM).



(Figure 2): The concentration of α-syn in the control saliva sample and its positive linear correlation with its absorbance reading (450 nM).



(Figure 3): Cortisol and α-synuclein in normal pooled saliva samples (N = 6) as a function of storage condition. NPS = Normal pooled (unspiked) saliva, Fresh = spiked pooled saliva, RT = storage at room temperature, 4C = storage in the refrigerator, -20C and -80C = storage in the freezers, F/T indicates the number of freeze-thaw cycles prior to testing. All stored samples were kept for a total of 24 hrs.

The storage condition of -80°C is the "gold-standard" for sample storage[27], [28], [29], [30] and it shows similar levels of concentration/ percentages of both biomarker samples compared to fresh saliva. However, ultra-low temperature storage can be limiting as it is not available to patients and many packaging/transport facilities [30]. Therefore, -20°C is a more attractive option as it is more cost, time, and technology-efficient and it is available in most home kitchen freezers. Additionally, such temperatures are mostly standard protocols for storage conditions in medical transport. The use of regular home freezers is part of the standard at-home collection protocols for commercial cortisol saliva tests. It is encouraging that this seems to also work for α-syn.

Another variable to consider is freeze-thaw cycles that may be incurred during sample handling and processing. If saliva samples need to be stored in a patient's freezer due to accessibility, or frozen and thawed at a packaging site, then need to be re-frozen, the issue of biomarker degradation in stability for both cortisol and α-syn must be addressed. Thus, we tested both biomarkers under 2 freeze-thaws at differing temperatures of -20°C and -80°C and found no significant changes in measured concentration. While some studies had previously shown that repeated freeze-thaw cycles did not affect cortisol levels measured in saliva [29], it is encouraging to see that this resistance to freeze-thaw can hold true for measuring α-syn levels in saliva as α-syn is a more complex structured protein when compared to a simple "small molecule" hormone like cortisol. Based on these results, we can safely determine that few freeze-thaws are not a detrimental variable in the packaging and transport of saliva samples from patients.

Conclusions

Senior citizens and elders in assisted facilities are a vulnerable, high-risk population for Parkinson's disease. This group is also one where self-collection of saliva samples is most appropriate. Our experience with COVID-19 sample self-collection in the assisted-living facility setting has been largely successful [31]. With samples being self-collected, caregivers are involved only in physically taking the samples and transporting them to the testing facility. In this realm, it is essential to ensure that sample quality is retained for analysis and there is no degradation or loss of the biomarkers during storage and transport. Our study indicates that cortisol and α-synuclein are stable under standard storage and handling conditions typically used during at-home sample collection. This is encouraging for the use of saliva as a sample type for biomarker assessment in Parkinson's disease.

Acknowledgements

The authors would like to thank Clemson Creative Inquiry for funding and support.

References

[1] "Parkinson's Foundation Statistics," https://www.parkinson.org/understanding-parkinsons/statistics. Accessed: Jun. 11, 2024. [Online]. Available: https://www.parkinson.org/understanding-parkinsons/statistics
[2] "Parkinson's Disease – Symptoms, Diagnosis and Treatment." Accessed: Jun. 11, 2024. [Online]. Available: https://www.aans.org/en/Patients/Neurosurgical-Conditions-and-Treatments/Parkinsons-Disease
[3] "Parkinson disease." Accessed: Jun. 11, 2024. [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/parkinson-disease
[4] "Living with Parkinson's Disease | American Parkinson Disease Assoc." Accessed: Jun. 11, 2024. [Online]. Available: https://www.apdaparkinson.org/resources-support/living-with-parkinsons-disease/

[5] N. Magdalinou, A. J. Lees, and H. Zetterberg, "Cerebrospinal fluid biomarkers in parkinsonian conditions: an update and future directions," *J Neurol Neurosurg Psychiatry*, vol. 85, no. 10, pp. 1065–1075, Oct. 2014, doi: 10.1136/JNNP-2013-307539.

[6] "Lumbar Puncture | Johns Hopkins Medicine." Accessed: Jun. 11, 2024. [Online]. Available: https://www.hopkinsmedicine.org/health/treatment-tests-and-therapies/lumbar-puncture

[7] A. E. Reis, M. Spano, C. Davis-Hayes, and G. R. Salama, "Lumbar Puncture Complications: A Review of Current Literature," *Curr Pain Headache Rep*, 2024, doi: 10.1007/S11916-024-01262-2.

[8] "Parkinson's Disease: Challenges, Progress, and Promise | National Institute of Neurological Disorders and Stroke." Accessed: Jun. 11, 2024. [Online]. Available: https://www.ninds.nih.gov/current-research/focus-disorders/parkinsons-disease-research/parkinsons-disease-challenges-progress-and-promise

[9] Z. Zhang *et al.*, "The role of peripheral inflammation-related biomarkers in distinguishing Parkinson's disease," *Parkinsonism Relat Disord*, vol. 123, Jun. 2024, doi: 10.1016/J.PARKRELDIS.2024.106102.

[10] M. I. De Bartolo *et al.*, "A systematic review of salivary biomarkers in Parkinson's disease," *Neural Regen Res*, vol. 19, no. 12, pp. 2613–2625, Dec. 2024, doi: 10.4103/NRR.NRR-D-23-01677.

[11] A. Siderowf *et al.*, "Assessment of heterogeneity among participants in the Parkinson's Progression Markers Initiative cohort using α-synuclein seed amplification: a cross-sectional study," *Lancet Neurol*, vol. 22, no. 5, pp. 407–417, May 2023, doi: 10.1016/S1474-4422(23)00109-6.

[12] T. Katayama, J. Sawada, K. Takahashi, and O. Yahara, "Cerebrospinal Fluid Biomarkers in Parkinson's Disease: A Critical Overview of the Literature and Meta -Analyses," *Brain Sci*, vol. 10, no. 7, pp. 1–14, Jul. 2020, doi: 10.3390/BRAINSCI10070466.

[13] S. Negi, N. Khurana, and N. Duggal, "The misfolding mystery: α-synuclein and the pathogenesis of Parkinson's disease," *Neurochem Int*, vol. 177, Jul. 2024, doi: 10.1016/J.NEUINT.2024.105760.

[14] S. Salaramoli, H. R. Joshaghani, and S. I. Hashemy, "Salivary Biomarkers: Noninvasive Ways for Diagnosis of Parkinson's Disease," *Neurol Res Int*, vol. 2023, no. 1, p. 3555418, Jan. 2023, doi: 10.1155/2023/3555418.

[15] N. S. Luthra, A. Clow, and D. M. Corcos, "The Interrelated Multifactorial Actions of Cortisol and Klotho: Potential Implications in the Pathogenesis of Parkinson's Disease," *Brain Sci*, vol. 12, no. 12, Dec. 2022, doi: 10.3390/BRAINSCI12121695.

[16] K. Pisanski and P. Sorokowski, "Human Stress Detection: Cortisol Levels in Stressed Speakers Predict Voice-Based Judgments of Stress.," *Perception*, vol. 50, no. 1, pp. 80–87, Jan. 2021, doi: 10.1177/0301006620978378.

[17] E. Aardal and A. C. Holm, "Cortisol in Saliva — Reference Ranges and Relation to Cortisol in Serum," *Clin Chem Lab Med*, vol. 33, no. 12, pp. 927–932, 1995, doi: 10.1515/CCLM.1995.33.12.927.

[18] M. I. De Bartolo *et al.*, "A systematic review of salivary biomarkers in Parkinson's disease," *Neural Regen Res*, vol. 19, no. 12, pp. 2613–2625, Dec. 2024, doi: 10.4103/NRR.NRR-D-23-01677.

[19] I. Devic *et al.*, "Salivary α-synuclein and DJ-1: potential biomarkers for Parkinson's disease," *Brain.*, vol. 134, no. 7, p. e17 8, Jul. 2011, doi: 10.1093/brain/awr015.

[20] W. Kang *et al.*, "Salivary total α-synuclein, oligomeric α-synuclein and SNCA variants in Parkinson's disease patients," *Scientific Reports 2016 6:1*, vol. 6, no. 1, pp. 1–8, Jun. 2016, doi: 10.1038/srep28143.

[21] G. Vivacqua *et al.*, "Abnormal Salivary Total and Oligomeric Alpha-Synuclein in Parkinson's Disease," *PLoS One*, vol. 11, no. 3, p. e0151156, Mar. 2016, doi: 10.1371/journal.pone.0151156.

[22] A. H. Simonsen *et al.*, "The utility of α-synuclein as biofluid marker in neurodegenerative diseases: a systematic review of the literature," *Biomark Med.*, vol. 10, no. 1, pp. 19–34, Jan. 2016, doi: 10.2217/bmm.14.105.

[23] A. Atik, T. Stewart, and J. Zhang, "Alpha-synuclein as a biomarker for Parkinson's disease," *Brain Pathol.*, vol. 26, no. 3, pp. 410–8, May 2016, doi: 10.1111/bpa.12370.

[24] L. Stendelyte, M. Malinauskas, D. E. Grinkeviciute, and L. Jankauskaite, "Exploring Non-Invasive Salivary Biomarkers for Acute Pain Diagnostics: A Comprehensive Review," *Diagnostics (Basel)*, vol. 13, no. 11, Jun. 2023, doi: 10.3390/DIAGNOSTICS13111929.

[25] S. A. Moyers and M. S. Hagger, "Physical activity and cortisol regulation: A meta-analysis," *Biol Psychol*, vol. 179, Apr. 2023, doi: 10.1016/J.BIOPSYCHO.2023.108548.

[26] C. F. Geißler, M. A. Friehs, C. Frings, and G. Domes, "Time-dependent effects of acute stress on working memory performance: A systematic review and hypothesis," *Psychoneuroendocrinology*, vol. 148, Feb. 2023, doi: 10.1016/J.PSYNEUEN.2022.105998.

[27] A. A. Nalla, G. Thomsen, G. M. Knudsen, and V. G. Frokjaer, "The effect of storage conditions on salivary cortisol concentrations using an enzyme immunoassay," *Scand J Clin Lab Invest*, vol. 75, no. 1, pp. 92–95, Jan. 2015, doi: 10.3109/00365513.2014.985252.

[28] J. J. Tonge, B. G. Keevil, J. N. Craig, M. J. Whitaker, R. J. Ross, and C. J. Elder, "Salivary Steroid Collection in Children Under Conditions Replicating Home Sampling," *J Clin Endocrinol Metab*, vol. 107, no. 11, pp. 3128–3136, Nov. 2022, doi: 10.1210/CLINEM/DGAC419.

[29] A. H. Garde and Å. M. Hansen, "Long-term stability of salivary cortisol," *Scand J Clin Lab Invest*, vol. 65, no. 5, pp. 433–436, 2005, doi: 10.1080/00365510510025773.

[30] J. Chen, X. Liu, Y. Hu, X. Chen, and S. Tan, "Cryopreservation of tissues and organs: present, bottlenecks, and future," *Front Vet Sci*, vol. 10, p. 1201794, May 2023, doi: 10.3389/FVETS.2023.1201794/BIBTEX.

[31] E. V Plumb *et al.*, "Implementation of a Rural Community Diagnostic Testing Strategy for SARS-CoV-2 in Upstate South Carolina," *Front Public Health*, vol. 10, p. 858421, 2022, doi: 10.3389/FPUBH.2022.858421.