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Bruno Hammerschlag *April 1, 2023*

Salivary Lactoferrin as a Robust Biomarker of Alzheimer's Disease

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

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Abstract

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Background: Pathological changes linked to Alzheimer's Disease (AD) emerge years before the behavioral symptoms do. Thus, research has focused on developing accessible biomarkers that can accurately differentiate individuals who are likely to develop AD and reliably measure signs of disease progression. Lactoferrin (Lf) is an iron-binding antimicrobial glycoprotein found in all biological fluids, and its concentration in saliva has been correlated with AD signs. Previous studies have found mixed results, so this pilot project aims to determine if the investigation of salivary lactoferrin (sLF) as a biomarker for AD is worth continuing. Moreover, understanding the variability of sLF across races is imperative to assess its clinical usefulness.

Methods: Participants were middle to older-aged African American (AA) and white individuals at risk for AD due to parental history of the disease. We collected saliva samples after an 8 hour fast, and administered a cognitive battery to assess executive function, memory, visuospatial ability, attention, and verbal fluency. We examined the relationship between sLF concentration and cognitive performance. We also examined differences in sLF levels across races.

Results: We enrolled 17 middle to older-aged (age = 60.29 ± 9.7 years) subjects. There was a 50-50 split between AA and whites. After controlling for age, sex, race, and years of education, we found a significant correlation between sLF and Digit Span Memory Test (DSMT) scores (P = 0.013). Furthermore, we found a correlation that is approaching significance between sLF and Mental Rotation Test scores (P = 0.194). Finally, we found no significant difference in average sLF concentration across AA and whites.

Conclusions: A decline in visuospatial ability and memory concerns are some of the earliest signs of cognitive deterioration in AD patients. Thus, we believe that the positive correlation found between sLF and measures of cognitive performance warrants more studies following sLF as a possible early biomarker for AD. Furthermore, the similarity in sLF concentration between AA and whites suggest a potential use as a biomarker in individuals regardless of their race. More studies measuring the relationship between sLF and other hallmark AD biomarkers, following

healthy subjects longitudinally, and measuring differences across other races are needed to assess its clinical usefulness as a robust biomarker for AD.

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1. Introduction

1.1 Alzheimer's Disease

Alzheimer's Disease (AD) is a progressive neurodegenerative disease characterized by memory loss, cognitive dysfunctions, and a decreased ability to carry everyday tasks. Currently, more than 5 million people suffer from AD in the United States, and the number is expected to triple to 14 million people by 2060 (Matthews, 2018). It is the 7th leading cause of death in the United States (Center for Disease Control, 2020). Therefore, understanding, assessing risk, and developing preventive mechanisms for AD is a top priority for healthcare research in the United States.

It is known that pathological changes linked to Alzheimer's Disease emerge many years before the behavioral symptoms do (Dubois, 2016). Thus, the asymptomatic stage (or preclinical phase) provides a crucial window of opportunity both to learn about the mechanisms in which AD evolves from pathogenesis to clinical symptoms, and to potentially treat a patient before the disease progresses.

Biomarkers are defined by the World Health Organization (WHO) as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (WHO, 2001). In other words, measurable changes in the physiology of a patient that can be linked to a specific disease can be classified as biomarkers. In 2011, biomarkers were recognized as a tool in the clinical diagnosis of AD by the National Institute on Aging (NIA, 2011). Specifically, the guidelines detail elevated levels of tau in CSF, decreased levels of beta-amyloid in CSF, decreased glucose uptake as determined by positron emission tomography (PET), and atrophy of certain brain regions as measured with magnetic resonance

imaging (MRI), as the current recognized biomarkers for AD, but purposely leave the guideline flexible for the possible addition of new, robust biomarker to assess risk, measure condition progression, or diagnose the disease.

The greatest known risk factor for AD is age, and a family history has been reported to increase risk by about 30% (Harvard Health, 2019). However, predicting with complete certainty which individuals with healthy cognition will develop an MCI or AD diagnosis in the future is not yet possible (NIA, 2011). Therefore, biomarkers research in the pre-clinical stages of AD is crucial to potentially lower the incidence and prevalence of the disease. Researchers aim to establish a set of accessible biomarkers that can accurately differentiate individuals that are likely to develop AD and that can benefit from potential preventative mechanisms or treatments against the disease.

1.2 Salivary Biomarkers

Cerebrospinal fluid (CSF) for amyloid–β (Aβ42), total tau (*t*-tau) and phosphorylated tau (*p*-tau) are hallmark biomarkers used as tools for the diagnosis of AD (Diniz, 2008). However, while highly promising, AD biomarker research and their implementation in a clinical setting can be expensive, uncomfortable, and impractical. Blood draws, lumbar punctures, and imaging technologies — such as PET and MRI — have currently taken center stage as the methods used to identify biomarkers. However, they are not easily accessible and all of them have at least one downside: lumbar punctures and venipuncture collection can be uncomfortable for the patient, and imaging technologies are expensive for the institutions (Ashton, 2021). So, is there a way of measuring biomarkers for AD that can be more accessible and less invasive than the methods currently used?

Saliva as a biofluid is a valuable alternative to investigate. Patients and research participants may favor it above other methods because it is simple and non-invasive; clinics and research institutions may favor it because it is cheaper and does not require expert personnel to collect samples — it could even be collected at home, by the patient. The remaining challenge about using saliva as a biofluid is determining if the biomarkers measured are disease specific. In other words, could a saliva sample tell if one is at risk of developing or already suffering from Alzheimer's Disease? It has been reported that salivary levels of total tau (t-tau) and neurofilament light chain protein (NfL) have no significant correlation to clinical symptoms of AD or other biomarkers (Ashton, 2018)(Gleerup, 2021). Salivary Lactoferrin, however, poses promising evidence.

1.3 Salivary Lactoferrin

Lactoferrin (Lf) is an iron-binding antimicrobial glycoprotein that can be found in most biological fluids, including in all exocrine secretions in mammals (Bermejo-Pareja, 2020). It was first isolated from bovine milk in 1939, and is now known as the main iron-binding protein in human milk (Sorensen, 1939). Its physiological function is modulating immune responses, while it also possesses anti-inflammatory properties (Ashton, 2019)(Actor, 2009). It has been previously described to inhibit pathogen proliferation through mechanisms such as iron sequestration and by inhibiting microbial adhesion to host cells (Fabian, 2012); it has also been described as a key nutrient for neurodevelopment and cognitive function during early periods of rapid brain growth (Chen et al., 2015).

In 1993, Kawamata et al. first reported that Lf is highly expressed in cortical tissue affected by AD pathology, specifically in senile plaques composed of aggregated β-amyloid (Aβ), extra-

and intracellular neurofibrillary tangles, and glial cells of AD patients (Kawamata, 1993). More recently, microbial infection and neuroinflammation have been proposed as possible explanations for the etiopathogenesis of neurodegeneration: studies have reported that systemic viral and bacterial infections are a significant risk factor for AD, and researchers have discovered increased levels of inflammatory markers in patients with AD (Leng, 2021)(Itzhaki, 2016). Therefore, it has been proposed that decreased Lf, a known antimicrobial and anti-inflammatory protein, could lead to less neurological protection, and therefore serve as an indicator of increased risk of AD (Welling, 2015)(Rousseau, 2013)(Gleerup, 2019). Saliva plays a key role as one of the body's main physical defense barriers against infection, and Lactoferrin is one of the major antimicrobial proteins found in it (Carro, 2017). Hence, salivary lactoferrin (sLF) has emerged as a promising candidate to investigate in relation to Alzheimer's Disease.

1.4 Animals Models

Lactoferrin has been investigated as a potential therapeutic agent with a role in AD progression (Mayeur et al, 2016). Initial animal studies have suggested that Lf can ameliorate mitochondrial dysfunction and reduce cellular damage (Park et al, 2013). Thus, researchers have investigated the effect of Lf administration on AD symptoms and its interactions with known AD biomarkers. In relation to cognitive decline, a group of researchers in China administered Lf by intragastric gavage for 3 months to 16-month-old mice, and noticed a significant improvement in spatial cognition in comparison to controls (Zheng et al., 2020). They also reported that serum levels of IL-1β and IL-6, known markers of neuroinflammation, were reduced in the experimental group of aged mice. Another study investigated the interaction of Lf administration on amyloid-β protein precursor transgenic mice (AβPP-Tg), a mouse model that over expresses amyloid precursor protein (APP) and is widely used as a model of AD pathogenesis (Abdelhamid, 2020).

Authors reported a significant reduction of neural Aβ40 and Aβ42 levels in the experimental group, and suggest that Lf drives the inhibition of amyloidogenic processing of APP by decreasing β-site amyloid protein precursor cleaving enzyme 1 (BACE1) levels.

The mechanism in which Lf produces these neural changes was further investigated in transgenic mouse models. A study published in *Nature Neuropharmacology* by Guo et al. described that administration of intranasal Lf to APPswe/PS1DE9 transgenic mice reduced Aβ deposition and improved spatial recognition by promoting the non-amyloidogenic processing of APP (Guo et al., 2017). Specifically, authors noted that Lf enhanced the expression of *α*-secretase a-disintegrin and metalloprotease10 (ADAM10), which metabolize APP and secrete soluble APP (sAPPα) and α-COOH-terminal fragments (α-CTF), consequently reducing Aβ production and apparent cognitive decline in the experimental group of transgenic mice.

Finally, in the specific case of lactoferrin *in saliva*, Antequera et al. reported a significant early and robust decrease of sLf in six- and twelve-month-old APP/PS1 mice that was not seen in their age-matched wild type counterparts (WT) (Antequera, 2021). Importantly, they did not see a decrease in total protein concentration in saliva. Furthermore, considering that the M3 muscarinic acetylcholine receptor has been linked to parasympathetic control of salivation in mice (Nakamura, 2004), authors measured muscarinic M3 receptor levels and acetylcholine (ACh) levels in submandibular glands, and found that both were decreased in the APP/PS1 mice in comparison to WT. They suggest that cognitive decline may be accompanied by a dysfunction of the ACh-mediated M3 signaling pathway in salivary glands of AD subjects, which may be leading to the reported reduction of sLf secretion.

1.5 Clinical and Translational Studies in Humans

With this evidence, sLF has been proposed as a possible biomarker for AD. Three pilot studies conducted by a research group in Spain give compelling evidence of a correlation between sLF and cognitive decline in both AD patients and patients at risk for the disease. The first study, published in 2017, found the concentration of salivary Lf to reliably discriminate between the cognitive status of subjects suffering from AD or amnestic mild cognitive impairment (aMCI) and healthy age-matched controls (Carro, 2017). Researchers also found the level of salivary Lf to be positively correlated with the Mini-Mental State Examination (MMSE) and Aβ42 in CSF, while inversely correlated with t-tau, in AD patients.

In that same paper, the researchers published results of a longitudinal analysis, where two blinded non-clinical cohorts with normal cognition (caregivers and family of AD patients) were followed from 2009 to 2014. The team set a concentration cut-off of lactoferrin in saliva at 7.43μg/mL, and compared baseline results of lactoferrin with yearly cognition updates in all subjects. From a cohort of 306 participants, 18 had a baseline lactoferrin concentration below the cutoff. After five years, 14 of those 18 had developed a diagnosis of MCI or AD, while no participant above the cut-off value of Lf concentration had changed clinical status within the same timeframe. Authors concluded that their cut-off value for sLF had 78% accuracy delineating which patients in their cohort would develop a clinical diagnosis of decreased cognition.

In the second study, published in 2020, the same Spanish group found that salivary Lf could differentiate between prodromal AD and AD, but had no correlation to frontotemporal dementia (FTD) in a cohort of 116 patients with different levels of cognitive deterioration (Gonzalez-

Sanchez, 2020). Authors reported that lactoferrin in saliva had 91% specificity and 86% sensitivity in making this differentiation.

The third study, published in 2021, reported that salivary Lf concentration was negatively correlated to cortical Aβ load and middle temporal cortex thickness, and positively correlated to poorer memory in a cohort of 74 cognitively normal participants (Reseco, 2015). In this study, cognitive realms of memory, working memory, attention, executive function, and language were measured, but only memory had a significant correlation to sLF levels.

A different research group in Egypt also published a paper supporting the relationship of sLF and AD signs. They described a beneficial alteration in hallmark markers of AD and improvement of cognitive function, specifically in memory, after AD patients took a daily dose intranasal Lf for three months (Mohamed, 2019).

While these studies describe promising results, the story of Lactoferrin is not completely straightforward. A research group in Copenhagen, Denmark, tried to replicate the evidence previously published by the Spanish researchers, and performed a study measuring the relationship between sLF and AD variables on a cohort recruited from a Danish mixed memory clinic (Gleerup, 2021). In their study, they could not replicate any results previously published by the group in Spain: they found no significant correlation between Lf concentration and hallmark AD biomarkers Aβ42, *t*-tau, and p-tau in CSF. Moreover, there was no significant concentration difference between healthy controls and patients with either MCI, AD, or any other non-AD neurodegenerative disease.

1.5 Rationale

Considering AD biomarkers start increasing years before cognitive symptoms are perceived, it is crucial to understand the behavior of disease biomarkers even in apparently healthy subjects — especially in those at risk for the condition. It is problematic that studies with very similar rationale and methodology, such as the ones from the groups from Spain and Denmark, can produce such different results (Ashton, 2021). However, it is important to analyze the differences between cohorts of research subjects and account for other variables that could be producing contrasting data. Firstly, the nature of the participant cohorts was different — the patients in the Denmark study were taken from a mixed memory clinic, and therefore common comorbidities of AD were not controlled for nor was there exclusion criteria accounting for them. It is known that factors such as cardiovascular disease can have significant effects on AD progression and presentation, so the effects of more heterogeneous individuals in terms of other diseases and conditions should be taken as a possible differentiating factor between cohorts (Tini, 2020). There was also a methodological difference in saliva collection: the researchers in Denmark asked subjects to restrain from eating 30 minutes before collection; the group in Spain asked participants to fast for at least 8 hours prior to collection. Further studies are needed to assess if the investigation of salivary lactoferrin as a biomarker for AD is worth continuing.

While the group in Denmark attempted to replicate a lot of data from the previous publications by the group in Spain, one major finding remains to be further tested: the correlation of lactoferrin levels and cognitive performance in cognitively normal participants. In the Denmark study, there were MCI and AD patients enrolled as subjects, and healthy participants were only enrolled as controls. However, there is always a possibility of non-diagnosed individuals having biomarker levels of an at-risk individual without showing cognitive symptoms. That is why the

results of the third study from the group in Spain, which enrolled healthy participants as research subjects and correlated sLF to their cognitive performance, specifically in the cognitive realm of memory, is so relevant to this discussion — it's imperative to know the behavior of the biomarker in the preclinical stage of AD to determine its reliability measuring disease progression and differentiating individuals who are likely to develop it. One of the best ways to do so, is correlating the sLF concentration to cognitive performance in healthy individuals *at risk for the disease*. To our knowledge, this experiment has not been performed before in that specific subset of subjects.

Another knowledge gap is the potential sLF differences across races. It has been documented that AD risk, symptoms and markers of disease appear and progress differently across races, with a marked disparity between whites and African Americans (AA)/Hispanics (Wharton, 2019)(Gauthier, 2016). To our knowledge, sLF has only been tested in cohorts with an overrepresentation of whites, and race has not been reported as a demographic variable in any previous study about neurological disorders in humans, nor its potential modifying effect on sLF levels has been described.

Finally, although various previously cited animal studies have reported that exogenous administration of Lf improves spatial cognition in aged mice, none of the studies that tested cognition in human subjects in relation to sLF measured performance on spatial cognition tasks (Zheng, 2020)(Guo, 2017). Considering this information and the results of the third study from Spain, we believe that memory and visuospatial cognition could be the cognitive areas most affected by decreased LF.

The present study will investigate a potential correlation between sLF and various categories of cognitive performance in a cohort of 17 cognitively normal older adults at risk for

Alzheimer's Disease enrolled in the Wharton Laboratory at Emory University. The cohort consists of an even split between white and AA individuals. Our hypothesis is that reduced levels of sLF will correlate to cognitive performance, specifically in the realms of memory and visuospatial cognition. One further exploratory aim of this study is to determine if there is a significant difference in general sLF levels across races.

2. Materials and Methods

2.1 Ethics

This paper represented results of a pilot project embedded into *E2: Sex Hormones and Alzheimer's Disease Prevention*, a host NIH-funded two-year observational study conducted by the Wharton Laboratory at Emory University, Atlanta, GA (Wharton Laboratory, 2022). Recruitment and cognitive testing were conducted by staff members of the Wharton Laboratory as part of the E2 study, while sLF collection, processing and analysis was conducted independently. All data presented in this study was collected between October 10th, 2022 and March 24th 2023. We also believe in the importance of diversity and inclusion in science — the population most at risk for AD is AA, but they represent a median of 2.8% of all subject enrolled in AD clinical studies (Franzen, 2021)(Raman, 2021). As such, ~50% of all subjects enrolled in this pilot study are AA, and we are analyzing race as a potential modifying variable for general sLF levels. All patients gave informed consent for participation. This project was supported by an Independent Research Grant provided by the Emory University URP Research Partners Program, the National Institute of Health, and the Alzheimer's Association.

2.2 Subjects

We enrolled 17 middle to older-aged (age = 60.29 ± 9.7 years) cognitively normal participants who have a biological parent with either autopsy-confirmed or probable AD as defined by NINCDSADRDA criteria (McKhann et al., 1984) and available medical records. Table 1 shows sample characteristics.

All individuals enrolled in this pilot study were already part of a clinical cohort from the Wharton Laboratory. Specifically, subjects from the E2 study, a longitudinal observational study in which 150 women and 50 men with normal cognition are being clinically and physiologically observed for 2 years (Wharton Laboratory, 2022). One of the main objectives of E2 is to measure if sex hormone levels influence the relationship between AD risk factors and AD biomarkers CSF Aβ, phosphorilated and t-tau, neuroimaging and cognition. The research participants visit the laboratory three times over the course of two years — Baseline, Year 1, and Year 2. In these visits there can be a blood draw, a lumbar puncture, an MRI, a series of cognition tests, a vascular ultrasound and a sleep assessment. We collected saliva samples at either Baseline or Year 1 visits, and the cognitive data used for this pilot study was taken from the cognitive tests that occurred on the same visit day as the saliva collection.

Exclusion criteria included active mouth lesions, a history of dry mouth, contraindication for lumbar puncture or magnetic resonance imaging, residence in a skilled nursing facility, significant neurological disease, stroke, or history of significant head trauma, untreated Major Depression within 2 years of study enrollment, history of alcohol or substance abuse, current participation in a study with an investigational agent, pregnancy, diagnosis of AD, and unwillingness to fast. Finally, the sLF data belonging to three subjects enrolled in this pilot study

was discarded from analysis, as their sLF levels were determined to be outliers due to methodological discrepancies in saliva collection and processing: two participants had a snack about 1 hour prior to collection, and there were inconsistencies with the saliva sample processing from the third participant.

2.3 Sample collection, Processing, and Protein Quantification

According to previous studies (Bartolome, 2021)(Reseco, 2021), participants were asked to fast and hold back from other oral stimulation (i.e., smoke, chew gum) for at least 8 hours prior to saliva collection. Saliva samples were collected between 8:00–11:00 AM through passive drooling. 1-2 mL of unstimulated whole saliva was collected in Falcon™ 15mL Conical Centrifuge Tubes (Corning, Massachusetts, United States). Samples were immediately placed in a cooler with ice packs and centrifuged at 1000 *rpm* for 5 min at 4 °C. Supernatant saliva was then aliquoted into 1.5 ml polypropylene tubes, treated with a protease inhibitor cocktail (cOmplete Ultra Tablets mini, Roche, Basel, Switzerland), and stored at − 81 °C until further analysis. Quantification of sLF concentration was performed with a commercially available Human Lactoferrin ELISA Kit (Abcam, ab200015, Cambridge, England), according to the manufacturer's instructions. All samples were diluted 1:10,000 into sample diluent, after which we added 50 µL of the diluted samples or a standard to the designated wells. All samples were tested in duplicate and the average of the two measurements was used for data analyses. Intra-assay CV values used for analysis differed by an average of two-hundredths of a decimal.

2.4 Neuropsychological Assessment

A cognitive battery of approximately 90 minutes of testing was administered to all subjects. It assessed several cognitive domains, including executive function, memory, visuospatial cognition, attention, and verbal fluency. Testing includes the Forwards and Backwards Digit Span Memory Test (Wechsler, 1981), Mental Rotation Test (Vandenberg & Kuse, 1978), Buschke Memory Test (Buschke, 1973), Consortium to Establish a Registry for Alzheimer's Disease (Fillenbaum, 1986), Trail Making Test (Bowie & Harvey, 2006), Animal Fluency (Rosen, 1980), Rey–Osterrieth Complex Figure (Rey, A., & Osterrieth, 1941), Judgment of Line Orientation (Benton, 1983), Digit Symbol Substitution Test (Wechsler, 1944), and the Montreal Cognitive Assessment (MoCA) (Nasreddine et al., 2005). In accordance with previous studies, the Forwards Digit Span Memory Test was used as a measure of memory and the Mental Rotation Test as a measure of visuospatial cognition (Richardson, 2007)(Shepard, 1971).

2.5 Statistical Analyses

Demographic variables were summarized using descriptive statistics. Multiple bivariate Pearson's correlations were performed to investigate statistically significant linear relationships between sLF and our various cognitive tests. To control for confounding factors, demographic variables, including age, gender, race, and years of education were adjusted for in all correlations that showed significance. No correlation lost significance level after adjustment. All statistical analyses were performed by SPSS® ver. 29.0.0.0 (IBM, Armonk, New York, USA).

3. Results

3.1 Demographics, Cognitive and sLF Measures

We analyzed saliva samples and cognitive test data from 14 subjects. Table 1 shows results from the total sample and stratified by race. Analyzed data consisted of cognitively normal middle to older-aged subjects at risk for AD, with a 50-50 split between AA and whites. Age, sex assigned at birth, and years of education were not significantly different between AA and whites. Those same variables were used as controls for correlation analyses of sLF and memory.

Table 1. Demographics and Cognitive Measures

	Total Sample ***N = 14***	**African American** ***N = 7***	**White** ***N = 7***
Age	60.29 ± 9.7	58.7 ± 7.9	61.22 ± 11.63
Sex Assigned at Birth (m/f)	4/10	2/5	2/5
Education Years	16.3 ± 2.2	15.7 ± 1.9	16.7 ± 2.5
Salivary LF (ug/ml)	18.7 ± 10.7	18.2 ± 9.1	19.2 ± 12.3
Cognitive Battery			
Digit Span Memory Test	10.5 ± 1.9	10.9 ± 2.0	10.2 ± 1.9
Mental Rotation Test	17.0 ± 6.1	14.4 ± 6.6	19.0 ± 5.3
BSRT delayed recall	5.4 ± 2.9	5.71 ± 2.5	5.2 ± 3.3
CERAD delayed recall	5.2 ± 3.0	5.1 ± 2.2	5.3 ± 3.6
TMT-B	91.6 ± 53.0	81.3 ± 20.5	99.6 ± 69.1
Animal Fluency	23.9 ± 6.0	21.9 ± 5.0	25.6 ± 6.4
ROCF	30.5 ± 5.8	28.2 ± 8.2	32.3 ± 2.3
JoLO	23.1 ± 4.7	21.7 ± 4.0	24.2 ± 5.0
Digit Symbol Substitution	59.2 ± 17.2	59.3 ± 6.8	59.1 ± 22.3
MoCA	26.9 ± 4.2	26.71 ± 2.4	27.0 ± 5.4

Results are expressed as mean ± standard deviation, unless otherwise stated. Abbreviations: *N* number, *F* female; *M* male, *TMT-B* Trails Making Test Form B, *LF* Lactoferrin, *CERAD* Consortium to Establish a Registry for Alzheimer's Disease, *MoCA* Montreal Cognitive Assessment, *JoLO* Judgment of Line Orientation, *ROCF* Rey–Osterrieth Complex Figure, BSRT Buschke Selective Reminding Test

3.2 Correlation Between Salivary Lactoferrin and Memory

We found a significant correlation between sLF and the Digit Span Memory Test (DSMT) scores in our cohort's participants (Pearson's = 0.607; $P = 0.013$; Fig. 1). The level of significance measured stands after adjusting for age, sex, race, and years of education ($P = 0.016$). The DSMT is used as an assessment of ability to momentarily store and handle information (Richardson, 2007). We suggest that the subjects who had higher levels of lactoferrin in saliva tend to have better memory. This indicates a possible protective effect of lactoferrin toward a cognitive area typically affected by Alzheimer's Disease.

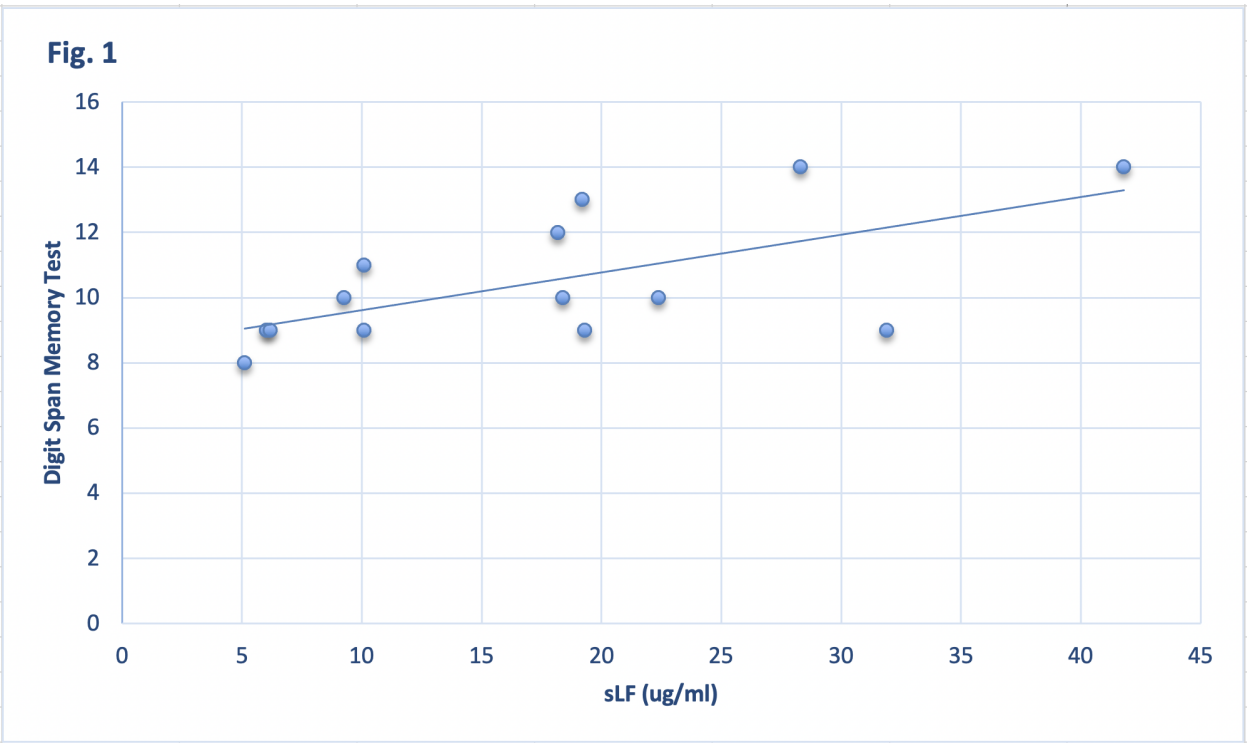

Fig 1. Digit Span Memory Test scores as a function of salivary lactoferrin concentration. We performed a bivariate Pearson's correlation, and found a positive relationship ($P = 0.013$).

3.3 Correlation Between Salivary Lactoferrin and Spatial Cognition

We found a correlation that is approaching significance between sLF and the Mental Rotation Test scores in our cohort's participants (Pearson's = 0.342; $P = 0.194$; Fig. 2). The MRT is used as an assessment of the ability to imagine spatial transformations of objects (Hegarty, 2018). We suggest that subjects who have lower levels of lactoferrin in saliva could have worse spatial cognition. Our small sample size might be restraining us from establishing a statistically significant correlation. It is also worth noting that no other cognitive realm besides memory and spatial cognition had significant or close to significant correlation values.

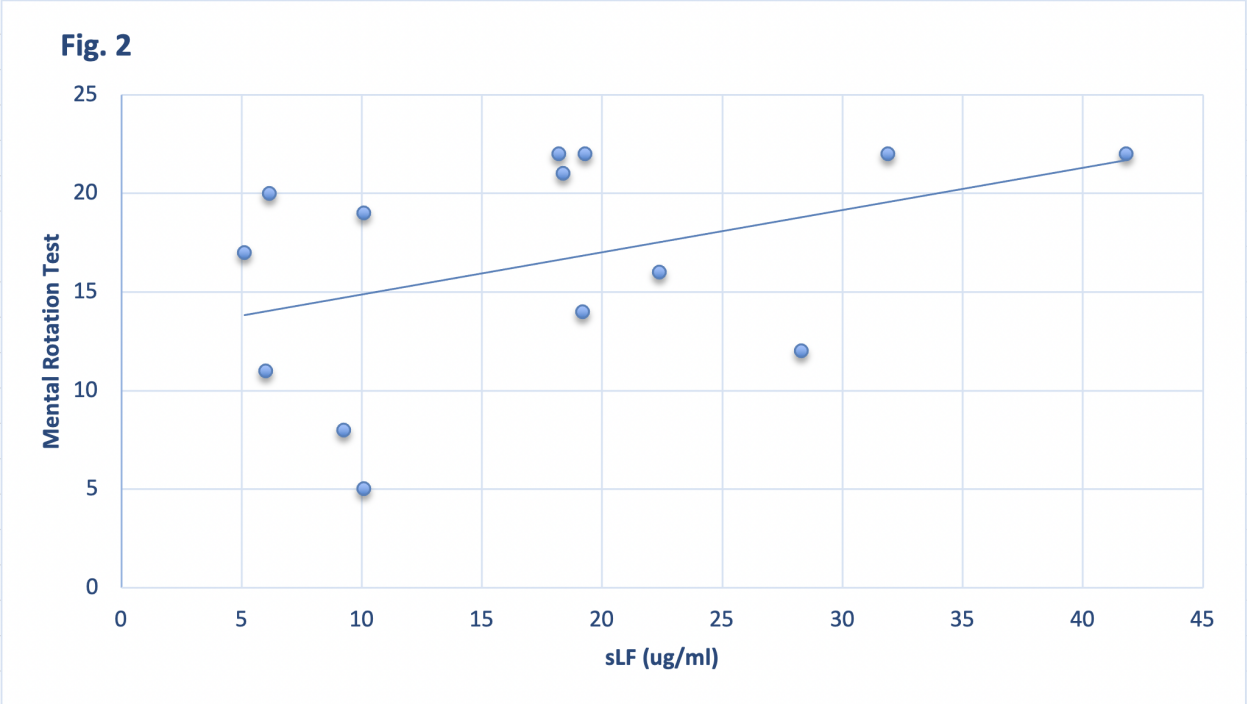

Fig 2. Mental Rotation Test scores as a function of salivary lactoferrin concentration. We performed a bivariate Pearson's correlation, and a found possible relationship that is approaching significance ($P = 0.194$).

3.4. Difference in Salivary Lactoferrin Levels Across Races

We found no significant difference in average sLF concentration between AAs and whites. While each group is composed of only 7 subjects, the average concentration difference is only of ~5%, as AAs had an average of 18.2 µg/mL and whites an average of 19.2 µg/mL. We do not believe that the increased standard deviation in whites is indicative of a larger phenomenon.

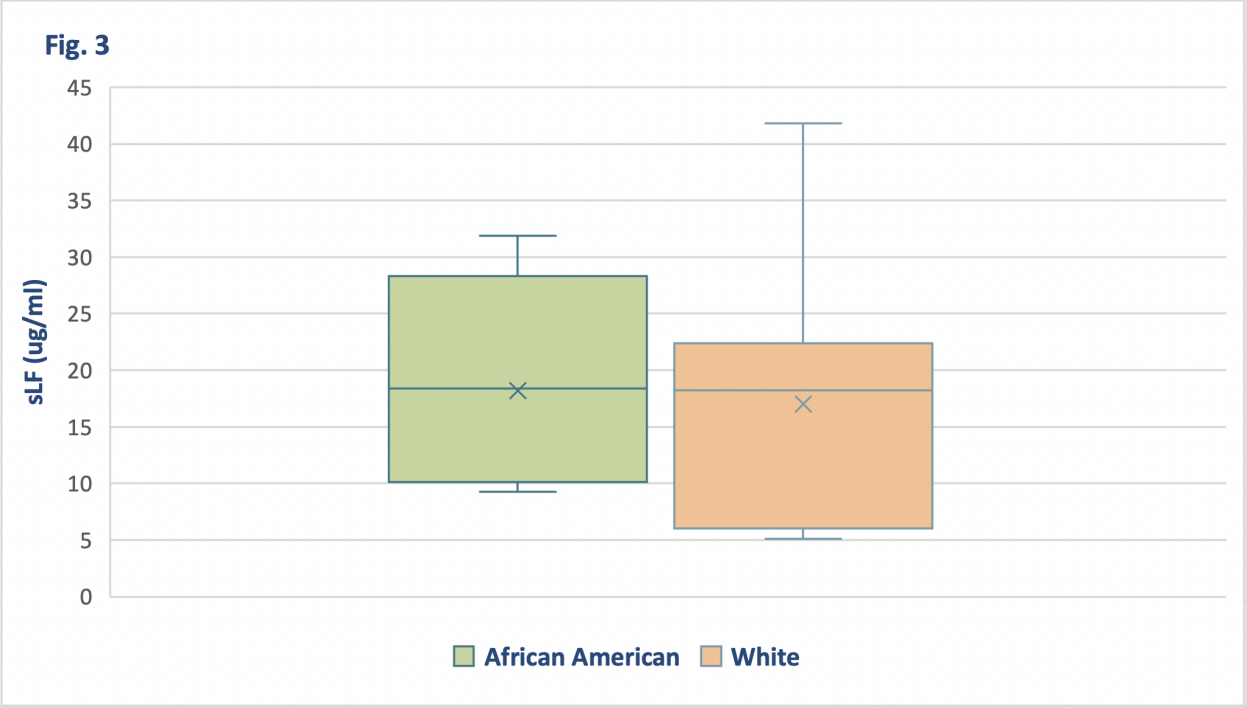

Fig. 3. Average salivary lactoferrin levels in African American and white participants. There appears to be no significant difference between both groups.

4. Discussion

A pilot project is carried out to test if an idea is worth pursuing and to evaluate the methodology of its implementation (Hassan, 2006). The scientific world was left divided when two manuscripts were published in The Lancet, the world's highest impact academic journal, describing what appeared to be the same rationale and methods but reporting completely opposite results, less than a year apart from each other (Gonzalez-Sanchez, 2020)(Gleerup, 2021). The purpose of this pilot project was to assess if the investigation of salivary lactoferrin as a biomarker for AD is worth continuing, as determined by positive, significant correlations between sLF and cognition in healthy individuals at risk for the disease. Furthermore, to explore if Lf, a commonly cited glycoprotein in scientific research, varied in average levels in saliva concentration between AA and whites.

The most important limitation of this pilot study is its small sample size. Also, the data presented is purely correlational. While inferences can be made about the validity of the protein as a robust biomarker for AD and about the mechanisms present in this correlation, results should be taken as preliminary and should be replicated in further studies.

Our conclusion about the significant correlation between sLF and memory in cognitively normal middle to older-aged adults at risk of AD was rather surprising considering the small sample size it was tested in. As such, it could be indicative of a strong association between the two. This conclusion alone seems to be enough to warrant more studies following sLF as a possible early biomarker for AD, since memory concerns are one of the earliest signs of cognitive decline in AD patients (Holger, 2013). It also points toward a possible neuroprotective effect of LF.

Our conclusion about the possible correlation of sLF with spatial cognition, while not statistically significant, we believe is still important. There was rigorous evidence from animal

studies pointing toward this possible correlation, and this study suggests a similar relationship in humans — one that, to our knowledge, had not been tested before. Difficulties in spatial cognition are common in AD patients, so this possible relationship should be studied further (Katherine, 2010).

These two conclusions are intriguing, because they raise questions about the reason why the study by Gleerup et al. could not replicate the positive results described by the group in Spain. A cohort from a mixed memory clinic can be more representative of the general AD population than a carefully controlled one, since AD patients not enrolled in research studies can also be suffering from heterogenous comorbidities possibly related to old age and general neurodegeneration. That may be one reason why the results were so contrasting, as the Danish research group proposed (Gleerup, 2021). *Spitting image: can saliva biomarkers reflect Alzheimer's disease?* is an open-peer commentary also published in The Lancet, that explores the conundrum between the two research groups and the variability in sLF concentration between cohorts (Ashton et al., 2021). The authors question the reproducibility of saliva collection, which can be sensitive to external stimuli and be affected by oral hygiene. One difference between the two cohorts that has not been previously reported in the literature, however, is the variation in the fasting times required for research participants — 30 minutes v. 8 hours by Danish and Spanish groups, respectively.

In our cohort at Emory University, we asked participants to fast for 8 hours prior to collection. Two participants, however, presented inconsistencies in fasting times (see *2.2 Subjects*). We still processed their samples and measured their sLF concentrations, and discovered them to be 3-4 standard deviations higher compared to the properly fasted participants, so their samples were considered outliers and discarded from analysis. While this experience produced a

smaller sample size than we expected for analysis, it also taught us a valuable lesson: a difference in 30 minutes v. 8 hours of fasting can produce a significant change in sLF concentration. One so significant — in our experience — that could possibly account for the contrasting results between Spain and Denmark. More studies investigating the interaction between fasting times and sLF levels are needed to confirm this.

Our final conclusion, less related to the previous studies of sLF and AD and more related to the field of LF in general, is the similarity in sLF concentration between AA and whites. To our knowledge, no previous studies investigating sLF in humans in relation to any neurological disease had reported on race as part of their analysis and/or cohort demographics. As such, our conclusions, which should be replicated across even more races, in other bodily fluids, and with a larger sample size, suggest that sLF *has potential* to be used as a biomarker for risk assessment in adults at risk for AD regardless of their race.

Our research group will continue collecting saliva samples from E2 subjects, with the aim of furthering the knowledge and statistical power of the conclusions reached in this pilot study. It will be interesting to correlate the sLF concentration of participants in our cohort to their levels of other known AD biomarkers in blood and CSF, such as t-tau, p-tau, and Aβ42. Furthermore, as proposed in the first study published by the group in Spain, sLF could serve as a measure of increased risk for developing AD if the concentration measured is below 7.43μg/mL (Carro, 2017). We had three participants fall below that cutoff, and it will be interesting to follow their cognitive status longitudinally to see if they follow the same trend.

The ultimate goal is producing easily accessible, disease specific biomarkers that can be clinically useful for determining risk, measuring progression, or diagnosing AD. We believe that the conclusions reached in this pilot study bring us closer to that goal, but more work is needed.

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