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Favorable Changes in Salivary Uric Acid and Acyl Peptide Enzyme Hydrolase Activity in a Photobiomodulation Pilot Study

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Abstract
Photobiomodulation (PBM) therapy has numerous documented health benefits, but the physiological mechanisms are still being elucidated. In this work, a pilot study of salivary biomarkers was investigated to potentially understand physiologic oxidative stress changes associated with PBM therapy. Eighteen participants completed six PBM sessions and provided saliva samples prior to session one and after each of the six sessions. Salivary uric acid, acyl peptide enzyme hydrolase (APEH) activity, and total protein were measured. While salivary uric acid and total protein have been measured before, APEH is a novel salivary marker. When grouped into 'heightened risk' categories, 67% of participants with high starting uric acid had a reduction in salivary uric acid and 57% of participants with low starting APEH activity had an increase in salivary APEH activity over the six PBM sessions. While a larger and more comprehensive study is required to make conclusions, this pilot study suggests that PBM therapy has beneficial outcomes to selected oxidative stress markers, thereby providing a physiological mechanism that may accompany reported positive biological outcomes.

Keywords: photobiomodulation (PBM) therapy; oxidative stress; salivary biochemical markers; uric acid; acyl-peptide enzyme hydrolase (APEH)

Introduction

Photobiomodulation (PBM) therapy, also called low-level laser therapy, is a form of light therapy that exposes participants to various wavelengths of light in the red (600-700 nm) and near-infrared (770-1200 nm) spectrum. This process elicits photophysical and photochemical events that have been shown to have beneficial biological effects such as alleviation of pain and inflammation, and promotion of wound healing and tissue regeneration [1–3]. While there are numerous studies on the effects of PBM, understanding the molecular, cellular, and systemic mechanisms involved is still being investigated. Continued studies elucidating such mechanisms are important for understanding the clinical potential and health benefits of PBM.

Oxidative stress and inflammation are well-known hallmarks of diseases such as cancer, cardiovascular disease, diabetes mellitus, and several neurodegenerative diseases [4–6]. PBM has been shown to modulate oxidative stress and inflammation in many studies such as in oral health issues [7], neurological disorders [8], and side effects of cancer therapy [9]. One issue with studying the physiologic mechanisms of PBM on biological outcomes is doing so in a non-invasive manner. As such, many mechanistic studies of PBM are performed in vitro using cell culture or in vivo using animal models.

Saliva has been demonstrated to be a promising bodily fluid for biomarker detection because it is easy, safe, cheap, and non-invasive [10]. PBM studies have employed saliva as a non-invasive biofluid for mechanistic effects with some success. For example, salivary cytokines such as CXCL8, Il-10, and TNFα have been measured and correlate to positive outcomes in PBM studies related to head and neck cancers [11]. Continuing to understand salivary biomarkers and their relationships to PBM is important for explaining physiological effects associated with reported positive outcomes. In this work, two biomarkers with ties to oxidative stress are investigated.

The first biomarker, uric acid, is the final product of purine metabolism in humans. It is produced in the liver and excreted in the kidneys. While uric acid is a waste product, it serves as an antioxidant and has some protective effects in the body at normal levels [12]. High levels of uric acid are most commonly associated with gout but are also observed in other diseases. For example, hyperuricemia is associated with disease severity in chronic heart failure and is associated with a chronic inflammatory response [13, 14]. Elevated uric acid is also observed in obesity, a

risk factor for cardiovascular disease and chronic inflammation [15]. Uric acids levels are typically measured from the serum, but salivary uric acid is a non-invasive tool for measuring the product [15–17].

The second biomarker, acyl peptide enzyme hydrolase (APEH), also called oxidized protein hydrolase, is a dual-function enzyme with both endopeptidase and exopeptidase activity [18, 19]. Recent work has shown that APEH has important roles in managing oxidative stress. For example, APEH degrades oxidatively damaged proteins including the clearance of N-formylated peptides from bacteria [20], pyruvylated-proteins caused by radical mediated damage [21], oxidized proteins from oxidative stress [22, 23] and amyloid-β peptides [24]. APEH is part of the cellular response to oxidatively-induced DNA damage [25] and part of the systemic response to oxidative stress and immune response [26]. APEH activity is decreased in diseases related to oxidative stress such as Alzheimer's disease [27] and diabetes [28]. Taken together, APEH activity is emerging as an important to response to oxidative stress and damage and decreased activity may lead to disease progression. APEH has been measured from serum and plasma [27, 28], but has yet to be reported in saliva. The salivary proteome has a 25% overlap with the plasma proteome and is enriched in peptidase and hydrolase activities [29]. APEH, a protease, is part of this proteasomal overlap and its salivary activity is reported in this study.

In this work, a pilot study was performed looking at selected salivary biomarkers to potentially understand physiologic oxidative stress changes associated with PBM therapy. This work is important to establish parameters for a larger, more comprehensive study of PBM therapy on modulating salivary oxidative stress biomarkers.

Methods

Participants and Saliva Collection

This study was performed with 18 volunteers (13 women and 5 men, average age is 55 years) in line with Weber State University's Internal Review Board #IRB-AY22-23-44. Participants were provided an informed consent document outlining the research project; participation was voluntary. Participant data was kept confidential from researchers and analyzed under a non-identifying letter. Participants donated saliva a total of seven times during the study, once prior to their first PBM therapy and once following each of six PBM sessions (Figure 1A). Saliva collections, referred to as 0-6, occurred within three weeks of the first PBM session. Participants were asked to refrain from eating 2 hours prior to and rinse mouth with water immediately before saliva donation. About 2 mL of saliva was collected by unstimulated drooling into a SpeciMAX saliva collection tube (Thermo Fisher Scientific, Waltham, MA, USA). The saliva samples were centrifuged at 4,000 g x 10 minutes at room temperature. The pellets were discarded, and the supernatant was aliquoted and stored in a -80ºC freezer until analysis, which was less than 6 months. Samples that were discolored or had insufficient supernatant were eliminated from the study.

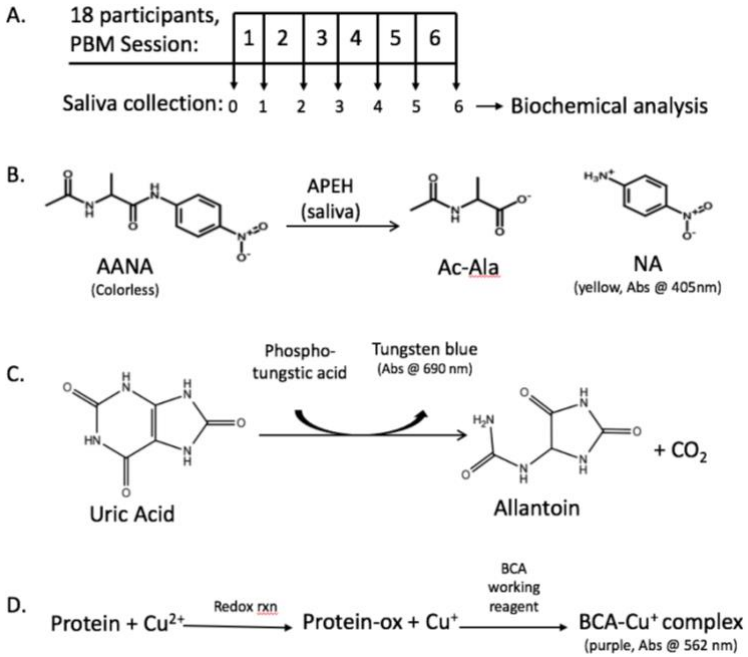

Figure 1. Summary of Study Set Up and Biochemical Assays Performed. (A) The study design where participants provided saliva prior to PBM session 1 and subsequently after PBM sessions 1-6 for a total of 7 saliva samples; (B) the biochemical basis of the APEH assay; (C) Uric acid detection; (D) Total protein measurement using a Bicinchoninic Acid (BCA) assay. Chemical structures were produced using Schrödinger Free Maestro (Release 2019-3) 2D sketcher program.

PBM Protocol

Participants received whole body PBM treatment using the proprietary protocol called WC Deep Relief™. Sessions were 20 minutes long and included pulsing of light at 630, 810, 850, and 940 nM with frequencies ranging from 4.56 to 584 Hz.

APEH Enzyme Activity

APEH assays were performed with 20 µL of 30% diluted saliva and 5 mM acetylalanine *p*-nitroanilide (AANA) (Biosynth International, Inc, San Diego, CA, USA) in 100 mM TRIS buffer (pH = 8.0). APEH hydrolyzes AANA into acetyl-alanine (AA) and 4-nitroanilide (NA); the reaction was monitored by NA production at 405 nm (Figure 1B) on a Tecan Spark 10 M plate reader every minute over 20 min as previously described [27, 28]. Slopes were converted to velocities (µM NA/min) using a 5-point standard curve of nitroaniline in Tris-buffer. Each saliva sample was analyzed in at least triplicate and activities averaged. A specific, competitive APEH inhibitor, AA74 (Sigma-Aldrich, St. Louis, Missouri, USA), was used at 1 µM as a control for APEH activity in saliva, which reduced APEH activity to <95% (data not shown).

Uric Acid Measurements

Uric acid concentrations in saliva were measured using a colorimetric assay kit (E-BC-K016-M, Elabscience, Houston, TX, USA). Briefly, 25 µL of undiluted saliva was used in the assay following manufacturer's protocol exactly. Uric acid in saliva reduces phosphotungstic acid to tungsten blue, allantoin, and carbon dioxide so that the absorption at 690 nm is proportional to the uric acid concentration (Figure 1C). The absorbance was determined on a Tecan Spark 10 M plate reader and Uric acid concentrations determined using a 5-point standard curve. Each saliva sample was analyzed in at least triplicate and concentrations were averaged.

Total Protein Measurements

Saliva samples were diluted to 30% saliva in 100 mM Tris buffer at pH = 8.0. Total protein was determined using a Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) as recommended by the manu facturer. Briefly, 10 µL of diluted saliva or 10 µL of BSA standard were mixed with 190 µL of BCA working reagent and incubated for 15 minutes at 37 ºC. The absorbance was read at 562 nM (Figure 1D) using a Tecan Spark 10M plate reader. Protein concentrations were determined using a 5-point BSA standard curve.

Statistical Analysis

Samples were analyzed in triplicate for each analysis. Results were averaged and a standard deviation determined. Linear regression analysis was used to evaluate individual participant changes over the course of the study.

Results

General Participant Data

The individual participant data for salivary Uric acid, APEH activity, and total protein for the course of the study are summarized in Figure 2. Each participant is shown with a non-identifying letter and color-coded box. The box plots represent an individual's salivary average and standard deviation for all seven saliva collections (0-6) for the duration of the study. The black lines indicate the average of all 18 participants in the study for relative comparison. The overall, combined participant average and standard deviation for tested salivary markers are as follows: APEH activity is 26.6 ± 16.1µM NA/min, Uric acid is 65.5 ± 19.5 µg/mL, and total protein is 430 ± 170 ng/µL.

Figure 2 illustrates that some participants have low variability in measurements over the study while some participants have a wider range in measurements. For example, participant E has a low variability in salivary Uric acid and protein but varies more in APEH activity. Figure 2 also illustrates which participants have below average (i.e. participant P) and above average (i.e. participant W) salivary measurements.

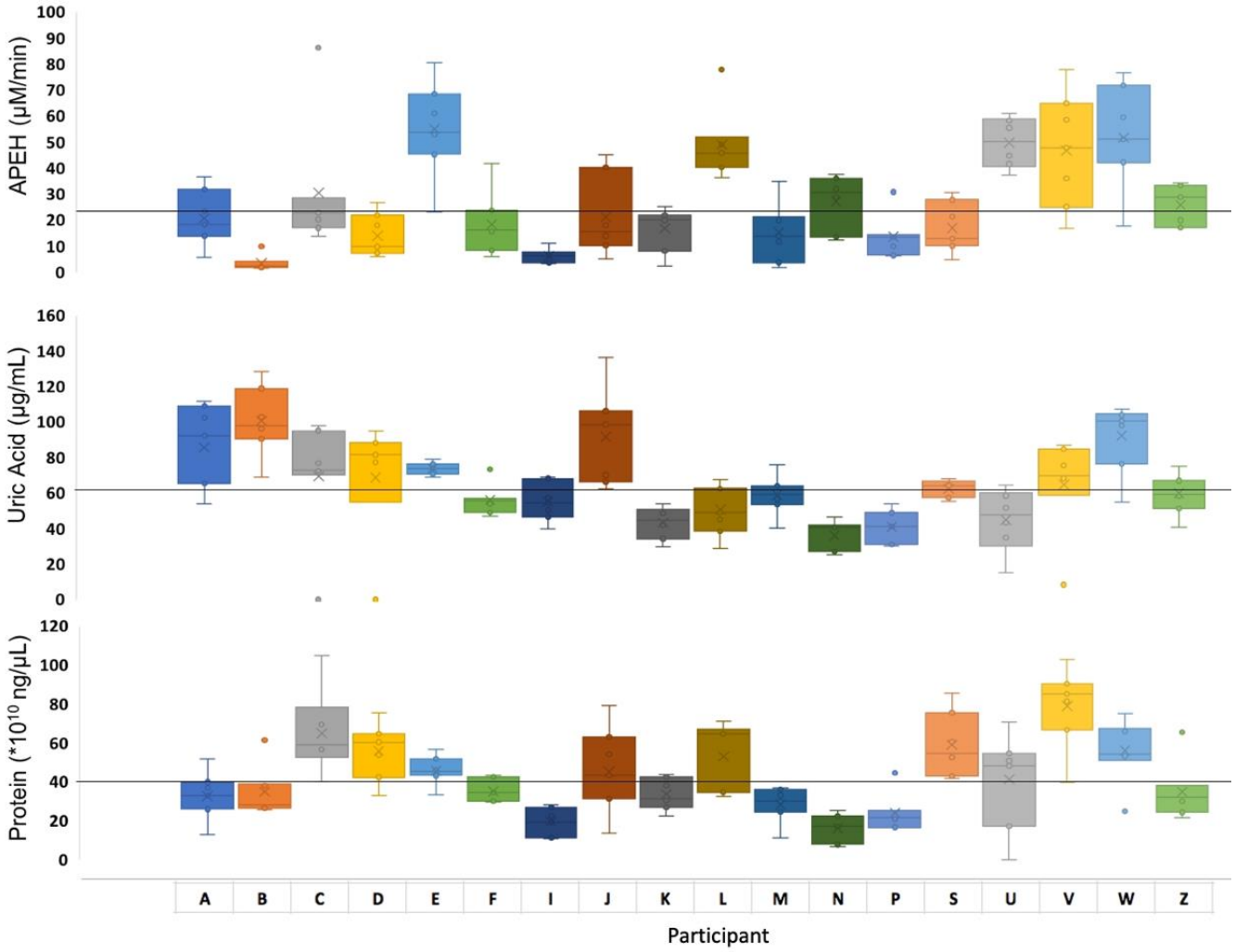

Figure 2. Summary of Individual Participant Data. Individual participant data is color-coded and represented by a letter for salivary APEH activity (top), Uric acid concentration (middle), and total protein (bottom). Box plots represent the individual's average with a standard deviation for the duration of the study (from PBM 0 to 6). The overall participant averages are shown with solid black lines for comparison. This figure was created using Microsoft Excel.

Individual Participant Data

The goal of this study was to investigate whether PBM therapy had an effect on salivary biomarkers during the course of the study. Participants provided saliva seven times during the study. Total protein is used as a control for salivary concentrations, as hydration status may affect measurements on different days. Figure 3 illustrates changes in selected participant measurements of APEH enzyme activity (µM NA/min)/total protein (ng/µL) for the course of the study. Some participants 'respond' with increasing salivary APEH activity (Figure 3A, C), while some participants have varied or no changes (Figure 3B, D). The slope indicates the magnitude and direction of change while linear regression analysis (R^2) is used to indicate the relationship between APEH activity/protein and the PBM treatments (Figure 3A, B, Table 1). Participants that 'respond' with increasing APEH activity trend toward low starting APEH activity/protein prior to PBM sessions (Figure 3C) whereas non-responders may have high APEH activity/protein prior to PBM session (Figure 3D).

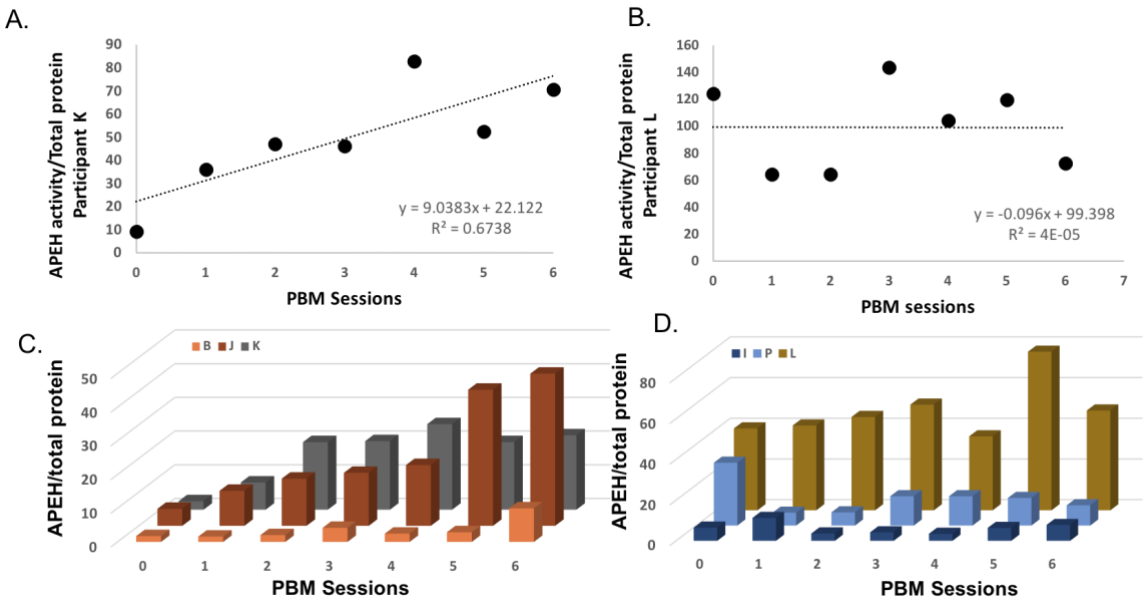

Figure 3. Analysis of APEH Activity 'Responders' vs. 'Non-Responders'. APEH activity/total protein is evaluated over the PBM sessions with A) indicating a 'responder' and B) indicating a 'non-responder'. A linear regression plot is shown with the equation for the line and R^2 value. Three-dimensional histograms showing three participant 'responders' in C) and 'non-responders' in D). This figure was created using Microsoft Excel.

Figure 4 illustrates the changes in Uric acid (µg/mL)/total protein (ng/µL) for course of the study. Some participants respond with decreasing Uric acid (Figure 4A, C), while some participants have varied or no changes (Figure 4B, D). The slope indicates the magnitude and direction of change while linear regression analysis (R^2) is used to indicate the relationship between Uric acid/protein and the PBM treatments (Figure 4A, B, Table 1). Participants responding to PBM therapy start with high Uric acid/protein (Figure 4C) whereas non-responders may have varied or lower starting Uric acid/protein (Figure 4D).

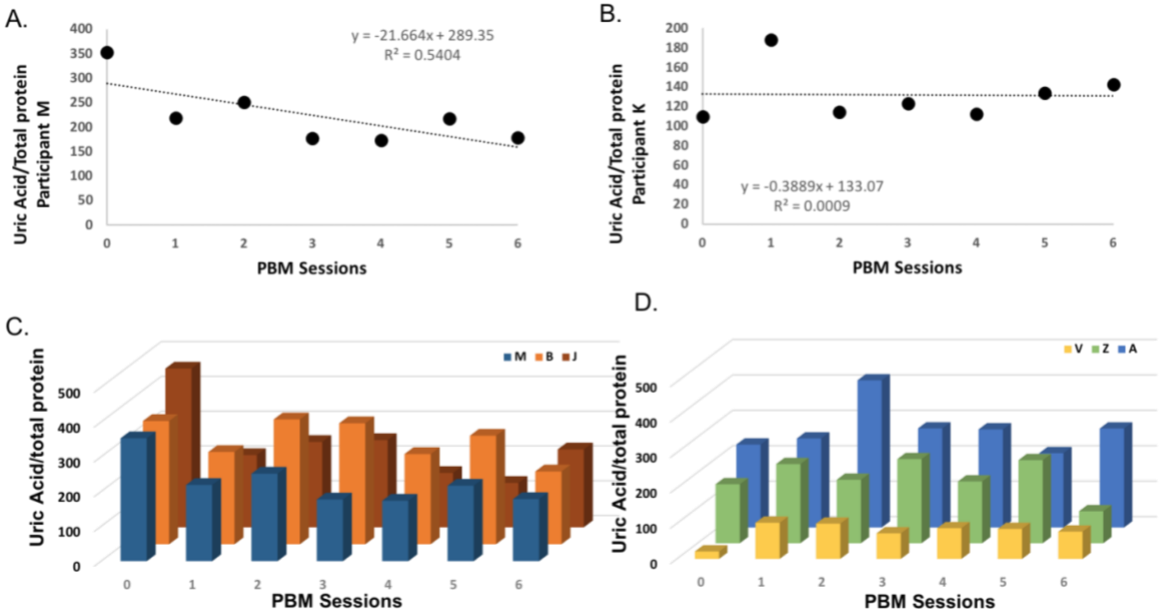

Figure 4. Analysis of Uric Activity 'Responders' vs. 'Non-Responders'. Uric acid/total protein is evaluated over the PBM sessions with A) indicating a 'responder' and B) indicating a non-responder. A linear regression plot is shown with the equation for the line and R^2 value. Three-dimensional histograms showing three participant 'responders' in C) and 'non-responders' in D). This figure was created using Microsoft Excel.

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Table 1. Summary of Participant Averages, Relative Categories, and Changes with PBM0-6

Participant Identifier	**A**	**B**	**C**	**D**	**E**	**F**	**I**	**J**	**K**	**L**	**M**	**N**	**P**	S	**U**	**V**	**W**	**Z**
Ave APEH (uM NA/min)/Protein (mg/mL)	63.5	10.1	47.1	25.0	120.5	52.4	31.1	46.9	50.2	92.3	54.2	166.8	56.7	28.8	92.5	59.1	92.3	74.0
Category (APEH/Prot)	ave	low	low	low	high	ave	low	low	low	high	ave	high	ave	low	high	ave	high	ave
Slope (change with PBM 0-6)	-4.0	**1.3**	-0.5	-1.2	3.5	-1.7	0.2	**12.7**	**9.0**	-0.1	-11.2	-3.4	-0.3	3.8	-0.1	-6.5	1.7	**-8.1**
R^2 (significance)	0.1	**0.4**	0.2	0.1	0.0	0.0	0.0	**0.4**	**0.7**	0.0	0.3	0.1	0.0	0.2	0.0	0.2	0.0	**0.4**
Ave Uric acid (ug/ml)/Protein (mg/mL)	262	287	125	143	162	160	279	202	129	96	209	221	169	105	93	82	165	171
Category (Uric Acid/Prot)	high	high	low	ave	ave	ave	high	high	low	low	high	high	ave	low	low	low	ave	ave
Slope (change with PBM 0-6)	-3.1	**-15.8**	**16.4**	-3.9	-5.5	-3.7	17.0	**-33.0**	-0.4	3.9	**-21.7**	**-34.1**	7.5	-5.0	0.5	4.3	**-22.2**	-7.6
R^2 (significance)	0.0	**0.4**	**0.7**	0.1	0.1	0.2	0.2	**0.5**	0.0	0.2	**0.5**	**0.5**	0.1	0.2	0.0	0.1	**0.5**	0.1

Table 1 summarizes each participant's average APEH/protein, the slope for direction and magnitude of individual changes during the PBM study, and the R^2 value for significance. R^2 values of 0.4 and above are bolded, indicating responders. While an R^2 value of 0.4 does not indicated a strong linear relationship, it does suggest a relationship between PBM treatments and specified biochemical markers (Figure 4A and 5A). Non-responders had R^2 values much less than 0.4, indicating a very unlikely relationship between PBM treatments and the biochemical markers (Figure 3B, 4B, Table 1).

Additionally, Table 1 categorizes participant averages as high, average, or low based on comparison to overall participant averages for APEH activity/protein and Uric acid/protein. The red color indicates 'heightened-risk', since low APEH activity and high Uric acid concentrations are associated with high oxidative stress. Yellow highlights a near-average measurement and green indicates a 'lowered-risk' based on the marker measurement level.

Categorizing The Data

To better understands the 'responders' vs 'non-responders', individual participant measurements were graphed from highest average measurement to lowest average (Figure 5). Participants with the highest Uric acid/protein are on the top left and participants with the highest APEH activity/protein are on the top right. The individual participant measurements from time 0-6 are shown in color-coded histograms and indicated by the arrow on the left of the figure (PBM0→6). Red boxes represent the 'heightened risk' category, yellow boxes indicate participants with measurements close to average, and green boxes suggest a 'lowered-risk' categories for Uric acid/protein and for APEH activity/protein. Individual 'responders' are shown with a black triangle in the direction of change. Most 'responders' fall in the high category (red box) for Uric acid/protein and are decreasing in salivary Uric acid (i.e. negative slope in Table 1). 'Responders' fall in the low category (red box) for APEH/protein and are increasing in salivary APEH activity/protein (i.e. positive slope in Table 1).

Figure 5 and Table 2 illustrate that most 'responders' fall in the high Uric acid/protein category and 'responders' fall in the low APEH activity/protein category. Notably, some participants are responders in both of these categories and are indicated with an asterisk (i.e. Participant J, I, B).

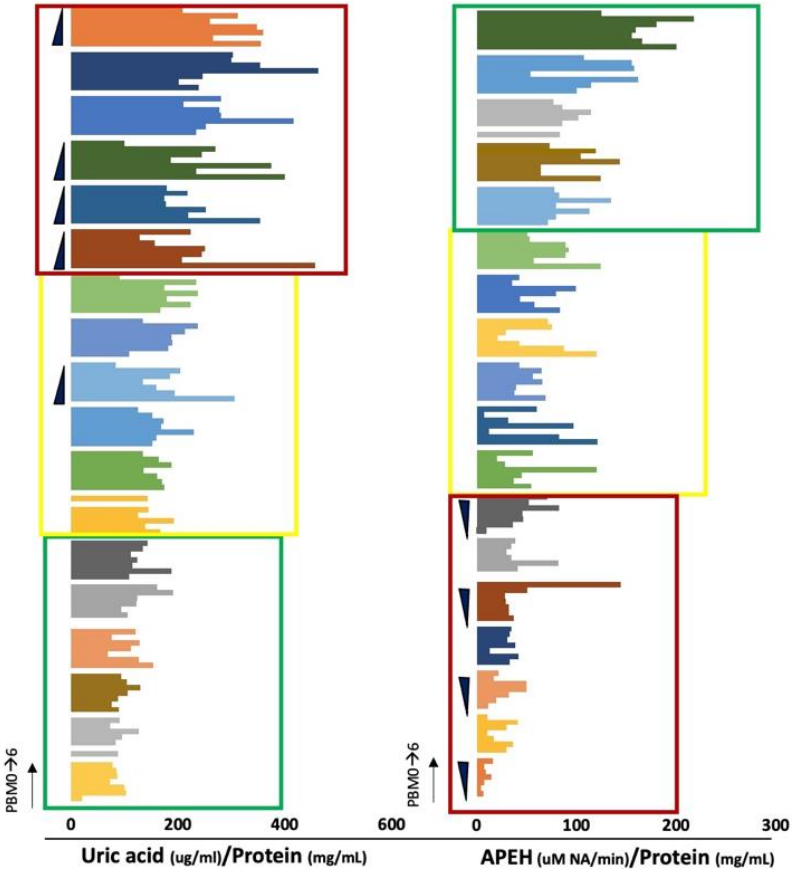

Figure 5. Participant Data Graphed by Category. Participant data is shown by color-coded histograms from PBM0-6 indicated by arrow. Ave participant data is organized from highest (top) to lowest Uric acid/protein (left) and APEH/protein (right). Red boxes indicate participants in 'heightened risk' category, yellow box is average, and green box is 'lowered-risk category'. Participant responders are indicated with black directional triangles. This figure was created using Microsoft Excel.

Table 2. Summary of Participant Data 'Risk' Categories and Where Responders Occur

Participant	Uric acid (ug/ml)/Protein (mg/mL)	Category	Participant	APEH (uM/min)/Protein (mg/mL)	Category
V	82	low	N	170	high
U	93	low	E	120	high
L	96	low	U	92	high
S	105	low	L	92	high
C	125	low	W	92	high
K	129	low	Z	74	ave
D	143	ave	A	63	ave
F	160	ave	V	59	ave
E	162	ave	P	57	ave
W	165	ave	M	54	ave
P	169	ave	F	52	ave
Z	171	ave	K	50	low
J*	202	high	C	47	low
M	209	high	J*	47	low
N	221	high	I*	31	low
A	262	high	S	29	low
I*	279	high	D	25	low
B*	287	high	B*	10	low

Notes: Green cells indicate 'responders', white cells indicate 'non-responders', and orange cells indicate an 'anti-responder' based on an R^2 value > 0.2. Asterisks (*) represent participants who are 'heightened risk' in both categories.

Discussion

In this study, our goal was to investigate whether salivary Uric acid and APEH activity might be biomarkers of physiologic changes occurring with PBM therapy. PBM therapy has been shown to modulate certain salivary biomarkers [11], and this data is consistent with the growing body of literature. While a small number of participant (n=18) data was analyzed, results show that salivary Uric acid and APEH activity have reasonably replicable ranges across the volunteer participants and individually over time to be used in future experiments. Overall, this study illustrated that 5/18 (28%) of the participants respond to PBM therapy by lowering salivary Uric acid/protein levels and 4/18 (22%) of the participants respond to PBM therapy by increasing salivary APEH activity/protein.

However, when participants are categorized by starting levels compared to overall participant averages, 4/6 (67%) of participants with high starting Uric acid/protein respond with decreasing levels upon PBM therapy. Likewise, 4/7 (57%) with low starting APEH activity respond to PBM therapy by increasing APEH activity/protein. This insight may be important, since high Uric acid and low APEH are associated with chronic inflammation and high reactive oxygen species [32-34]. By extension, high Uric acid and low APEH activity may be considered 'heightened-risk' biomarkers. In this work, PBM therapy resulted in favored salivary changes to the categories considered 'heightened-risk'. It is possible that participants that aren't in the 'heightened-risk' category have normal levels of Uric acid and APEH activity, and as such do not exhibit changes in their biomarker levels. The goal of this study was not to determine risk categories of these specific biomarkers, but rather to observe what changes were happening to these biomarkers during the course of the study.

While these results are potentially exciting in showing that PBM therapy can lower inflammation and redox imbalances, a larger and more comprehensive study is required. When extending this study, participants with low salivary APEH activity and high Uric acid should be recruited, similar to participants B, J, and I. While it was beyond the scope of this study to include participant health data, it would be beneficial to include additional health information to further study correlations. This study does

highlight the measurement of salivary APEH activity as a new avenue for assessing APEH. Additionally, a non-PBM therapy control group is required to compare non-intervention salivary changes over the same timeframe.

Conclusions

In this work, a pilot study of salivary biochemical markers was analyzed for changes associated with PBM therapy. Salivary Uric acid, APEH enzyme activity, and total protein were measured and analyzed over time with the following ranges: Uric acid is 65.5 ± 19.5 µg/mL, APEH activity is 26.6 ± 16.1µM/min, and total protein is 430 ± 170 ng/µL. While salivary Uric acid and total protein have been measured before, APEH activity is a novel salivary marker. 67% of participants with high starting Uric acid had a reduction in salivary Uric acid over the six PBM sessions. 57% of participants with low starting APEH activity had an increase in salivary APEH activity over the six PBM sessions. While a larger and more comprehensive study is required to make statistically sound conclusions, this pilot study suggests that PBM therapy may have beneficial outcomes to oxidative stress markers, thereby providing an important physiological response to PBM therapy.

List of Abbreviations Used

AANA: acetylalanine *p*-nitroanilide
APEH: acyl peptide enzyme hydrolase
NA: 4-nitroanilide
PBM: photobiomodulation

Conflicts of Interest

UMD and TMC declare that they have no conflict of interests. CHW is the manager of The Wellness Center, a business that provides red-light therapy. The Wellness Center collaborated on this project to provide saliva samples to researchers at Weber State University. CHW was not involved in the research experiments nor data analysis.

Ethics Approval and/or Participant Consent

This work received Institutional Review Board approval from Weber State's College of Science IRB. The title of the approved study is: Investigating oxidative stress markers in saliva following photobiomodulation, approval number IRB-AY22-23-344, approval date 05-17-2023. Participants were provided an informed consent document prior to volunteering for the study.

Authors' Contributions

UMD: Validated and performed experiments, analyzed data, made figures, revised manuscript, and gave final approval of the version to be published.
CHW: Contributed to study design and planning, assisted with participant recruiting, collected the saliva samples, and gave final approval of the version to be published.
TMC: Contributed to study design, wrote IRB, assisted with experiments and data analysis, drafted and revised manuscript, and gave final approval of the version to be published.

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