

INVESTIGATING ECCRINE SWEAT AS A NONINVASIVE BIOMARKER RESOURCE

by

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ABSTRACT

Background: Recent interests in continuous biomonitoring and the surge of wearable biotechnology demand a better understanding of sweat as a noninvasive biomarker resource. The ability to use sweat as a biofluid provides the opportunity for noninvasive early and continuous diagnostics. This thesis serves to help fill the existing knowledge gap in sweat biomarker discovery and applications.

Experimental Design: In part one of this study, exercise-induced eccrine sweat was collected from 50 healthy individuals and analyzed using mass spectrometry, protein microarrays, and quantitative ELISAs to identify a broad range of proteins, antibody isotypes, and cytokines in sweat. In part two of this study, cortisol and melatonin levels were analyzed in exercise-induced sweat and plasma samples collected from 22 individuals.

Results: 220 unique proteins were identified by shotgun analysis in pooled sweat samples. Detectable antibody isotypes include IgA (100% positive; median $1230 \pm 28\,700$ pg/mL), IgD (18%; 22.0 ± 119 pg/mL), IgG1 (96%; 1640 ± 6750 pg/mL), IgG2 (37%; 292 ± 6810 pg/mL), IgG3 (71%; 74.0 ± 119 pg/mL), IgG4 (69%; 43.0 ± 42.0 pg/mL), and IgM (41%; 69.0 ± 1630 pg/mL). Of 42 cytokines, three were readily detected in all sweat samples ($p < 0.01$). The median concentration for interleukin-1 α was 352 ± 521 pg/mL, epidermal growth factor was 86.5 ± 147 pg/mL, and angiogenin was 38.3 ± 96.3 pg/mL. Multiple other cytokines were detected at lower levels. The median and standard deviation of cortisol was determined to be 4.17 ± 11.1 ng/mL in sweat and 76.4 ± 28.8 ng/mL in plasma. The correlation between sweat and plasma cortisol levels had an R-squared value of 0.0802 (excluding the 2 highest sweat cortisol levels). The median and standard deviation of melatonin was determined to be 73.1 ± 198 pg/mL in sweat and 194 ± 93.4 pg/mL in plasma. Similar to cortisol, the correlation between sweat and plasma melatonin had an R-squared value of 0.117.

Conclusion: These studies suggest that sweat holds more proteomic and hormonal biomarkers than previously thought and may eventually serve as a noninvasive biomarker resource. These studies also highlight many of the challenges associated with monitoring sweat content including differences between collection devices and hydration, evaporation losses, and sweat rate.

TABLE OF CONTENTS

CHAPTER		
1	INTRODUCTION	1
2	IMMUNE BIOMARKERS IN ECCRINE SWEAT	2
3	STRESS AND SLEEP HORMONES IN ECCRINE SWEAT	3
4	CONCLUSIONS AND FUTURE DIRECTIONS	4
REFERENCES		5

Chapter 1

INTRODUCTION

Eccrine and apocrine glands in the epidermis produce sweat, a clear and hypotonic biofluid ^[1]. Sweat is slightly acidic (pH 5.5 – 6.5) and is composed of mainly water, containing electrolytes (e.g. sodium, chloride, and potassium), urea, pyruvate, lactate, and peptides. In lower concentrations, antigens, antibodies, cytokines, and some xenobiotics (e.g. drugs, cosmetics, and ethanol) are also present ^[2]. These substances are stored in eccrine and apocrine glands, secreted into the sweat, and transported through a sweat pore to the epidermal surface. Partial reabsorption of sodium and chloride occurs during transportation in the reabsorptive duct ^[3]. Disease states can change sweat composition by altering the concentration of common components or reporting new components that may function as biomarkers for a given disease ^[4].

Historically, eccrine sweat has been used to diagnose cystic fibrosis in infants and children by measuring the chloride and sodium content via pilocarpine stimulated iontophoresis. Because cystic fibrosis is the result of a defective chloride membrane transporter, reduced transport of chloride and sodium occurs in the reabsorptive duct leading to saltier sweat ^[5]. Beyond electrolyte monitoring, sweat has not been commonly used as a biofluid for disease detection. The contents of sweat and the mechanisms of biomarker transport from blood and interstitial fluid remain relatively unexplored compared with other diagnostic biofluids.

Recent interests in continuous biomonitoring and the surge of wearable biotechnology demand a better understanding of sweat as a noninvasive biomarker resource. The ability to use sweat as a biofluid provides the opportunity for noninvasive early and continuous diagnostics. Noninvasive sampling is also critical for frequent sampling from neonates or elderly individuals and avoiding infections to patients who need daily analysis. Sweat shows promise as a biofluid

providing a source of plasma proteins, interstitial proteins, and metabolites. Due to the nominal presence of impurities, sample preparation of sweat is simpler than other biofluids. Studies have also indicated that sweat samples can also be stored for long periods of time making it relatively stable for analyte detection.

This thesis serves to fill the existing knowledge gap in sweat biomarker discovery and applications. Chapter 2 presents a broad proteomic analysis of eccrine sweat, mainly focusing on immune biomarkers. Chapter 3 presents a study on cortisol and melatonin levels in tandem sweat and plasma samples. These preliminary studies aim to demonstrate the potential of sweat as a diagnostic biofluid and help inform the design and development of wearable biosensors.

Chapter 2

IMMUNE BIOMARKERS IN ECCRINE SWEAT

2.1 Introduction

The identification and validation of immune biomarkers in eccrine sweat may allow researchers, clinicians, and epidemiologists to monitor patients without the need for laborious hospital visits and delays in disease diagnosis and management. Several studies have evaluated the presence of immune biomarkers, such as certain antibody isotypes and cytokines, in the sweat of healthy and diseased individuals. Antibody isotypes reported in sweat include IgA, IgE, and IgG [6-8]. Secretory IgA was detected in sweat of healthy individuals. Allergen-specific IgE was detected in sweat of patients with atopic dermatitis, and Hepatitis B-specific IgG was detected in sweat of Hepatitis B seropositive patients. Cytokines reported in sweat of healthy individuals include IL-1 α , IL-1 β , IL-6, IL-8, IL-31, TNF- α , and TGF- β [9, 10]. Additionally, mass spectrometry-based proteomic studies report an abundance of apolipoprotein D, clusterin, prolactin-inducible protein, and serum albumin in eccrine sweat [11].

Proteomic analyses also suggest that other immune-related, proteomic biomarkers in sweat may correlate with certain disease states. The P.O.W.E.R. study detected elevated levels of neuropeptide Y, substance P, and calcitonin gene-related peptide in the sweat of premenopausal women with major depressive disorder in remission [12]. Another proteomic analysis of pooled sweat samples from patients with schizophrenia showed a 2-fold or greater concentration of kallikrein, prostatic-binding protein, and thioredoxin compared with controls [13]. And finally, Adewole et al demonstrated 26 proteins to be uniquely detected in pooled sweat samples from patients with active tuberculosis [14]. However, the study of sweat-based immune biomarkers is still in its infancy compared with that of other biofluids, such as blood, urine, and saliva.

In this study, exercise-induced eccrine sweat was collected from 50 healthy individuals and analyzed using mass spectrometry, protein microarrays, and quantitative ELISAs to identify a broad range of proteins, antibody isotypes, and cytokines in sweat. LC-MS/MS was used to perform a global proteomic analysis on pooled sweat samples from 10 healthy individuals. To establish the feasibility of utilizing sweat as a source of biomarkers, antibody isotypes were measured in individual sweat samples using quantitative antibody isotyping microarrays. Expanding upon previous work that demonstrates the presence of cytokines in sweat, 42 cytokines were probed for in sweat using semi-quantitative cytokine microarrays. Based on the results of the cytokine arrays, interleukin-1 α (IL-1 α), epidermal growth factor (EGF), and angiogenin (ANG) were selected for further analysis by quantitative ELISA. This study provides quantitative data on specific antibody isotypes and cytokines present in eccrine sweat.

2.2 Experimental Procedures

2.21 Sweat Collection.

16 male and 34 female individuals (age range: 18 – 69) were recruited into the study. Informed, written consent was obtained from all participants in accordance with the ASU IRB. Sweat samples were collected using the Macroduct sweat collection device (WESCOR, Logan, UT) and the PharmChek[®] sweat patch (PharmChem, Fortworth, TX). The volar region of each participant's forearm was first cleaned with 70% ethanol. The sweat collection devices were then fitted to each forearm following the manufacturer's instructions. Each participant was instructed to perform 30 – 60 min of moderate aerobic exercise indoors (stationary bike, treadmill, or elliptical). Following aerobic exercise, the sweat collection devices were removed. Sweat samples were immediately de-identified, processed, and stored at -20°C.

Samples collected via the Macroduct required liquid transfer into a microcentrifuge tube. The samples were centrifuged for 5 min at 5,000 rpm in order to remove shed skin cells and cellular debris and were stored at -20°C until further analysis. Samples collected via the PharmChek[®] sweat patch required centrifugation to extract the liquid sample and remove shed skin cells and cellular debris. Each patch was placed in a 15 mL tube and centrifuged for 5 min at 6,000 rpm. The extracted sample was transferred to a microcentrifuge tube and stored at -20°C until further analysis.

For mass spectrometry analysis, two pooled sample groups, collected using the Macroduct sweat collection device, were created by randomly selecting 5 samples from each the male (Group 1) and female (Group 2) individuals. The samples were pooled together to make Group 1 and Group 2. 100 µL of each liquid sample, in Group 1 and Group 2, were desalted on a 0.5 mL Zeba[™] Desalt Column (Thermo Fisher Scientific). The protein concentration of each sample was determined using a Pierce[™] BCA Protein Assay and 15 µg equivalent volumes were lyophilized and stored at -80°C. All pooled samples were prepared for mass spectrometry analysis by rehydration with 200 mM Tris pH 8.2. The rehydrated samples were run on a 4 – 20% Criterion[™] TGX[™] precast gel (Bio-Rad Laboratories, Hercules, CA) and stained with Bio-Safe[™] Coomassie Blue (Bio-Rad Laboratories). Samples were then destained with 50 mM Tris pH 8.1/50% acetonitrile and reduced with 50 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP)/50 mM Tris pH 8.2. Alkylation of samples was performed with 50 mM Iodoacetamide/50 mM Tris pH 8.2 at room temperature in the dark for 30 min. Overnight enzymatic digestion was performed at 37°C on 50 µL samples with 0.0025 µg/µL sequencing grade trypsin (Promega, Madison, WI) in 20 mM Tris pH 8.2. Digestion was quenched with addition of 0.2% TFA and desalting was performed with C-18 Zip Tips (Millipore, Burlington,

MA). Finally, samples were dried in a vacuum concentrator and reconstituted in 5 μ L of 0.2% formic acid.

2.22 Mass Spectrometry.

The pooled extracts were concentrated, and the proteins were identified by nano-flow liquid chromatography electrospray tandem mass spectrometry (nano LC-ESI-MS/MS) using a Thermo Scientific Q-Exactive Plus Mass Spectrometer (Thermo Fisher Scientific) coupled to a Thermo Ultimate 3000 RSLC nano HPLC system. The digested peptide mixture was loaded onto a 250 nL OPTI-PAK trap (Optimize Technologies, Oregon City, OR) custom packed with Michrom Magic C18, 5 μ m solid phase (Michrom Bioresources, Auburn, CA). Chromatography was performed using 0.2% formic acid in both the A solvent (98% water/2% acetonitrile) and B solvent (80% acetonitrile/10% isopropanol/10% water), and a 5% B to 45% B gradient over 78 min at 400 nL/min through a PicoFrit (New Objective, Woburn, MA) 100 μ m x 35 cm column hand-packed with Agilent Poroshell 120 EC C18 packing. The Q-Exactive mass spectrometer experiment was a data dependent set up with a MS1 survey scan from 340 – 1500 m/z at resolution 70,000 (at 200 m/z), followed by HCD MS/MS scans on the top 15 ions having a charge state of +2, +3, or +4, at resolution 17,500. The ions selected for MS/MS were placed on an exclusion list for 30 s. The MS1 AGC target was set to 1.0×10^6 and the MS2 target was set to 1.0×10^5 with max ion inject times of 50 ms for both.

2.23 Bioinformatics and Gene Ontology Analysis.

Tandem mass spectra were searched against the Uniprot human database, version Oct2016, using the MASCOT database search engine (Matrix Science, Boston, MA). To

determine gene ontological annotations for the selected proteins, we used the Gene Ontology Consortium, PANTHER Classification System^[15].

2.24 Antibody Isotyping.

Antibody isotyping was performed on all 49 sweat samples collected via the Macroduct collection device using the Quantibody Human Ig Isotyping Array 1 (RayBiotech, Norcross, GA) according to the manufacturer's instructions with the following modifications. On day 1, each slide was brought to room temperature and allowed to dry for 1 hr. 100 μ L of blocking buffer was added to each well and allowed to incubate for 30 min at room temperature. Sweat samples and standards were diluted in blocking buffer (10 μ L of sweat into 75 μ L of blocking buffer). The blocking buffer was removed from the arrays, and the diluted samples and standards were added to each well. The slides were incubated for 16 hrs on a rocking shaker at 4°C. On day 2, the slides were washed according to the manufacturer's instructions. Eighty μ L of diluted biotinylated anti-human Ig's were added to each well and incubated for 16 hrs on a rocking shaker at 4°C. On day 3, the slides were washed according to the manufacturer's instructions. Eighty μ L of diluted Cy3 equivalent dye-conjugated Streptavidin was added to each well and incubated for 2 hrs on a rocking shaker at room temperature. Slides were washed, the gaskets removed, and the slides were dried using filtered compressed air. All slides were imaged using a Tecan Power Scanner (Tecan, Männedorf, Switzerland) and analyzed using Array-Pro Analyzer (Meyer Instruments, Houston, TX).

Each slide contained 16 identical subarrays, in which human antibody isotypes (IgA, IgD, IgE, IgG1, IgG2, IgG3, IgG4, and IgM) were printed in quadruplicate. Prior to data analysis, each array was normalized by removing the background signal estimated by the first quartile of

the nonspots and taking the log-transforming median-scaled raw intensities to bring the data to the same scale and stabilize the variance across the range of signals. One subarray on each slide was used to calculate the standard curve for each antibody isotype and fitted to a 2-parameter polynomial curve. For each participant, the quadruplicate spots were averaged and the standard deviation was calculated. The mean intensity values were used to determine the concentration of each antibody isotype present.

2.25 Cytokine Arrays.

Cytokine profiling was performed using the Human Cytokine Array G-Series 3 (RayBiotech) according to the manufacturer's instructions with the following modifications. Due to limited sample volumes, 16 sweat samples collected via the Macroduct collection device and 14 sweat samples collected via the PharmChek[®] sweat patch were analyzed. On day 1, each slide was brought to room temperature and allowed to dry for 1 hr. 100 μ L of blocking buffer was added to each well and allowed to incubate for 30 min at room temperature. Sweat samples were diluted in blocking buffer (15 μ L of sweat into 75 μ L of blocking buffer). The blocking buffer was removed from the arrays, and the diluted samples were added to each well. The slides were incubated for 16 hrs on a rocking shaker at 4°C. On day 2, the slides were washed according to the manufacturer's instructions. 70 μ L of diluted biotinylated anti-cytokines was added to each well and incubated for 16 hrs on a rocking shaker at 4°C. On day 3, slides were washed according to the manufacturer's instructions. 70 μ L of diluted Streptavidin-Fluor was added to each well and incubated for 16 hrs on a rocking shaker in the dark at 4°C. Slides were washed, the gaskets removed, and the slides were dried using filtered compressed air. All slides were imaged using a Tecan Power Scanner and analyzed using Array-Pro Analyzer.

Each slide contained 8 identical microarrays in which 42 unique human cytokines were printed in duplicate. Prior to data analysis, each array was first normalized to 6 positive control spots on the first subarray. The duplicate cytokine spots and 14 negative control spots on each subarray were averaged and standard deviations calculated. Mean intensity values were considered positive if they were 2 standard deviations above the mean negative control intensity. An unpaired t-test was performed for each cytokine and the cut off value to identify positive cytokines.

2.26 Quantitative ELISAs for IL-1 α , EGF, and ANG.

Sweat samples collected via the Macroduct device were selected based on sufficient sample volume and high cytokine array signals for IL-1 α (n=16), EGF (n=6), and ANG (n=7) quantitative analysis. The Human IL-1 alpha Platinum ELISA kit (eBioscience, San Diego, CA), Human EGF Immunoassay Kit (Invitrogen, Carlsbad, CA), and Human ANG ELISA Kit (Thermo Fisher Scientific) were used for the quantitative ELISAs. Assays were performed according to the manufacturer's instructions with the following modifications. All samples and standards were performed in duplicate. Sweat samples and standards were diluted 1:10 in the sample diluent. The standard concentrations for each quantitative ELISA ranged from 200 – 1 pg/mL for IL-1 α , 250 – 1 pg/mL for EGF, and 400 – 1 pg/mL for ANG. 75 μ L of each diluted sweat sample and standard were dispensed into the microtiter plate and incubated according to the manufacturer's instructions. No sample controls served as the background control, which were subtracted from the sample and standard wells. The mean intensity values of the standards were fit to a 4-parameter logistic regression to generate a standard curve. The standard curve was used

to calculate the concentrations of the unknowns. All samples' duplicate OD values were within 15% of the mean; thus, no data points were omitted from the study.

2.3 Results and Discussion

2.31 Identification of proteins by Mass Spectrometry.

Two pools of sweat samples from 5 male participants (Group 1) and 5 female participants (Group 2) collected via the Macroduct were analyzed by LC-MS/MS. Due to the limited volume of sweat and the low overall protein abundance in individual samples, it was necessary to pool sweat samples for analysis. This resulted in two separate sample sets with equal total protein amount. A total of 311 proteins were identified in the male set and 189 proteins in the female set using a minimum of 2 peptides identified per protein. 93% of the proteins identified between the two sample sets were the same, indicating a strong degree of similarity between the male and female sweat proteome. Only minor discrepancies were observed in certain peptides and proteins between the two groups in this limited analysis (Figure 2.31B).

To classify the proteins identified through LC-MS/MS, gene ontology analysis was performed on the combined list of identified proteins using the publicly available classification system, PANTHER (Protein Analysis Through Evolutionary Relationships). Of the 334 proteins submitted, 220 fit the criteria for classification using the PANTHER system "biological process" (Figure 2.31A). Similar to previous reports, high abundance of proteins involved in metabolic process (40.4%), cellular process (39.5%), and cellular component organization or biogenesis (15.9%) was observed (Figure 2.31A). Within the "biological process," 22 (10%) proteins were identified as responding to stimulus, of which the majority are involved in innate or adaptive immune responses (Figure 2.31A).

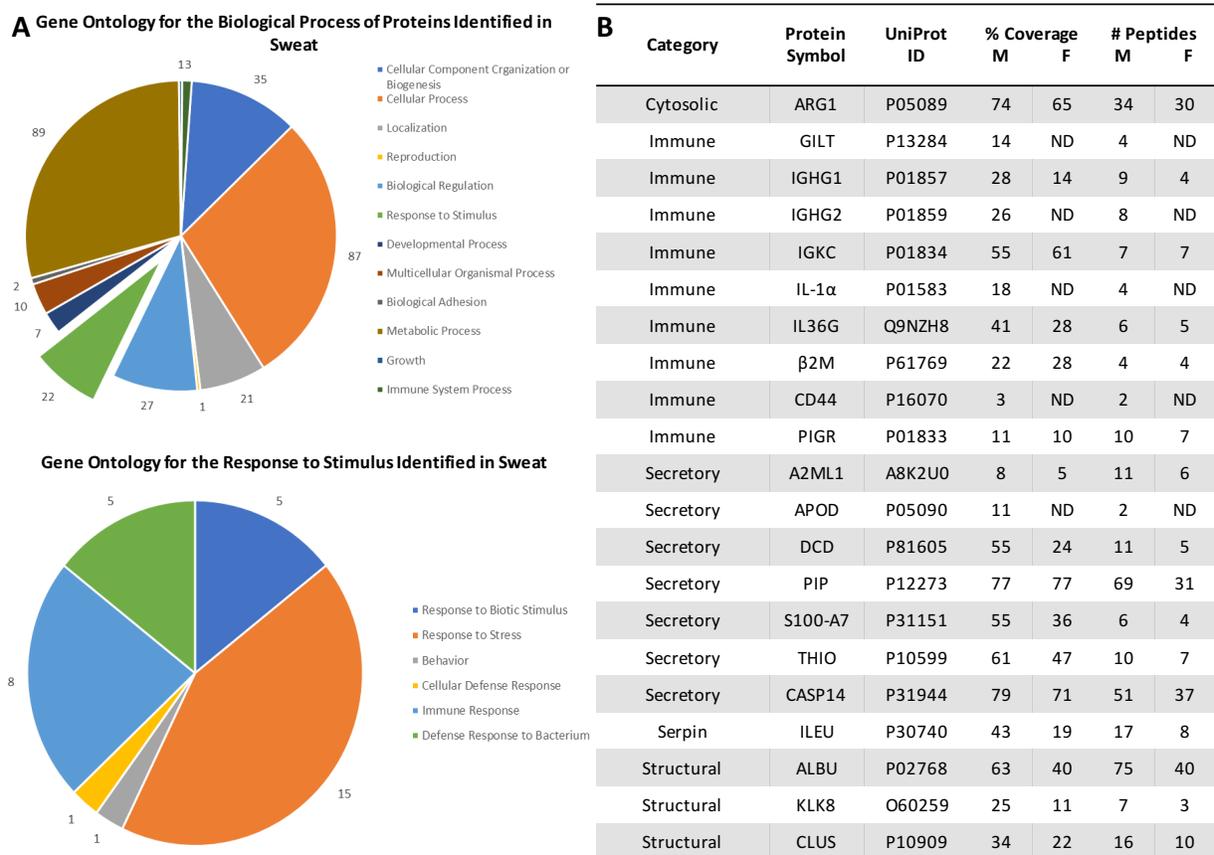


Figure 2.31. Gene ontology analysis for the biological function of proteins identified in sweat. Sweat samples from 10 individuals (5 males and 5 females) were pooled by gender and evaluated by LC-MS/MS. (A) Detected proteins were categorized based on biological processes and response to stimuli using gene ontology analysis. (B) The sweat proteome contains a broad array of proteins, including innate and adaptive immune markers. ND means the protein was not detected.

In previous sweat proteomic studies, the sweat proteomes of males and females, both healthy and diseased, were compared to serum in an effort to understand the differences in the two biofluids [16]. The most significant difference between serum and sweat were reported in the categories of catalytic activity, enzyme regulation, and structural molecular activity. It was also observed that there was a significantly higher amount of proteins involved in structural molecular activity. A similar pattern was observed in this study as highlighted by glutaredoxin-1, peroxiredoxin-1, and quiescin sulfhydryl oxidase 1 [11, 13, 14, 16] (Figure 2.31B). In addition, the detection of membranous, cytoskeletal, microsomal, mitochondrial, ribosomal, and nuclear proteins in eccrine sweat support the hypothesis that proteins are secreted into sweat in a merocrine-like manner, including abundant defense proteins, such as, apolipoprotein D,

dermcidin, and prolactin-inducible protein [11, 13, 14]. These results confirm the findings of previous studies and support the conclusion that these collection methods provide material representative of sweat proteomes.

2.32 Quantitative Analysis of Antibody Isotypes.

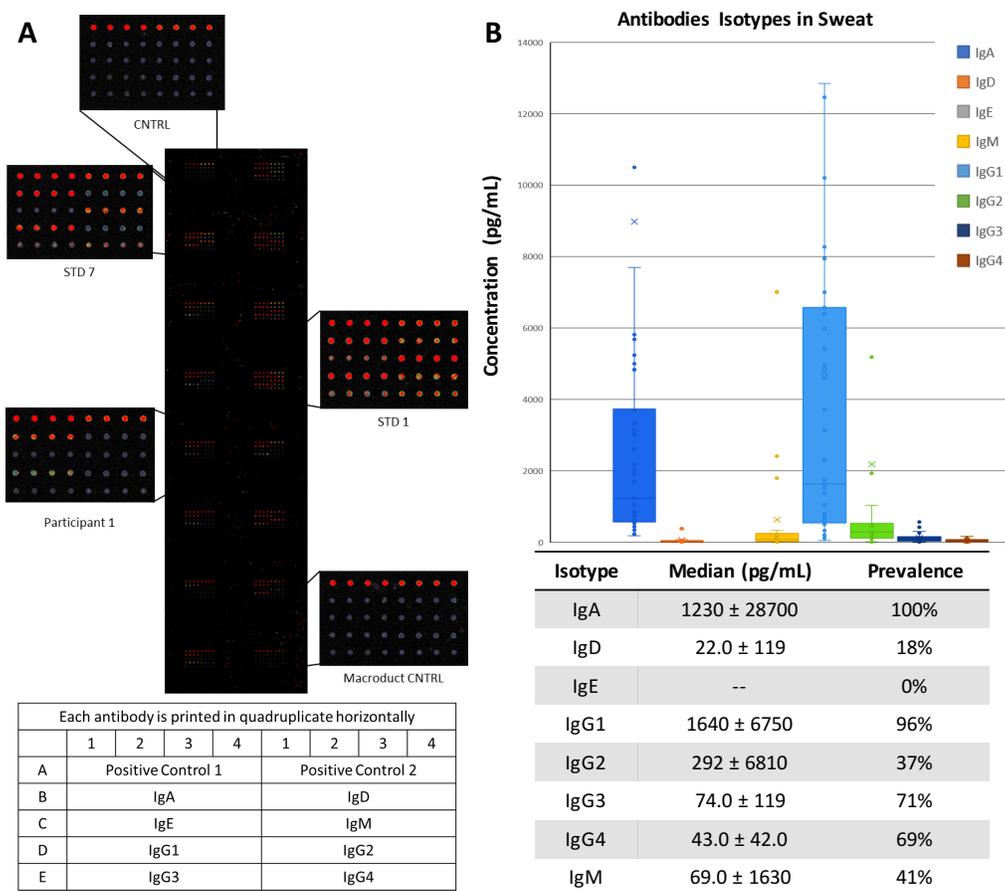


Figure 2.32. Antibody isotyping. Sweat from 50 healthy individuals were evaluated for the concentration of human antibody (Ig) isotypes. (A) An example antibody isotyping array is presented displaying the high (STD 1) and low (STD 7) concentration standards, background control sample (CNTRL), Macroduct collection device control sample (Macroduct CNTRL), and a representative participant image (Participant 1). (B) The box and whisker plot represents the median, interquartile range, and range for each isotype. The table represents the median concentrations of isotypes with the standard deviations and prevalence among the participant group. The antibody isotypes quantified were IgA, IgD, IgE, IgG1, IgG2, IgG3, IgG4, and IgM.

To determine the concentration of the antibody isotypes in the eccrine sweat of 49 participants, samples were run on human antibody isotyping arrays from RayBiotech containing IgA, IgD, IgE, IgG1, IgG2, IgG3, IgG4, and IgM (Figure 2.32A). All samples analyzed were

collected via the Macroduct collection device. Each antibody isotype was printed in quadruplicate, and standards were run on each individual slide to determine the concentration of each antibody isotype. All antibody isotypes except IgE were detected in sweat (Figure 2.32B). A sample was considered positive if the mean raw value was 2 standard deviations higher than the mean background control (Macroduct Control, Figure 2A). The antibody isotypes IgA (100% positive; median 1232 ± 28700 pg/mL; range 37843-1832631.8 pg/mL) and IgG1 (96%; 1639 ± 6746 pg/mL; range 14246-380735.5 pg/mL) were present at significantly high levels in majority of individuals regardless of ethnicity, gender, or age. The concentration of antibody isotypes IgD (18%; 22 ± 119 pg/mL; range 13873-16802 pg/mL), IgM (41%; 69 ± 1627 pg/mL; range 10548.8-185040.5 pg/mL), IgG2 (37%; 292 ± 6806 pg/mL; range 13472-30549.8 pg/mL), IgG3 (71%; 74 ± 119 pg/mL; range 8646.5-15444.3 pg/mL), and IgG4 (69%; 43 ± 42 pg/mL; range 10548.8-185040.5 pg/mL) were widely variable. These results demonstrate that all antibody isotypes, except IgE, were detectable in eccrine sweat.

Previous studies have detected IgA, IgE, and IgG antibodies secreted in sweat. These results required large volumes of sweat and have detected vastly different concentrations among men and women ^[1]. Okada et al demonstrated the presence of IgA in the sweat of healthy men (13 ± 0.9 μ g/mL) and women (1.6 ± 0.9 μ g/mL) ^[6]. This nearly ten-fold difference in sweat secreted IgA levels between men and women was not observed in our study. Additionally, while IgE was not detected in our study, another group had previously reported the IgE to be secreted in the sweat of patients with atopic dermatitis at concentrations ranging from 1.0 – 75.5 ng/mL ^[17]. Lastly, one study even showed Hepatitis B-specific IgG to be in eccrine sweat and eccrine glands of seropositive patients ^[7]. Taken together, these preliminary studies suggest that IgG and

IgA levels in eccrine sweat, in particular, may be sufficient to measure the seroconversion of patients with infectious diseases.

2.33 Identification of Human Cytokines.

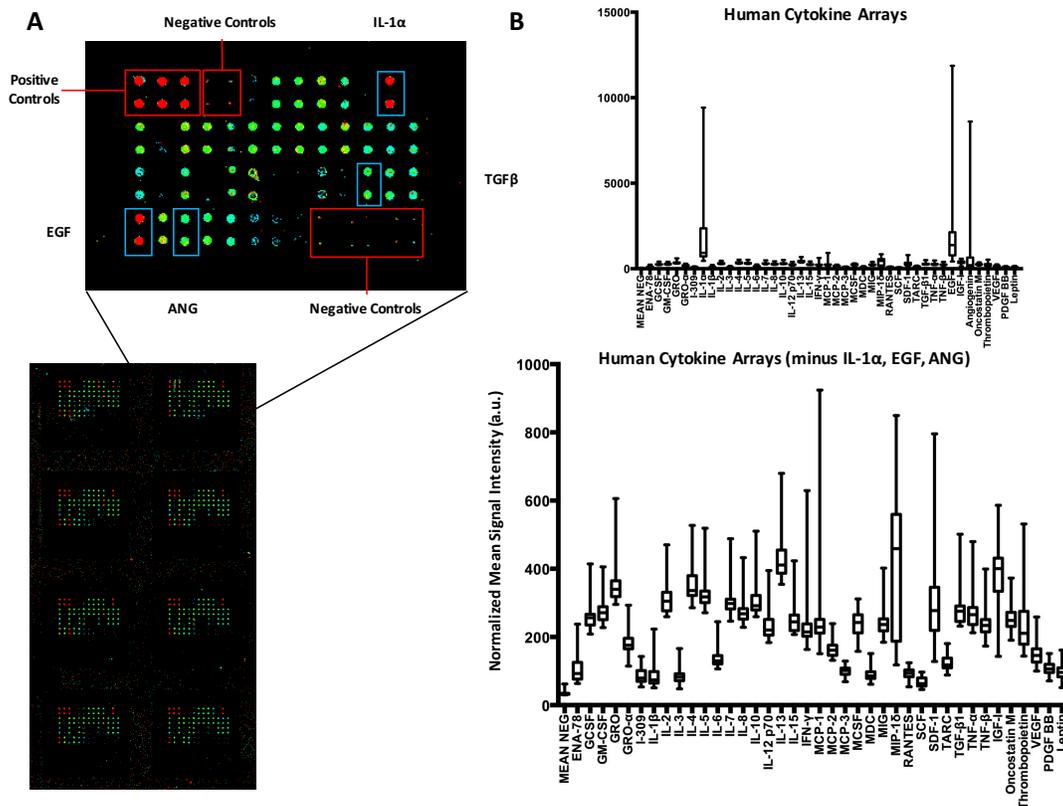


Figure 2.33. Human cytokine antibody arrays. Human cytokine arrays were used to probe for 42 cytokines in 30 human sweat samples (16 Macroduct samples, 14 PharmChek® samples). (A) The intensities of different cytokines (red – high, green – low) were quantified using ArrayPro Analyzer and normalized to the intensities of the internal positive controls for signal comparison. (B) The normalized mean signal intensities for each cytokine and the negative control spot were plotted for each participant as a box and whisker plot representing the median, interquartile range, and range. The top graph represents all 42 cytokines and the bottom graph represent 39 of the cytokines excluding IL-1 α , EGF, and ANG.

42 human cytokines were probed for in 30 sweat samples using the Human Cytokine Array G-Series 3. Of the 30 samples, 16 samples were collected via the Macroduct and 14 collected via the PharmChek®. The arrays indicated whether a cytokine was present or not but not absolute concentration, and 42 different human cytokines were printed in duplicate on the arrays (Figure 2.33A). Cytokines were considered positive if the mean signal intensity was two standard deviations above the negative control signal intensity. IL-1 α ($p < 0.01$), epidermal

growth factor ($p < 0.01$), and angiogenin ($p < 0.01$) were identified as positive for all sweat samples. Other human cytokines detectable on the arrays included IL-2, IGF-1, IL-13, IFN- γ , and MIP-1 δ ($p < 0.01$) (Figure 2.33B).

Few studies have broadly evaluated cytokines in sweat. Marques-Deak et al and Dai et al performed extensive studies on identifying cytokines and immune response proteins in sweat. Marques-Deak et al identified IL-1 α , IL-1 β , IL-6, IL-8, TNF- α , and TGF- β in sweat samples from 9 healthy women collected via a sweat patch^[10]. Dai et al also identified IL-1 α , IL-1 β , and IL-31 in 11 healthy volunteers collected via tissue paper^[9]. These human cytokine array results validate those findings as IL-1 α , IL-1 β , IL-6, IL-8, IL-31, TNF- α , and TGF- β were all considered positive on the arrays. Further, our results identify additional human cytokines in sweat, including EGF, ANG, IL-2, IGF-1, IL-13, IFN- γ , MCP-1, MIP-1 δ , SDF-1, and IGF-1. These results indicate that sweat contains a wider range of inflammatory and innate immune response proteins than previously observed.

2.34 Quantitative Analysis of IL-1 α , EGF, and ANG cytokines.

To determine the concentration of IL-1 α , ANG, and EGF, participant samples with sufficient sample volume and high signal intensities on the cytokine arrays to perform on quantitative ELISAs (Figure 2.34). 16 Macroduct samples were evaluated for human IL-1 α on a quantitative ELISA. The median concentration was 352 ± 521 pg/mL. IL-1 α is a cytokine that is part of the interleukin 1 family, which is responsible for producing inflammatory responses, fever, and sepsis. IL-1 α has also been investigated as a biomarker in serum for neurodegenerative disorders and alcohol abuse, and in saliva for oral lichen planus, an inflammatory condition of the oral cavity^[18]. In previous studies, Marke-Deak et al reported a

mean concentration of 7.6 ± 3.4 pg/mL of IL-1 α in sweat samples of 9 healthy participants determined by recycling immunoaffinity chromatography [8]. In contrast, our study demonstrates the detection of IL-1 α in a broad range of samples using a standard ELISA.

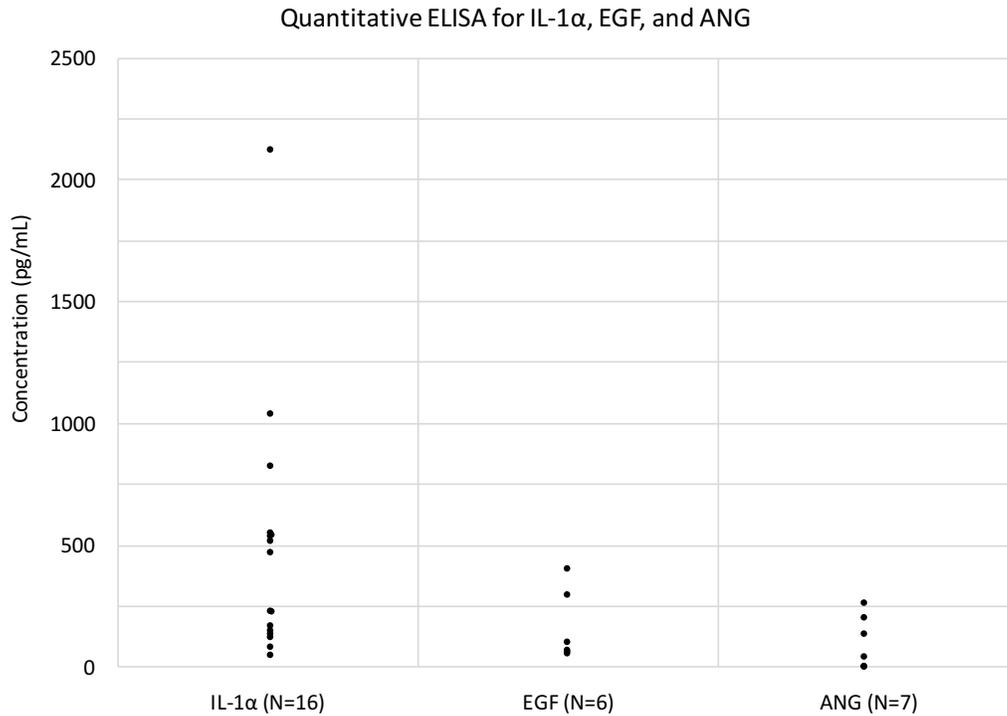


Figure 2.34. Quantitative human cytokine ELISAs for IL1 α , EGF, and ANG. Macroduct sweat samples with high signal intensities on the cytokine arrays and sufficient volumes were analyzed on quantitative ELISAs for IL-1 α , EGF, and ANG. The median concentrations were 352 ± 521 pg/mL for IL-1 α (n=16), 86.5 ± 147 pg/mL for EGF (n=6), and 38.3 ± 96.3 pg/mL for ANG (n=7). Of the 7 samples tested for ANG, 3 sample results were below the detectable range of the standard curve and thus, included as the LOD of the commercial ELISA (1.5 pg/mL) in the data summary.

To determine the concentration of EGF, 6 Macroduct samples were probed for EGF using a quantitative ELISA. The median concentration was 86.5 ± 147 pg/mL. EGF stimulates cell growth and has been associated with interstitial cystitis in urine specimens [19]. Serum EGF levels have also been found to be elevated in active psoriasis vulgaris (mean 323.0 vs. 36.6 pg/mL) [20]. For ANG detection, 7 Macroduct sweat samples were evaluated. Three samples were below the detectable range of the standard curve and thus included as the lower limit of detection (1.5 pg/mL) in the data summary. The median concentration was 38.3 ± 96.3 pg/mL. Elevated ANG levels in serum have been associated with heart failure with preserved ejection fraction, bladder

carcinoma, and ALS, and ANG expression in prostatic epithelial cells have been shown to increase during transition from benign to invasive prostate cancer ^[21, 22]. ANG levels in plasma have been reported to be 308 ng/mL of 208 healthy control participants ^[22]. To our knowledge, this study is the first to demonstrate EGF and ANG in eccrine sweat. The ability to monitor IL-1 α , EGF, and ANG in real-time may provide a valuable diagnostic metric in specific disease settings.

Chapter 3

STRESS AND SLEEP HORMONES IN ECCRINE SWEAT

3.1 Introduction

Hormones regulate circadian rhythms, metabolism, and the immune system. Understanding how these biomolecules fluctuate on a regular basis via continuous monitoring in sweat may provide meaningful insight to a person's health and well-being. Commonly monitored hormones include stress and sleep hormones—cortisol and melatonin, respectively. Cortisol, a steroid hormone, is released by the adrenal cortex in response to stress or low blood sugar. It functions to increase blood sugar through gluconeogenesis, suppress the immune system, and aid in metabolism ^[23]. Cortisol serum levels generally peak in the early morning and decline throughout the day; they are also used to diagnose Addison's disease (hypocortisolism) and Cushing's syndrome (hypercortisolism) ^{[24] [25]}. Melatonin, a derivative of tryptophan, is released by the pineal gland and controls circadian rhythms including sleep-wake timing, blood pressure, and seasonal reproduction ^[26]. Its levels peak during the night or sleep and serve as a biomarker for circadian dysregulation ^[27]. The ability to detect cortisol and melatonin in sweat may allow for real-time monitoring of sleep patterns, stress, immune-compromised states, and metabolic fluctuations without the need of a laboratory test.

However, these hormones are traditionally measured only in blood, saliva, and urine. Russell et al reported sweat cortisol levels in healthy subjects within the range of salivary cortisol levels, indicating that sweat may be a reasonable biofluid for cortisol detection ^[28]. However, further studies should be done to confirm these results and compare sweat cortisol levels to those in serum or saliva. To our knowledge, melatonin levels in sweat have yet to be reported. In this study, cortisol and melatonin levels were analyzed in sweat and plasma samples

collected from 22 individuals. This study aims to better understand cortisol and melatonin levels in sweat compared to those in plasma.

3.2 Experimental Procedures

3.2.1 Sweat and Plasma Collection.

13 male and 10 female individuals (age range: 18 – 58) were recruited into the study. Informed, written consent was obtained from all participants in accordance with the ASU IRB. Sweat samples were collected using the Eccrine Systems G32 sweat collection device (Eccrine Systems, Cincinnati, OH), Macroduct sweat collection device (WESCOR, Logan, UT) and the PharmChek[®] sweat patch (PharmChem, Fortworth, TX). The volar region of each participant's forearm was first cleaned with 70% ethanol. The sweat collection devices were then fitted to each forearm following the manufacturer's instructions. Each participant was instructed to perform 30 – 60 min of moderate aerobic exercise indoors (stationary bike, treadmill, or elliptical). Following aerobic exercise, the sweat collection devices were removed and 8 mL of venous blood were collected using BD Vacutainer lithium heparin tubes (BD, Franklin Lakes, NJ). All venous blood draws were performed by a trained phlebotomist at ASU Health Services in Tempe, AZ. Sweat and venous blood samples were immediately de-identified, processed, and stored at -20°C.

Sweat samples collected via the Macroduct required liquid transfer into a microcentrifuge tube by a sterile syringe and were stored at -20°C until further analysis. Samples collected via the Eccrine Systems G32 device and PharmChek[®] sweat patch required centrifugation to extract the liquid sample and remove shed skin cells and cellular debris. Each patch and filter paper from the G32 device was placed in a 15 mL tube with a honeycomb filter and centrifuged for 5 min at 5,000 rpm. The extracted sweat sample was transferred to a microcentrifuge tube and stored at -

20°C until further analysis. Venous blood samples were transferred to a 15 mL tube and centrifuged for 10 min at 10,000 rpm. The separate plasma was then aliquoted into 1.5 mL Eppendorf tubes and stored at -80°C until further analysis.

3.22 Bicinchoninic Acid (BCA) Assay for Total Protein Measurement in Sweat.

All sweat samples regardless of the collection device used were analyzed for total protein content using the Pierce BCA Protein kit (Thermo Scientific, Waltham, MA). An 8-serial dilution BSA standard was used ranging from 2 – 0 mg/mL. All standards and samples were performed in duplicate on a microplate, according the manufacturer's instructions.

3.23 Quantitative ELISAs for Cortisol and Melatonin.

For analyzing sweat cortisol, 21 sweat samples collected via the Eccrine Systems G32 device and 1 sample via the collected Macroduct were run on the Salimetrics Salivary Cortisol ELISA kit (Salimetrics, State College, PA). Samples were diluted 1:10 and performed in duplicate according to the manufacturer's instructions. 17 sweat samples collected via the Eccrine Systems G32 device and 2 samples via the collected Pharmchek[®] patch were run on the Salimetrics Salivary Melatonin ELISA kit. Samples were diluted 1:3 and performed in duplicate according to the manufacturer's instructions. For analyzing bound and unbound cortisol in plasma, the matching plasma samples to each analyzed sweat sample were run on the DetectX Cortisol EIA kit (Arbor Assays, Ann Arbor, MI). Samples were diluted 1:100 and run in duplicate according to the manufacturer's instructions. For analyzing plasma melatonin, the matching plasma samples to each analyzed sweat sample were run on a Melatonin ELISA kit (LSBio, Seattle, WA). Samples were diluted 1:1 and run in duplicate according to the

manufacturer’s instructions. For all ELISAs, the mean intensity values of the standards were fit to a 4-parameter logistic regression to generate a standard curve. The standard curve was used to calculate the concentration of the unknowns. All samples’ duplicate OD values were within 10% of the mean; thus, no data points were omitted from the study.

3.3 Results and Discussion

3.31 Sweat Yield and Protein Concentration.

Sweat Yield		Approximate Volume (uL)			Time (min)	Exercise
ID	Sex	Eccrine Systems Device	Macroduct Tube	Pharmcheck® Patch		
1	F	220	80	NA	50	Jogging
2	M	200	40	NA	40	Stairmaster
3	M	1500	0	1000	90	Biking-outdoors
4	M	500	100	NA	90	Rock climbing-indoors
5	F	80	0	NA	40	Walking
6	M	500	80	NA	60	Stairmaster
7	F	400	0	NA	60	Jogging
8	F	250	150	NA	60	Jogging
9	M	1000	250	NA	60	Jogging
10	F	250	100	NA	60	Jogging
11	M	360	0	NA	60	Biking-outdoors
12	M	500	50	NA	60	Biking-outdoors
13	F	200	100	500	30	Jogging
14	F	400	40	500	60	Jogging
15	F	200	100	1000	60	Weights/Cycling-indoors
16	M	480	100	NA	60	Jogging
17	M	400	40	NA	60	Jogging
18	F	480	100	NA	60	Stairmaster
19	M	570	80	NA	60	Biking-outdoors
21	M	200	100	NA	45	Jogging
22	F	50	100	NA	60	Jogging
23	M	1000	50	NA	60	Stairmaster

Figure 3.31. Sweat Yield by each participant and activity log. The average volume collected by the Eccrine Systems G32 device was 431 uL and the Macroduct device was 74 uL. NA indicates the collection device was not used on the participant.

Sweat yield in volume was measured for each collection device used per participant (Figure 3.31). The average volume was 430 µL for 22 samples collected by the Eccrine Systems G32 device and 70 µL for 22 samples collected by the Macroduct device. The Pharmchek® patch

was only used on 4 participants due supply constraints. The maximum volume capacity of each device is 1 mL for the G32 and 150 μL for the Macroduct. Categorizing the G32 samples by gender, an average of 568 μL was collected from males compared with 253 μL from females.

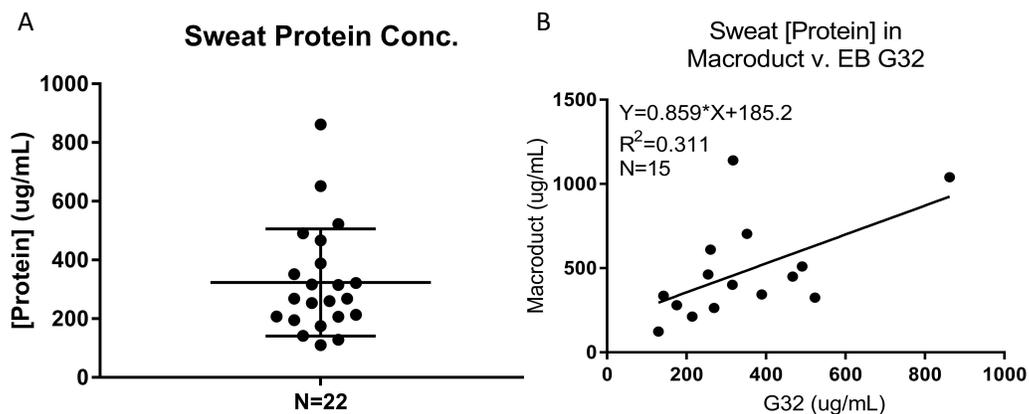


Figure 3.32. Sweat protein concentration determined by BCA assay. (A) Total protein concentration was determined in 22 sweat samples collected via the Eccrine Systems G32 device. The median protein concentration was 269 ug/mL with a standard deviation of 183 ug/mL. (B) For 15 participants, the total protein concentration was compared between sweat samples collected using the Eccrine Systems G32 and Macroduct devices.

Next, a BCA assay was performed on all 22 sweat samples collected by the Eccrine Systems G32 device and 15 of the sweat samples collected by the Macroduct device. For the G32 sweat samples, the median protein concentration was determined to be 269 $\mu\text{g}/\text{mL}$ with a standard deviation of 183 $\mu\text{g}/\text{mL}$ (Figure 3.32A). Comparing 15 tandem G32 and Macroduct samples, total protein concentrations showed poor correlation with an R-squared value of 0.311 (Figure 3.32B). These differences may result from different evaporation rates that occur within each device and/or varied sweat rates and content from different areas of the epidermis. The density of sweat glands can also significantly vary across the epidermis affecting the sweat rate and thereby, the concentration of proteins. The collection device, sweat rate, and epidermis location are important factors that should be further investigated in establishing sweat as biofluid for biomonitoring of proteins.

3.32 Sweat and Plasma Cortisol Analysis.

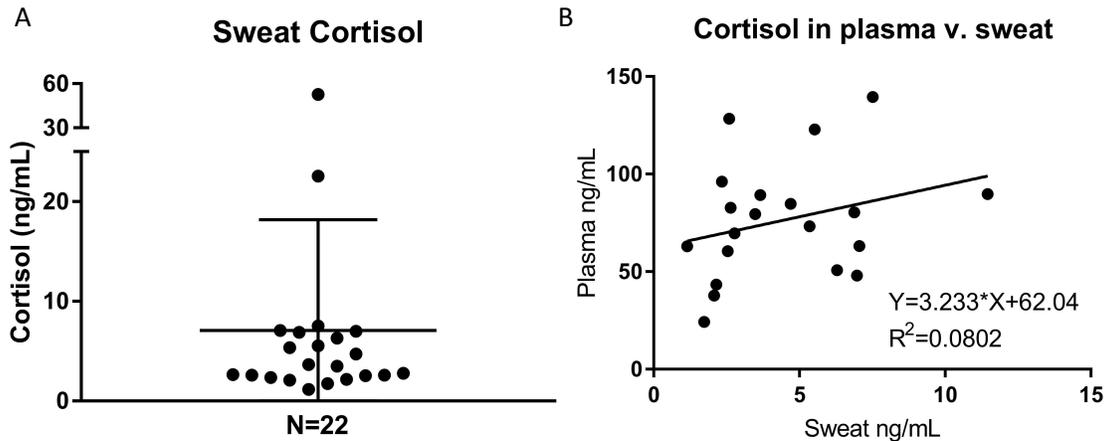


Figure 3.33. Sweat and Plasma Cortisol. (A) Cortisol levels in 22 sweat samples. Median concentration was 4.17 ng/mL with a standard deviation of 11.1 ng/mL. (B) Cortisol levels compared in plasma and sweat samples.

Sweat and plasma cortisol levels were determined from 22 participants. Of the 22 sweat samples, 21 were collected via the Eccrine Systems G32 device and 1 via the Macroduct due to limited sample volumes. The median and standard deviation of cortisol was determined to be 4.17 ± 11.1 ng/mL in sweat and 76.4 ± 28.8 ng/mL in plasma (Figure 3.33A). Cortisol detected in sweat represents only free cortisol while cortisol detected in plasma represents both free or bound cortisol. Approximately 5% of plasma cortisol is free and may be secreted and detected in sweat^[29]. While the median sweat cortisol level is approximately 5% of the median plasma cortisol level, the correlation between sweat and plasma cortisol levels remains low with an R-squared value of 0.0802 (excluding the 2 highest sweat cortisol levels). In contrast, the correlation between salivary and plasma cortisol has reported to have an R-squared value of 0.35 in adults and 0.45 in preterm infants^[30,31].

The poor correlation observed between sweat and plasma cortisol may result from sweat rate, hydration, and evaporation altering the sweat cortisol concentration. Additionally, the sweat cortisol levels represent an accumulated concentration as the samples were collected over an extended period of time, ranging from 30 to 90 minutes. The plasma cortisol levels represent the

concentration at a single time point when the venous blood was drawn. Thus, changes in the cortisol levels throughout the sweat collection time period would also contribute to the poor correlation.

In order to establish sweat cortisol as a useful biomarker, further studies will need to be performed to demonstrate that fluctuations in sweat cortisol levels correlate with physical symptoms and provide meaningful data to individuals. Additionally, a major challenge to this study was not understanding how sweat rate changes throughout a collection period and impacts the analyte concentration. For the field of sweat diagnostics as a whole, methods for sweat rate and analyte measurement in real-time must be established to enable the development of clinically useful wearable biosensors.

3.33 Sweat and Plasma Melatonin Analysis.

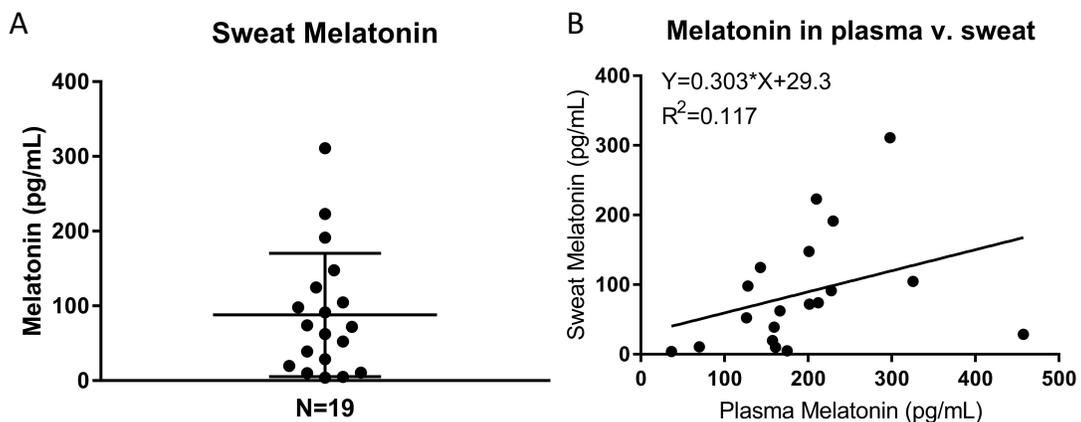


Figure 3.34. Sweat and Plasma Melatonin. (A) Melatonin levels in 19 sweat samples. Median concentration was 73.1 pg/mL with a standard deviation of 198 pg/mL. (B) Melatonin levels compared in plasma and sweat samples.

Sweat and plasma melatonin levels were determined from 19 participants. Of the 19 sweat samples, 17 were collected via the Eccrine Systems G32 device and 2 via the Macroduct. The median and standard deviation of melatonin was determined to be 73.1 ± 198 pg/mL in sweat and 194 ± 93.4 pg/mL in plasma (Figure 3.34A). Similar to cortisol, poor correlation was

observed between sweat and plasma melatonin with an R-squared value of 0.117. The same reasons mentioned for cortisol are likely contributing factors to the concentration differences.

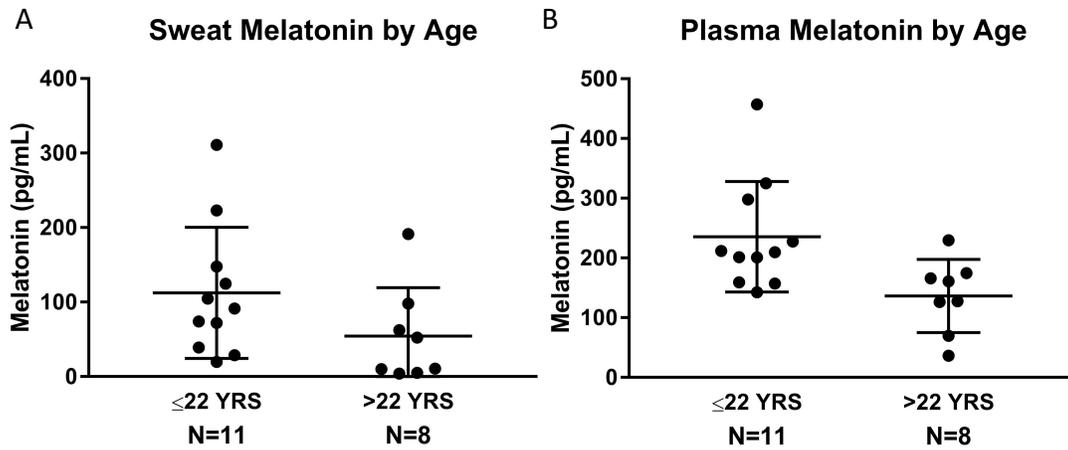


Figure 3.35. Sweat and Plasma Melatonin levels by age. (A) The median and standard deviation of sweat melatonin levels was 112 ± 88.1 pg/mL in participants ≤ 22 years old and 54 ± 65.0 pg/mL in participants > 22 years old. (B) The median plasma melatonin levels was 236 ± 92.4 pg/mL in participants ≤ 22 years old and 137 ± 61.3 pg/mL in participants > 22 years old.

Since melatonin is known to decrease with age, sweat and plasma melatonin levels were categorized based on participant age. For participants 22 years of age or below (group 1), the average melatonin concentration and standard deviation was 112 ± 88.1 pg/mL in sweat and 236 ± 92.4 pg/mL in plasma. For participants over 22 years of age (group 2), the average melatonin concentrations and standard deviations decreased to 54 ± 65.0 pg/mL in sweat and 137 ± 61.3 pg/mL in plasma (Figure 3.35A-B). In both sweat and plasma, a nearly two-fold difference in melatonin levels was observed between age group 1 and group 2. Similar trends have been reported in plasma and urine showing decreased melatonin production with age^[32].

Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

Chapter 2 presented an exploratory study characterizing the antibody isotypes, cytokines, and other proteins in eccrine sweat. This study suggests that there is a plethora of proteomic biomarkers in sweat that largely remains untapped. Many of the antibody isotypes (IgA and IgG) and some specific cytokines (IL-1 α , EGF, and ANG) were detectable in the pg/mL range, making them possible target analytes for wearable biosensors. Chapter 3 presented a more focused study on cortisol and melatonin levels in sweat and compared those levels to plasma samples, which were collected in tandem. Cortisol and melatonin were both detectable in sweat in the ng/mL and pg/mL range, respectively. Correlation between sweat and plasma levels were poor; however, much of this variation likely resulted from the cumulative collection method and inability to account for the sweat rate. Taken together, these studies demonstrate that sweat holds more proteomic and hormonal biomarkers than previously thought and may eventually serve as a noninvasive biomarker resource. These studies also highlight many of the challenges associated with monitoring sweat content including differences between collection devices and hydration, evaporation losses, and sweat rate. It appears that the field of sweat diagnostics requires an intimate collaboration between biochemists, engineers and others not just to develop the future of wearable biosensors but also to simply better understand the basic science underlying how biomarkers are reported in sweat.

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