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# Biomarker Signature Classification of Various Stress Forms

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# Biomarker Signatures of Various Stress Forms

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A Paper Submitted in Partial Fulfillment of the Requirements for the University of Connecticut  
University Scholars Program in Molecular and Cell Biology

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MCB4997W: Honors Thesis

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**ABSTRACT**

Various types of stressful conditions can have unique and important effects on immunity and can lead to dramatic consequences to health. For my University Scholar project, the characteristic biomarker signatures produced from a set of diverse stressors (e.g. psychological, biological and chemical) are being investigated. A biomarker signature is a distinctive biological indicator of a specific condition. High-throughput tools for the measurement of different cellular products have the potential to further our understanding of human disease and facilitated the identification of new biomarkers in all areas of medicine. The hypothesis that each form of stress, psychological, chemical and physical, will elicit different biomarker signatures is being tested. In order to examine the biomarker signatures associated with various stressors, samples from individuals who have experiences one of several forms of stress will be evaluated. The biological samples to be tested include human saliva samples taken from patients who had experienced psychological trauma. We hypothesize that distinctive changes to the biomarker signature found in these patients' saliva may facilitate the identification of those with post traumatic stress disorder (PTSD) and ultimately may enable a more effective treatment regimen. To quantify the differential expression profiles of the salivary components of the biomarker signature (including interleukins, chemokines, TNF, interferon, heat shock proteins, melatonin, and procalcitonin) traditional enzyme-linked immunosorbent assay (ELISA) as well as Surface Plasmon Enhanced Fluorescence (SPEF) techniques will be utilized. By categorizing the unique endogenous responses that are associated with each stressful condition, better diagnosis could be made and the effects of different stresses may be better predicted and controlled. Categorizing and quantifying different biomarker signatures of individuals experiencing different stressors

will help provide insight into some of the health consequences which may result from these stressors.

## INTRODUCTION

### *Biomarker Signatures*

Biomarkers are features that can be objectively measured and evaluated as an indicator of a normal biological process, a pathogenic process, or a pharmacological response to a therapeutic intervention (Biomarkers Definitions Working Group. 2001). A biomarker can be used as an indicator of a biological state; it is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Limits to using biomarkers as a diagnostic tool exist. Using only a single indicator leaves room for false positives in the population of healthy individuals with high levels of that specific substance, or false negatives, diseased individuals with lower levels of the indicator. This inaccuracy can be reduced by use of biomarker signatures. A biomarker signature is an integration of simultaneous biomarker measurements that generate a predictive pattern or signature of a biological state. Analysis of large analyte sets can generate biomarker signatures of disease or stress exposure that has diagnostic potential.

In addition to diagnostic uses, biomarker signatures have potential implications with therapeutics. Certain therapeutic interventions can only be effective in subpopulations (Trusheim, Berndt, Douglas 2007). Biomarker signatures have the potential to be useful for identifying patients that are more likely to benefit or to experience an adverse reaction in response to a given therapy; thereby more effectively treat diseases. For example, clinicians now commonly test to determine which breast tumors over-express the human epidermal growth

factor receptor type 2 (HER2), which is associated with a worse prognosis but also predicts a better response to the medication trastuzumab (Hamburg and Collins 2010). . The development of tools for high-throughput analysis has increased the determination of biomarkers that can be used for personalized medicine. Characterization of various biomarkers could aid in the avoidance of harmful adverse effects and maximize treatment success. Additionally, assessment of an individual's susceptibility to certain diseases before they become apparent should also be possible. The use of biomarker signatures for predicting onset and prognoses of diseases allows time to set out a plan for prevention, treatment and monitoring that is specific for an individual patient.

### *The Links between Stress and the Immune Response*

The immune system is a highly regulated system which is divided into innate and adaptive responses. The nervous system plays a role in the regulation of immunity through neuroendocrine responses which control inflammation at a systemic level via two axes; the hypothalamic-pituitary-adrenal axis (HPA axis) and the sympathetic-adrenal medullary (SAM) axis (Sternberg 2006). The central cells of the immune system (lymphocytes, monocytes, etc.) display receptors for some neuroendocrine products of the HPA and SAM axes (Padgett and Glaser 2003). Chronic stress is associated with the activation of the hypothalamic–pituitary–adrenal (HPA) axis, as well as with the depression of immune function. Acute stress is associated with activation of the sympatho–adreno–medullary system, which is reflected by salivary  $\alpha$ -amylase and chromogranin A (Soo-Quee Koh and Choon-Huat Koh 2007).

Psychological stressors increase glucocorticoid levels through increased adrenal activity which leads to an inhibition of the functions of lymphocytes, macrophages and monocytes.

Chronic activation of the HPA axis and the SAM axis, results in chronic production of glucocorticoid hormones and catecholamines (Padgett and Glaser 2003). Glucocorticoid receptors expressed on a variety of immune cells bind cortisol which indirectly regulates the activity of immune cells capable of producing cytokines. Adrenergic receptors bind catecholamines (epinephrine and norepinephrine) and activate the transcription of genes encoding for a variety of cytokines. The changes in gene expression mediated by glucocorticoid hormones and catecholamines can lead to immune system dysregulation. The relationship between stress, the HPA endocrine system and immunity is complex. The multi-directional relationship between the different systems of the body provide the foundation to my hypothesis that different forms of stress will affect the nervous, endocrine, and immune systems in unique ways that are reflected in the unique biomarker signatures associated with each stress.

### **Biomarker Signatures of Various Stress Forms: Literature Review**

An enormous amount of work has been done linking conditions such as post-traumatic stress disorder (Gill et al. 2009), psychologically stressful situations (Depke et al. 2009) and various chemical exposures (Pruett et al. 2009) to various immune-altering effects. As high throughput technology becomes more popular and the technology more accurate, biomarker signatures of many conditions have been studied as potential diagnostic tools, prognosis predictors, as well as means to determine the most effective treatment options.

#### *Biomarker Signature of Traumatic Brain Injury*

Traumatic brain injury (TBI) is a complex injury with a broad spectrum of symptoms, disabilities, and causes. Brain damage occurs as a consequence of an external force in the form

of a direct impact or by acceleration or deceleration alone. The nature of the forces which cause trauma to the brain determine both the pattern and extent of damage, for example blast waves from an explosion have been identified as a cause of TBIs with specific characteristics (Maas, Stocchetti, Bullock 2008)(Maas, Stocchetti, Bullock 2008)(Maas, Stocchetti, Bullock 2008)(Maas, Stocchetti, Bullock 2008)(Maas, Stocchetti and Bullock 728-741)(Maas, Stocchetti, and Bullock 2008, 728-741)(Maas, Stocchetti, and Bullock 2008, 728-741). In addition to the damage caused at the time of injury, brain trauma causes secondary injury, a variety of events that take place in the hours and days following the injury. These events include neurotransmitter release, free-radical generation, calcium mediated damage, gene activation, mitochondrial dysfunction, and inflammatory responses (Maas, Stocchetti, Bullock 2008). This secondary damage creates opportunities for medical intervention and targeted therapies to improve the outcome for the patient. Currently the common treatment of TBI revolves around standardized approaches that follow predetermined guidelines for all patients. Knowledge of biomarker signatures of TBI diagnosis and potential outcome predictors could steer interventions and targeted therapies in the right direction for TBI victims.

Biomarkers of physical brain injury can classify injury severity level as well as provide a prediction of the secondary damage which may occur in a specific patient. A number of studies have shown that the levels of a multitude of brain-specific proteins are altered both in cerebrospinal fluid (Buttram et al. 2007) and in blood (Berger et al. 2007)(Hergenroeder et al. 2008)(Honda et al. 2010)(Rhind et al. 2010) are altered following a TBI. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), 31 proteins whose serum levels were altered were identified in TBI patients (Hergenroeder et al. 2008). In the three days following a TBI, serum levels of glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE) and S-



100B proteins are increased in patients who had suffered a TBI when compared to the control group (Honda et al. 2010). Mean serum concentrations of Hsp70 were significantly higher in TBI patients than in the control group (da Rocha et al. 2005). Many inflammatory and coagulation proteins may serve as candidate biomarkers of different characteristics of a TBI soon after the injury takes place.

In the period following the initial injury, biomarker levels may serve as indicators of injury severity and prospective outcome. At all post-injury time points, higher serum levels of NSE, S100B, and myelin basic protein (MBP) were associated with worse outcome for children who had suffered from a TBI (Berger et al. 2007). In addition, serum tau protein levels in TBI patients at the time of hospital admission correlated with poor outcome (Liliang et al. 2010). Serum retinol binding protein 4 (RBP4) levels may serve as a predictor of a subsequent increase in intracranial pressure (Hergenroeder et al. 2008). Increased levels of Hsp70 are also seen in TBI victims with an increased possibility of death indicating a direct correlation between this biomarker and severity of the TBI (da Rocha et al. 2005).

TBI provokes marked differences in an abundance of serum protein levels. In many studies, three biomarkers were individually measured using enzyme-linked immunosorbent assays. Using sensor-chip technology, multiple biomarkers associated with various time points of TBI, severity, and likely outcome could be simultaneously assessed. Classification of serum biomarkers in TBI patients may be useful in the prediction of secondary pathologies as well as in estimating the effectiveness of various therapeutic agents. Further research on the biosignatures of traumatic brain injuries of all severity levels could lead to better injury classification and therefore patient-specific therapies to improve the outcome of TBI patients.

*Biomarker Signatures of Rheumatoid Arthritis*

Rheumatoid arthritis (RA) is a condition characterized by persistent synovitis, systemic inflammation, and the presence of specific autoantibodies (particularly to rheumatoid factor (RF) and citrullinated peptide (CCP)) which affect many tissues and organs, but mainly attack synovial joints. Uncontrolled active rheumatoid arthritis causes joint damage, disability, decreased quality of life.

Characterizing the biomarkers associated with RA onset can be useful in predicting the disease before symptoms become apparent and selecting the most advantageous treatment. Studies using enzyme-linked immunosorbent assays have shown that anti-citrullinated peptide (ACPA), anti- mutated citrullinated vimentin (MCV) and IgM RF can be detected up to 10 years before RA diagnosis (Turesson et al. 2010). Additional prognostic markers studied include C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR), ACPA, and rheumatoid factor (RF), or matrix metalloproteinase-3 (a proteolytic enzyme which is thought to play a pivotal role in joint destruction in RA) (Klareskog et al. 2008)(Mamehara et al. 2010). Biomarkers have also been used to predict the effectiveness of specific treatments on patients with RA. Results suggest T-lymphocyte expression of CD91 is a biomarker that signifies unresponsiveness to anti-TNF therapy, a biological disease modifying anti-rheumatic drugs (DMARDs) used to slow down the progression of joint destruction. Therefore CD91 expression may be used to identify potential responders and non-responders (Eriksson, Rantapaa-Dahlqvist, Sundqvist 2010). Developing a method to identify biomarker signatures associated with RA can be useful in preventing the appearance of disease symptoms and providing effective treatment.

*Biomarker Signatures Associated with Alzheimer's Disease*

Alzheimer's disease (AD) is the most common form of dementia. The disease is incurable, degenerative, and terminal. Studies have been done using cerebrospinal fluid to identify biomarker signatures of the disease. Establishing a profile for the biomarkers of this disease might serve as signatures for the presence of Alzheimer's pathology, and aid in the identification of patients suffering from Alzheimer's among elderly individuals with late-life cognitive impairment.

Using the Luminex assay, it was found that the CSF biomarker signature of AD are defined by A $\beta$ 1-42 and t-tau. This signature appears to predict conversion from mild cognitive impairment to Alzheimer's (Shaw et al. 2009). Increased levels of tau in CSF are thought to occur after its release from damaged and dying neurons and tangles, large-scale accumulation of this least soluble of A $\beta$  peptides into insoluble plaques in the AD brain, lead to decreased CSF levels of A $\beta$ 1-42. The combination of increased CSF concentrations of tau proteins, t-tau and phosphotau (p-tau), and the decreased concentrations of A $\beta$ 1-42 in a patients CSF are considered to be a pathological biomarker signature that can be used as a diagnostic tool for Alzheimer's (Frank et al. 2003).

Among the CSF biomarkers studied, CSF A $\beta$ 1-42 concentration appears to be the most sensitive analyte for the detection of AD, thereby indicating that CSF A $\beta$ 1-42 is the most informative single biomarker (Shaw et al. 2009). The use of the single biomarker A $\beta$ 1-42 is inadequate as there is a portion of the healthy population who has higher levels of this indicator. A panel of biomarkers would be more useful means of diagnosing Alzheimer's, predicting which individuals will progress to AD, as well as for monitoring the response of patients to various therapies in the hopes of developing improved treatment methods.

*Additional Uses of Biomarker Signatures*

In addition to the previously mentioned conditions, biomarker signatures are being characterized for multiple other uses. Biomarker signatures are being studied as a potential method to predict allograft tolerance for the identification of patients who can lower their amounts of immunosuppressive therapy, and as predictors of allograft rejection, so that damage can be prevented (Hernandez-Fuentes and Lechler 2010). Also, microarray analysis showed over 1,500 genes exhibited significantly different expression level in saliva between oral cancer patients and controls. Seven of these cancer-related mRNA biomarkers exhibited at least a 3.5-fold increase in the saliva of oral squamous cell carcinoma (OSCC) patients. Combinations of salivary RNA biomarkers allowed for distinguishing patients suffering from OSCC from the control group (Li et al. 2004).

**EXPERIMENT BACKGROUND***Post Traumatic Stress Disorder*

Biomarker signatures will be sought in human saliva samples taken from patients who have experienced traumatic situations. This model will allow us to determine unique and distinctive changes to the biomarker signature in exposed individuals before the onset of post traumatic stress disorder (PTSD). PTSD is a severe anxiety disorder that can develop after exposure to any event (physical or psychological) that result in psychological trauma. The Posttraumatic Stress Diagnostic Scale (PDS) is a questionnaire used to aid in the detection and diagnosis of PTSD. The PDS yields a total severity score (ranging from 0 to 51) that largely reflects the incidence of the symptoms of PTSD. Symptoms of PTSD include experiencing recurrent and intrusive recollections of the stressful event and persistent avoidance of things that

remind them of the traumatic event. A PDS score provides diagnostic status, a count of the number of symptoms experienced, a rating of symptom severity, and a rating of the level of impairment of functioning. A drawback to the PDS is that administration of the questionnaire takes one to two hours. Symptoms of PTSD can last a long time and can be significant enough to cause impairment in social, occupational, or other areas of functioning.

Efforts to prevent PTSD have been classified as either primary (intervention prior to the traumatic event) or secondary (intervention after the trauma) (Fletcher, Creamer, Forbes 2010). Due to the effect PTSD has on the lives of people who suffer from it as well as the effects PTSD has on the family of sufferers and on society, prevention and early treatment would be of extremely advantageous. In addition, the frequency of treatments which are ineffective is far too great, therefore having an early indicator of PTSD would be of great benefit to individuals (Fletcher, Creamer, Forbes 2010). PTSD creates an increased risk for illness in an individual as patients with a history of PTSD have long-lasting immune dysfunction. It has been shown that the number of lymphocytes, NK cell activity, and total amounts of certain immune related chemokines were significantly lower in patients with a past history of PTSD (Kawamura 2001). Characterizing biomarker signatures associate with the development of PTSD following a traumatic event would be greatly useful in determining when treatment would be helpful and in improving the quality of life for the PTSD patient.

### *Saliva as a Diagnostic Tool*

Saliva samples are easily accessible and have been shown to serve as a useful analyte for protein markers because saliva composition is influenced by many factors (Esser et al. 2008). The use of saliva as a medium for biomarker signatures has not been popular in the past due to

the low concentration of these markers in saliva when compared to serum. This has changed due to the development of highly sensitive and high-throughput assays such as microarray, mass spectrometry, reverse transcriptase polymerase chain reaction (RT-PCR), and other technologies which can be used to measure proteins even at concentrations found in saliva. Many biomarkers are measurable in saliva, including heavy metals, hormones (e.g. cortisol and dehydroxyepiandrosterone (DHEA)), toxins and their metabolites, enzymes, immunoglobulins, other proteins and DNA (Soo-Quee Koh and Choon-Huat Koh 2007).

About 18% of the saliva protein composition is involved in the immune response (Esser et al. 2008). It has also been found that there is an increase in the secretion of salivary stress biomarkers (salivary alpha amylase and salivary cortisol) under different stressful conditions (Wagner et al. 2009). Chronic stress is associated with an increase in salivary cortisol, as well as with the depression of immune function seen by a decrease in by salivary IgA and lysozyme levels. Acute stress is associated with activation of the sympatho-adreno-medullary system, measured by salivary a-amylase and chromogranin A (Soo-Quee Koh and Choon-Huat Koh 2007). These features of human saliva make it a simple and useful analyte for diagnostic purposes. Possible limitations to using saliva as a diagnostic biofluids are normal variations among the population and difficulty storing the sample.

## **TECHNOLOGIES FOR BIOSIGNATURE ANALYSIS**

Surface Plasmon Resonance (SPR) assays represent a novel approach to signature measurements. A surface plasmon is a traveling, temporary electromagnetic wave caused by charge-density oscillation at a metal-dielectric interface. Surface plasmon resonance can be employed to make sensitive measurements of molecular binding interactions. One way in which

SPR technology is utilized is by protein detecting microarrays. These assays utilize a wide variety of capture agents (antibodies, fusion proteins, DNA/RNA aptamers, synthetic peptides, carbohydrates, and small molecules) immobilized on a solid surface. (Tomizaki, Usui, Mihara 2010). Each capture agent binds selectively to its target protein in a complex mixture, such as serum or saliva samples. Captured proteins are subsequently detected and quantified in a high-throughput fashion, with minimal sample consumption.

SPR technology is used in high content assays with improved sensitivity compared to standard fluorescent microarrays (Unfricht et al. 2005). This high sensitivity assay allows the measurement of immune system biomarkers that are normally found in very low concentrations in body-fluid samples that are easily obtained, such as saliva. By measuring  $10^2 - 10^3$  biomarkers simultaneously, it is possible to reveal patterns of biomarker expression that are indicative of stressed or diseased states.

#### *Grating-Coupled Surface Plasmon Enhanced Fluorescence (GCSPEF)*

The optical phenomenon of surface plasmon resonance can be employed to make sensitive measurements of molecular binding interactions. For detection of small molecular weight or low concentration biomarkers, the generated surface plasmon can be used to enhance fluorophore excitation (Surface plasmon enhanced fluorescence; SPEF) and improve optical collection efficiency from fluorescently labeled secondary antibodies or biomarkers directly labeled in a sample. GCSPEF introduces the use of fluorescent tags attached to a captured analyte (Figure 1). This technology was developed by the Lynes laboratory in collaboration with the biotechnology company Ciencia Inc. GCSPEF can be used for cell-based assays, protein assays, and many other functional purposes i.e. to obtain a measurement of cell function by

means of measuring the soluble proteins secreted from stimulated cells (Jin et al. 2006).

Advantages of GCSPEF over more widely used techniques include its need for small sample size ( $\leq 1$  ml), microvolumes of detection reagents, and for its ability to measure hundreds of simultaneous real-time measurements of different analytes.

In GCSPEF the analyte is flowed over a sensor chip which has been prepared with specific immobilized receptors (which can be antibodies or other proteins). The sensor chips are covered with a gold surface. During an experiment, the captured analytes are illuminated with light that couples with the electrons on the grated surface of the gold plated GCSPEF chip to form a surface Plasmon (Jin et al. 2006). Because plasmons do not propagate far into gold, hundreds to thousands of interactions can be analyzed on one chip. Sensor chips will be configured with capture reagents that measure proteins of interest (e.g. cytokines, stress hormones, antibodies, and other soluble factors). Antibodies or capture ligands are covalently attached to the gold surface of sensor chips on spots, called Regions of Interest (ROIs) which allows for specific binding of analytes or cells to occur which changes the refractive index at the interface.

GCSPEF is able to detect and measure the presence of hundreds of different analytes simultaneously from a small sample volume. ROIs are spatially organized on a gold-plated sensor chip so that the specific binding of different analytes in a mixed sample can be measured simultaneously by passing the sample over the sensor chip and recording changes in the SPR angle at different ROIs. As an analyte is captured on a ROI, the SPR angle (the angle of minimal reflection due to maximum coupling) increases. The camera within the GCSRPI machine scans through a variety of angles to find the SPR angle, which is dependent upon the index of refraction at the metal interface. The SPR angle can be increased due to interactions between



analytes and immobilized capture molecules on the chip which that cause an increase in the index of refraction. The SPR angle can be correlated to the amount of analyte that has bound. GCSPEF will be specifically useful in my project since hundreds of different regions of the chip can be independently analyzed simultaneously using sub-milliliter sample volumes (Unfricht et al. 2005).

### *Surface Plasmon Enhanced Fluorescence (SPEF)*

The Surface Plasmon Enhanced Fluorescence, SPEF, assay allows for increased sensitivity for detection and quantification of analytes that are too small or too dilute to be detected by traditional GCSPEF. For detection of small molecular weight or low concentration biomarkers, the generated surface plasmon can be used to enhance fluorophore excitation to improve the optical collection efficiency from fluorescently labeled secondary antibodies (Figure 3). Due to the sensitivity of the SPEF assay, it is ideal for highly sensitive fluorescent protein microarrays. Gold plated chips are used in the SPEF assay because a metal dielectric interface is needed for SPR. The two platforms (GCSPEF and SPEF) utilize the same chip and the same machine; therefore independent measurements can be made using both methods from the same ROIs in sequence during a single experiment.

The SPEF assay can utilize a traditional sandwich immunoassay format (Figure 2) which enables the large-scale analyses of protein interactions across the entire proteome in a high-throughput fashion with less consumption of the sample. Capture agents are immobilized on a gold surface and target proteins in the sample are captured. Fluorescently conjugated detection antibodies are then flowed over the chip which allows for the measurement of captured proteins in specified ROIs. The SPEF assay is a high content technology, many measurements from the

same sample can be done simultaneously. The technology is versatile in that many different types of biomarkers can be measured in conjunction with one another. The high sensitivity of the system allows for the use of small sample volume, a very useful feature of the technology in diagnostic sciences since sample sizes are often extremely limited.

In the future, new instruments can be used in order to reduce both cost and size of instruments so that these technologies can move from the laboratory to the field to be used in clinical diagnostics. There are also opportunities to improve the sensitivity of the system by increasing the amounts of immobilized capture molecules, properly orienting the capture molecule for maximum accessibility of analyte to binding sites, and minimizing non-specific binding which affects the net sensitivity of the assay. One current approach is the coating of chips with various surface chemistries that allow for more predictable orientation of binding of the capture molecule (Jin et al. 2006).

#### *Salivary Biomarkers of Stress Project*

Saliva samples were collected from trauma patients at two different time points following the initial injury. In order to quantify the differential expression profiles of interleukins, chemokines, TNF and interferon families, and other related stress proteins (e.g. heat shock proteins, melatonin, and prolactin) traditional enzyme-linked immunosorbent assay (ELISA) as well as surface Plasmon enhanced fluorescence (SPEF) techniques were utilized.

## **MATERIALS AND METHODS**

Saliva samples were collected by our collaborators at UCLA from patients who had been assaulted (either shot or stabbed). Saliva samples were collected using a “lollipop” style device

that patients suck on. The device has a filter and the salivary fluid is absorbed through it and collected. Roche complete protease inhibitor cocktail tablets were used for stable storage of the samples. Saliva samples were frozen in microcentrifuge tubes. The saliva samples were shipped frozen to the University of Connecticut. Samples were stored in the -80°C ultrafreeze until ten to fifteen minutes before they were analyzed.. Samples were allowed to thaw by remaining at room temperature for around ten minutes.

The enzyme-linked immunosorbent assay (ELISA) can also be used to detect the presence and concentrations of an antibody or antigen analyte in a sample. An unknown amount of the analyte is captured by specific antibodies which have been fixed to a well surface. Secondary antibody linked to an enzyme is then washed over the surface so that it may bind to antigen captured previously. A substrate is then added which the enzyme can convert to a detectable, colored signal whose absorbance can be measured by spectrophotometer.

#### *ELISA Protocol for Salivary Biomarkers-Alpha Amylase and Salivary Cortisol*

To determine the saliva sample dilution with PBS-T (dissolve 8g of NaCl, 0.2g of KCl, 1.44g of Na<sub>2</sub>HPO<sub>4</sub>, 0.24g of KH<sub>2</sub>PO<sub>4</sub>, and 2ml of tween-20 in 800 ml of distilled H<sub>2</sub>O, adjust pH to 7.2 and adjust volume to 1L with additional distilled H<sub>2</sub>O) that should be used for SPEF analysis, enzyme-linked immunosorbent assays were done on various analytes including salivary alpha-amylase and cortisol. 96-well Immulon 2 HB plates (Dynatech Laboratories, Inc.) were coated in triplicate with capture antibody (mouse anti amylase at 1 µg/mL from Santa Cruz or mouse anti cortisol from ABD serotech) with 100ul per well. Plates were incubated overnight at 4°C. Plates were then washed with PBS-T (PBS with 0.2% NaN<sub>3</sub> and 0.005% Tween 20, pH 7.2) using the ELx405 autoplater washer. The wells were then blocked with 2%BSA in PBS-T using

200µl per well and incubated for 1 hour at 37°C. Plates were washed again before adding standard human salivary amylase or human salivary cortisol dilutions starting with 1 mg/ml in PBS-and continuing with an 11-step doubling dilution series (100ul/well). Plates were incubated for 2 hours at 25°C and then washed again as done previously. The detection antibody cocktail, (detection anti-amylase, goat polyclonal IgG from Santa Cruz or polyclonal rabbit anti-cortisol from ABD Serotech) 1 ug/mL in PBS-T, was added (100µL/well) and incubated for 2 hours at 25°C. 100 µL of detection anti-goat IgG-biotin antibody (Rabbit Anti-Goat IgG-Biotin from Southern Biotech at 0.5 mg/mL) at 1/5000 in PBS-T was added to each well and incubated for 1 hour at 37°C. Plates were washed then Streptavidin-conjugated alkaline phosphatase at a dilution of 1/500 (100µL/well) was then added to each well and plates were incubated for 20 minutes at 37°C before a final wash. Wells were then coated with 100 µL substrate buffer (9.7 mL diethylamine, 0.01 g of MgCl<sub>2</sub>·6 H<sub>2</sub>O, 0.02 g NaN<sub>3</sub> and 90 mL H<sub>2</sub>O, pH 9.8) containing one mg para-nitrophenylphosphate (PNPP) per mL. A kinetic plate read at 405nm was performed for ten minutes using a SpectraMax M2 plate reader (Molecular Devices). Nonlinear regression software (SOFTmax PRO from Molecular Devices) was used to generate a best-fit curve and its equation.

#### *ELISA Protocol for IL-1beta*

To determine the saliva sample dilution (with PBS-t) that should be used for SPEF analysis, enzyme-linked immunosorbent assays was also done on IL-1 beta. 96-well Immulon 2 HB plates (Dynatech Laboratories, Inc.) were coated in triplicate with capture antibody (Mouse anti-human IL-1β with 2 ug/mL PBS-t from R&D Systems with 100ul per well. Plates were incubated overnight at 4°C. Plates were then washed with PBS-T (PBS with 0.2% NaN<sub>3</sub> and 0.005% Tween 20, pH 7.2) using the ELx405 autoplater washer. The wells were then blocked

with 2%BSA in PBS-T using 200ul per well and incubated for 1 hour at 37° C. Plates were washed again before adding standard human salivary IL-1 beta dilutions from 2,000 pg/ml in PBS-t and unknown dilutions in halves from 1:5 for an 11 step dilution series (100ul/well). Plates were incubated for 2 hours at 25°C and then washed again as done previously. The detection antibody cocktail, biotinylated goat anti-human IL-1 $\beta$ -biotin at 400 ng/mL with PBS-T 1 ug/mL was added (100 $\mu$ L/well) and incubated for 2 hours at 25°C. Plates were washed then Streptavidin-conjugated alkaline phosphate at a dilution of 1/500 (100 $\mu$ L/well) was then added to each well and plates were incubated for 20 minutes at 37°C before a final wash. Wells were then coated with 100  $\mu$ L substrate buffer (9.7 mL diethylamine, 0.01 g of MgCl<sub>2</sub>·6 H<sub>2</sub>O, 0.02 g Na<sub>3</sub>N and 90 mL H<sub>2</sub>O, pH 9.8) containing one mg mg para-nitrophenylphosphate (PNPP) per mL. A kinetic plate read at 405nm was performed for ten minutes using a SpectraMax M2 plate reader (Molecular Devices). Nonlinear regression software (SOFTmax PRO from Molecular Devices) was used to generate a best-fit curve and its equation.

### *SPR Chip Preparation*

SPR sensor chips were washed with 95% ethanol (EtOH) exhaustively for 30 sec. The chips were then washed exhaustively with ddH<sub>2</sub>O for 60 sec. Chips were dried under filtered pressurized air and placed into a plastic chip holder to keep clean until printing. In order to print capture antibodies onto the gold surface of the chips, a 384 well plate was used, v-bottomed wells (Table 1) were filled with their respective antibody solutions to spot capture antibody microarray on the cleaned gold chip. The printing program used for biomarker signature analysis in all human saliva samples was written using the Spotblot II software. Chips were printed under

high humidity using a robotic spotter by Arrayit Microarray Technology. Chips were kept in the humidity chamber of the robotic spotter for 60 minutes before use.

To analyze sample set two saliva samples, the chemical cross-linker dithiobis(succinimidyl propionate), DSP, was coated on the gold surface of the chips in order to improve the method for immobilizing proteins on the chip surface. 4 mg of DSP, dithiobis(succinimidyl propionate) from Thermoscientific were dissolved in 1 mL of dimethylsulfoxide (DMSO) from Fisher Scientific. 300  $\mu$ L of the solution was spread over the gold surface and allowed to sit for 30 minutes. The chips were then washed by rinsing them with DMSO followed by a rinse with ddH<sub>2</sub>O. Spotting proteins to the activated gold surface was done immediately following the DSP coating.

SPEF sensor chips with was washed with 95% EtOH exhaustively for 30 seconds then washed exhaustively with ddH<sub>2</sub>O for 60 sec. Chips were dried under filtered pressurized air and placed into a plastic chip holder to keep clean until printing. In order to print capture antibodies onto the gold surface of the chips, a 384 well plate was used, v-bottom wells were filled with their respective antibody solutions (Table 1) to spot capture antibody microarray on the cleaned gold chip. The printing program was made using the Spotblot II software for this experiment to be used for biomarker signature analysis in all human saliva samples. Chips were printed under high humidity using a robotic spotter from Arrayit Microarray Technology. Chips were allowed to stand for 60 min in the humidity chamber of the robotic spotter before proceeding.

### *Sample Analysis Protocol*

Bench top chip fluidic device was washed by circulating PBS-T through the fluidics at high speed for 5 min. Fluidics were then primed with 2% BSA in PBS. Dry, printed chips were

inserted into the chip-holding chamber and were blocked using 2% BSA in PBS at 0.5 ml/min for 30 min. The chips were then washed by running PBS-T at 0.5 ml/min for 5 min. Saliva samples were thawed immediately before use. Human saliva sample (0.25 ml) was diluted with PBS-T up to a total volume of 1.5 ml (1:5 dilutions). One ml of sample was drawn into the fluidics before shunting the end of the waste tube back into the sample holder. The sample was recirculated at 0.5 ml/min for 60 minutes. The chip was then washed by running PBS-T at 0.5 ml/min for 5 min. 1.5 ml of detection antibody cocktail in PBS-T was recirculated at 0.5 ml/min for 60 minutes (use Table 2 for antibody information and dilution factors). The chips were then washed by running PBS-T at 0.5 ml/min for 5 min. Streptavidin-alexa647 cocktail was recirculated at 0.5 ml/min for 30 min. Chip was washed by running PBS-T at 0.5 ml/min for 10 min. To obtain fluorescent data from the cortisol and amylase secondary detection antibodies, 1.5 ml of anti-goat IgG-alexa647 and anti-rabbit IgG alexa-647 cocktail in PBS-T was recirculated at 0.5 ml / min for 30 min (See Table 3 for reagent dilution factors). Chips were washed by running PBS-T at 0.5 ml/min for 10 min.

To analyze saliva sample set two, an eight chamber bench top chip fluidic device (a device with eight separate inflow tubes, chip chambers, and outflow tubes with uniform fluid movement controlled by a central pump) from Masterplex was primed with 2% BSA in PBS. Dry, printed chips were inserted into the chip-holding chambers and were blocked using 2% BSA in PBS at 0.5 ml/min for 30 min. The chips were then washed by running PBS-T at 0.5 ml/min for 5 min across the sensor chip surface. Saliva sample were thawed immediately before use. 0.25 ml of human saliva sample was diluted with PBS-T to 1.5 ml (1:5 dilution). 1 ml of diluted sample was drawn into the fluidics before moving the end outflow tube back into the sample chamber enabling recirculation with the 0.5 ml reserve in the sample tube. The sample

was recirculated at 0.5 ml/min for 60 minutes. The chip was then washed with PBS-T at 0.5 ml/min for 5 min. 1.5 ml of detection antibody cocktail (Table 2) in PBS-T was recirculated at 0.5 ml/min for 60 minutes (use Table 2 for antibody dilution factors). Chip was washed by running PBS-T at 0.5 ml/min for 5 min. Streptavidin#alexa647 (from, anti-goat IgG#alexa647 and anti-rabbit IgG alexa#647 (from Invitrogen Molecular Probes) cocktail in PBS-T was recirculated at 0.5 ml / min for 30 min (Table 3). Chips were washed by running PBS-T at 0.5 ml/min for 10 min. Fluorescent data was read using the SPEF dualmode instrument from Ciencia.

### *Data Analysis*

Saliva samples were divided into low stress or high stress groups by our collaborators at UCLA according to their post traumatic stress diagnostic scale (PDS) score (Figure 4). Samples were collected 2-3 weeks apart and called “visit 1” and “visit 2”. All samples were measured for the presence of immune/stress associated biomarkers using SPEF microarray. Using both parametric and non-parametric T-tests, groups were analyzed for differences between single biomarkers or for changes in levels of a single biomarker. Parametric tests were useful in examining the levels of individual biomarkers among groups, while non-parametric tests were used to compare differences in biomarker levels between groups. Differences in biomarker levels between groups, and the change in biomarker levels between visits were analyzed for statistically significant ( $p \leq 0.05$ ) differences.



## RESULTS

### *ELISA Validation of Reagents*

Enzyme-linked Immunosorbent Assays (ELISAs) were conducted in order to determine the saliva sample dilution that should be used for GCSPEF and SPEF assays. A saliva sample was analyzed in comparison to a standard 11-step dilution series of each biomarker. Alpha-amylase was diluted in halves from 1 mg/mL, IL-1 beta from 2000 pg/mL, and salivary cortisol from 100ng/mL. Control saliva samples and PTSD saliva samples were diluted from a 1:1 dilution to a 1:5 dilution in each experiment. Based on the ELISA results, it was determined that physiologically relevant measurements of these biomarkers could be made with a saliva sample dilution of 1:5 in PBS-T.

### *PTSD Saliva Samples: Sample Set 1*

Surface Plasmon enhanced fluorescence (SPEF) was utilized in order to quantify the differential expression profiles of interleukins, chemokines, TNF and interferon families, and other related stress proteins (e.g. heat shock proteins, melatonin, and procalcitonin) in saliva samples obtained from PTSD patients at two different time points. The samples were divided into four categories, high stress visit 1, high stress visit 2, low stress visit 1, and low stress visit 2. Several patterns are apparent among the biomarkers examined. A complete heat map of all of sample set 1 can be seen in Figure 6.

Analysis of 39 stress exposed individuals for the presence of 26 immune and/or stress associated biomarkers revealed one biomarker, MMP-9, that was significantly different between high and low stress groups (Figure 6). Dividing stress groups samples into 2 visits indicated that IL-2 levels are significantly higher in the high stress visit 1 group (Figure 7).

Sample set 1 revealed no significant intergroup variations in the additional biomarkers examines (as listed in Table 1). Because direct analysis of biomarker levels revealed few differences between groups, we looked at the changes in the levels of biomarkers that occurred between visit 1 and visit 2 in each patient. This integrative analysis revealed that levels of C-reactive protein (Figure 9) declined more in the low stress group and that levels of cortisol increased more in the low stress group (Figure 8). Ultimately, our analysis is limited by sample size and the ability of the PDS to accurately group individuals into high and low stress groups. However, the observed differences indicate molecular patterns of stress exposure may exist.

#### *PTSD Saliva Samples: Sample Set2*

36 saliva samples were analyzed in sample set 2 with the use of DSP surface chemistry (Figure 10). Dividing stress groups samples into two time points, visits 1 and 2, indicated significant differences in levels IL-18 binding protein (IL-18 Bpa) among the groups in sample set 2 (Figure 11). Additionally, we found that levels of IL-8 (Figure 12) and alpha-amylase (Figure 13) decreased more in the low stress group. Again, our analysis is limited by sample size, inter-assay variability and the ability of the PDS to accurately group individuals into high and low stress groups. However, these variations in biomarker level, or changes in biomarker level, indicate that biomarker signatures of psychological stress exposure may be present.

## **DISCUSSION**

MMPs are a family of enzymes which have the ability to degrade components of the extracellular matrix. These degrading enzymes are upregulated in inflammatory processes. Our sample set 1 data revealed levels of salivary MMP-9 that were significantly higher in the low

stress group when compared to the high stress group . This observation implies that there is an association between MMP-9 and psychological factors. It was found that there are significant positive associations between MMP-9 and depression, hostile affect, cynicism, and significant negative association with a sense of coherence (Garvin et al. 2009). Cancer patients with elevated symptoms of depression, chronic stress, and low social support also express elevated MMP-9 levels in tumor associated macrophages as well as in CD68+ cells (Lutgendorf et al. 2008). An explanation for the connection between elevated MMP-9 levels and stress is through the effects of stress hormones, such as norepinephrine and cortisol. Isolated human macrophages stimulated with these stress hormones *in vitro* increased MMP-9 production (Lutgendorf et al. 2008).

Our sample set 1 data also showed higher CRP levels in the high stress when compared to the low stress group. C-reactive protein (CRP) is a protein found in blood, levels of which rise in response to inflammation. It has been shown that there is a greater CRP concentration in lower socioeconomic status (SES) groups defined by occupational status implying an increase in CRP levels associated with a more stressful lifestyle (Owen et al. 2003). Low SES is associated with increased risk of coronary heart disease and immune-related disorders which could in part be reflected by the greater CRP concentration (Owen et al. 2003).

Our sample set 1 data suggests that levels of cortisol increased more in the low stress group from visit 1 to visit two when compared to the high stress group. Previous research suggests that a stronger cortisol response at the time of exposure to a traumatic event has a protective effect against posttraumatic stress disorder symptoms. It was shown that a lower rise in salivary cortisol levels was associated with an increase in risk of PTSD at both one month and 6 months (Yehuda 2002). However, conflicting data has been found in a separate study. In that

work, patients who were PTSD positive at 1 month were found to have lower salivary cortisol levels on the day 2 assessment when compared to those who did not have PTSD (McFarlane et al. 2010).

In sample set 2, IL-18 binding protein (IL-18 Bpa) was found in increased levels in the low stress, visit when group when compared to the other three groups. IL-18 binding protein binds to IL-18 and blocks its biological activity. Therefore, IL-18 is an inhibitor of the Th1 cytotoxic T cell response of the immune system (Novick et al. 1999). Interleukin-18 (IL-18) is a pro-inflammatory cytokine which plays a role in a variety of conditions and diseases including infections, autoimmunity, and cancer. IL-18 levels are elevated by activation of the hypothalamic–pituitary–adrenal (HPA) axis and may be down-regulated by the activation of the para-sympathetic nervous system (Sugama and Conti 2008). Data obtained in humans or in animal models demonstrated an association between IL-18 levels and psychiatric disorders (Sugama and Conti 2008). It has been shown in humans that serum levels of IL-18 were elevated in depression, panic disorders and other stressful conditions (Kokai et al. 2002). Our data shows increased levels of an IL-18 inhibitor in the low stress group. Further data analysis and a final PTSD diagnosis in the patients will be needed to evaluate the linkage between IL-18 binding proteins and PTSD.

Sample set 2 results indicated that levels of IL-8 and alpha-amylase decreased more in the low stress group (between visits 1 and 2) when compared to the high stress group. IL-8 is a chemokine produced by macrophages and other cell types. IL-8 functions as a chemoattractant, and is also a potent angiogenic factor. It has been shown that serum IL-8 concentration in women with psychological symptoms was significantly higher than that in women without psychological symptom indicating a link between IL-8 and psychological stress (Yasui et al. 2007).

Amylase is a calcium-containing metalloenzyme that hydrolyzes starch in the oral cavity and is also considered to play an important role in binding to oral bacteria (Nater et al. 2005). Salivary alpha-amylase levels were found to respond to both physical and psychological stress. While alpha-amylase levels seem to rise following physical stress, the response to a psychological stress appears inconsistent (Nater et al. 2005). This inconsistency might be due to the psychological nature of the stressors employed or due to experimental details, such as measuring alpha-amylase levels at inappropriate time points. However, other studies have shown marked increases in salivary alpha-amylase following psychosocial stress, indicating a stress-dependent activation of salivary alpha-amylase (Nater et al. 2006).

Our sample set 2 results indicate an increase in salivary alpha amylase 2-3 weeks following the traumatic event in the high stress group when compared to the low stress group. The increase in alpha-amylase between visit 1 and visit 2 in the high stress group may be associated with the greater psychological trauma experienced.

### *Further Experiments*

Further analysis of the data already generated will include combining data from multiple biomarkers into potential biomarker signatures. Additionally, grouping suites of biomarkers will be done to assess the presence of immune response types (ex. TH1 vs. TH2 responses, pro-inflammatory vs. anti-inflammatory responses, etc.). It is possible that an integrative analysis will reveal patterns or signatures that are characteristic of different types of psychological stress. Having a molecular mechanism of diagnosing PTSD, quicker diagnosis can be made without filling out a time-consuming questionnaire (the PDS). A molecular analysis of serum or saliva

could be used in place of the more traditional psychological screening exams to improve diagnosis of PTSD and lead to better therapeutic outcomes.

SPEF is a high content assay with improved sensitivity (with detection limits as low as 500 fg/ml) compared to standard fluorescent microarrays. There are other protocol modifications that could still be examined as ways to further improve the speed and accuracy of the GCSPEF and SPEF technologies. These modifications include experimentation with different surface chemistries, different blocking reagents, and altering the antibody and sample concentrations. Variation between chips, reagents, and assays pose a potential difficulty among inter-assay comparisons of SPEF experimental results.

Our current research focuses on the measuring salivary biomarkers associated with psychological stress and trauma. We have already observed significant observable differences between affected populations and controls for multiple immune biomarkers. By grouping these differences into patterns we hope it identify signatures of stress associated with disease, trauma, or toxicant exposure that could be used for clinical diagnosis. Additional statistical analysis needs to be done to further analyze our data obtained from psychologically stressed patients. SPEF could be used in the diagnostic world to speed up the diagnosis of many diseases by looking for many markers of the disease in real time without a requirement for extra labeling steps, such as fluorescence. SPEF technology may be useful in predicting the development of post traumatic stress disorder. Utilizing knowledge of biomarker signatures for PTSD, it may be possible to determine what initially triggered the psychological disorder, making it feasible to control PTSD before it progresses in severity.

In addition to the previously mentioned purposes, the data collected through our experiments will provide research supporting the accuracy and wide range of uses for GCSPEF

and SPEF technologies. Additionally, the saliva experiments further supposed the possibility of utilizing saliva as a safer, less invasive diagnostic biofluid. To further develop the use of saliva as a diagnostic biofluid, more research needs to be done to determine the differences in salivary content throughout the day and night as well as normal differences among the population. The stability and ideal storage methods of saliva also need to be further examined for ideal accuracy in the results of studies using saliva as a biofluid.

### *Future Directions*

The use of biomarker signatures to diagnose psychological or chemical stress is a rapidly expanding field of research. Ultimately, using biomarker signatures, effects of different stresses may be better predicted and controlled and insight can be provided as to some of the health consequences which may result. Our experimental protocols and technologies utilized can be expanded to include a wider variety of stress forms including traumatic brain injury, rheumatoid arthritis, toxicant exposure, cancer and many other forms of stress. In addition, other biofluids may be assayed for biomarker signatures (i.e. blood plasma from patients who have been exposed to various chemical agents). Another application of SPEF technology, in addition to protein interactions, is cell capture using antibodies to cell-surface protein which provides the possibility of cell surface biomarker signature studies.

In summary, we have shown that variation in biomarker levels exist among different stress levels in patients diagnosed with post traumatic stress disorder. These differences in biomarker levels allow for further statistical analysis to develop patterns of biomarker signatures of post traumatic stress disorder diagnosis, onset, severity, and the most effective treatment options. The assays remain to be optimized, and any analysis of the data must take into

account the differences between protocols utilized, individual experimental assay conditions, and differences among the samples used. These assays may be used in the future for the study of additional biological signatures of disease and could provide a novel method of diagnosis.

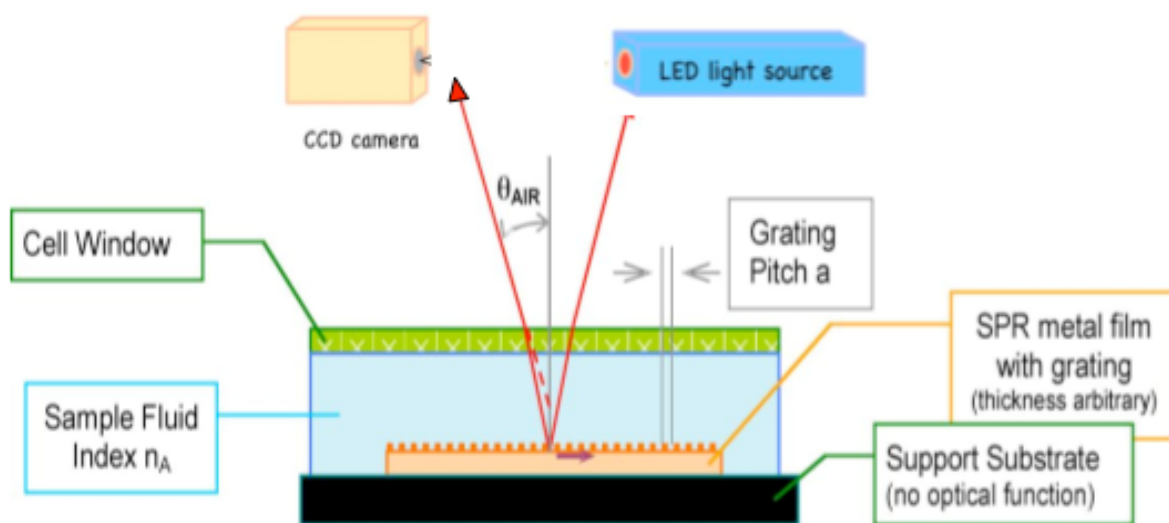
## **ACKNOWLEDGEMENTS**

Saliva samples were provided from the emergency department at UCLA by Dr. Vivek Shetty. Financial support for this project is provided by a grant from the Genes and Environment trans-institutional program of the NIH, the Phillip Austin Summer Undergraduate Research Fund Grant, and the Life Sciences Thesis Grant.

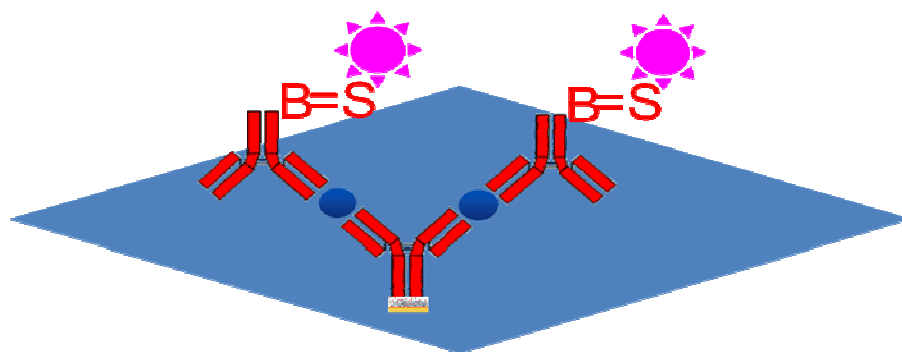


## FIGURES

## Grating Coupled Surface Plasmon Resonance Imaging

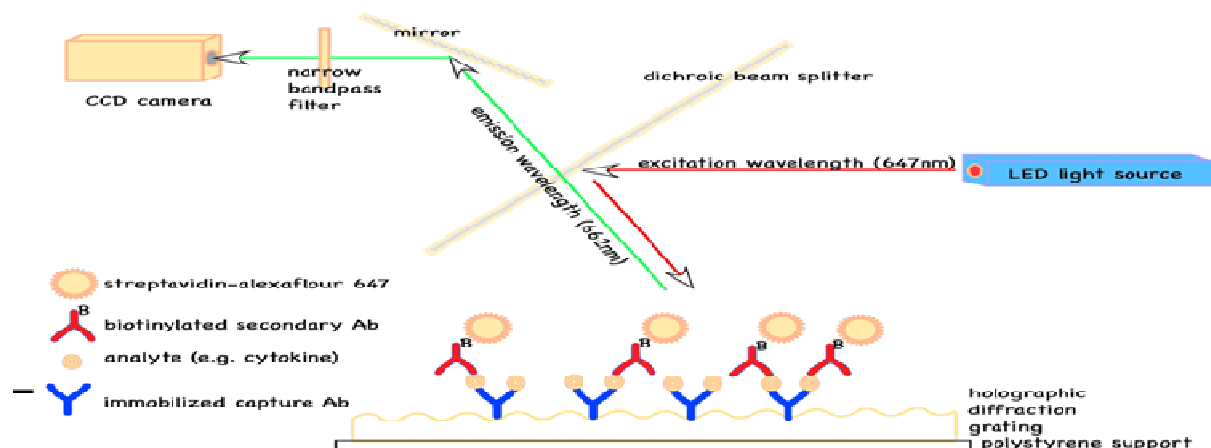


**Figure 1:** GCSPEF Technology Design

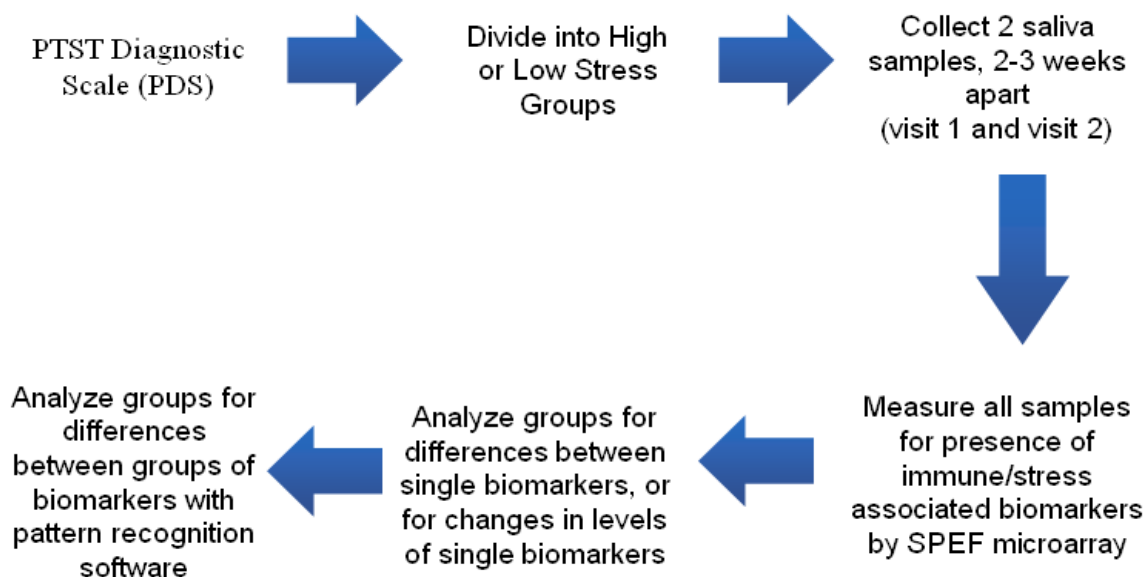


**Figure 2:** Traditional Sandwich Immunoassay Format

## Surface Plasmon Enhanced Fluorescence

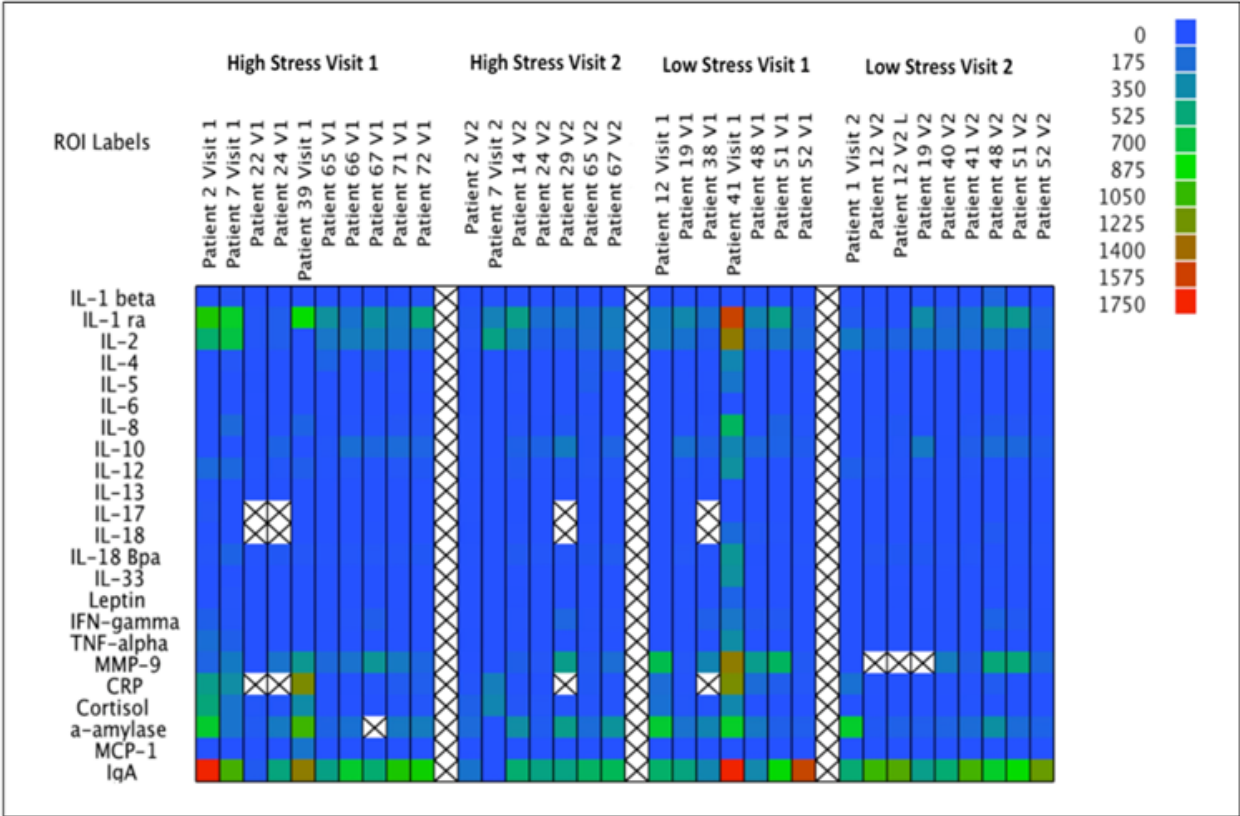


**Figure 3:** SPEF Technology Design



**Figure 4:** Biomarker signatures of PTSD Experimental Design

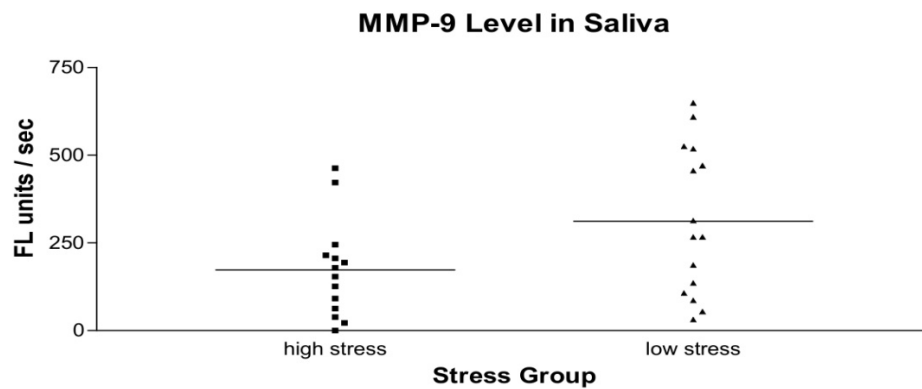
Sample Set 1



**Heatmap display of biomarker levels in saliva from high or low stress groups.** Salivary levels of 26 stress and/or immune associated biomakers were measured from saliva by SPEF microspot assay from high or low stress groups defined by PTSD Diagnostic Scale (PDS).

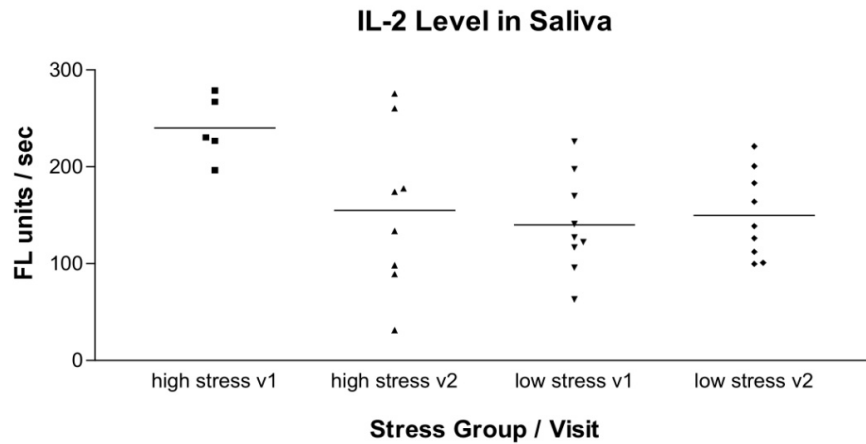
**Figure 5:** Results of Sample Set 1 PTSD Saliva Samples; Heatmap

\*Experiments completed from 06/08/10-09/10/10



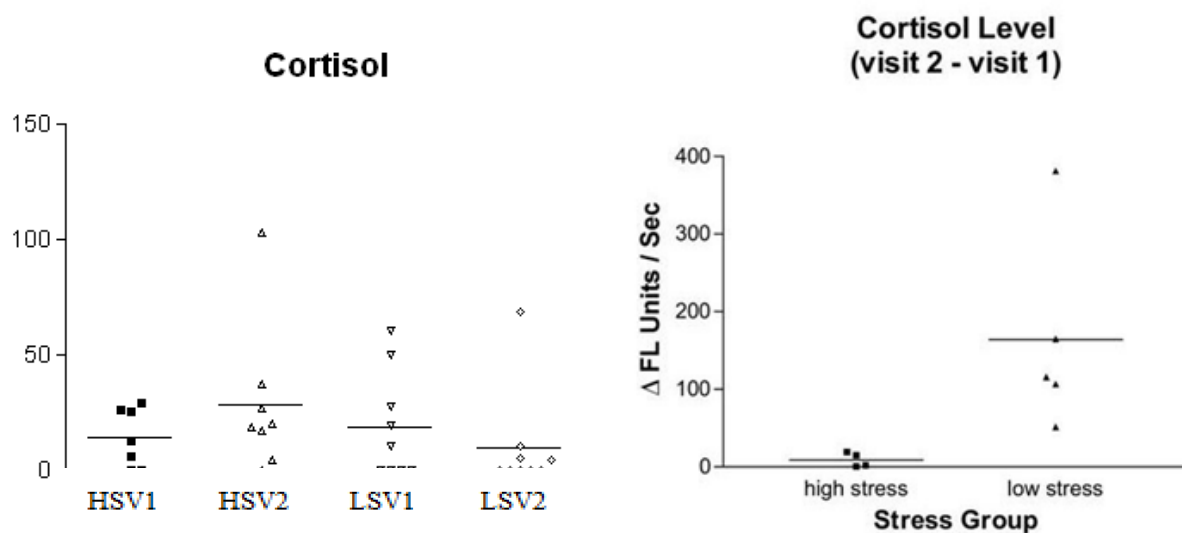
**Salivary stress associated biomarker expression.** Salivary levels of 26 stress and/or immune associated biomarkers were measured from saliva by SPEF microspot assay from high or low stress groups defined by PTSD Diagnostic Scale (PDS). Levels of salivary MMP-9 were significantly higher in the low stress group compared to the high stress group ( $p \leq 0.05$ ).

**Figure 6:** Sample Set : 1MMP-9 Data



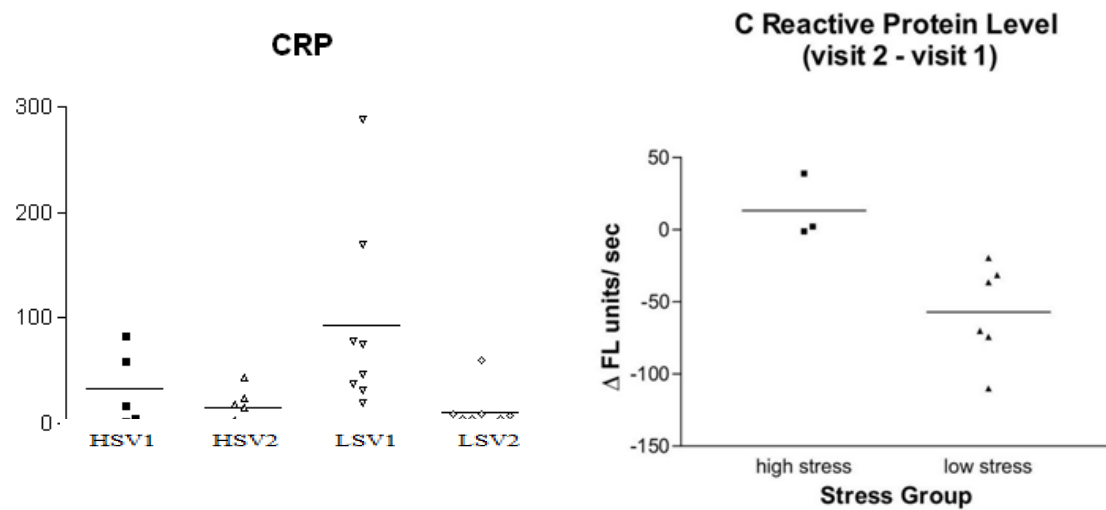
**Salivary stress associated biomarker expression.** Salivary levels of 26 stress and/or immune associated biomarkers were measured from saliva by SPEF microspot assay from high or low stress groups defined by PTSD Diagnostic Scale (PDS). Sample groups were further divided to include visit number and stress condition. Levels of salivary IL-2 were significantly higher in the high stress visit 1 group compared to the other 3 groups ( $p \leq 0.05$ ).

**Figure 7:** Sample Set 1: IL-2 Data



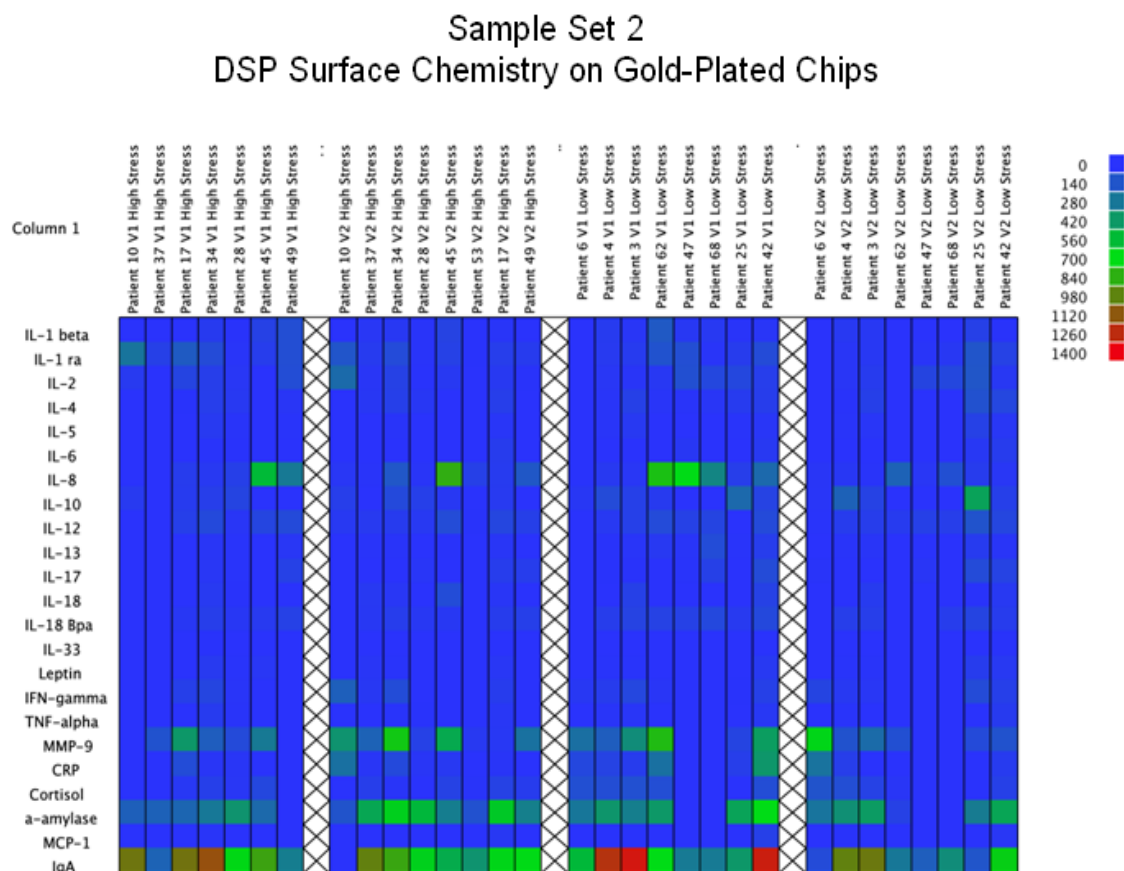
**Changes in the levels of salivary stress associated biomarker expression over time.** Salivary levels of 26 stress and/or immune associated biomarkers were measured from saliva by SPEF microspot assay from high or low stress groups defined by PTSD Diagnostic Scale (PDS). 2 samples were taken two weeks apart after stress exposure and used to identify differences in longitudinal expression of stress associated biomarkers. Changes in the levels of CRP and cortisol were significantly different between the two stress groups in sample set 1 ( $p \leq 0.05$ ).

**Figure 8:** Sample Set 1: Salivary Cortisol Data



**Changes in the levels of salivary stress associated biomarker expression over time.** Salivary levels of 26 stress and/or immune associated biomarkers were measured from saliva by SPEF microspot assay from high or low stress groups defined by PTSD Diagnostic Scale (PDS). 2 samples were taken two weeks apart after stress exposure and used to identify differences in longitudinal expression of stress associated biomarkers. Changes in the levels of CRP and cortisol were significantly different between the two stress groups ( $p \leq 0.05$ ).

**Figure 9:** Sample Set 1: CRP Data

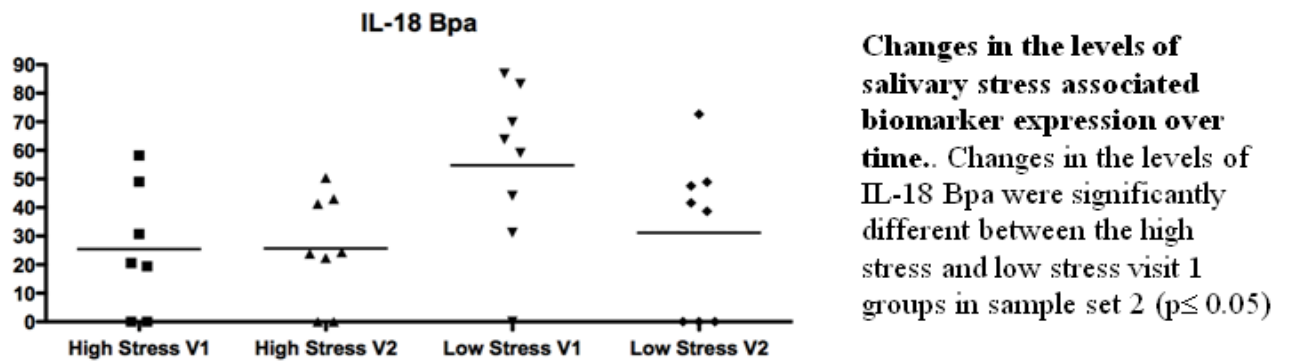


**Heatmap display of biomarker levels in saliva from high or low stress groups.** Salivary levels of 26 stress and/or immune associated biomarkers were measured from saliva by SPEF microspot assay from high or low stress groups defined by PTSD Diagnostic Scale (PDS). Chips were coated with DSP prior to printing.

**Figure 10:** Results of Sample Set 2 PTSD Saliva Samples; Heatmap

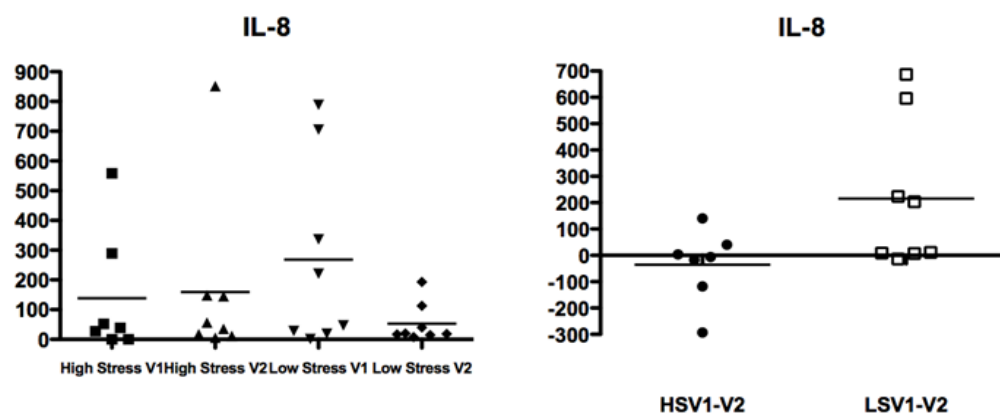
\*Experiments completed from 11/17/10-01/10/11





\* Mann-Whitney U test used

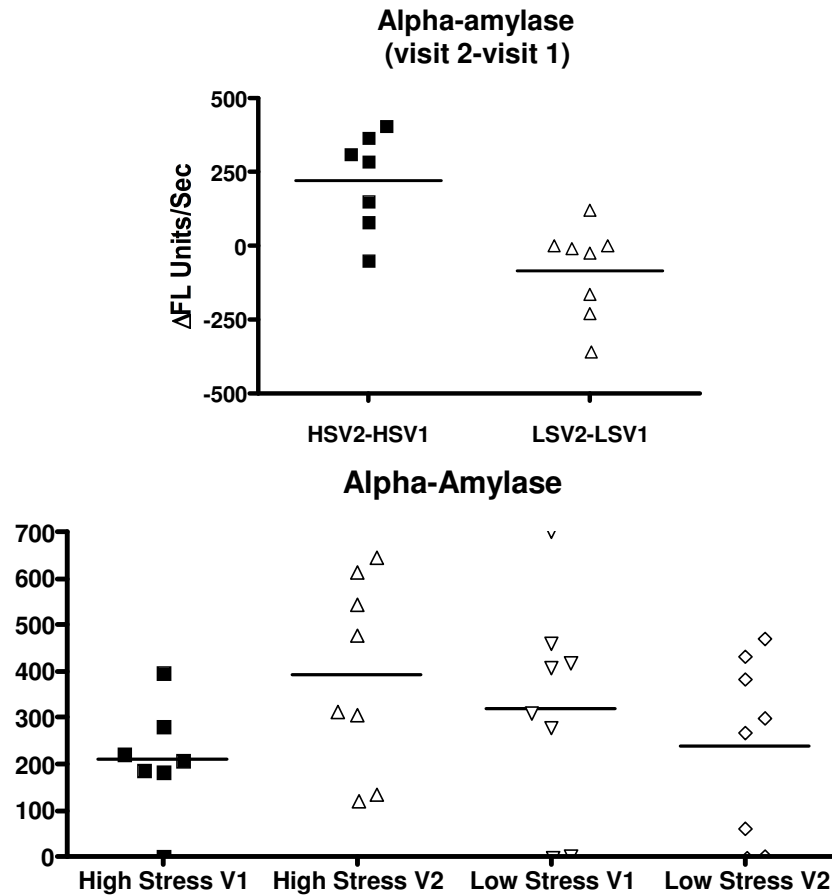
**Figure 11:** Sample Set 2: IL-18 Binding Protein Data



**Changes in the levels of salivary stress associated biomarker expression over time.** Salivary levels of 26 stress and/or immune associated biomarkers were measured from saliva by SPEF microspot assay from high or low stress groups defined by PTSD Diagnostic Scale (PDS). 2 samples were taken two weeks apart after stress exposure and used to identify differences in longitudinal expression of stress associated biomarkers. Changes in the levels of IL-8 and alpha-amylase were significantly different between the two stress groups in sample set 2 ( $p \leq 0.05$ ).

\* Mann-Whitney U test used

**Figure 12:** Sample Set 2: IL-8 Data



**Changes in the levels of salivary stress associated biomarker expression over time.** Salivary levels of 26 stress and/or immune associated biomarkers were measured from saliva by SPEF microspot assay from high or low stress groups defined by PTSD Diagnostic Scale (PDS). 2 samples were taken two weeks apart after stress exposure and used to identify differences in longitudinal expression of stress associated biomarkers. Changes in the levels of IL-8 and alpha-amylase were significantly different between the two stress groups in sample set 2 ( $p \leq 0.05$ ).

\*Paired T-test and Mann-Whitney U tests used

**Figure 13:** Sample Set 2: Alpha-amylase Data

**TABLE 1** Microarray Strategy and Design

Analyte (human)	Type	Rationale	Detection Strategy	Ab Vendor	Plate position
Dilution buffer	Control				O1
BSA	control				O2
IL-1 beta	Inflammatory Cytokine	Increased in stress {Miller, 2006 #4}	Goat – poly $\alpha$ -IL-1 $\beta$ -biotin Str. – Alexa 647	R&D systems	O3
IL-1 ra	Anti-inflammatory Response		Goat – poly $\alpha$ -IL-1ra-biotin Str. – Alexa 647	R&D systems	O4
IL-2	Th1 Cytokine	{Chiappelli, 2006 #5}	Goat – poly $\alpha$ -IL-2-biotin Str. – Alexa 647	R&D systems	O5
IL-4	Th2 Cytokine	{Chiappelli, 2006 #5}	Goat – poly $\alpha$ -IL-4-biotin Str. – Alexa 647	R&D systems	O6
IL-5	Th2 Cytokine		Goat – poly $\alpha$ -IL-5-biotin Str. – Alexa 647	R&D systems	O7
IL-6	Inflammatory cytokine		Goat – poly $\alpha$ -IL-6-biotin Str. – Alexa 647	R&D systems	O8
IL-8	Inflammatory cytokine		Goat – poly $\alpha$ -IL-8-biotin Str. – Alexa 647	R&D systems	O9
IL-10	Anti-inflammatory cytokine		Goat – poly $\alpha$ -IL-10-biotin Str. – Alexa 647	R&D systems	O10
IL-12	T cell stimulating factor		Goat – poly $\alpha$ -IL-12-biotin Str. – Alexa 647	R&D systems	O11
IL-13	Anti-inflammatory cytokine		Goat – poly $\alpha$ -IL-13-biotin Str. – Alexa 647	R&D systems	O12
IL-17	Pro-inflammatory cytokine		Goat – poly $\alpha$ -IL-17-biotin Str. – Alexa 647	R&D systems	O13
IL-18	Cell mediated immunity cytokine		Rat poly $\alpha$ -IL-18-biotin Str. – Alexa 647	MBL	O14
IL-18Bpa	IL-18 binding protein		Goat – poly $\alpha$ -IL-18-biotin Str. – Alexa 647	R&D systems	O15
IL-33	Th2 stimulating cytokine		Goat – poly $\alpha$ -IL-33-biotin Str. – Alexa 647	R&D systems	O16
Leptin	Adiposity signal		Rat – poly $\alpha$ -Leptin-biotin Str. – Alexa 647	R&D systems	O17
Interferon- $\gamma$	Th1 cytokine	{Chiappelli, 2006 #5}	Goat – poly $\alpha$ -IFN- $\gamma$ -biotin Str. – Alexa 647	R&D systems	O18
TNF- $\alpha$	Inflammatory cytokine	{Chiappelli, 2006 #5}	Goat – poly $\alpha$ -TNF- $\alpha$ -biotin Str. – Alexa 647	R&D systems	O19
MMP-9	gelatinase	{Menon, 2006 #11}	Goat – poly $\alpha$ -MMP-9-biotin Str. – Alexa 647	R&D systems	O20
C-reactive protein	Inflammatory Biomarker		Rat– poly $\alpha$ -CRP-biotin Str. – Alexa 647	R&D systems	O21
Cortisol	Corticosteroid hormone	{Wagner, #8}{Takatsuji, 2008 #10} increased with stress	Rabbit – poly $\alpha$ -Cortisol  Dk $\alpha$ -rabbit-Ig – alexa647	AbD Serotec	O22
$\alpha$ -amylase	Salivary amylase	{Wagner, #8} increased with stress	Goat – poly $\alpha$ -amylase Rabbit $\alpha$ -goat IgG-alex647	Santa Cruz Biotechnol ogy	O23
MCP-1	Monocyte, T <sub>m</sub> chemokine	{Jiang, 1999 #1}, indirect LPS response biomarker in	Goat – poly $\alpha$ -MCP-1-biotin Str. – Alexa 647	AbD Serotec	O24

		saliva			
IgA	Secretory Ig	{Takatsuji, 2008 #10}	M.mono. $\alpha$ -human kappa light chain- biotin M.mono $\alpha$ -human lambda light chain – biotin Str. – Alexa 647	AbD Serotec	P1
IgG	Human Ig		M.mono. $\alpha$ -human kappa light chain- biotin M.mono $\alpha$ -human lambda light chain – biotin Str. – Alexa 647		P2
IgM	Human Ig		M.mono. $\alpha$ -human kappa light chain- biotin M.mono $\alpha$ -human lambda light chain – biotin Str. – Alexa 647		P3
IgE	Human Ig		M.mono. $\alpha$ -human kappa light chain- biotin M.mono $\alpha$ -human lambda light chain – biotin Str. – Alexa 647		P4
MOPC	Mouse IgG Negative control	control			P5
Goat anti-mouse IgM	Anti-goat IgG-alexa647 positive control	control	Rabbit $\alpha$ -goat Ig – Alexa647		P6
Rabbit anti-mouse CCR3	Anti-rabbit IgG-alexa647 positive control	control	Donkey $\alpha$ -rabbit Ig – Alexa647		P7
Biotin-mIgG	Streptavidin alexa647 – positive control	control	Str.-Alexa647		P8
BSA	Protein Block NSB control	control			P9
Dilution Buffer	Negative Control	control			P10

**TABLE 2** Detection Antibody Cocktail: Antibody Dilution Factors

Analyte (human)	FL Detection Strategy	Ab Vendor / stock ab concentration	Recommended working conc. / dilution range	Working concentration / dilution used	Volume of stock antibody in 1.5 ml PBS-T
Dilution buffer					
BSA					
IL-1 beta	2 <sup>o</sup> - biotin* / avidin-alexa647	R&D systems 50 $\mu$ g /mL	0.1- 0.4 $\mu$ g /mL	0.25 $\mu$ g /mL	7.5 $\mu$ l
IL-1 ra	2 <sup>o</sup> - biotin* / avidin-alexa647	R&D systems 50 $\mu$ g /mL	0.1- 0.4 $\mu$ g /mL	0.25 $\mu$ g / mL	7.5 $\mu$ l
IL-2	2 <sup>o</sup> - biotin* / avidin-alexa647	R&D systems 50 $\mu$ g /mL	0.1- 0.4 $\mu$ g /mL	0.25 $\mu$ g /mL	7.5 $\mu$ l
IL-4	2 <sup>o</sup> - biotin* / avidin-alexa647	R&D systems 50 $\mu$ g /mL	0.1- 0.4 $\mu$ g /mL	0.25 $\mu$ g /mL	7.5 $\mu$ l
IL-5	2 <sup>o</sup> - biotin* / avidin-alexa647	R&D systems 250 $\mu$ g /mL	0.5- 2 $\mu$ g/ mL	1.25 $\mu$ g/ mL	7.5 $\mu$ l

IL-6	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 50µg /mL	0.1- 0.4 µg /mL	0.25 µg /mL	7.5 µl
IL-8	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 3.6 µg /mL	20 ng /mL	20 ng /mL	8.3 µl
IL-10	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 50µg /mL	0.1- 0.4 µg /mL	0.25 µg /mL	7.5 µl
IL-12	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 50µg /mL	0.1- 0.4 µg /mL	0.25 µg /mL	7.5 µl
IL-13	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 50µg /mL	0.1- 0.4 µg /mL	0.25 µg /mL	7.5 µl
IL-17	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 50µg /mL	0.1- 0.4 µg /mL	0.25 µg /mL	7.5 µl
IL-18	2 <sup>o</sup> - biotin* / avidin- alexa647	MBL 1 mg/mL	1:1000 dillution	1:1000 dillution	1.5 µl
IL-18Bpa	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 36µg /mL	200 ng/ml	200 ng/ml	8.3 µl
IL-33	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 50µg /mL	0.1- 0.4 µg /mL	0.25 µg /mL	7.5 µl
Leptin	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 250µg /mL	0.5- 2 µg/ mL	1.25 µg/ mL	7.5 µl
Interferon-γ	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 9µg / mL	50 ng/ mL	50 ng / mL	8.3 µl
TNF-α	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 50µg / mL	0.1- 0.4 µg /mL	0.25 µg /mL	7.5 µl
MMP-9	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 50µg / mL	0.1- 0.4 µg /mL	0.25 µg /mL	7.5 µl
C-reactive protein	2 <sup>o</sup> - biotin* / avidin- alexa647	R&D systems 22.5µg / mL	125 ng / mL	125 ng / mL	8.3 µl
Cortisol	2 <sup>o</sup> - rabbit IgG / anti- rabbit IgG- alexa647	AbD Serotec 5mg / mL	?	1:1000	1.5 µl
α-amylase	2 <sup>o</sup> - goat IgG / anti- goat IgG- alexa647	Santa Cruz Biotechnology 200 µg / mL	1:30 – 1:3000	1:1000	1.5 µl
MCP-1	2 <sup>o</sup> - biotin* / avidin- alexa647	AbD Serotec 50 µg/ mL	2- 4µg / mL	0.3 µg / mL	9 µl

IgA	2° $\alpha$ - $\kappa$ + $\alpha$ - $\lambda$ biotin* / avidin-alexa647	AbD Serotec 1mg / mL	1:2000 – 1:4000	1:2000	1 $\mu$ l
IgG	2° $\alpha$ - $\kappa$ + $\alpha$ - $\lambda$ biotin* / avidin-alexa647	eBioscience 500 $\mu$ g / ml	1 $\mu$ g / ml	0.5 $\mu$ g / ml	1 $\mu$ l
IgM	2° $\alpha$ - $\kappa$ + $\alpha$ - $\lambda$ biotin* / avidin-alexa647	eBioscience 500 $\mu$ g / ml	1 $\mu$ g / ml	0.5 $\mu$ g / ml	1 $\mu$ l
IgE	2° $\alpha$ - $\kappa$ + $\alpha$ - $\lambda$ biotin* / avidin-alexa647	eBioscience 500 $\mu$ g / ml	1 $\mu$ g / ml	0.5 $\mu$ g / ml	1 $\mu$ l
MOPC					
Goat anti-mouse IgM	Donkey $\alpha$ -goat Ig – Alexa647	Invitrogen 2mg / ml	1-10 $\mu$ g / ml	4 $\mu$ g/ml	1 $\mu$ l
Rabbit anti-mouse CCR3	Donkey $\alpha$ -rabbit Ig – Alexa647	Invitrogen 2mg / ml	1-10 $\mu$ g / ml	4 $\mu$ g/ml	1 $\mu$ l
Biotin-mIgG	Str.- Alexa647	Invitrogen 2mg / ml	?	400 ng / ml	2 $\mu$ l stock -> 1 ml PBS-T, mix, add 150 $\mu$ l to final of 1.5 ml
BSA					
Dilution Buffer					

Antibodies from R&D Systems

**Table 3** Detection Antibody Cocktail: Reagent Dilution Factors

Analyte (human)	FL Detection Strategy	Detection reagent stock concentration	Detection reagent working concentration	Volume of stock det. reagent in 1.5 ml PBS-T
Dilution buffer				
BSA				
IL-1 beta	2° - biotin* / avidin-alexa647	Streptavidin-alexafluor 647 2mg /ml	200 ng / ml	1 $\mu$ l of stock in 1 ml of PBS, mix, add 150 $\mu$ l
IL-1 ra	2° - biotin* / avidin-alexa647	Streptavidin-alexafluor 647 2mg /ml	200 ng / ml	1 $\mu$ l of stock in 1 ml of PBS, mix, add 150 $\mu$ l
IL-2	2° - biotin* / avidin-alexa647	Streptavidin-alexafluor 647 2mg /ml	200 ng / ml	1 $\mu$ l of stock in 1 ml of PBS, mix, add 150 $\mu$ l
IL-4	2° - biotin* / avidin-alexa647	Streptavidin-alexafluor 647 2mg /ml	200 ng / ml	1 $\mu$ l of stock in 1 ml of PBS, mix, add 150 $\mu$ l
IL-5	2° - biotin* / avidin-alexa647	Streptavidin-alexafluor 647 2mg /ml	200 ng / ml	1 $\mu$ l of stock in 1 ml of PBS, mix, add 150 $\mu$ l

IL-6	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
IL-8	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
IL-10	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
IL-12	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
IL-13	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
IL-17	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
IL-18	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
IL-18Bpa	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
IL-33	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
Leptin	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
Interferon-γ	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
TNF-α	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
MMP-9	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
IgA	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
C-reactive protein	2 <sup>o</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
Cortisol	2 <sup>o</sup> - rabbit IgG / anti-rabbit IgG-alexa647	Donkey anti- rabbit IgG (H+L) alexa647 2mg/ml	5 ug / ml	3.7 µl
α-amylase	2 <sup>o</sup> - goat IgG / anti-goat IgG- alexa647	Rabbit anti-goat IgG (H+L) alexa647 2mg/ml	5 ug / ml	3.7 µl



MCP-1	2 <sup>O</sup> - biotin* / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
<i>E. coli</i>	2 <sup>O</sup> - biotin / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
<i>Listeria monocytogenes</i>	2 <sup>O</sup> - biotin / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
Metallothionein				
RF (IgM anti- h IgG)	Anti-h IgM 2 <sup>O</sup> - biotin / avidin- alexa647	Streptavidin- alexafluor 647 2mg /ml	200 ng / ml	1 µl of stock in 1 ml of PBS, mix, add 150 µl
MOPC				
Goat anti-mouse IgM				
Rabbit anti-mouse CCR3				
Biotin-mIgG				
BSA				
Dilution Buffer				

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