Integration of Salivary Bioscience into Behavioral, Health, and Sports Sciences:

How Biomarkers in Saliva can Benefit Your Research Program

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Disclosure Statement

In the interest of full disclosure, Douglas A. Granger is the founder (or co-founder), serves as the Chief Scientific and Strategy Advisor, and holds equity in Salimetrics LLC and Salivabio LLC.

These relationships are managed by the policies of the Conflict of Interest Committee at the Johns Hopkins University School of Medicine.
Effects of context on development moderated through individual differences in stress responsive biological systems.

Biology and behavior have reciprocal effects and expression of bio-behavioral relationships is dependent on context.

Probability that individual differences in biological reactivity and regulation linked to outcomes of interest highest when studied in meaningful social contexts.

Biological systems are networked and multi-system measurement of stress response is critical.
Perturbing the organism: The biology of stressful experience

Environmental Demands (physical, social, cultural)

Behavioral Surface (emotion regulation, coping, flight-fight, tend-befriend)

Fast Acting—Physiological Processes (neural, HPA, ANS activity)

Slow Acting—Physiological Processes (genetic activity)

Individual Development

Biological Susceptibility to Context, Adaptive Calibration

Intra-individual patterns of stress-reactivity

Normative-adaptive pattern assumes habituation of reactivity and recovery over time.
<table>
<thead>
<tr>
<th>Advantage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Minimally Invasive”</td>
<td>Considered “acceptable and non-invasive” by research participants. Collection is quick, non-painful, uncomplicated.</td>
</tr>
<tr>
<td>“Safety”</td>
<td>Reduces transmission of infectious disease by eliminating the potential for accidental needle sticks. CDC does not consider saliva a class II Biohazard unless visibly contaminated with blood.</td>
</tr>
<tr>
<td>“Economics”</td>
<td>Eliminates the need for a health care intermediary (e.g., phlebotomist, nurse). Resources for collection and processing samples are low cost and available.</td>
</tr>
<tr>
<td>“Accuracy”</td>
<td>Salivary levels of many analytes represent the “free unbound fraction” or biological active fraction in the general circulation.</td>
</tr>
<tr>
<td>“Ecological Validity”</td>
<td>Enables biological reactivity and regulation to be monitored in everyday social world.</td>
</tr>
<tr>
<td>“Multiple Participants”</td>
<td>Enables samples to be collected from groups of individual participants simultaneously in real time.</td>
</tr>
</tbody>
</table>

Slavkin /Mandel/Malamud
Oral fluid subtypes that compose “Whole Saliva”

- Sublingual saliva (4%)
- Parotid saliva (23%)
- Submandibular saliva (65%)
- Minor saliva glands (7%)
- Cervicular fluid (1%)
Movement of Biomarkers into Saliva

- Passive diffusion
- Ultrafiltration
- Active transport
- Synthesis

Acinar cells

Capillaries surrounding glands
Numerous capillaries surround the saliva glands. Hormones and other compounds pass through the capillary walls and bathe the salivary glands.

Cortisol is secreted into the bloodstream from the adrenal cortex. About 95% of it binds to carrier proteins (binding globulin). Only the remaining 5% is available for use by target tissues.

Unbound cortisol is lipid soluble and can move through the lipo-protein cell membranes of the secretory cells by passive diffusion. It is then released into the saliva.
Many analytes are released locally into oral fluids and are not from blood.

Knowing whether an analyte measured in oral fluid is or is not from blood is key to interpretation of the meaning of individual differences.
Analytes in Oral Fluid of Interest to Health Sciences

- Cortisol
- Dehydroepiandrosterone (s)
- Testosterone, androstenedione
- Estradiol, estrone, estriol
- sIgA
- Progesterone and 17-alpha-hydroxy-Progesterone
- Cotinine
- Drugs of Abuse and Environmental Chemicals
  - C-Reactive Protein, Neopterin, Beta-2-microglobulin
  - Melatonin, Oxytocin, NPY, VIP
  - Cytokines (e.g., IL-6, TNFa, IL1b), soluble cytokine receptors (sTNF-I, -II)
  - Disease specific antibodies (e.g., HIV, HSV, EBV, CMV) or antigens
  - Alpha-amylase, Chromogranin A
  - Metalloproteinases (MMP-8)
  - Heat Shock Proteins, aldosterone
  - Adiponectin, Leptin, Ghrelin
  - Electrolytes
  - DNA and Methylation, RNA
  - Microbiome
  - GFAP, Neurogranin
Salivette Module with Oral Swab

Easy to use
Excellent Recovery
1.5 mL capacity

Restricted analysis potential
Small size-choking hazard
Large head space
Location of placement
BD Hydrocellulose Microsponge

Handle
Highly absorbent
Recovery of low volumes
Sleeping participant

High surface area
Evaporation hazard
Use two
Restricted analysis

Handling, Transport, Storage

- Biosafety
- Bacteria growth and specimen stability
- Freeze thaw
- Field storage
- Shipping rules and regulations
- Long term storage and archiving
Enzyme Immunoassay Competitive Binding

Conjugate
Standard and/or sample are added
Antibody on EIA plate

Unbound analytes are washed away
Bound analytes

Substrate (TMB)

4 parameter

Concentration

4 Parameter (y = CA - D) / (1 + C/CPB * D)
Axes 2D

Standards
Separated Data
Standard Curve

G:\2011\B2\1887\10372\0.5 DP 4M 0.5 s 9600
Multiplexing Technology
Dampened Salivary Alpha-amylase and Cortisol Reactivity to Psychosocial Stress: Maltreated versus Comparison Adolescents

Cortisol, Alpha-amylase, and subjective emotional reactivity in women with Borderline Personality Disorder (BPD)

(Scott, Granger, Levy, 2011)

The BPD group had higher average baseline and overall average NA than both NTM and TM groups.

Both the BPD and TM groups demonstrated attenuated stress-related sAA reactivity as compared to the NTM group.

The BPD group had higher average baseline and overall average NA than both NTM and TM groups.
Developmental differences in sAA Response to Inoculation Stress

- 2-month old infants did not show an sAA response.
- 6 and 12-month old infants displayed a significant sAA increase.
- 24-month old infants displayed an anticipatory rise and decrease in sAA.

Internalizing problem behavior and sAA reactivity to social exclusion task

Allwood et al., (2011). Biological Psychology, 88, 57-64
Maternal Engagement in Early Infancy Predicts Children’s Cortisol Reactivity at 15 months

Figure 2. Cortisol reactivity and regulation in response to emotion challenge in the toddler period as a function of maternal engagement in infancy. Dashed line = low maternal engagement; solid line = high maternal engagement as defined by median split; post = post–peak arousal. Error bars represent the sampling error of the mean.
# Effects of early adversity on stress reactivity in adulthood

<table>
<thead>
<tr>
<th>Birth Group</th>
<th>Neonatal Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Term</td>
<td>Full Term; medically &amp; neurologically healthy</td>
</tr>
<tr>
<td>Healthy Preterm</td>
<td>Premature; no medical/neurological complications</td>
</tr>
<tr>
<td>Medical Preterm</td>
<td>Premature; neonatal medical illness (BPD, RDS, NEC, sepsis)</td>
</tr>
<tr>
<td>Neurological Preterm</td>
<td>Premature; neonatal neurological illness (Grade III &amp; IV IVH, meningitis, shunted hydrocephalus)</td>
</tr>
<tr>
<td>Small for Gestational Age</td>
<td>Premature; birth weight for gestational age &lt; 10th percentile</td>
</tr>
<tr>
<td>Preterm</td>
<td>Premature; birth weight for gestational age &lt; 37 weeks gestation</td>
</tr>
</tbody>
</table>

*Full Term (>37 weeks gestation) *

Sullivan, Mills, Winchester, Granger (in prep)
**NPT group**: highest post-stressor cortisol levels, with the sharpest decline. Recovery does not fall below baseline.

**HPT group**: most dysregulated pattern for stress reactivity.

**SGA group**: blunted pattern marked by lower than normal baseline, lower peak cortisol levels 15-minutes post-stressor, and a slow recovery to social stress.
Army Nurse Combat Casualty Stress Task

McGraw et al. (2012) Psychoneuroendocrinology
Salivary Cortisol Response to Rowing Ergometer Competition

Men and women’s endocrine responses were more different than alike and varied by level of experience.

Individual differences associated with social affiliation rather than dominance or competitiveness.

Kivlghan, Granger, Booth (2005). *Psychoneuroendocrinology*
Salivary sAA Response to Rowing Ergometer Competition

sAA higher for varsity than novice, and associated with performance.

sAa reactivity associated with perceived dominance

sAA reactivity specific to competition, and levels Higher in men than women

Kivlighan & Granger (2006) Psychoneuroendocrinology
Mucosal IgA and URTI in American College Football Players: A Year Longitudinal Study

Immunological differences in English Premier League football players with infections

28 players in total were sampled on up to 4 occasions (total of n=94); 5 players had viral infections on 11 occasions.
A longitudinal study of changes in s-IgA and respiratory illness in athletes

- 38 members of America’s Cup yacht crew
- Studied over 50 weeks of training and sailing
- Morning saliva samples collected weekly
- Clinically confirmed illness

Relative s-IgA before and after an infection episode

Gleeson
Conclusions

• On a group basis, relative s-IgA determined a substantial proportion of the variability in weekly infection incidence

• Significant reduction in s-IgA in the 3 weeks prior to infection

• Relative s-IgA value <40% of healthy baseline value indicated a 50% chance of contracting an infection within 3 weeks

Hence, possible predictive value with regular monitoring
Monitoring hormones

- With stress: ↑ C and ↓ T
- C/T ratio ↑ 30% = Overreached/Stressed
- Salivary C/T (1000*nM/pM) ratio > 40 = Stressed or Not Recovered
Monitoring recovery in RFU players

- 10 England International RFU Players
- 29 1 years, 102.4 3.9 kg, 1.86 0.02 m
- 4 saliva samples collected each wk for 3 weeks
- Morning collection, before breakfast
- IgA, Cortisol, Testosterone measured by ELISA
- Transferrin (blood contamination) by ELISA
- Training load monitored
- 3 international matches
- Players’ perceptions of fatigue and stress monitored using the REST-Q questionnaire
Training Load

Gleeson
Cortisol / Testosterone Ratio

Effect of time (P<0.001)

* P<0.05; ** P<0.01 vs #1

Effect of time (P<0.001)
Conclusions

• On a group basis, saliva C/T ratio appears to be indicative of stress or inadequate recovery
• May help in recovery prescription for individual players
• S-C/T >40 likely associated with underperformance

Hence, possible value with monitoring during intensive phases of season
Momentary Cortisol - Behavior Associations in Everyday Contexts

Diurnal variation determined by fixed sampling at specific times

Momentary Determination

Residual above curve

Residual below curve

Cortisol \( \text{ug/dL} \)
# Dyadic Similarity in Cortisol Levels

<table>
<thead>
<tr>
<th></th>
<th>Mother</th>
<th>Father</th>
<th>Middle Child</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>.34**</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(326)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle Child</td>
<td>.19**</td>
<td>.21**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(320)</td>
<td>(326)</td>
<td></td>
</tr>
<tr>
<td>Adolescent</td>
<td>.34**</td>
<td>.24**</td>
<td>.22**</td>
</tr>
<tr>
<td></td>
<td>(320)</td>
<td>(322)</td>
<td>(318)</td>
</tr>
</tbody>
</table>

** P<.01

400 Mothers, Fathers, Older and younger sibling Saliva sampled at home two mornings Relationship Quality Moderates Martial satisfaction and conflict, spouse who are congruent have higher covariation in cortisol levels

Booth, Johnson, Goslin, & Granger (in prep)
Approach would theoretically enable the exploration of factors that contribute to each individual member's contribution to the family level construct, and contribute to differences between groups.
Social Networks: Families, Teams, Classes

Self-reported Friendship Nominations in 6th Grade

Thick blue lines are reciprocated Friendships

Thin grey lines are asymmetric Friendships

Data provided by Scott Gest, PhD, Penn State University
Cortisol, Testosterone, and the Social Network: Friendship Nominations
Assessing genetic polymorphisms using DNA extracted from cells present in saliva samples.

Are the typical volumes of saliva collected in studies of child development sufficient to enable isolation of DNA for genetics analyses?

Do collection device materials affect the isolation of sufficient quantity and quality of DNA from saliva?

What are the effects of room temperature (RT) storage of saliva samples on the quality and quantity of DNA recovered?

Do archived saliva samples that have been exposed to multiple freeze-thaw cycles yield sufficient quantity and quality of DNA for genetic analyses?

When oral fluids are collected from specific areas in the mouth associated with different salivary glands, is there a difference in DNA quantity and quality?

Nemoda, et al. (2012). *BMC Medical Research Methodology*. 
OXTR and the Social Network: Friendship Nominations

AA – Black; AG – Red; GG - Blue
Epigenetics

- **Global Methylation** -- Levels correlated in DNA extracted from cells from oral fluid compared to DNA extracted from cells from whole blood
Cytokines

- **Design**: 113 Healthy Adolescent Girls, multiplex EC Immunoassay, 3 waves of annual samplings
  - GM-CSF, IFNγ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL12-p70, and TNFa.

- **Cytokine levels**: all cytokines, except IFNγ and IL-10, were detectable in the majority of saliva samples; non-normal distributions; levels of all cytokines lower in saliva than serum except IL-8 and IL-1β; saliva levels of IL-8 and IL-1β were >20x higher than other salivary cytokines;

- **Cytokine Inter-correlations**: in saliva were lower than in serum; individual differences in cytokine levels were more stable across years when measured in serum than in saliva;

- **Stability**: there were strong associations between salivary adiponectin and cotinine with salivary but not serum cytokines; and

- **Serum-saliva correlation**: only IL-1β

- **Correlates**: Salivary cytokine levels were not associated with age, pubertal development, or gynecological age,

- **Conclusion**: Variation in salivary cytokine levels largely reflects compartmentalized local activity of the oral mucosal immune system raising questions about the role they play, if any, in intracellular and hormonal signaling within the neuro-endocrine-immune network.

Granger et al (in prep)
**Prospective Markers of TBI in Saliva**

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Primary Source</th>
<th>Primary Function/Use</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>filament and cytoskeleton protein</td>
<td>astrocytes</td>
<td>marker of astrocytes, astrocyte differentiation, astrogliosis, tumor marker</td>
<td>45 kDa</td>
</tr>
<tr>
<td>S100B</td>
<td>calcium-binding protein</td>
<td>cytoplasm of astroglia and Schwann cells</td>
<td>participates in intracellular activities, marker of pathological conditions and injury</td>
<td>20 kDa</td>
</tr>
<tr>
<td>NSE</td>
<td>glycolytic enzyme</td>
<td>cytoplasm of neurons, neuroendocrine cells</td>
<td>released under pathological conditions, marker of cell damage, tumor marker</td>
<td>78 kDa</td>
</tr>
<tr>
<td>Neurogranin</td>
<td>neuroprotein, protein kinase C substrate</td>
<td>neurons</td>
<td>participates in synaptic signaling, marker of dendrite spine density and synaptic plasticity</td>
<td>7.5 kDa</td>
</tr>
<tr>
<td>BDNF</td>
<td>neurotrophin</td>
<td>widespread in CNS</td>
<td>marker related to neuron growth and survival</td>
<td>14 kDa</td>
</tr>
<tr>
<td>Beta-Synuclein</td>
<td>soluble protein</td>
<td>presynaptic brain tissues, astrocytes</td>
<td>development and maintenance of synaptic function, tumor marker</td>
<td>19 kDa</td>
</tr>
<tr>
<td>ICAM-5</td>
<td>transmembrane glycoprotein</td>
<td>neurons</td>
<td>synapse development, immune and inflammatory responses</td>
<td>130 kDa</td>
</tr>
<tr>
<td>MT-III</td>
<td>metal-binding protein</td>
<td>astrocytes</td>
<td>protects neurons and other tissues against toxic factors, oxidative stress</td>
<td>6 kDa</td>
</tr>
</tbody>
</table>
Future Directions

- Salivary bioscience is poised to enable and facilitate advances in multiple fields

- Emerging applications have implications for screening and diagnostics, and decisions related to personal health, well-being, and performance

- Prevention and intervention science

- Community of interdisciplinary scholars linked together by an international network of centers of excellence
Questions and Discussion

Johns Hopkins University
Center for
Interdisciplinary
Salivary Bioscience
Research