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Biological Measurement in Intervention Research

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BIOLOGICAL MEASUREMENT IN INTERVENTION RESEARCH

Donna McCarthy

> "What is the use of repeating all that stuff, if you don't explain it as you go on?"—*Alice in Wonderland*

Studies to test an intervention will have greater impact if there is also a measure indicating the mechanism of treatment effect. Integrating biologic measures into biobehavioral research increases our understanding of how an intervention produced or did not produce the anticipated outcome. The selection of biologic measures (see Table 9.1) should be based on a clear understanding of the biology contributing to the intervention effect. However, human biology is complex, with considerable cross talk between body systems, such as the sympathetic nervous system (SNS), the hypothalamus-pituitary-adrenal axis (HPA), immune function, and the inflammatory response. Each of these systems affects each other, and each is impacted by behavioral, social, and psychological factors. Thus, it is important to consider these potential factors when designing an intervention study.

This chapter will focus on biologic measures (biomarkers) of HPA activity, SNS activity, immune function and inflammation that have great potential for intervention research based on their sensitivity to changes within individuals over time, and between groups. These biomarkers include cortisol, salivary amylase, antibody titers, and proinflammatory cytokines. They can be measured in blood, saliva, or urine, which are easily obtained in clinical and field settings with low risk to human subjects. Some of these biomarkers can be measured in samples of hair and sweat, which may be easier to obtain in some settings or with some populations.

Reagents and kits for measuring these biomarkers are commercially available and can be used in a clinical or research laboratory. The manufacturers often provide step-by-step protocols that have been carefully developed to help you obtain high-quality results using their product. However, you should do extensive pilot testing to be sure the

TABLE 9.1 Biological Measures

	BIOMARKER	BLOOD	URINE	SALIVA	HAIR	SWEAT
HPA	Cortisol	x	x	x	x	?
SNS	Alpha-amylase			x		
Inflammation	Cytokines/cytokine receptors	x	?	?		?
	Neopterin	x	x	?		?
	C-reactive protein	x		?		?
Immune function	IgA (total or antigen specific)			x		
	IgG, IgM (antigen specific)	x				

Note. HPA = hypothalamus-pituitary-adrenal axis; SNS = sympathetic nervous system; IgA = Immunoglobulin A; IgG = Immunoglobulin G; IgM = Immunoglobulin M.

biomarker can be detected in your study population, and that it is sensitive to change caused by the planned intervention. For example, if you hypothesize that an intervention will improve sleep quality by promoting relaxation, you might include a subjective measure of sleep quality and a biological measure of SNS activity at bedtime. The work of Barbara Andersen's group is an excellent example of a program of research that included subjective measures of emotional distress and biological measures of HPA activity, SNS activity, and immune cell function to determine the effects of a psychoeducational support intervention in women with breast cancer (Andersen et al., 2010).

BIOLOGICAL MEASURES COMMONLY USED IN BIOBEHAVIORAL RESEARCH

HPA Activity: Cortisol Secretion

Cortisol is a steroid hormone that plays a significant role in regulating the availability of glucose to support cellular metabolism. It is secreted from the adrenal gland continuously, and 90% of the cortisol in the circulation is bound to carrier proteins. Only free cortisol is able to interact with its receptors and is considered to be the biologically active fraction. Because it can diffuse out of capillaries to reach its target tissues, unbound cortisol can also be measured in saliva or urine. Salivary cortisol is an accurate reflection of the unbound, biologically active cortisol circulating in the blood stream at any given time point. Because only the unbound fraction of cortisol can be detected in saliva, salivary cortisol cannot be used to assess adrenal gland function. It should also be noted that less than 1% of unbound cortisol is excreted in urine; 70% is excreted as cortisol metabolites over the course of several hours, making it insensitive to changes in cortisol secretion during the course of the day.

The HPA axis is a dynamic, self-regulated feedback system that matches plasma cortisol levels to environmental demands. Circulating levels of cortisol are highest in the morning and lowest in the late evening. Measurement of morning levels must account for a surge in cortisol levels that occurs within 30 minutes of awakening, called the

cortisol awakening response (CAR), after which levels decline to their nadir at night. The magnitude of change between morning and evening cortisol levels declines with age, as does the CAR. Infants do not have a detectable morning–evening rhythm until after 3 months of age, and circadian rhythms of cortisol secretion vary greatly between children under the age of 3. A circadian rhythm of cortisol secretion similar to adults is evident by age 6.

Several factors influence the secretion of cortisol and need to be taken into account when designing a study using this biomarker of HPA activity. The normal circadian rhythm of cortisol secretion can be overridden by the central nervous system to produce an abrupt increase in cortisol secretion. This surge in cortisol secretion characterizes an acute stress response. Athough the stress-induced increase in cortisol secretion in adults has been well characterized, there is sufficient evidence that it also occurs in infants and children. School-aged children (McCarthy et al., 2009) and infants (Davis & Granger, 2009) exhibit an increase in cortisol levels in response to painful medical procedures. If an intervention should reduce HPA activity, it may be important to ask the subjects to diary when the cortisol levels are being measured. For example, in adults, evening cortisol levels are higher on weekdays/workdays versus weekends/nonwork days, and afternoon cortisol levels in toddlers are higher on days they are in child care than on days when they are not (Sumner, Bernard, & Dozier, 2010; Watamura, Coe, Laudenslager, & Robertson, 2010).

There is a substantial body of literature describing dysregulation of the HPA axis with chronic stress. This is characterized by smaller stress-induced spikes in cortisol secretion, a smaller CAR, and a smaller difference (flatter slope) between morning and evening levels of cortisol. The dysregulation of the HPA axis is likely caused by changes in intracellular expression of glucocorticoid receptors, so that elevated levels of cortisol fail to exert negative feedback on the HPA axis. Therefore, research to demonstrate that an intervention reduces stress in treated subjects compared to untreated subjects requires serial sampling of cortisol before and after the expected surge in cortisol secretion and comparison to basal levels on "normal" days in the same time frame of the circadian rhythm. Changes in circadian patterns of cortisol secretion require serial measures of cortisol levels over 24 hours before and after completion of the intervention. Changes in the circadian pattern of cortisol secretion can be captured by computing the difference between morning and evening cortisol levels (8 a.m.–8 p.m.), the ratio of nocturnal to morning levels (8 p.m./8 a.m.), or as percent variation in cortisol levels ([8 a.m.–8 p.m./8 a.m.] x 100).

When the population of interest has a chronic illness such as cancer or depression, it may be difficult to sort out the effects of psychoemotional distress on cortisol levels from that of the disease process. In a study of 177 women suspected of having ovarian cancer, the women who were later diagnosed with ovarian cancer had significantly higher evening cortisol levels the days before the biopsy than women who were subsequently diagnosed with benign disease. In the women subsequently diagnosed with cancer, the evening cortisol levels were related to functional disability and stage of disease and not to measures of stress, distress, or negative mood (Weinrib et al., 2010). These and similar findings in other groups of cancer patients (Andersen et al., 2010; Jehn et al., 2010) suggest that aberrations in cortisol secretion in patients with cancer or major depressive disorders may have different physiological drivers than in healthy individuals. However, there is evidence that yoga can improve mood, function, and the CAR in women with chronic pain syndromes (Curtis, Osadchuk, & Katz, 2011; Field, 2011), which suggests that cortisol is a sensitive biomarker of changes in HPA activity in populations with chronic illnesses characterized by dysregulation of the HPA axis.

SNS Activity: Salivary Alpha-Amylase

Salivary alpha-amylase (sAA) is an enzyme produced by the salivary glands and secreted into saliva. The concentration of this enzyme in saliva does not reflect gastrointestinal levels of alpha-amylase. However, the concentration is affected by activity of the autonomic nervous system (Bosch, Veerman, de Geus, & Proctor, 2011). Norepinephrine that is released from sympathetic nerve terminals directly stimulates beta adrenergic receptors in the salivary glands, which increases sAA secretion. Increased SNS activity also decreases blood flow to the oral mucosa, which decreases saliva flow. The result of increased SNS activity is an increased protein, fluid ratio in saliva, and one of these proteins is sAA. With reduced SNS activity, there is an increase in saliva flow, less protein per volume of saliva, and less sAA secreted into the saliva. Like heart rate, sAA is increased following strenuous exercise. There is a strong correlation between stress-induced spikes in sAA, heart rate, and systolic and diastolic blood pressure; the stress-induced spike in sAA, heart rate, and blood pressure are all blunted by pretreatment with beta blockers (Nater & Rohleder, 2009).

A stress-induced increase in sAA is distinct from a stress-induced increase in salivary cortisol. This corresponds with physiological differences in the SNS and HPA response to acute stress. The SNS directly enervates the salivary glands, so sAA secretion peaks in 10 minutes after the onset of the stress response and returns to baseline quickly following removal of the stressor. In contrast, the adrenal gland must secrete additional cortisol, and the concentration of free cortisol in the plasma must increase before the concentration of cortisol in saliva increases. The stress-induced increase in salivary cortisol peaks 20–30 minutes after the onset of stress response and returns to baseline 40–60 minutes following cessation of the stressor. Thus, measures of sAA and salivary cortisol at specific time points are not correlated, and individual differences in sAA levels are not mirrored by similar differences in salivary cortisol levels.

There is a diurnal pattern of sAA secretion, and it is opposite to that of cortisol; sAA secretion is lowest in the morning, increases during the day, and peaks before bedtime. This diurnal pattern in sAA secretion does not differ by gender or age and is stable within individuals over time. Basal levels of sAA are higher in individuals with higher levels of self-reported stress or stress reactivity and in persons with chronic back pain. Men tend to have higher basal levels throughout the day and a greater stress-induced spike than women. In infants, stress-related spikes in sAA secretion rate are evident by 12 months (Davis & Granger, 2009), and a diurnal pattern of sAA secretion may be seen as early as age 3. Stress-induced spikes in sAA are smaller than in adults (Yim, Granger, & Quas, 2010), and the diurnal rhythm of sAA secretion in adolescents is similar in magnitude to that of adults (Adam, Hoyt, & Granger, 2011).

A growing body of literature suggests that sAA may be a useful biomarker of arousal because of pain. In one study, presurgical levels of sAA were significantly higher in patients with chronic back pain than in patients without chronic pain and fell to control group levels with induction of anesthesia (Shirasaki et al., 2007). In another study, investigators used a randomized crossover design to test the effects of massage on agitation in dementia patients. Data collectors, blinded to the assigned treatment condition, observed each subject and collected saliva samples 1 day a week at four time points during the day. They reported that behavioral indicators of pain were reduced, and daily average sAA levels were reduced 50% by the end of the 4-week intervention compared to week one levels and compared to levels during the attention-only control intervention, which did not change across the 4-week intervention period (Hodgson & Andersen, 2008).

There is a great deal of debate as to how the saliva specimen should be collected to account for effects of the autonomic nervous system on the salivary flow rate and sAA

secretion rate (Beltzer et al., 2010; Bosch et al., 2011; Nater & Rohleder, 2009). Given the wide range of factors that can affect SNS activity in any one individual, it is important to conduct pilot work to optimize the study design and inclusion criteria for subject recruitment. Subjects should be instructed to avoid caffeine and strenuous exercise for 1 hour before the saliva sample is obtained. There is appreciable variability in basal levels of sAA between individuals, but the secretion pattern tends to be stable within an individual. Thus, the change in sAA may be more informative than absolute values for quantifying the intervention effect on SNS activity. The distribution of sAA tends to be positively skewed, and square root or log-10 transformations of the data prior to statistical analysis of the findings may be required.

Immune Function: Antibody Titer

Chronic stress has widespread effects on the immune system, which have deleterious effects on health. There is a large body of literature demonstrating that interventions to reduce psychological distress have positive effects on immune cell function. Bioassays of immune cell function are based on culturing peripheral blood leukocytes ex vivo and measuring cell responses to specific immune stimuli. The most common bioassays are mitogen-induced lymphocyte proliferation, stimulated cytokine production, or natural killer cell activity. However, these ex vivo (or in vitro) assays may not reflect immune cell function in vivo. Thus, researchers have been searching for reliable and valid in vivo measures of immune function.

The enumeration of subpopulations of circulating leukocytes is not usually informative unless you want to look for changes in expression of a cell surface molecule, such as HLA-DR, on a subpopulation of leukocytes, such as monocytes. In contrast, antibody production is an excellent biomarker of in vivo immune function (Burns & Gallagher, 2010). Antibody production in response to vaccination is reduced with aging and chronic stress. The study design must account for the baseline measurement of antibody titer and the timing of the intervention, the vaccination, and testing for the postvaccine titer to coincide with the peak antibody response. These considerations for study design are demonstrated in a report describing the positive effects of Tai Chi on the antibody response to vaccination in older adults (Irwin, Olmstead, & Oxman, 2007).

Antibody titers, in response to vaccination, are measured in serum samples. However, acute and chronic stress suppresses secretion of (immunoglobulin A) IgA by the oral mucosa, reducing levels of salivary IgA (sIgA). Stress-induced suppression of sIgA has been demonstrated in elder caregivers of dementia patients, children in day care, and in healthy college students taking final examinations. Salivary IgA secretion could be a high-value biomarker in studies to test the effectiveness of interventions to reduce stress (Burns & Gallagher, 2010).

Inflammation: Proinflammatory Cytokines

Cytokines were originally called interleukins for their role in activating leukocytes to mount an immune response. Thus, stimulated cytokine production by cultured peripheral blood leukocytes is one of the several laboratory methods used to evaluate immune function ex vivo, as discussed in the previous section. Proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF) are low molecular weight proteins produced in large part by cells involved in the innate or nonspecific immune response. Cytokines have local effects in cells and tissues that help to contain the

offending event. Later, these same cells will produce anti-inflammatory cytokines, cytokine receptor antagonists, or soluble cytokine receptors, which block the effects of the proinflammatory cytokines and reduce the inflammatory response. These anti-inflammatory biomarkers include IL-10, IL-1 receptor antagonist (IL-1RA), and soluble IL-6 receptor (sIL-6R) or soluble tumor necrosis factor (sTNF) receptor type II (sTNF-RII).

With increased or more widespread production, proinflammatory cytokines enter the circulation. Once in the blood stream, cytokines act throughout the body to cause the classic signs and symptoms of acute illness, such as fatigue, myalgia, fever, and anorexia. They also increase hepatic production of acute phase proteins, such C-reactive protein (CRP), a classic nonspecific biomarker of inflammation. Of particular note, activated macrophages excrete a small molecule called neopterin that is also a reliable but nonspecific biomarker of inflammation and activation of cell-mediated immunity (Sucher et al., 2010). In general, there are no specific biomarkers of inflammation, only nonspecific biomarkers of the inflammatory response.

There is convincing evidence that chronic inflammation plays a major role in the development of arthritis, cancer, atherosclerosis, and many other chronic diseases. We also know that chronic stress, sleep deprivation, smoking, high-fat intake, and adiposity increase production of proinflammatory cytokines, which may explain why these lifestyle factors increase the risk for chronic inflammatory diseases. Because increased circulating levels of proinflammatory cytokines are associated with symptoms of pain, fatigue, and depressed mood, one might assume that interventions to cytokine production should also reduce symptom burden in some populations with chronic inflammatory disease. Theoretically, behavioral interventions to reduce the stress response, increase physical activity, alter the diet, or reduce body fat could reduce cytokine production by virtue of their ability to affect the pathobiology of the inflammatory response at the cellular level (Phillips, Flynn, McFarlin, Stewart, & Timmerman, 2010; Kiecolt-Glaser, 2010). However, it also plausible that cytokine production will prove to be an insensitive biomarker of intervention effects because of the underlying disease process.

Although many disease processes involve increased production of proinflammatory biomarkers, other factors can affect the production of cytokines (O'Conner et al., 2009; Zhou, Fragala, McElhaney, & Kuchel, 2010). Cytokine production increases with sleep deprivation and smoking. IL-6 exhibits a strong diurnal rhythm, levels increase in the late evening and during sleep, and decline sharply with onset of morning activity. The diurnal rhythm is maintained even though circulating levels increase with age and body weight. The challenge for investigators is whether to control for these factors in the analysis plan or to narrow the subject inclusion criteria. Subjects should be instructed to refrain from exercise, high-fat intake, and caffeine for a minimum of 4 hours before the blood sample is collected. Given the strong relationship between sleep deprivation and inflammatory markers, subjects who have experienced 3–4 hours of sleep loss the night before the blood draw should be excluded from testing. Blood should be drawn at the same time each day to account for diurnal variations in cytokine production.

Probably the most important consideration when planning to include a biomarker of inflammation is the very short half-life of proinflammatory cytokines in the blood. Alternatively, cytokine antagonists, such IL-1RA, sIL-6R, or sTNF-RII, which are produced as part of the counter-inflammatory response, have a longer half-life, as do CRP and neopterin, and have a stronger association with symptom ratings than do serum levels of the proinflammatory cytokines (Bower et al., 2011).

CRP, neopterin, and proinflammatory cytokines can also be detected in saliva and urine. However, the concentration of the biomarker should be normalized to the total protein concentration in the saliva or urine. Urine levels are not sensitive to daily fluctuations in production of these biomarkers, but can be used to track changes over time.

Salivary CRP is highly correlated with plasma levels (Ouellet-Morin, Danese, Williams, & Arseneault, 2011). Basal levels of IL-6 and sIL-6R in saliva may not reflect those in plasma (Fernandez-Botran, Miller, Burns, & Newton, 2011), but levels do increase with exercise (Ives et al., 2011) and examination stress in healthy adults (Lester, Brown, Aycock, Grubbs, & Johnson, 2010). However, salivary levels of cytokines are increased in individuals with oral cancers, periodontal disease, and infections in the oral cavity, which requires you to include oral health in the screening of potential subjects.

CONSIDERATIONS FOR COLLECTING BIOLOGICAL SAMPLES

Circulating Biomarkers (Blood)

Measuring biomarkers in the blood poses unique considerations. Many proteins and lipids are not freely circulating in the blood, but are bound to carrier proteins like albumin or soluble receptors, which may require special processing of the sample before the biomarker can be measured. A bioassay could require a sample of whole blood, serum, or plasma. It is important to carefully read the laboratory protocol before deciding on how to obtain and process the blood sample. In general, a vacutainer tube is the best collection system to prevent contamination of the blood. This is especially important if you intend to measure circulating cytokine levels without stimulating additional cytokine production by contaminated leukocytes. Vacutainer tubes are easily centrifuged to obtain serum or plasma from the blood sample. If you need both whole blood and cell-free serum or plasma for different assays, it is best to obtain separate tubes of blood. The tubes may need to contain different anticoagulants, so again, read the manufacturer's instructions to be sure you make the best choice before you begin the study.

Serum is the fluid portion extracted from clotted blood. The blood is drawn into a tube without an anticoagulant and kept at room temperature for 10–30 minutes to allow it to clot in the tube. The serum is separated from the clotted blood by centrifugation. Because clotting can activate leukocytes and increase cytokine production, the clotted blood should be stored at 4 °C or on ice if the serum cannot be separated from the clotted blood within an hour. After centrifugation, the clear serum layer at the top of the tube is removed from the formed blood elements at the bottom by careful pipetting. The serum should be aliquoted into small single-use vials or tubes and stored at −80 °C until analysis. The volume in each tube should be enough for one assay, which prevents having to thaw and refreeze the specimen.

Plasma is the fluid collected from whole, unclotted blood. The blood is drawn into a tube with an anticoagulant and chilled (not frozen) until separated by centrifugation. After centrifugation, the straw-colored plasma is removed from the formed elements by careful pipetting, aliquoted into smaller, single-use tubes, and promptly stored at −80 °C until analysis. Plasma tends to look like partially thickened gelatin when thawed and should be thoroughly agitated before used in an assay. Using ethylenediaminetetraacetic acid (EDTA) as the anticoagulant when the blood is collected is thought to reduce "gelling" of the sample and to provide more consistent interassay results if the plasma is going to be frozen and thawed for later analysis. Plasma and serum samples can be stored at −80 °C for up to 2 years.

If the bioassay requires *whole blood*, the sample should be anticoagulated when it is obtained and should be placed on ice or stored at 4 °C if it cannot be processed for several hours. Before opening the tube, the blood should be gently agitated to remix the formed elements that settle with gravity. EDTA is a calcium chelator, so it is not a good choice if you are going to stimulate the blood cells ex vivo.

Saliva

There are more than 2,000 different molecules in human saliva. Some have diffused from the blood via the capillaries in the oral mucosa and are washed into the saliva. Examples include cortisol, androgens, and reproductive hormones (progesterone, estradiol), and metabolites of medications and nicotine. In fact, saliva contains many reliable biomarkers of tobacco consumption and nicotine metabolism (Ahijevych, 2009). Other molecules found in saliva are produced by cells in the oral mucosa, such as IgA, and others are secreted from the salivary glands, such as sAA. Saliva should be centrifuged to remove any debris before being used in an assay. Any mucous in the sample can be broken up by freezing and thawing, which does not affect the integrity of most biomarkers in the saliva. However, careful reading of the assay protocol is needed to be sure this is not contraindicated.

Saliva flow is described as unstimulated or stimulated, and collection methods include passive drooling, spitting, or an absorbent plug. The collection method depends on the information needed and how well the method will be accepted by the subjects. For each method, the saliva is collected over a specific time interval, usually 2–5 minutes. Most importantly, all subjects and study team members should be trained in the collection method so it is consistent between subjects. If the time of day the sample is obtained in the home or work environment is important, you can use caps embedded with a microchip to record when the cap was removed for collection of the sample. Alternatively, you can call or send text messages to a cell phone or pager to remind the subject to collect the sample. Both of these approaches bring extra cost to the study design, but improve the likelihood of collecting samples within the desired time frame.

Obtaining a saliva sample by passive drool may not be well accepted by subjects, especially if samples have to be collected when the subject is out of their home setting. However, this is the preferred method if you want to determine the saliva flow rate (milliliters per minute). Saliva flow rate is reduced when SNS activity is increased. Alternatively, the subject can spit into the collection device for several minutes. Again, this method of collection may be poorly accepted by subjects when samples have to be collected outside the home setting. Because spitting may increase saliva production, this method does not allow you to accurately calculate the saliva flow rate as a biomarker of SNS activity. If the rate of saliva flow is not an issue, the subject can suck or chew on paraffin to shorten the sample collection time or increase the sample volume. Paraffin is easily removed from the sample by centrifugation. It is not advisable to use citric acid to stimulate saliva production because it can decrease the pH of the sample, which could affect the sensitivity of some assays.

A well-accepted method of collecting saliva is placing an absorbent cotton or hydrocellulose plug in the mouth for 1–5 minutes. The plugs can stimulate saliva flow, so you cannot accurately calculate the saliva flow rate. For this reason, 2 minutes is often recommended to avoid diluting the concentration of some biomarkers in a greater volume of saliva (Beltzer et al., 2010). If a larger volume of saliva is needed, then the plug must be held in the mouth for a longer time. The plug is placed inside a collection device that is centrifuged to extract the saliva. A particular advantage of this method is that any mucous in the sample is retained in the plug. There are several types of plugs commercially available, including hydrocellulose microsponges for collecting saliva from infants. Because different salivary glands have different secretion rates, it is important that all subjects be instructed to place the plug in the same place in the mouth for the same duration each time a saliva sample is collected.

Salivary cortisol, sAA, and sIgA are stable at room temperature for up to 4 days, stable at 4 °C for up to 3 months, and stable for 1 year at −20 °C. Assay results are usu-

ally reported as concentration of the biomarker per milliliter of saliva (nanograms or micrograms/milliliter) or secretion rate (micrograms/minute). The saliva flow rate is calculated based on the volume collected in a fixed time interval (milliliter/minute).

Urine

Urine samples are often used to test for metabolites of hormones, drugs, and nicotine. Neopterin can be measured in urine, and there is some evidence that cytokines may be measured also. The assay may require the first void of the day or a 24-hour collection, neither of which are useful for detecting circadian or acute fluctuations in circulating levels of the biomarker. Collecting and storing urine for 24 hours may be burdensome and inconvenient for the subject, and for that reason unreliable. Metabolites in the urine are usually stable for several hours at room temperature or for several days at 4 °C. The urine sample should be centrifuged to remove any sediment before proceeding with the assay. Because the individual's hydration status can affect the volume of urine produced, the concentration of the biomarker in the urine should be normalized to the concentration of creatinine or total protein in the same sample of urine.

On the Horizon

In many cases, biomarkers that are normally found in the blood and detected in saliva can also be found in hair and sweat. Hair samples are used to measure drug and tobacco metabolites over an extended time. The concentration of cortisol in hair may prove to be a very useful biomarker of increased HPA activity over a period of months in pregnant women (D'Anna-Hernandez, Ross, Natvig, & Laudenslager, 2011) and individuals with chronic pain (Gow, Thomson, Rieder, Van Uum, & Koren, 2010). Thus, hair cortisol may be a convenient way to determine the long-term impact of psychosupportive interventions over an extended time. The hair sample should be taken close to the scalp because repeated exposure to water can reduce the amount of the biomarker in the hair shaft. The hair sample is accurately weighed and then pulverized or minced. Several different extraction processes have been described, and the concentration of the biomarker in the extract can be measured using the same methods used for analysis of serum or saliva samples.

Skin patches worn for 24–48 hours are another option for measuring biomarkers in settings where blood or saliva collection is not feasible. They have been used to test for the presence of drug and tobacco metabolites, and several commercial laboratories specialize in the extraction and analysis of soluble metabolites in the sweat. The concentrations of proinflammatory cytokines and neuropeptides in sweat patches worn for 24 hours by women with major depressive disorder were significantly greater than in patches worn by nondepressed women and were strongly correlated with plasma concentrations within that 24-hour period (Cizza et al., 2008).

MEASUREMENT METHODS

Proteins and lipid molecules have mass, charge, and structure that determine their movement through porous frameworks, which is the basis of chromatography. Mass and structure also determine light wave absorbance, which is the basis of spectropho-

tometry. Variations on these basic principles of mass and structure include high performance liquid chromatography (HPLC) and gas chromatography, which is the preferred method for measuring fatty acids in body fluids (McDaniel, Massey, & Nicolaou, 2011).

For the most part, if the biomarker is a protein, it can be measured using techniques based on antigen–antibody binding. If the protein is in fluid, its concentration per volume of fluid can be determined using an enzyme-linked immunosorbent assay (ELISA). If the biomarker is a protein expressed on the membrane of peripheral blood leukocytes, the proportion of cells expressing the protein can be detected using flow cytometry.

ELISA

This method is used to measure the concentration of a protein in a small sample of fluid such as serum, plasma, saliva, and urine. Basically, the wells of a microtiter plate are coated with an antibody to a specific antigenic site in the protein of interest. The sample fluid is added to the well, and the plate is agitated to allow the antibody on the plate to bind to the antigen in the fluid. The fluid is then removed from the plate, and a second antibody to another antigenic site in the protein is added to each well. Again, the plate is agitated so the second antibody can bind to the protein that was bound by the first antibody—this is sometimes called a "sandwich" assay. However, the second antibody is conjugated to an enzyme or other type of molecule that will change color during final step of the assay: The greater the color, the greater the concentration of the test protein in the well. The color is quantified using a spectrophotometer with a light filter of appropriate wavelength to detect that color. It is important to note that a purified form of the protein biomarker is provided with the ELISA kit, and serial dilutions are added to several wells. A standard dilution curve based on the light wave absorbance of the known standards is generated, against which the concentration of the test protein in the samples is calculated.

Refinements in antigen–antibody specificity and enzyme-linked fluorescent dyes have led to a vast number of commercially available ELISA kits for detection of numerous different proteins in most types of body fluids or extraction buffers. New ELISA-based technology also allows you to measure as many as eight cytokines simultaneously in the same fluid sample. This "multiplex" approach gives a more comprehensive picture of levels of one cytokine relative to others, such as a proinflammatory and anti-inflammatory cytokines. You can compare expression profiles between groups or within groups over time. One method uses microtiter plates containing antibody-coated microelectrodes, whereas others use antibody-coated beads. There are no meaningful differences between these methods and the sensitivity is similar to that of the single-cytokine ELISA (Chowdhury, Williams, & Johnson, 2009; Codorean et al., 2010; Richens et al., 2010).

An ELISA plate can have from 96 to 384 wells, although the 96 well-plate is more commonly used. The samples are usually run in duplicate, and of these "samples" are serial dilutions of a known amount of the biomarker, which is called the standard. The standard is provided with the kit and is serially diluted 5- or 10-fold according to the published protocol instructions. The standards must be included in all assays to ensure that assay properties remain the same. The serial dilutions of the standard provide the basis for calculating the concentrations of the biomarker in the test samples. If there are five dilutions, the standards will take 10 wells on the plate. Two wells might be left blank, and two might contain a laboratory sample that will be run on every plate as an internal control. That leaves 84 wells for testing of 42 samples in duplicate on each plate.

Samples obtained as part of a repeated measures design should be "batched" so that all samples from same subject are assayed together. This allows you to detect subtle

changes in the biomarker concentration in samples from the same individual at different time points. Data from several different assay "runs" can be pooled, but you should never assay all samples from control subjects at one time and all samples from experimental subjects at another time. It is best to distribute interassay variables evenly across groups to increase the likelihood of detecting true between-group differences.

Flow Cytometry

If the protein of interest is attached to cells in the blood, it can be detected using techniques of flow cytometry. Basically, a thin stream of cells is propelled through a special light beam, and reflection of the light beam varies with the size of the cells, for example, lymphocytes are smaller than monocytes. Thus, you can determine the percent of any given subpopulation of cell in the sample of cells. The cells in the sample can be stained using antibody-conjugated dyes to reflect specific light waves, allowing you to detect the proportion of the subpopulation of cells that are expressing the protein of interest. Alternatively, the cells can be treated to allow an antibody to permeate the membrane of the cells and to bind with specific proteins inside the cell. This approach is used to quantify the proportion of cells producing the protein of interest.

GETTING STARTED

Study team members who will perform the bioassays should be trained in the laboratory by persons who routinely perform the assay you plan to use in your study. Every institution has a laboratory safety training program that must be completed before team members set foot in the laboratory.

How do I go about incorporating a biomarker into my program of research? Find collaborators with extensive experience measuring the biomarker you think could improve the scientific impact of your research. None of us work in a vacuum, nor can we be a jack of all trades.

1. Attend research seminars in your setting to see who is working in a related area or using the bioassay you are interested in learning.
2. Use the websites of agencies that fund research, such as the National Institute of Health (NIH) Reporter (http://projectreporter.nih.gov/reporter.cfm), to search for funded investigators in your area of science and in your location. For example, this writer found an excellent collaborator using the search terms of cytokine, depression, mice, and Ohio.
3. Search PubMed (http://www.ncbi.nlm.nih.gov/) using your key words. Most published papers will include contact information for one of the authors. If the investigator is in an academic setting, one can go to the organization's website and verify if the person is still conducting research in this setting.

My Research Setting Does Not Include Lab Facilities

Funded investigators make the best collaborators because they have more laboratory space, equipment, reagents, and personnel to assist you with your research. Tenured investigators are no longer struggling to establish their own program of research

and can "risk" spending time developing additional lines of research if the research question is intriguing and could result in an interesting paper. The growing pressure to conduct interdisciplinary or transdisciplinary research can also be helpful for gaining access to laboratory space in another department or college or for obtaining money from your department to support your access to another investigator's laboratory.

How Do I Know the Bioassay Will Work in My Study With My Population?

The study team should always do pilot work to optimize the protocol for their setting, study conditions, and population before beginning to collect samples from human subjects enrolled in the study. This will establish whether you can collect sufficient samples from subjects, get the samples to the laboratory in a timely fashion, and get high-quality data from the bioassay. This is the time to test the effects of storage conditions on the quality of the samples and reproducibility of the data. Failure to detect the biomarker in your samples may require "spiking" some samples with a commercially available form of the biomarker and testing them in parallel with the unspiked samples to be sure nothing is interfering with detection of the biomarker in the samples. Once the protocol has been optimized, the research team should establish the interassay variability (values obtained for a known sample measured at different times), and intra-assay variability (values obtained from one sample tested more than once in the same assay).

How Do I Control for Within-Subject Variations When I Am Doing Serial Measurements?

Always consult a statistician for development of the analysis plan before beginning the actual research project. The statistician will need to see the data obtained during your pilot work to determine the best analysis plan and sample size needed to test the study hypothesis. Often, biological measures are positively skewed, so the statistical analysis is done using log-10 or square root transformations of the data. Values that are three standard deviations outside mean are likely anomalies and can be discarded as "outliers." When serial measures of the outcome variables are part of the study design, data analysis should include methods of hierarchical linear modeling, which will control for the nonindependence of multiple observations nested within the individual, and for missing data points.

● ● ○ Key Points From This Chapter

Integrating biologic measures into the study increases our understanding of how an intervention produced or did not produce the desired outcome and accelerates the translation of effective interventions in clinical practice.

Many intervention-induced changes in subject behavior are accompanied by changes in neuroendocrine or immune system activity. Biomarkers of neuroendocrine-immune changes caused by an intervention can be measured in blood or saliva.

Pilot work must be done to determine the feasibility of obtaining biological samples from subjects in your research setting, and to optimize the laboratory protocol to capture treatment-induced changes in the biomarker of interest.

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