Modern approaches to understanding stress and disease susceptibility: A review with special emphasis on respiratory disease

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Abstract: Studies in animals and humans link both physical and psychological stress with an increased incidence and severity of respiratory infections. For this manuscript we define stress as the physiological responses an individual undergoes while adjusting to a continually changing environment. It is known that stressors of various types (psychological/physical) can alter the physiological levels of certain hormones, chemokines and cytokines. These alterations send information to the central nervous system to take necessary action which then sends messages to appropriate organs/tissues/cells to respond. These messages can either activate or suppress the immune system as needed and failure to compensate for this by the body can lead to serious health-related problems. Little is known how stress affects disease susceptibility, yet understanding this mechanism is important for developing effective treatments, and for improving health and food quality. The current review focuses on (a) the effects of psychological stressors in humans and animals, (b) various methodologies employed to understand stress responses and their outcomes, and (c) the current status of the attempts to correlate stress and disease with respiratory disease as model system. The methodologies included in this review span traditional epidemiological, behavioral and immunological studies to current high throughput genomic, proteomic, metabolomic/metabonomic approaches. With the advent of various newer omics and bioinformatics methodologies we postulate that it will become feasible to understand the mechanisms through which stress can influence disease onset. Although the literature in this area is limited because of the infancy of this research area, the objective of this review is to illustrate the power of new approaches to address complex biological questions. These new approaches will also aid in our understanding how these processes are related to the dynamics and kinetics of changes in expression of multiple genes at various levels.

Keywords: stress, disease, biomarker discovery, systems biology, omics, respiratory disease

Definition of stress

Stress is a very broad concept and difficult to define in a concise way to capture all its connotations. Greek philosopher Hippocrates perhaps was the first to attempt to define the word stress in terms of 'balance' which was conceived as an essential state of health and 'disharmony' which manifested as disease when perturbed.[1] In the early 20th century Hans Seyle proposed the general adaptation syndrome which provided the first comprehensive biological theory of stress.[2] In a veterinary context this was identified as an abnormal or extreme adjustment in the physiology of the animal to cope with adverse changes in environment and management.[3] Within the modern physiological context, internal balance as described by Hippocrates is recognized as homeostasis. The definition of stress is an evolving process and for this review we accept the definition of stress as the state of threatened balance, equilibrium, or harmony and threats to homeostasis are called stressors.[1]

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From the preceding discussion it is clear that stress can be defined in many different ways depending upon the objectives or perspective of the researcher. All these definitions, however, share a common component of adaptive physiological responses following challenges to homeostasis. The adaptive reactions to stressors may involve mobilization of a wide variety of physiological responses including the immune response. Stress responses usually include physical perturbations that can encompass either the entire body or specific cellular compartments. Considering the volume of work in various areas of stressors and their effects the main objective of the current review is to focus on one type of stress, which includes psychological stressors.

For our purposes, stress can be defined as a psychologically perturbing condition occurring in response to adverse external influences capable of affecting physical health. Transportation, fear (ie, fright and flight response), overcrowding and weaning in the form of social reorganization are a few of the important types of psychological stressors identified in the literature. These stressors have been linked to many conditions including immune suppression, disease susceptibility, hypertension and reproductive dysfunctions.[4–6] Stress is a major concern because it is ubiquitous, recurring in nature and has detrimental effects on health. In this review we will primarily deal with important psychological stressors and their influence on respiratory disease which is one of the most widely studied models.

Stress and disease

For many years, psychological stress has been shown to significantly increase disease susceptibility.[6,7] Until 20 years ago, researchers investigating the psychological factors contributing to human disease focused primarily on coronary heart disease and cancer and neglected studies on infectious diseases.[8] However, interest in this area started to shift with the publication of evidence that psychological factors influenced immune function.[9] Furthermore, there was an increasing awareness that stress and other psychological factors played a role in the onset and progression of acquired immunodeficiency syndrome (AIDS).[10] These studies demonstrated a significant role for psychological stressors in compromising immunity and interest in the effects of stressors in other diseases was initiated. Considerable emphasis has been placed on respiratory diseases in understanding the onset and severity of the disease as a result of psychological stress.[11]

Viral–bacterial synergy

Increased risk of fatal bacterial respiratory infections following a primary viral infection has been observed in a wide variety of species. This phenomenon is called viral–bacterial synergy and was first established following human influenza epidemics when a variety of secondary bacterial respiratory infections were associated with increased mortality.[12] Studies have also linked a variety of psychological stressors with an increased incidence and severity of respiratory infections in humans[13,14] and animals.[15,16] It is known that respiratory disease has a huge economic impact in the areas of human health care, animal welfare and the food industry.[17,18] To focus the review, we will restrict our discussion to research related to a comparative analysis of the effects of psychological stress on respiratory disease in humans and cattle.

Stress and viral infection

There are reports which have shown direct connections between stress and immune system function.[19] Similarly, other studies have shown that social stressors could also increase the risk for upper respiratory infection.[20] A viral challenge study provides the strongest evidence for a link between stress and susceptibility to the common cold.[21] Other studies have extended these results by considering a wider range of psychosocial factors.[22] The effects of stress on health are often mediated by a number of psychological factors. Social support can often act as a buffer against the effects of stress as Cohen and colleagues showed that social support reduced viral replication rate and increased mucociliary clearance of infection.[11,13] In another report, they examined the effects of stress and social support in a routine study of upper respiratory tract illnesses.[23] Under low levels of stress, high levels of social support were associated with a decreased risk of infection, whereas social support had no effect when levels of stress were high.

A separate study was done to examine the associations between psychosocial factors (stress, social support, fluctuations in mood) and viral exacerbations of asthma. The study involved naturally occurring illnesses rather than experimentally-induced infections but it maintained several important features of the methodology used by Cohen and colleagues.[21,24] For example, stress was measured at the start of the study by measuring the immune responses in terms of leukocytes present in peripheral blood in order to determine the extent to which stress could predict subsequent illness. In addition, effects of personality,[25] smoking status, and alcohol consumption[21] were also studied as possible predictors of susceptibility to respiratory viral infections.

Before considering the effects of psychosocial factors on respiratory virus-induced exacerbations of asthma, it is essential to have strong evidence that these viruses play a

direct role in asthma. Until recently, it appeared to be only a weak association between asthma and upper respiratory tract infection in adults.[26,27] The absence of a stronger association in these epidemiological studies of adult asthmatics could, at least in part, have been due to difficulties in isolating human rhinoviruses and coronaviruses. Indeed, results from a study using enzyme-linked immunosorbent assays for antibodies to human coronavirus and semi-nested reverse transcriptase polymerase chain reactions for detections of rhinovirus suggested a stronger association between these viruses and asthma in adults.[28] In summary, this study confirmed that psychosocial factors and health-related behaviors were associated with increased susceptibility to colds, which then led to an exacerbation of asthma. This conclusion was made in the context of a study where both diseases (cold and asthma) were verified using objective measures. The well established buffering effect of social support was observed in the high stress group and possible confounders such as demographics, health-related behaviors or personality could not account for this effect. Alcohol consumption, personality and demographic factors were also shown to be important predictors of susceptibility. In contrast smoking was related to illness severity but not disease susceptibility. These results show that one must consider a range of psychosocial factors, personality traits, demographic factors, and health-related behaviors in studies of individual differences in susceptibility and severity of upper respiratory tract infections.

Stress and immunity

While studies have correlated stress-related behavior with disease susceptibility, research has also been performed to determine the immunological basis of the relationship between stress and disease. Exposure to viral agents that cause upper respiratory disease provokes illness in some individuals but not others. Moreover, the severity of clinical symptoms among those who develop illness can vary substantially. Evidence from prospective epidemiological studies[20,23,29] and from experimental viral-challenge studies[21,30] show that individuals reporting greater psychological stress have both a higher incidence and a greater severity of illness. However, past attempts to identify the behavioral patterns and biological responses linking psychological stress with upper respiratory viral illness have been unsuccessful.[20,21,30]

Other studies reported that psychological stressors acutely activate the production of interleukin-6 (IL-6), a pro-inflammatory cytokine. IL-6 release in response to infection is thought to be mediated by glucocorticoids, thus providing a hypothetical pathway by which stressors (via the induction of glucocorticoid production) could control cytokine release.[31] In addition, IL-6 triggers additional release of glucocorticoids, possibly exacerbating the stress response through positive feedback. At least one source of IL-6 is epithelial cells as evidenced by their production of IL-6 *in vitro* and *in vivo* when exposed to rhinovirus.[32] Because a local increase in the concentration of this pro-inflammatory cytokine precedes the development of acute signs and symptoms of illness, it has been implicated as a mediator in the pathway for symptom expression. In fact, IL-6 concentrations in nasal secretions were associated with upper respiratory tract symptoms among persons infected with influenza A virus (A/Texas and A/Kawasaki) and rhinovirus (strain Hanks' and type 39).[13]

Cohen and colleagues addressed the hypothesis that IL-6 production in response to influenza A virus infection represents a viable pathway through which psychological stress influences the severity of illness. To achieve that goal, they measured levels of psychological stress in a group of adult subjects before experimentally infecting them with influenza A virus. Self-reported respiratory symptoms, mucus weights, and local IL-6 concentrations were then measured on the day before and for seven days after virus exposure. The data collected supported the conclusion that the level of psychological stress predicts the severity of illness and also the magnitude of the cytokine response. The data were then examined for evidence that IL-6 mediated the association between stress and illness. These analyses suggested that most of the effects of psychological stress on clinical symptoms and mucus weights could be accounted for by changes in IL-6. However, it is possible that IL-6 itself is not the causal link but rather just a marker (covariate) for other pro-inflammatory cytokines which were elevated during the course of experimental infection. For example, in another related study[33] reported that interferon α and IL-6 levels (but not tumor necrosis factor [TNF], IL-8, IL-1, or IL-2) increased early in the course of infection and both correlated with viral titers, increases in body temperature, mucus production and symptom scores. There is also an issue regarding the correlational nature of the mediational analysis. Although consistent with the hypothesis that the association between stress and illness was mediated by IL-6, the data do not permit causal inference. For example, this pattern of data is also consistent with increases in IL-6 occurring in response to tissue damage associated with illness symptoms. Even with these reservations, this was the first

study to provide evidence consistent with the hypothetical model that psychological stress influences upper respiratory infections through a biological pathway.[13]

Axes of stress response

There are two parts to the stress response: sympathetic–adrenal–medullary (SAM) and the hypothalamic–pituitary–adrenal axis (HPA). The HPA is the core stress axis in mammals and together with the SAM system co-ordinates response to the diverse range of stressors from psychological to physical. There is considerable interplay between both neuronal systems especially between the noradrenergic nucleus locus ceruleus which provides central regulation of the SAM and the parvocellular neurones which regulate the HPA. The SAM, by triggering catecholamine release, provides the acute stress response whilst the HPA governs longer term stress defence mechanisms. Together those systems regulate energy utilisation and metabolic activity throughout the body.[34] The SAM system produces the immediate shock response by acting on the hypothalamus, which activates the adrenal medulla and the sympathetic autonomic nervous system (ANS) (Figure 1). The SAM produces the "fight-or-flight" response which increases alertness, blood flow to muscles, heart rate, blood pressure, respiration rate, etc. and might decrease activity in the digestive system. The HPA system regulates release of the hormone CRF to activate the anterior pituitary and uses another hormone, adrenocorticotropic hormone (ACTH), to activate the adrenal cortex to release a group of hormones including cortisol (Figure 2). Cortisol and other glucocorticoid hormones have various effects such as conservation of glucose for neural tissues, elevation or stabilization of blood glucose levels, mobilization of protein reserves, conservation of salts and water, suppression of wound healing and the immune system. According to Seyle's general adaptation syndrome (GAS) theory the general adaptation syndrome divides the stress response into three stages: Stage 1: Alarm reaction (SAM and HPA activity increases and result in the "fight-or-flight" response); Stage 2: Resistance (HPA activity takes over, bodily resources are at maximum and if the stress is experienced for short term the body returns to normalcy); Stage 3: Exhaustion (with very prolonged stress, bodily systems are ineffective. Sympathetic ANS action reappears. Adrenal cortex damage causes parasympathetic action,

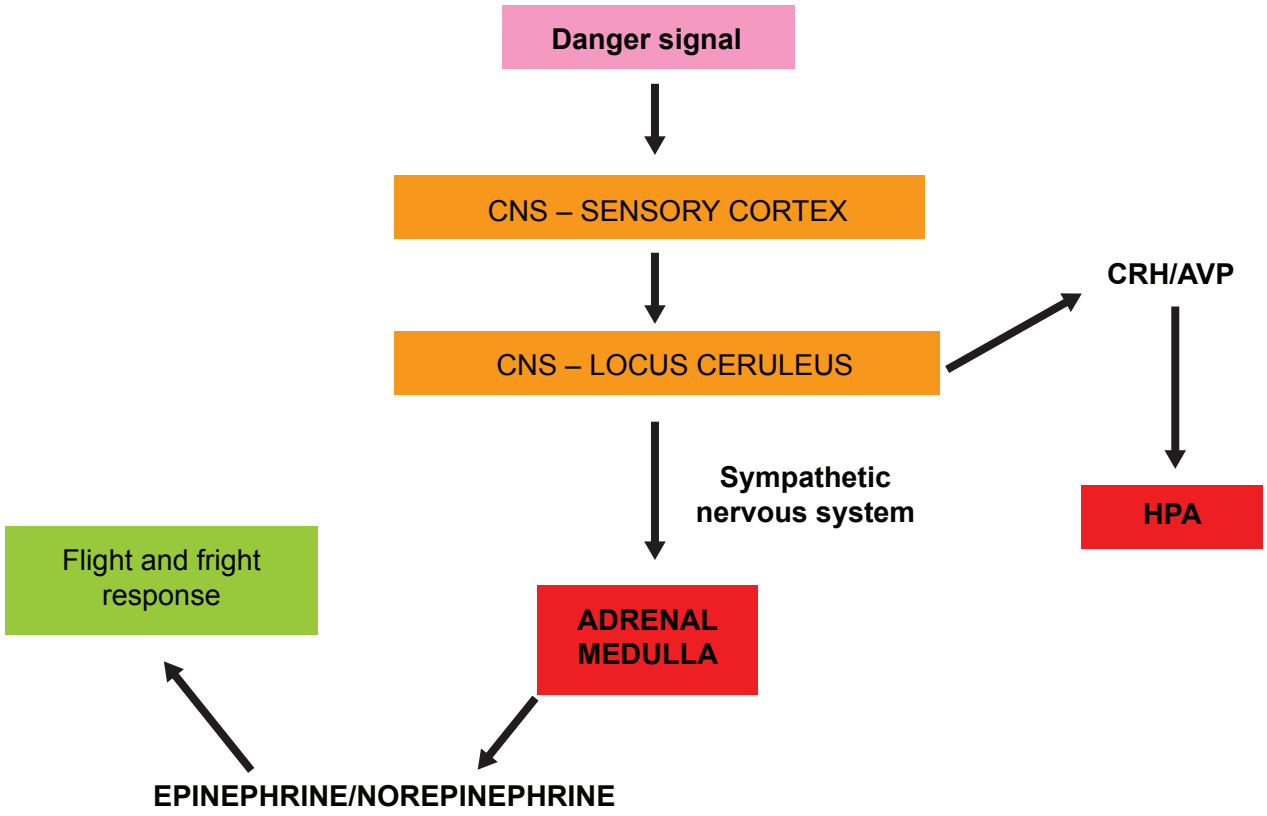

Figure 1 Schema of sympathetic–adrenalin–medullary axis of stress response.
Abbreviations: CNS, central nervous system; CRH/AVP, corticotrophin-releasing hormone/arginine vasopressin; HPA, hypothalmic–pituitary–adrenal axis.

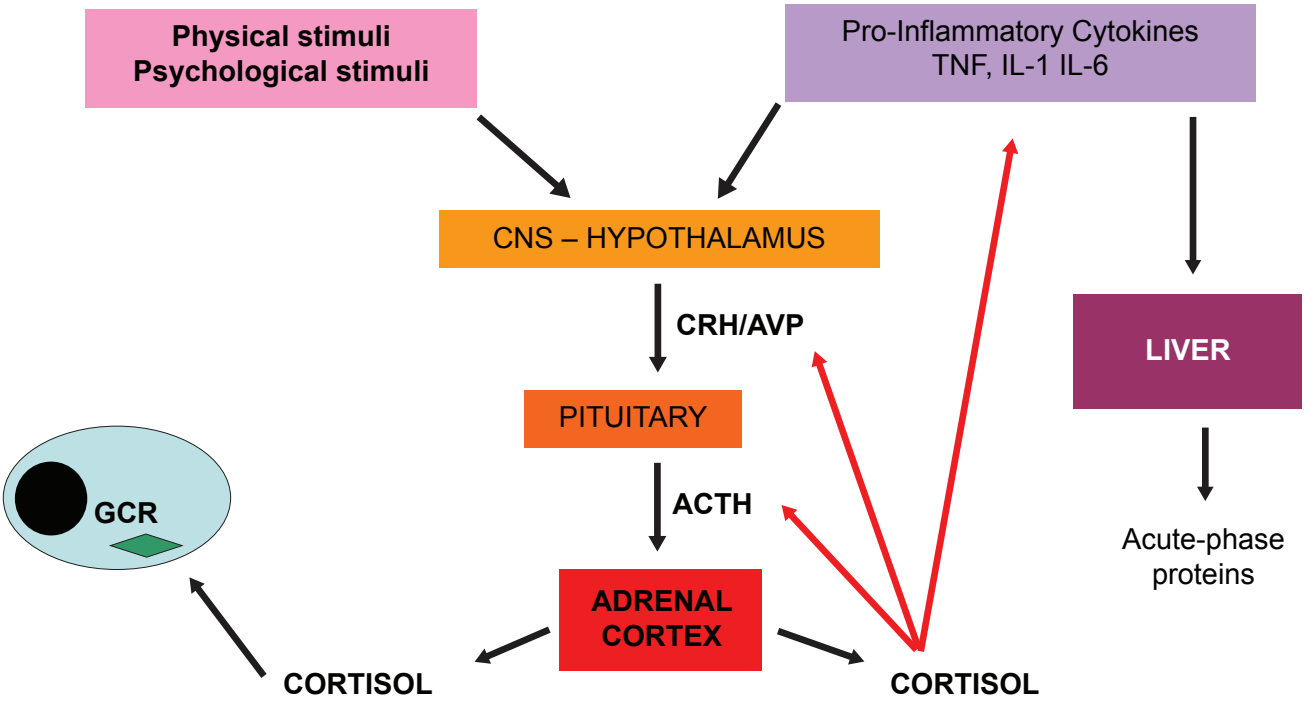

Figure 2 Schema of hypothalamus–pituitary–adrenal (HPA) axis of stress response.
Abbreviations: ACTH, adrenocorticotropic hormone; CNS, central nervous system; CRH/AVP, corticotrophin-releasing hormone/arginine vasopressin; GCR, glucocorticoid receptor; IL, interleukin; TNF, tumor necrosis factor.

eg, energy storage failure. The immune system collapses, and stress-related diseases increase).

Mechanism of stress-induced infection susceptibility

While it has been assumed that psychological (psychogenic) and physical (neurogenic) stressors are most closely aligned with depression, the suspicion has arisen that systemic stressors, including immune alterations, may also act in such a provocative capacity.[35] Communication occurs between the immune, endocrine, autonomic and central nervous systems[36] such that psychological events that affect central neurochemical processes may affect immune activity. Conversely, immune activation may affect hormonal processes and the activity of central neurotransmitters. Thus, by virtue of the neurochemical effects imparted, immune activation may affect behavioral outputs and may even be related to behavioral pathology such as depressive illness.[37]

The hypothesis that altered immune activation may occur as a result of various stressors emerged over time. The initial theory came from Hans Seyles' GAS, which was derived from observation and experimentation on laboratory animals. Using a variety of stressors (ie, pain, thermal extremes and starvation), Seyle described a common nonspecific stress response pathway.[38] After initial perception of a stressor, the animal mounts an emergency alarm or fight-or-flight response. This catecholamine-driven reaction results in increased cardiovascular function and an overall increase in metabolism. If the stressor persists, the resistance phase or 'conservation withdrawal reaction' is initiated, which is a physiological coping reaction to the increased demands of maintaining homeostasis. Chronic stress leads to the exhaustion phase and may lead to pathology. Seyle's theory unified the stress phenomena because it provided a common response pathway to all the varied stressors encountered.

This pathway, the HPA axis, involves perception in the brain with release of hypothalamic corticotropin-releasing factor (CRF) and vasopressin which stimulates the anterior pituitary to secrete ACTH (Figure 2). Circulating ACTH causes the adrenal cortex to produce glucocorticoids (GC). Glucocorticoids cause gluconeogenesis with conversion of lipid to glucose for the central nervous system (CNS) and other functions. Thus, GAS allowed the identification of stressors (and presumably the status of well being of the animal) by measurement of GC levels. It was an attractive theory because GC levels were relatively easy to measure.[2]

Unfortunately the GAS theory has proven to be too simplistic. Mason's experiments with Rhesus monkeys

exposed to different types of stressors revealed a disparity in neuroendocrine responses provided by specific stressors.[38] For instance, monkeys subjected to emotional stress had elevated serum GC levels but those subjected to a heat-stress regime failed to show GC elevation. In addition to GC, other neuroendocrine mediators were characteristically produced in response to specific stressors.[38] Emerging information on the response to stressors suggests that there are at least four different avenues of neuroendocrine responses. These involve the autonomic nervous system, the HPA axis, neuropeptides, neurotransmitters and neuroimmunological peptides and receptors.[2]

Immunity and CNS

Interactions between the immune and nervous systems play an important role in modulating host susceptibility and resistance to inflammatory disease. Neuroendocrine regulation of inflammatory and immune responses as well as onset of disease can occur at multiple levels: (a) systemically through the anti-inflammatory action of GCs released via HPA axis stimulation, (b) regionally through production of GCs within the affected tissue as well as by sympathetic enervation of immune organs such as the thymus and (c) locally at sites of inflammation. Estrogens also play an important role in immune modulation and contribute to the approximately 2- to 10-fold higher incidence of autoimmune/inflammatory diseases in females of all mammalian species.[39] During inflammation, cytokines from the periphery activate the central nervous system through multiple routes. This results in stimulation of the HPA axis which in turn, through the immunosuppressive effects of the glucocorticoids, generally inhibits inflammation. Recent studies indicate that physiological levels of glucocorticoids are immunomodulatory rather than solely immunosuppressive causing a shift in patterns of cytokine production from a TH1- to a TH2-type pattern. Interruptions of this loop at any level and through multiple mechanisms, whether genetic, or through surgical or pharmacological interventions, can render an inflammatory resistant host susceptible to inflammatory disease. Over-activation of this axis, as occurs during stress, can also affect severity of infectious diseases through the immunosuppressive effects of the glucocorticoids. These interactions have been clearly demonstrated in many animal models using a variety of species and infectious agents.[39] The results from these models are also relevant to human inflammatory, autoimmune and allergic illnesses including rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome, allergic asthma and atopic skin disease. While many genes and environmental factors contribute to susceptibility and resistance to autoimmune/inflammatory diseases, a full understanding of the molecular mechanisms by which a combination of neuropeptides, neurohormones and neurotransmitters can modulate immune responses is essential for effective design of future interventions.[39]

Stress and disease re-visited

It is clear from the previous discussion and review of previous research that the relationship between stress and disease susceptibility is very complex and intertwined with cascades of events. In order to understand or characterize stress-induced disease susceptibility one needs to identify various biological or functional pathways and interaction networking involved at any given time. To follow the cascade of molecular events happening over time one needs to employ methodologies which are holistic in nature and can provide global information related to the kinetics of multiple changes in gene expression and multiple biomolecular interactions. With the advent of various high-throughput genomic approaches it has become possible to explore complex biological processes and in one step obtain information about gene expression at the transcriptional (genomics) and translational (proteomics) level, as well as to identify metabolites arising from these responses (metabolomics/metabonomics). These methodologies together are often referred to as 'omics' approaches. Although these methodologies are still in their infancy they have started showing promise in understanding systems' biology and various disease processes. Work employing omics approaches to understand mechanism of stress and disease susceptibility is limited at this time. There are reports which describe the effects of oxidative stress on cellular apoptosis[40] but there are no reports on the effects of psychological stressor and disease susceptibility in animals using holistic methodologies such as various omics approaches. The literature in this area is very scarce because these methodologies are very new and few results are currently available. The main studies to correlate stress and disease susceptibility was done either in plant systems[41] or a correlation between oxidative stress and infectious disease.[42] The review by Hirai and Saito established, using liquid chromatogram based mass spectrometric proteomic and metabolomic and cDNA microarray based transcriptomic analyses, that these omics studies can reveal the genomic networking involving several pathways related to oxidative stress response and key metabolic pathways.[41]

Omics at a glance

During last 10-years a variety of tools have been developed to expedite large-scale analyses of gene expression at

the level of individual cells, tissues, or whole organisms. Among these tools the ones which have attracted much of attention and have been best developed include SNP analyses, oligonucleotide microarrays,[43,44] cDNA microarrays,[45] serial analysis of gene expression,[46] proteomics[47] and bioinformatic methodologies to deal with the vast amount of information generated. Microarray technology has developed rapidly and numerous text books and reviews have been published addressing the critical issues of microarray experimental design,[48] data analyses,[49] and the application of microarray technology to investigate normal physiology[50] and disease pathogenesis.[51,52] Microarrays have been developed for a wide variety of microbial pathogens and host systems, but the application of this technology to functional genomic studies is very recent.[53–58] A variety of commercial microarrays are now available for different species and microarrays representing specific cell signaling pathways or biological functions are being used as routine tools to address hypotheses in basic research and clinical trials. However, there has been a substantial delay in the application of microarray technologies to the investigation of biological questions in species of veterinary importance as well as in the area of the current review. In brief, microarray technology compares the relative abundance of mRNA over time or under different conditions or treatments which can lead to studies of comparative genomics of host responses following different pathogenic infections, stress situations or time-dependent kinetic studies of host–pathogen interactions. Bioinformatics and statistical approaches specific for detailed transcriptomics or cluster analyses of expressed genes have been developed and are useful for extracting information on various functional genomic responses.[59–61] It is important, however, to understand the translation of transcriptomic information into protein expression and modifications since it is the protein that acts as biomarkers for various disease processes or conditions.

Several proteomic tools have been developed. These include gel-based two-dimensional (2D) gel electrophoresis (GE),[47] 2D fluorescence difference gel electrophoresis (2DIGE)[62] or gel-free multidimensional protein identification technology (MudPIT),[63,64] isotope-coded affinity tag (ICAT™),[65] Surface-enhanced laser desorption–ionization time-of-flight (SELDI-TOF) MS[66,67] or isobaric tag for relative and absolute quantitation (iTRAQ)[68] methodologies. While these technologies are being matured more appropriate biological questions can be addressed.

In 2D-GE whether traditional or newer DIGE system, proteins are first focused (first dimension) in terms of their pI values followed by SDS-PAGE (second dimension). In contrast to traditional 2D-GE, DIGE system utilizes the fluorescence labeling of protein samples and two-to-three different samples can then be run on a single gel which makes it superior to traditional 2D-GE in terms of eliminating gel to gel variation and comparative analysis. Although 2D-GE is a very good way of separating complex protein mixtures, it is limited in terms of high-throughput analysis and sensitivity. Because of low sensitivity it is mostly limited to the analysis of high abundant proteins of a system. Efforts have been made to improve the sensitivity and analysis capacity of proteomics techniques. As a result, other methodologies such as 2D-HPLC, MudPIT, ICAT, and iTRAQ have been developed and are gaining popularity. Although various bioinformatic and data analyses approaches have been developed to analyze gel images and mass spectrometric based protein characterization a high-throughput methodology in this area has yet to be developed.

Functional genomic techniques of transcriptomics and proteomics and available bioinformatic and statistical analyses promise unparalleled global information during the analyses of complex biological responses. However, if these technologies are used in isolation, the large multivariate data sets produced are often difficult to interpret and have the potential to ignore key metabolic events to understand the true biology. High resolution ^{1}H NMR spectroscopy used in conjunction with pattern recognition provides one such tool for defining the dynamic phenotype of a cell, organ, or organism in terms of a metabolic phenotype. In a recent review the benefits of this metabonomics/metabolomics approach to problems in toxicology have been discussed.[69] One of the major benefits of this approach is its high-throughput nature and cost-effectiveness on a per sample basis. Using such a method, the consortium for metabonomic toxicology (COMET) is currently investigating approximately 150 model liver and kidney toxins. This investigation will allow the generation of expert systems where liver and kidney toxicity can be predicted for model drug compounds, providing a new research tool in the field of drug metabolism. The review also included how metabonomics may be used to investigate co-responses with transcripts and proteins involved in metabolism and stress responses such as during drug-induced fatty liver disease. By using data integration to combine metabolite analysis and gene expression profiling, key perturbed metabolic pathways can be identified.

Bioinformatics, stress, and disease

Detailed omics-based bioinformatics studies, to correlate stress-dependent disease susceptibility, have yet to analyze

this complex biological response. A few preliminary studies have been reported in the literature. A recent study has shown the adverse effects of using mechanical ventilators for respiratory support.[70] This acute lung injury because of mechanical stretch can lead to high mortality and this study has utilized recent advances in bioinformatic-intense candidate gene searches to correlate the lung injury and gene expression profiles in a rodent model analyzed with microarrays.[70] The authors used 2,769 mouse/rat orthologous genes identified on RG_U34A and MG_U74Av2 arrays and the expression profiles were simultaneously analyzed by significance analysis of microarrays. This combined ortholog and significance analysis of microarrays approach identified 41 up- and 7 downregulated ventilator-induced stress-related candidate genes. Results were validated by comparable expression levels obtained by either real-time or relative RT-PCR for 15 randomly selected genes. K-mean clustering of 48 candidate genes clustered several well-known lung injury associated genes (IL-6, plasminogen activator inhibitor type 1, CCL-2, cyclooxygenase-2) with a number of stress-related genes (Myc, Cyr61, Socs3). The only unannotated member of this cluster (n = 14) was RIKEN_1300002F13 EST, an ortholog of the stress-related Gene33/Mig-6 gene. The authors speculated that the ortholog-Significant Analysis of Microarray approach is a useful, time- and resource-efficient tool for identification of candidate genes in a variety of complex disease models such as ventilator induced lung injury.[70] Using microarray-based genomic approaches work has also been initiated to identify hypertension-related genes in rat model.[71]

Host responses

Though it is a common belief that the stressors mentioned earlier can compromise host immune responses and enhance susceptibility to various diseases, very little work has been done in this area to understand the mechanism of stress dependent disease susceptibility in mammals. Kelley in 1980 identified eight stressors that typically occur in modern livestock production units: heat, cold, crowding, mixing, weaning, controlled-feeding, noise and restraint. All these stressors were shown to alter components of the immune system in animals and these changes in immune function may ultimately explain the physiological basis of disease-environment interactions.[72] Another study in 1993 showed that neurotransmitters and neuroendocrine hormones can modify the function of immune cells. Conversely, cytokines produced by immune cells can alter brain homeostasis.[73] This connection is further manifested by experimental studies showing a relationship between stress and resistance to infection. Human subjects under high stress were shown to be more susceptible to infection with common cold viruses. Furthermore, a diversity of experimental animal models confirmed that laboratory stressors such as forced exercise, avoidance learning, restraint, isolation and cold exposure made animals more susceptible to primary infection with a variety of viruses and bacteria.

The cellular and molecular basis for the observed modulation of host resistance is not fully understood but involves altered functioning of both T-lymphocytes and cells of the hypothalamic-pituitary adrenal axis. Also involved is the altered production of cytokines and hormones produced by the immune system and brain.[73] Emau and colleagues in 1987 showed that the onset of immunodepression by stress or viral infection in the pathogenesis of bovine pneumonia permits super infection of the lungs with Mannheimia *haemolytica* (formerly called *Pasteurella haemolytica*) which results in exudative fibrinous pneumonia.[74] Although these studies provide a preliminary basis of stress-induced bovine respiratory disease (BRD), there was a lack of necessary information to determine the molecular basis of such dependence, which can be found by studying changes at the level of gene expression (transcriptional and proteomic) and metabolites. A recent study compared proteomics of epithelial lining fluid from lungs of weaned and nonweaned cattle.[75] The study was done over a shorter period of time (36 h) with a combination of stressors (weaning, transportation) and important questions such as to determine specificity and duration (stability) of the protein biomarkers are yet to be addressed. The group also studied the proteomic profile of bronchoalveolar lung lavage fluid following treating the calves with dexamethasone to determine the effects of glucocorticoids, which is elevated following induction of stress.[76,77] More studies are however needed (a) to find biomarkers associated with stressors and infection, (b) to determine the duration of the biomarkers so that these are sustainable enough to identify and predict stressed animals (which might be more susceptible to infection in real life situation such as in feedlot cattle).

Bovine respiratory disease

Disease models have been developed to study the molecular mechanisms underlying the viral-bacterial synergy which results in fatal BRD infections.[78–80] We have confirmed with the infectious BRD model that the stress of weaning[81] significantly enhances the viral–bacterial synergy leading to fatal bacterial respiratory infection.[82] The major stressors young

cattle experience include maternal separation or weaning, dietary changes, transportation, social reorganization and other environmental effects (Figure 3). With this in mind, we focused our initial studies to understand the role of weaning on the clinical response to BRD. Fifteen suckling calves were removed from their mothers 24 h prior to viral infection (abruptly weaned/stressed, AW). A second group of 15 calves was weaned 2 wks prior to viral infection (pre-conditioned/control, PC); 2 wks was chosen as an appropriate interval to eliminate psychological and physiological effects associated with breaking the maternal/nutritional bond and to adapt to the dietary change and social re-organization that follows weaning (Figure 4). All calves were transported to Vaccine and Infections Disease Organization VIDO, and aerosol challenged with bovine herpesvirus-1 (BHV-1) followed by *M. haemolytica*; this combined viral–bacterial infection induces fatal pneumonia in 50%–70% of calves.[83]

Our clinical analyses revealed a significant difference in BRD clinical disease when comparing freshly weaned calves (80% mortality) versus pre-conditioned calves (40% mortality).[84,85] Increased mortality associated with fresh weaning was characterized by a decrease in both survival time post-infection and, interestingly, decreased lung pathology which suggested a systemic reaction. Contrary to expectations, transportation induced a significant cortisolemia in pre-conditioned but not freshly weaned calves (Figure 5). Transportation is known as an important stressor which increases blood cortisol level.[86–89] However, the stressor combination of weaning and transportation may have different physiological markers than commonly expected. Cortisol is a potent regulator of pro-inflammatory cytokine transcription by acting as a negative regulator of NFκB activation, and can be used as an indicator of stress.[90] Fatal bacterial respiratory infection in calves was usually associated with an accelerated decline in serum cortisol levels. Therefore, we hypothesized that increased respiratory disease susceptibility was associated with enhanced pro-inflammatory responses in freshly weaned calves. Elevated body temperature and interferon-gamma (IFN-γ) levels in nasal secretions during BHV-1 infection of freshly weaned calves were similar to previous reports,[82,91] supporting this hypothesis. These immune responses were significantly increased despite no significant difference in virus shedding. Furthermore, increased IL-10 expression, during BHV-1 infection of pre-conditioned calves, was consistent with reduced pro-inflammatory responses.[82]

Genomics and proteomics

We conducted bovine microarray analyses of RNA isolated from blood mononuclear cells to determine if changes in gene expression correlated with either stress or the severity of BRD infection; results support the conclusion that differential regulation of pro-inflammatory responses is a major mechanism contributing to increased disease susceptibility. Conserved responses included an enhanced potential to

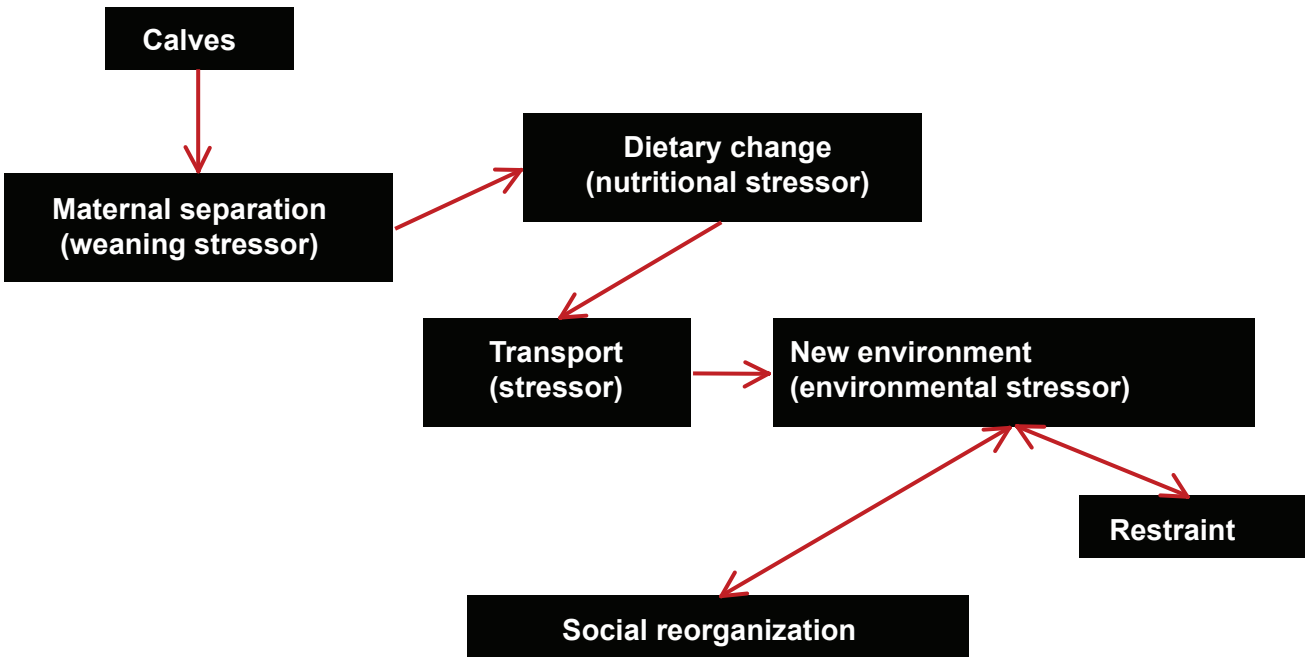

Figure 3 Schematic diagram of various important stressors of feedlot cattle.

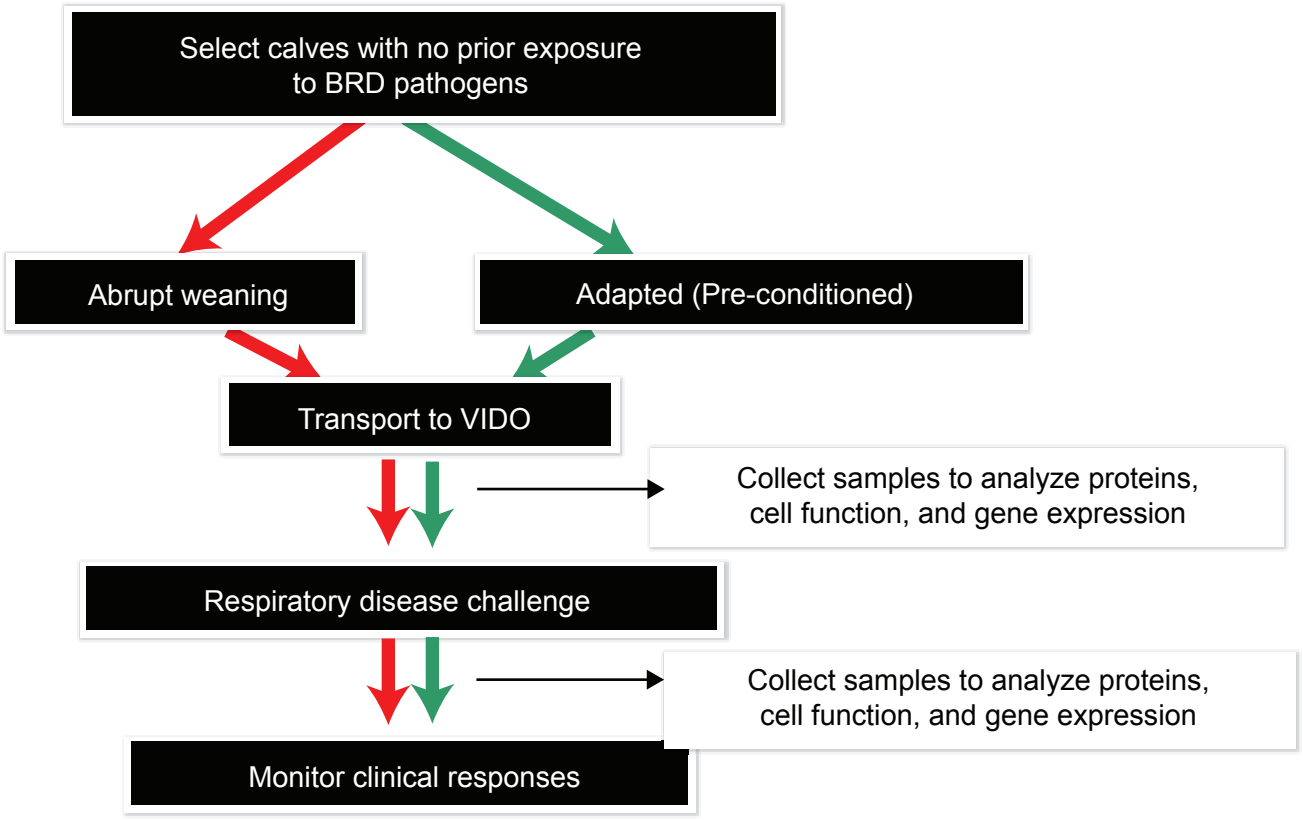

Figure 4 A schematic BRD stress model diagram for BHV-1 followed by *M. haemolytica* aerosol challenge of weaned and pre-conditioned calves.
Abbreviations: BRD, bovine respiratory disease; BHV-1, bovine herpesvirus-1; VIDO, Vaccine and Infections Disease Organization.

respond to pathogen-associated molecular patterns through increased expression of toll-like receptors (TLRs). TLR induction of innate immune responses is mediated primarily through activation of nuclear factor kappaB (NFκB). We also observed differential expression of β-defensin 5 (a host defense peptide induced by TLR4 signaling) in freshly weaned calves, consistent with reduced cortisol levels.[92] In addition, when we compared expression of select innate immune genes (eg, TLR2, TLR4, IFN-γ and 2'5' O-adenylate synthetase) between day 4 (post BHV-1 infection) and day 0 (prior to BHV-1 infection) for AW- and PC-groups, results revealed a trend of increased expression of the select genes on day 4 irrespective of the stress situation (Table 1). This trend of activation of select immune genes clearly revealed that following BHV-1 infection the innate immune response was enhanced. This observation contradicts the current hypothesis that the primary viral enhances the secondary bacterial infection by compromising the immune system.[78,93] Our studies revealed that the anti-inflammatory gene IL-10 was upregulated on day 4 in PC groups while β-defensin was activated on day 4 in AW groups (Table 1). IL-10 which acts as an anti-inflammatory gene should be activated on day 4 to control pro-inflammatory responses as a result of BHV-1 infection in either AW or in both groups; instead it was only over expressed in control (PC) groups. On a similar token β-defensin on day 4 should also be observed in both groups to control BHV-1 infection. These results are particularly interesting to explore further to understand the detailed mechanism of stress-induced disease correlation.

Although advances have been made in understanding various disease processes, successful intervention often depends upon the stage at which disease is detected. Thus, identifying markers for disease or its cause, as well as understanding the mechanism of disease onset and susceptibility, are very important. Systems biology approaches such as omics (genomic, proteomic, metabonomic) and multivariate analysis to understand mechanisms and to identify biomarkers provide potential tools to address these questions.[69,94–96] It is particularly important to employ a combination of molecular approaches such as omics to understand complex processes such as stress and its correlation with disease susceptibility. With such goals in mind, we identified and characterized a group of protein, metabolite and elemental biomarkers using serum samples from BHV-1

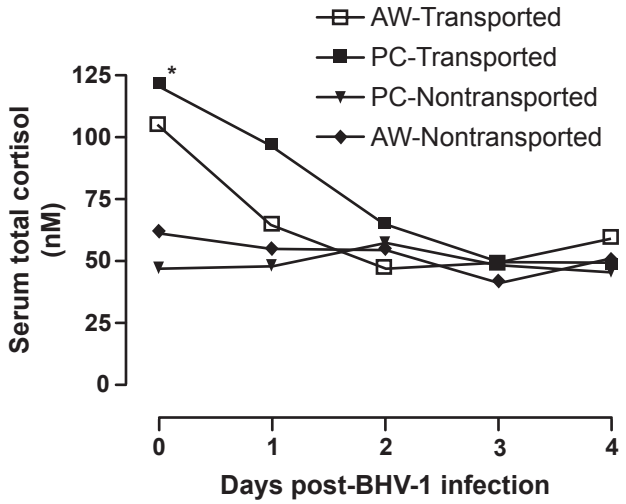

Figure 5 Serum cortisol levels following transport and BHV-1 infection. Serum cortisol levels were significantly elevated ($p < 0.01$) in both groups of calves following transport (Day 0) compared to respective group of calves which were not transported. Cortisol levels remained significantly elevated in pre-conditioned calves at 24 h post-BHV-1 infection. Data presented are median values for each group (n = 10). Symbols associated with each group are defined in the figure.
Abbreviation: BHV-1, bovine herpesvirus-1.

infected as well as from stressed animals. The trend of each group of biomarkers distribution was analyzed to correlate with the groupings based on stress condition or infection.[53] Multivariate analysis revealed distinct differential trends in the distribution profile of proteins, metabolites and elements following a stress response both before and after primary viral infection. A group of acute phase proteins, metabolites and elements could be specifically linked to either a stress response (decreased serum amyloid A and Cu, increased apolipoprotein CIII, amino acids, low-density lipoprotein [LDL], P and Mo) or a primary viral respiratory infection (increased apolipoprotein A1, haptoglobin, glucose, amino acids, LDL and Cu, decreased lipid and P). Thus, combined OMICS analysis of serum samples revealed that multimethod analyses could be used to discriminate between the complex biological responses to stress and viral infection.

Table 1 Expression trend of select innate immune genes are listed as observed by quantitative real time-polymerase chain reaction analysis

Gene	AW	PC
TLR2	day 4 > day 0	day 4 > day 0
TLR4	day 4 > day 0	day 4 > day 0
IFNγ	day 4 > day 0	day 4 > day 0
2'5' OAS	day 4 > day 0	day 4 > day 0
IL-10	day 4 = day 0	day 4 > day 0
β-defensin	day 4 > day 0	day 4 = day 0

Abbreviations: IFN, interferon; IL, interleukin; TLR, toll-like receptor.

There are reports which suggest that transport can be an important stressor which alters blood leukocyte populations and sets the stage for BRD.[97,98] Currently we are conducting experiments in which transportation of the animals is eliminated, such that stressors related to a new environment are removed. It was documented that abrupt weaning (separation of suckling calves from their dams) causes a prolonged psychological stress response.[81] This stress response manifests as an increase in vocalization by both calves and cows and a significant increase in time spent walking by the calf. Consequently, time spent eating and resting also decreases for abruptly weaned calves. Statistically, these changes in behavior return to baseline values after four to five days of weaning.

Pros and cons

As is evident from the review of the literature that traditionally effects of stress have been studied by attempting to understand behavioral patterns of the subject and cellular immune response of the host. It is, however, clear that amount of data to understand such a complex condition (such as stress and stress-induced diseases) is not adequate and enough. Behavioral studies may not always be correlated to a particular stressor as there might have been other parameters affecting the observations. Attempts have also been made to use cortisol as a unique biomarker for stress, but cortisol is diurnal hence cannot be a reliable marker. While it is important to understand cellular immunology to establish the mechanism of stress-induced disease susceptibility, however without identifying and establishing the immune markers specific for stressor it will not lead into any meaningful inference. Systems biology approaches using various omics and bioinformatics methodologies can identify a group of biomarkers at various levels of host immune response, gene expression and metabolism. Association of the identified biomarkers and the patterns in changes in their expression level above or below a determined threshold level with specific stressor(s) could then be used to define stress situation, identification of individuals susceptible to stress-induced disease. Moreover, establishing a pattern in changes of biomarkers is more reliable than a single biomarker. Duration of identified biomarkers can further strengthen the methodology for applying in real life situation.

Perspective

The current review established the importance of psychological stress and its effects on infectious disease severity

and susceptibility with emphasis on respiratory disease. As evident from the literature, initial work focused mainly on understanding the roles of various stressors on physiology and behavior. Theories emerged as a consequence of these studies which implicated the HPA axis and the sympathetic nervous system (SNS) in altered immunity. However, physiological responses studied in a laboratory may not necessarily be consistent with real life phenomena, especially when attempting to analyze heterogeneous populations such as cattle or humans. Experiments with human subjects cannot always be properly controlled because of ethical concerns and therefore using appropriate animal models to mimic natural disease outcomes may be more informative. With the use of high-throughput omics approaches it should be possible to extract more detailed information out of these complex biological systems and understand the complex biology and mechanisms by which stress augments disease susceptibility. More studies are needed to understand the physiological differences between acute versus chronic stress as well as the combined effects of various stressors. It is becoming more apparent that stress not only makes an individual more susceptible to a disease but can also enhance disease severity. Therefore, it is very important to understand the kinetics, specificity and stability or duration of biomarkers associated with a specific stressor or combination of stressors before we can predict disease susceptibility in individual people or animals.

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