Review

Rhythm Definition of Biomarkers for an Objective Measure of Autism

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A review of the literature noted 16 biomarker candidates that could be utilized to develop an objective measure of autism, and we found that 11 of them were quantifiable in saliva collected twice in the evening from 12 neurotypical adults. These biomarkers (and body systems) in which autism is manifest were: Glutamine and Glutamic acid (ubiquitous), CD26 (gastrointestinal); C4B and IFNγ (immunologic); Cortisol, Melatonin, Testosterone (neurologic); and GSH, GSSG, Metallothionein-2 (toxicologic). These 11 biomarkers also monitor the three unifying concepts of the cause of autism: oxidative stress, immune glutamatergic dysfunction, and pineal gland malfunction. Saliva was used since, of the typical body fluids, its collection is least stressful to the individual. Because the concentrations of biomarkers can vary dramatically over 24-hours, circadian studies are being planned, since defining the circadian rhythm of each biomarker will allow future testing using as few assays at appropriate times as necessary in order to obtain a valid objective measure of autism. A diagnosis based on chemical measurements can then be made, resulting in a patient-specific profile that will rank the biomarkers in the order of their difference from normal. It is hoped that this profile will provide a guide for biomarker replacement therapy to improve the symptoms of autism, as has been demonstrated for melatonin and several components of the methionine cycle (involved in detoxification).

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BACKGROUND

In preparation for research into biomarkers of autism, reviews of theoretical aspects regarding causes and biomarkers of autism have been published by one of us (HVR).[1,2] The current overview article briefly summarizes an initial study of biomarkers in saliva of neurotypical adults and discusses future planned 24-hour studies using both neurotypical and autistic subjects.

To date, there are still no objective measures of autism. Autism is characterized by impaired communication, social interaction dysfunction, and repetitive behaviors, and is diagnosed by observing behavior using the *Diagnostic and Statistical Manual of Mental Disorders* IV published by the American Psychiatric Association (1994).[3] Using a list of diagnostic criteria, at least 6 of 12 factors must be exhibited, including at least two relating to social abnormalities and one each regarding impaired communication and range of interests and activities. Onset of the condition must have been prior to age three as shown by delay or abnormal functioning in social interaction, language as used in social interaction, and symbolic/imaginative play.[3]

There are more than 70 biomarkers documented to be in significantly different concentrations in blood or urine from autistic individuals compared to age- and sex-matched controls,[2] and these biomarkers can be organized according to the system of the body in which autism is manifest. These systems were defined by Jepson to be: ubiquitous, gastrointestinal, immunologic, neurologic, and toxicologic.[4] It was very problematic to choose which of these biomarkers to measure in saliva in the preliminary study using neurotypical adults. The rationale for the 16 biomarkers eventually chosen for measurement is given in references: *Ubiquitous:* [Glutamine,[5] Glutamic Acid,[5] Gamma Amino Butyric Acid (GABA),[5] Carnitine[6]]; *Gastrointestinal*: [Dipeptidyl Peptidase IV / CD26[7]]; *Immunologic:* [Complement Component C4B,[8] Interferon-gamma (IFN-γ),[9] Interleukin-12 (IL-12)[9]]; *Neurologic*: [Cortisol,[10,11] Melatonin,[11,12] Testosterone,[13] Serotonin[14]]; and *Toxicologic*: [Cystine,[15] Glutathione (GSH),[15] Oxidized Glutathione (GSSG),[15] Metallothionein-2[16]]. [Note: GABA, Carnitine, IL-12, Serotonin, and Cystine could not be measured in the majority of the saliva samples, leaving 11 for the next phase of our research.] Each system of the body in which autism is manifest is represented by at least one biomarker. In addition, the chosen biomarkers monitor three unifying concepts of the cause of autism: oxidative stress, immune glutamatergic dysfunction, and pineal gland malfunction.[2]

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Saliva is considered a filtrate of the blood, and concentrations of biomarkers are usually lower in the saliva than in the blood. The circadian rhythms for two of the biomarkers, cortisol and melatonin, are well known, and it has been determined that measurement of these hormones in saliva, blood, or urine will give an adequate estimate of an individual's synchronization status, i.e., times of daily highs and lows in relation to the sleep-wake schedule (see Fig 11.3 in ref [17]). Thus, to ensure our ability to measure the lowest expected concentrations, the times of measurement for a preliminary study were chosen in the evening (7:30 & 8:30 pm) when these two biomarkers are usually at their lowest throughout a 24-hour day.[17]

Indications that synchronization of the sleep-wake cycle is a problem in people with autism come from reports of aberrant circadian rhythms of cortisol and melatonin in autistic individuals.[11,18,19,20] The next step in our research will be to measure the circadian rhythm of the eleven biomarkers, including cortisol and melatonin, in the saliva of adults diagnosed with autism and/or Asperger's, and of neurotypical adults matched for age, sex, ethnicity, and place of residence.

In order to assess the correlation of the concentration of the biomarkers with the severity of autism, it is first important to determine the concentration of the biomarkers in neurotypical controls. It is also essential to assess how many measurements are needed. Accordingly, it is critical to establish proper controls, so that a definition of "normal" can be made. Once the normal concentration of each biomarker is established, the amount of difference from normal can be measured in the saliva from the autistic subject.

DEFINITION OF NORMAL

Although the establishment of 'normal' is complex, it is customarily defined as: typical; usual; healthy; according to the rule or standard; and in psychiatry and psychology, denoting a state of effective function which is satisfactory both to the individual and his social milieu.[21] Furthermore, it is important to consider the setting when "normal" is defined. For example, measurements and characteristics may be greatly influenced by genetics and the environment. In addition, the seasons and time of day greatly influence all living things. An organism, as well as its parts, does not function at a constant rate along a 24-hour scale. There are repetitive sequences of events, with organization in time evolving through genetic adaptation of body metabolism to a terrestrial environment.[22] The concentrations of several biomarkers have been shown to vary significantly at different times of day, seasons of the year, or over years. Therefore, the normal concentration of a biomarker would be different at different times and settings. To standardize those settings, it is thus appropriate to consider biological rhythms and define normal according to time of day, season, year, etc., in addition to qualifying for age, sex, ethnicity, and perhaps for location of residence. For the purposes of the next phase of our research, in addition to selecting appropriate sampling time(s), we will limit the place of residence to within 200 miles of Boston because the original laboratory that performed analyses of the biomarkers in saliva is near Boston. Because autism is a complex disorder with many facets,[1] choosing appropriate subjects will be of prime importance. Adults with a diagnosis of autism and/or Asperger's will be included in the study, and a ratio of sampling of 4:1 for males:females (typical sex distribution of autism)[1] for these subjects and neurotypical controls will be maintained. The means of diagnosis used will be noted if available and we will attempt to correlate the circadian rhythm findings with the scoring system used for diagnosis. Ethnicity will also be noted, in addition to any co-morbidities. These factors will be included in criteria used for discriminate analysis.

RHYTHMICITY OF LIFE

The rhythmic nature of life influences the very existence of organisms, commencing before conception. The 'period' (time required to complete a cycle) of the rhythm for different organisms can vary from less than a second (EEG activity in the human, delta frequency) to > 100 years (flowering of Chinese bamboo).[23]

It has been known for many centuries that body time is important. The 8th century BC Greek poet Hesiod wrote that "Of themselves, diseases come among men, some by day and some by night" and "Observe due measure: and best in all things is the right time and right amount". In the 4th century BC, the ancient Greek physician Hippocrates wrote "Whoever wishes to pursue the science of medicine in a direct manner must first investigate the seasons of the year and what occurs in them". The Hippocratic school defined health as a balance of four humors (extracellular fluid of the body): blood, yellow bile, black bile, and phlegm.[24] Similarly, Chinese classical medicine defines health as a balance of opposites: cold and warm, moist and dry, passive and active, moon and sun, night and day, etc., which represent the concepts of Yin and Yang.[25]

DIFFERENT TYPES OF RHYTHMS

Examples of different types of rhythms include a period of 13 or 17 years for cicadas. 2013 is the year for the Brood II 17-year cicadas to emerge in the spring in the eastern United States, where billions are expected in neighborhoods from New England to North Carolina.[26,27] A shorter period of about 10 to 11 years has been documented for sunspots.[28-30] Although cycles in sunspot numbers were not found to have a significant, consistent relationship with some biological growth patterns, such as tree-rings,[31] the dynamics of sunspots were documented to be reflected in human physiology and pathophysiology. Notably, six annually rhythmic Pap smear-detected infectious, premalignant and malignant cervical epithelial pathologies showed strong 10-year and weaker 5.75-year cycles, as did six self-measured, annually rhythmic, physiologic functions: oral temperature, pulse, respiratory rate, peak expiratory flow, systolic blood pressure and diastolic blood pressure.[32]

The American chronobiologist Franz Halberg introduced the term *circadian* in 1959 as follows: "… the term "circadian" was derived from "circa" (about) and "dies" (day); it may serve to imply that certain physiologic periods are close to 24

hours, if not of exactly that length. Herein, "circadian" might be applied to all "24-hour" rhythms, whether or not their periods, individually or on the average, are different from 24 hours, longer or shorter, by a few minutes or hours. "Circadian" thus would apply to the period of rhythms under several conditions. It would describe: 1. rhythms that are frequency synchronized with "acceptable" environmental schedules (24-hour periodic or other) as well as 2. rhythms that are "free-running" from the local time scale, with periods slightly yet consistently different from 24 hours (e.g., in relatively constant environments).[33] 'Circadian' therefore defines the most obvious rhythms associated with the daily alternation of activity and rest. All life-forms on Earth have built-in circadian rhythms, with organisms adapting their behavior in such a way that their survival is enhanced.

Biological rhythms are well established for each body process or parameter,[17,34] are reproducible, and can be unique for the strain of animal, providing evidence of the effects of genetics on physiological function. For example, with regard to a yearly cycle, in a circannual (period of about a year) study, it was shown that antibody production in the B6C3F1 mouse was highest in the summer, and lowest in the winter. In contrast, for the CD1 mouse, antibody production was greatest in the spring and lowest in the fall. This seasonal difference was predictable, with similar results documented in three separate years for the CD1 mice, and in two years for the B6C3F1 mice. It should be pointed out that, although the rhythm was predictable, the magnitude of the response varied from year to year.[35]

Circadian rhythms are typically quantified by a 24-hour cosine curve, with the 'amplitude' being the magnitude of the variable from the baseline (usually the middle value or mean) to the peak (acrophase) or trough (bathyphase) of the curve. There can be a great difference of magnitude between the peak and the trough of many substances in the body. For example, the range of change of the mean value in percent was 502% for cortisol (greatest concentration at 07:47 h), and >241,000% for melatonin (greatest concentration at 02:55h).[34] These examples and many others in clinical medicine show that the magnitude of change throughout 24 hours can be very great, and establishing a "normal" value must be set for different times of day.[17,34]

There is also a feed-sidewards effect of the hormones on each other. For example, data document that when one hormone is out of synchronization, other hormones of the body are also out of synchronization.[36] This desynchronization of biological rhythms increases the risk of disease or dysfunction. Pattern abnormalities, trend shifts, or loss of timing may also presage the occurrence of pathology.[37]

Because biological clocks are built-in, most circadian rhythms will persist without the daily alternation of light and dark for many days before beginning to dampen. Some high-amplitude rhythms that are remarkably stable, such as for cortisol and melatonin, are used as 'marker' rhythms to confirm light-dark synchronization, since they exhibit their characteristic rhythm consistently during health. Indeed, molecular clocks present in organs and individual cells throughout the body are central for the temporal coordination of rhythms in internal biological processes among themselves and with external environmental cycles. Of interest, it has been shown that altered function of specific clock gene (CG) components can have significant impact relevant to health and disease.[38] For example, several articles have referred to the role of CGs in the etiology and setting of the clock in autism spectrum disorders.[39,40,41,42] It is therefore important to note that some individuals with autism have an aberrant rhythm for cortisol and for melatonin.[11,18,19,20] The ongoing research described herein will readdress these rhythms in autism and hopefully add information about other biomarkers important for the disorder.

SUMMARY

The biomarkers we have chosen to study are important to maintaining a "normal" state, and they do so when in proper concentrations (balance) and exhibiting typical circadian rhythms. We now have data proving we can measure 11 biomarkers that are pertinent to autism in the saliva: Glutamine, Glutamic Acid (ubiquitous); CD26 (gastrointestinal system); C4B, IFN-γ (immunologic); Cortisol, Melatonin, Testosterone (neurologic); GSH, GSSG and Metallothionein-2 (toxicologic). These biomarkers represent three unifying concepts of the cause of autism: oxidative stress, immune glutamatergic dysfunction, and pineal gland malfunction. In our next step, we plan to measure the concentration of the 11 biomarkers every 4 hours for a total of 6 collections from autistic and neurotypical adults, in order to measure the circadian rhythm of each biomarker. It is hoped that comparisons of the mean concentration and amplitude of the rhythm between these two groups will provide a way for us to determine the severity of autism and to provide a patient-specific panel of biomarker concentrations. In addition, defining the circadian rhythm of each biomarker will allow future testing using as few assays at certain standardized times as necessary (e.g., upon arising, mid-morning, early evening, and/or bedtime, etc.) in order to obtain a valid objective measure of autism. The biomarkers will be ranked in accordance to the difference from normal, which in theory will allow a physician to have a guide for which biomarkers to add to the body and which to antagonize. The concept that adding a biomarker to an autistic individual who is deficient in that biomarker helps the autistic individual approach normalcy has been documented for melatonin[43-45] and for the components of the methionine cycle involved in detoxification.[15] This approach will need to be validated by a larger study before any attempt is made to use any new biomarker data therapeutically.

CONFLICT OF INTEREST

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