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The systems medicine of neonatal abstinence syndrome

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1. ABSTRACT

This review will focus on a systems medicine approach to neonatal abstinence syndrome (NAS). Systems medicine utilizes information gained from the application of "omics" technology and bioinformatics (1). The omic approaches we will emphasize include genomics, epigenomics, proteomics, and metabolomics. The goals of systems medicine are to provide clinically relevant and objective insights into disease diagnosis, prognosis, and stratification as well as pharmacological strategies and evidence-based individualized clinical guidance. Despite the increasing incidence of NAS and its societal and economic costs, there has been only a very modest emphasis on utilizing a systems medicine approach, and this has been primarily in the areas of genomics and epigenomics. As detailed below, proteomics and metabolomics hold great promise in advancing our knowledge of NAS and its treatment. Metabolomics, in particular, can provide a quantitative assessment of the exposome, which is a comprehensive picture of both internal and external environmental factors affecting health.

2. INTRODUCTION

NAS is a rapidly emerging and costly global public health problem arising from opioid use during pregnancy and resulting in newborns with withdrawal signs caused by the cessation of maternal opioid exposure. This review will address NAS through a systems medicine approach and is intended for general health care providers, health care policymakers, and researchers. Systems medicine is an integrative approach utilizing "omics" information from genomics, epigenomics, proteomics, and metabolomics, as well as conventional evidenced-based clinical data. One branch of systems medicine is the "bench-to-bedside" aspect, which refers to utilizing information from basic science research to treat patients, i.e., translational research. There has been a dramatic revolution in bench research that is now impacting patient care: this revolution is the result of "big data" from "omics" technology. Omics technology refers to the ability to measure, in a biofluid/biopsy, all of the small organic molecules (metabolomics) and proteins (proteomics), and much of the genetic information (genomics and epigenomics). Integrating "omics" data with conventional clinical information is a major goal of systems medicine. Moreover, systems medicine can go beyond the bench-to-bedside axis and attempt to integrate information from healthcare and societal systems, i.e., community-translation. Systems medicine is, therefore, from "bench-to-bedside and beyond."

Most clinical omic studies attempt to use the results of a single platform, such as proteomics, to initially discover a set of parameters (e.g., the levels for a set of proteins) that correlate and predict the pathological parameters that characterize a disease. This "mono-omic" approach is a good initial start, but using parameters from more than one omic platform, i.e., multi-omics, is likely to have considerably more power. Software for the analysis of multi-omic data is now freely available

(mixomics.org/mixdiablo/). An specific example of multi-genomics is proteogenomics ,which combines information from proteomics, genomics and transcriptomics.

3. THE HEALTHCARE IMPACT AND COSTS OF NEONATAL ABSTINENCE SYNDROME (NAS).

The increasing incidence of NAS in the USA has been well documented (2, 3). Over the decade ending in 2013, the rate of NAS admissions to neonatal intensive care units (NICUs) has almost quadrupled (to 27 cases per 1000 admissions). Similarly, the median length of stay (LOS) in NICUs has increased from 13 days to 19 days during this period (3). For infants covered by Medicaid, the NAS incidence increased at least 5-fold from 2004 to 2014 (2). Newborns with NAS are at increased risk of having low birth weight (LBW) and respiratory complications (4). The healthcare expenses associated with NAS have also been well-characterized: the mean hospital charges associated with NAS increased from $39,400 in 2000 to $53,400 in 2009 (4). The total hospital costs for NAS births covered by Medicare were $65 million in 2004 and $462 million in 2014, a seven-fold increase (2).

4. THE ETIOLOGY AND DIAGNOSIS OF NAS

4.1. Opiates, opioids and opioid use disorder (OUD)

The terms opiates and opioids are often used interchangeably, but they are not equivalent. Opiates refer to naturally occurring alkaloids derived from the opium of the poppy plant (*Papaver somniferum),* whereas opioids refer to both opiates and synthetic drugs that bind to opioid receptors (see below) and affect pain perception (www.drugabuse.gov/publications/research-reports/prescription-drugs/opioids/how-do-opioids-affect-brain-body). Schumacher *et al.* have published an excellent online review of opioid pharmacology (5). The terms “substance abuse” or “substance dependence” have been replaced with the term “substance use disorders” which, in turn, are further defined as mild, moderate, or severe based on the degree of severity (www.drugabuse.gov/publications/media-guide/science-drug-use-addiction-basics). A substance use disorder occurs “when the recurrent use of alcohol and/or drugs causes clinically and functionally significant impairment, such as health problems, disability, and failure to meet major responsibilities at work, school, or home” (www.drugabuse.gov/publications/media-guide/science-drug-use-addiction-basics). The term “opioid use disorder” is now used in place of “opioid addiction.”

4.2. Is there a distinction between opioid physical dependence and addiction?

As detailed below, it is important to make careful functional definitions in the area of opioid pharmacology since this approach will avoid confusion and foster research progress. There are some schools of thought, e.g., The National Alliance of Advocates for Buprenorphine Treatment (NAABT), asserting a marked distinction between physical dependence and addiction. The NAABT (see www.naabt.org/-addiction_physical-dependence.cfm) defines physical dependence as meaning “that the body relies on an external source of opioids to prevent withdrawal,” whereas addiction is viewed as manifesting “uncontrollable cravings, inability to control drug use, compulsive drug use, and use despite doing harm to oneself or others.” This distinction could be interpreted as implying that addiction is “psychological” and “non-physical” and is similar to the question of whether or not psychology is “biological.” As stated by Joshua A. Gordon at National Institute of Mental Health, “All psychology works through biology” with the divide being “artificial at the level of neurocircuits” (6). It is likely that both physical dependence and addiction have a physiological basis, with addiction having a long-term behavioral component. Alavi *et al.* (7) note that all “entities capable of stimulating a person can cause addiction.” It is possible, therefore, to have a “behavioral” addiction without a “substance” addiction. Pharmaceuticals and toxins can influence behavior, and a whole journal is devoted to this topic, i.e., *Pharmacology Biochemistry & Behavior.*

4.3. What criteria are used to diagnose NAS and its severity?

NAS is defined by the group of signs and symptoms that can affect the central nervous, autonomic nervous, and gastrointestinal systems of a newborn exposed to an opioid during gestation. A number of recent and excellent reviews address NAS diagnosis in detail (8-13). For newborns, a key issue is the criteria used to diagnose the severity and guide treatment. This is not a straightforward task since NAS, being a syndrome, is a group of signs and symptoms that can be further complicated by polydrug use, poor maternal health, and poor nutrition (14, 15). The diagnosis of NAS is often described as being "clinical," meaning that clinical signs provide the main criteria for diagnosis, rather than a specific diagnostic test (8). Moreover, the signs of NAS may not become evident for more than 48 hours after delivery; this is problematic since most mothers are discharged from the newborn nursery in less than 48 hours if there are no delivery complications (16).

Both the treatment protocols and the criteria for diagnosing NAS are highly variable (17). A commonly utilized tool is the Finnegan Scoring System which relies on 31 signs of opioid withdrawal such as loud, high-pitched crying, sweating, yawning and gastrointestinal disturbances. Scoring is most often performed by nurses. A recent study of scoring consistency and accuracy (18) concluded that there is a need for "more objective tools to quantify withdrawal severity given that assessments are the primary driver of pharmacological management in neonatal drug withdrawal."

Most diagnosing paradigms for NAS require evidence for intrauterine exposure to a neuroactive substance but self-reporting is often sufficient to meet this requirement. While useful (see below) some diagnosing paradigms do not require additional documentation of opioid (or other neuroactive substance) use during pregnancy or obtaining drug screening or drug testing information. In addition to opioids, exposure to other drugs such as selective serotonin reuptake inhibitors (SSRIs) can contribute to NAS (19). A systems medicine approach, as discussed below, is likely to provide a useful framework for guiding NAS differential diagnosis, stratification of severity, prognosis and individualized treatment.

4.3.1. Opioid treatment for NAS infants based on the Finnegan tool may not be optimal

Finnegan scores have been widely used as a criterion for administering opioids to infants suffering from NAS to prevent withdrawal signs (13). A recent study by Grossman *et al.* (13) at Yale New Haven Children's Hospital explored a novel approach for accessing NAS infants utilizing the Eat, Sleep, Console (ESC) tool and compared it to the standard Finnegan tool. The ESC approach is less intrusive to the NAS infant than the Finnegan scoring tool and emphasizes breast feeding, consoling interventions such as skin-on-skin contact, checks to ensure adequate feeding, and making sure there is at least one hour of sleep between feedings. In the Yale retrospective study, it was determined that infants managed by ESC were treated with morphine significantly less often than would have been predicted using Finnegan scores. Moreover, the average LOS was significantly reduced in the ESC approach compared to the Finnegan approach (13). Other Children's hospitals have also experienced very positive results with the ESC approach (20).

5. TESTING FOR OPIOID USE DURING PREGNANCY COULD GREATLY IMPROVE THE DIAGNOSIS AND TREATMENT OF NAS.

Since opioid use is the key etiological factor in NAS, it is reasonable to suggest that the diagnosis and subsequent treatment of NAS would be greatly improved by detailed knowledge of maternal drug/polydrug use during pregnancy, as well as by fetal drug exposure, e.g., timing, dose, and duration (emedicine.medscape.com/article/978763-workup?). For prescription opioids, this is not an issue, but for illicit opioids, this is complicated by ethical and legal issues. Accurate maternal self-reporting would be ideal if it could provide detailed information on dose, timing, and duration of drug use (15). In practice, maternal self-reporting has not proven to be very accurate, with underreporting

being very common (15). Some hospitals have, therefore, embraced dual screening for both the mother and newborn. A screening study in the region of Cincinnati, Ohio, found that 5.4% of all mothers had a positive urine drug test on admission and 3.2% tested positive for opioids (21). In the Cincinnati universal screening program, mothers were asked for consent; when not provided, their newborns were tested instead. Urine immunoassays were used for the initial screening since these assays are fast, sensitive and relatively inexpensive. If a positive test was obtained with the initial urine immunoassays, the Cincinnati program rapidly followed-up with mass spectrometry (MS) testing, which provided details on 47 drugs of abuse. The rationale for rapidity was based on the notion that early diagnosis and treatment could provide optimal outcomes following NAS therapy.

5.1. Legal/ethical issues on maternal and infant drug testing

As recently pointed out by Hamdan (22), substance abuse testing in the USA is complicated by state laws, with some states requiring testing if prenatal drug abuse is suspected. In 2014, Tennessee passed a statute making substance abuse during pregnancy a crime (23); this statute expired in 2016, although about 100 women were arrested during its enforcement. Moreover, some states require that women with a positive prenatal drug abuse test be reported to child protective services. Some Alabama hospitals have tested new mothers without obtaining informed consent (www.advisory.com/daily-briefing/2015/10/06-/hospitals-test-new-moms-for-drugs-without-their-explicit-consent). Universal maternal testing for opioids is, therefore, enmeshed in a complicated set of ethical and legal issues (22). Although controversial, it is generally accepted that a maternal consent form is not required to perform drug testing on a newborn if there is: (1) evidence of risk indicators for *in utero* drug exposure; and (2) the purpose of such testing is to determine appropriate medical treatment. Many states do not have a uniform policy or state law regarding newborn drug testing, and policies can vary among hospitals in the same state.

5.2. A positive maternal urine drug test for opioids does not necessarily mean a newborn will develop NAS

A positive maternal urine drug test for opioids does not necessarily mean that a newborn will develop withdrawal signs (16, 21): between 55% and 94% of newborns will develop withdrawal signs, and between 30% and 80% of these may require pharmacologic treatment (21). As discussed below, a variety of factors (e.g., pharmacogenetics) in newborns may influence the degree to which newborns develop withdrawal signs in response to *in utero* opioid exposure.

5.3. What is the best body fluid/tissue to test for opioid exposure and omic analyses?

The timing and duration of fetal opioid exposure are important for understanding the underlying molecular pathology for opioid fetal toxicity, and its consequences at different stages of fetal development. The biosamples typically used for evaluating opioid exposure are equally useful for omic analyses. Metabolomics, in particular, has the potential for measuring not only opioids but a comprehensive panel of metabolites that could be altered as a consequence of opioid exposure (24, 25). Polydrug exposure could also be evaluated by metabolomics.

5.3.1. Plasma and urine

Most opioids have a plasma half-life (in adults) in the range of minutes to about one-and-a-half days. Maternal plasma is, therefore, not a particularly good body fluid for measuring long-term opioid exposure. Urine is the most commonly used biofluid for neonatal drug testing, with opioid levels generally higher than those observed in plasma (22). Nevertheless, like plasma, urine testing provides exposure information only over the last few days, and maternal urine samples must be obtained very close to birth to be a meaningful measure of recent fetal drug exposure. Moreover, urine and plasma are systemic biofluids and not likely to yield direct information about the potential neurotoxicity of opioids/metabolites. Newborn urine samples (after the first day of life) are readily obtainable from cotton

balls inserted into diapers and have great advantages in both omics (see below) and drug toxicology. These samples can be obtained serially by noninvasive means and are underutilized in NAS studies.

5.3.2. Meconium

Meconium, the earliest infant stool, is a useful alternative to urine since its composition reflects materials ingested during the time the infant spends *in utero* and is, therefore, able to assess long-term fetal drug exposure (as early as the second trimester)(22). Work by Gray *et al.* (26-28) has proven the clinical utility of meconium drug analyses in assessing prenatal drug exposure. These investigators found, for example, that meconium levels of methadone (and its metabolite) did not predict NAS severity as well as the presence of opioids (28). Meconium can, however, be contaminated with urine, and its collection may require as many as three days after birth (22). Moreover, the passage of meconium may be delayed in preterm infants compared to term infants (29).

5.3.3. Umbilical cord

Umbilical cord tissue is rapidly becoming the biosample of choice for monitoring *in utero* drug exposure, since it is available at birth without delay, is easily collected, and has a clear chain of custody (22). Results obtained from meconium samples compare very well to those obtained from umbilical cord samples (30). While umbilical cord testing provides a long-term window of *in utero* drug exposure, it is not yet clear how well it reflects maternal drug use in the days immediately before delivery (31). Urine testing combined with umbilical cord testing covers all bases.

5.3.4. Cord blood

The levels of opioids and their metabolites in cord tissue and cord blood are particularly informative. As schematically indicated in Figure 1, maternal drugs and their metabolites that are detected in cord blood have crossed the placenta and can, therefore, be potentially toxic to the developing fetus. The general factors affecting the placental transfer of maternally administered drugs and their potential short- and long-term effects have been reviewed (32, 33). It is generally accepted that all opioids, particularly lipophilic opioids, can cross the placenta at significant levels. Methadone, which is very lipophilic, has been found in cord blood at levels about half that found in a paired maternal blood sample (34). Buprenorphine is widely used to treat OUD in pregnant women. Both buprenorphine and its active metabolite, norbuprenorphine, have been measured in cord blood by LC-MS and shown to useful predictors of NAS onset in exposed newborns (35). Although only a small population was studied, each 5 ng/ml increment in norbuprenorphine increased the odds of treating a NAS newborn with morphine by a factor of 2.5 (35).

Opioids and their metabolites can have adverse effects by multiple mechanisms. In the case of NAS, it is the cessation (or abstinence) of opioid exposure at birth that is thought to give rise to the behavioral changes collectively termed “withdrawal symptoms.” As detailed below, prenatal opioid exposure can also influence fetal brain development with potentially long-lasting consequences.

6. FETAL OPIOID TOXICITY DURING PREGNANCY

The toxicology of opioids during pregnancy is relevant to the etiology of NAS. Most of what we know about opioid toxicity is from adult studies, which are primarily focused on acute toxicity with respiratory inhibition being a key contributor to mortality (36). Unfortunately, we know less about fetal opioid toxicity and its potential impact on prenatal and postnatal development. It is increasingly clear, however, that a diagnosis of NAS is associated with poor school performance (37).

Opioids are often prescribed during pregnancy for pain-relief or for treating maternal OUD. Studies relating prescription opioids to birth outcomes are particularly useful since they are generally on stronger footing than studies on exposure to elicit opioids (38, 39). Although there is considerable state-to-state variation, about four-in-ten to one-in-ten pregnant women in the USA are prescribed at least one opioid (39). The review by Yazdy *et al.* (38) concluded that the effects of opioid use during pregnancy on birth weight and preterm birth were inconclusive and required further study.

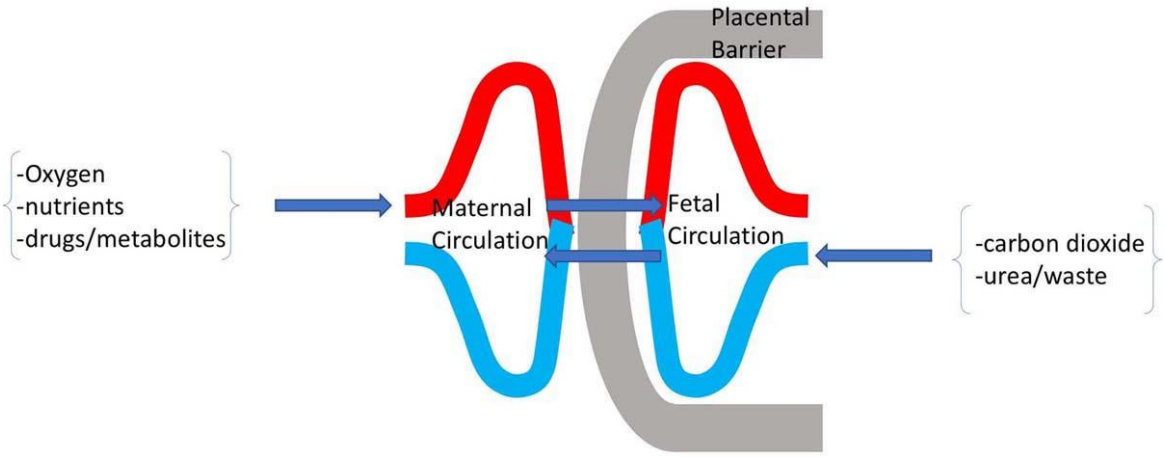

Figure 1. A simplified diagram of placental-maternal circulation. Maternal blood (red, oxygenated blood) arrives at the placental via the uterine artery and returns via the uterine veins (blue, deoxygenated). Fetal blood (blue, deoxygenated) arrives at the placenta via the umbilical arteries (blue, deoxygenated) and leaves via the umbilical vein (red, oxygenated). Maternal drugs/metabolites from the uterine artery that cross the placental barrier enter the fetal circulation via the umbilical vein.

Nevertheless, a well-designed and large-scale study by Patrick *et al.* (39) found that prescription opioid use during pregnancy was associated with LBW. Moreover, a multivariate statistical model showed that cumulative prescription opioid exposure, opioid type, tobacco use, and use of a SSRI were all associated with an increased NAS risk.

6.1. Fetal growth, newborn head circumference, and brain development are affected by opioids

The data on fetal growth reviewed by Yazdy *et al.* (38) suggests that neither head circumference nor birth length was decreased by opioid use. In contrast, data from Visconti *et al.* (40) show that chronic maternal OUD is associated with a significantly decreased newborn head circumference. About one-third of the NAS infants had a head circumference less than or equal to the 10th percentile for gestational age compared to control. Moreover, the femur and humerus lengths in the NAS infants were also decreased (40). These investigators did not find the use of any specific opioid to be associated with a reduced head circumference in the NAS infants but rather found an association with all types of opioids. Moreover, many of the NAS mothers ingested more than one opioid, and many were also cigarette smokers. Particularly troubling was the association of a small head circumference with buprenorphine and methadone use, since these drugs are often used to treat pregnant women with OUD (41). Visconti *et al.* (40) are justified in asserting that their findings need to be extended to include long-term follow up to determine if the small head circumference is reversible post-delivery. Moreover, the underlying molecular mechanism for their findings needs elucidation.

A recent study by Monnelly *et al.* (42) found that prenatal methadone exposure was associated with altered newborn brain development. In this study, maternal drug use was determined from medical records as well as biological screenings, if performed: the vast majority of the women prescribed methadone (mean dose of 55 mg per day) also smoked tobacco and showed polydrug/illicit drug use. Diffusion nuclear magnetic resonance imaging (MRI) was used to study neonatal brain development (see (43). The MRI data were collected before the newborns were exposed to any postnatal opioids to treat NAS. The MRI results showed that maternal methadone exposure was associated with neonatal microstructural alterations in large segments of the brain white matter (primarily myelinated axons). These alterations were independent of head growth. The mean head circumference of the methadone-exposed newborns was, however, significantly

smaller than the controls. As mentioned above, it would also be informative to have a cohort in which opioid/drug/metabolomic testing was performed to help determine the most dangerous opioids or opioid/other drug combinations. A complete metabolomic analysis would be optimal.

6.2. Fetal opioid exposure increases newborn birth defects

Fetal opioid exposure can also elicit adverse effects by increasing newborn birth defects. Broussard *et al.* (44) used a case-control study design to evaluate birth defects in pregnant women using prescription opioid analgesics between one month before pregnancy and the first trimester. In this study, opioid use was determined by self-reporting rather than by toxicology reports. The results showed a significant association between exposure to opioids, early in pregnancy, and birth defects, particularly congenital heart defects. As detailed in an informative "letter-to-the-editors", Broussard *et al.* (44), did not address the issues of dose, duration, and frequency of opioid use, all of which could be informative in providing evidence-based advice when prescribing pain medication to pregnant women. The smoking rates were, however, about the same in the cases (20.9%) and controls (18.9%).

7. THE MOLECULAR MECHANISMS UNDERLYING OPIOID USE DISORDER AND THE IMPORTANCE OF GENETICS

Identifying the molecular players in opioid use disorder is a necessary first step in a systems medicine approach. As detailed below, genetic variants in these the molecular players are likely determinants of OUD heritability and could also inform the development of new therapeutic interventions (45). More specifically, the genes involved in opioid metabolism, mechanism of action, and opioid-induced signal transduction events are all "candidate genes" for investigating the genetics of OUD and NAS. The molecular mechanism underlying either dependence or withdrawal remains an active area of research, but it is generally accepted that opioid receptors are essential via their roles as G-protein-coupled receptors (GPCRs). Excellent reviews are available on the molecular biology of opioid receptors (46, 47). Here, we will only touch upon the key areas necessary to advance a systems medicine approach to OUD/NAS.

7.1. Opioid receptors are G-protein-coupled receptors

G-proteins (or guanine nucleotide-binding proteins) are a large family of proteins controlling many cellular signal transduction pathways. G-protein-coupled receptors, also termed seven-transmembrane domain receptors, are a group of protein receptors that modulate the activity of G-proteins. Opioid receptors are G-protein-coupled receptors activated by opioid/agonist binding and are located on neuronal membranes in the central (brain and spinal cord) and peripheral nervous system. The sensory neurons of the peripheral nervous system transmit information concerning pain (and other external stimuli) to the central nervous system (CNS). Opioid receptors are widely distributed throughout the brain.

There are two major classes of opioid receptors: classical receptors and non-classical receptors. The classical receptors include the mu-, delta- and kappa-receptor subtypes and the non-classical receptors include the opioid-like-subtype-1 (or ORL1). The genes for the three classical opioid receptors (48) and ORL1 (49) have been cloned, thereby enabling the utilization of many molecular biology tools. There are also several types of G-proteins, and the type regulated by opioid receptors are called heterotrimeric since they have three different protein subunits, i.e., alpha-, beta-, and gamma-subunits. There are a variety of naturally occurring opioid peptides (e.g., beta-endorphin, enkephalins, endomorphins and dynorphins) that are released by neurons and subsequently bind to and activate opioid receptors.

As indicated in Figure 2, opioid receptors are transmembrane proteins that can bind to a presynaptic membrane and, in the absence of an agonist (the resting state), bind the trimeric $G_{alpha,\ beta,\ gamma}$ complex with the G_{alpha} subunit binding a guanosine diphosphate (GDP). In the resting state, the Ca^{2+}-channels are open permitting Ca^{2+} to enter the presynaptic neuron. In contrast, K^{+}-channels are

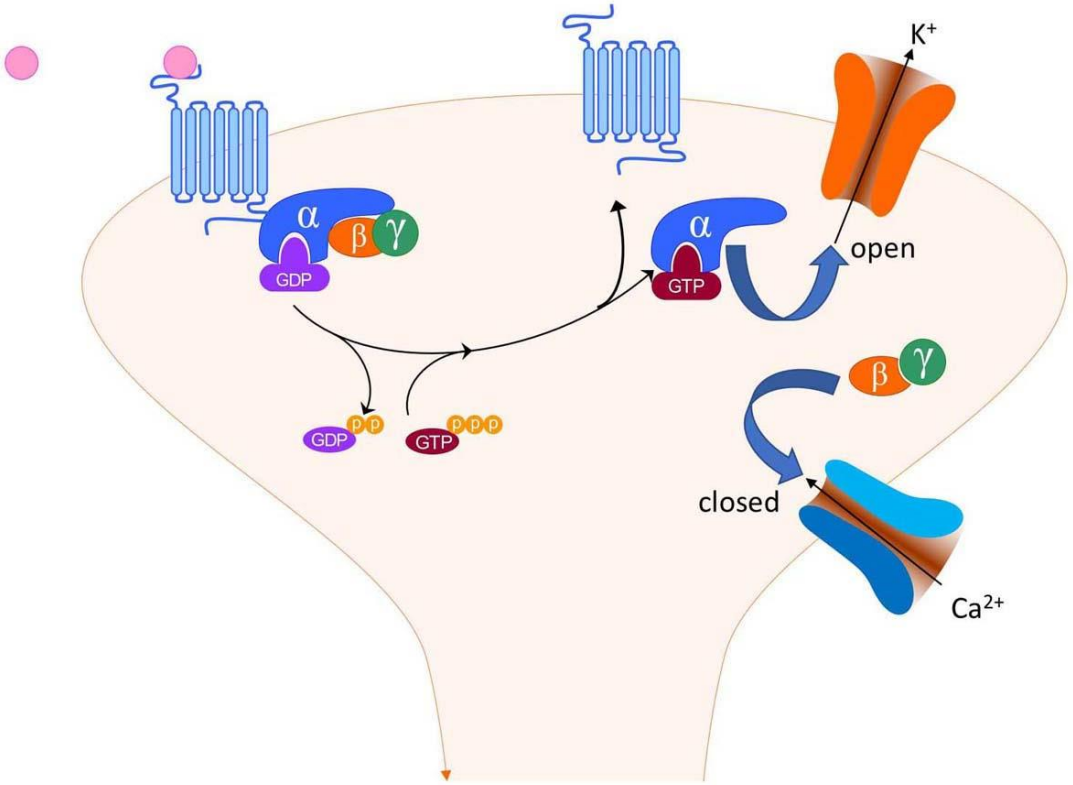

Figure 2. Opioid receptors**.** Opioid receptors are transmembrane proteins (shown in upper left with seven blue transmembrane helices) that can bind to a presynaptic membrane and, in the absence of an agonist (the resting state), bind the trimeric Galpha, beta, gamma complex with the Galpha subunit binding a guanosine diphosphate (GDP). In the resting state, the Ca^{2+}-channels are open permitting Ca^{2+} to enter the presynaptic neuron. In contrast, K^+-channels are closed in the resting state blocking the exit of K^+ ions from the intracellular to the extracellular space. In the presence of an agonist (shown in the upper left as pink sphere) the Galpha subunit dissociates from the Galpha, beta, gamma subunit and the GDP bound to the Galpha subunit is replaced by guanosine triphosphate (GTP). The G alpha subunit opens K^+-channels and the G beta, gamma subunit closes Ca^{2+}-channels.

closed in the resting state blocking the exit of K^+ ions from the intracellular to the extracellular space. The K^+-channels illustrated in Figure 2 are specifically termed G-protein-gated inwardly rectifying potassium (GIRK) channels. In the presence of an agonist, such as morphine, the G_{alpha} subunit dissociates from the $G_{alpha,\ beta,\ gamma}$ subunit and the GDP bound to the G_{alpha} subunit is replaced by guanosine triphosphate (GTP). As indicated in Figure 2, the $G_{\ alpha}$ subunit opens K^+-channels and the $G_{\ beta,\ gamma}$ subunit closes Ca^{2+}-channels, with the net effect of producing a hyperpolarization of the presynaptic membrane which inhibits neurotransmitter release into the synaptic cleft. Glutamate and substance P are two important neurotransmitters whose release is modulated by opioid receptors.

Opioids induce analgesia and euphoria by inhibiting neurotransmitter release. In a prescient paper, Nestler and Landsman (45) emphasized the importance of genomics in helping to elucidate the biology of addiction. These authors suggested that genetic variants in G-coupled opioid receptors and GIRKs could be important in modulating opioid-dependence.

7.2. The mu-opioid receptor (MOR) is a major molecular player in OUD

In a key article, Matthes *et al.* (50) used genetically modified mice lacking the mu-opioid receptor to study how the opioid system effects: (1) analgesia; (2) the award effect and; (3) withdrawal

signs. These investigators found that mice lacking the mu-receptor showed a loss of all three of these morphine-induced effects. This result provides very strong evidence that the mu-receptor is a key target of morphine and a "mandatory component of the opioid system for morphine action" (50).

Although we do not fully understand the complex molecular mechanisms linking opioid receptors to complex behaviors like OUD and NAS, it is very likely that opioid receptors, and the downstream events they modulate, play critical roles. A systems medicine approach can help unravel these complex mechanisms and thereby provide clinically useful information. Moreover, a systems medicine approach could help explain the wide variability observed in the severity of NAS (51).

7.2.1. Phosphorylation and trafficking of the of mu-receptor

Given the unique importance of the mu-opioid receptor, it is likely that factors influencing its expression, subcellular localization, recycling, and activity are important in OUD/NAS. Phosphorylation of the of mu-receptor has emerged as critically important in this respect (52, 53). The temporal sequences of events following phosphorylation of the mu-receptor are critically important for understanding its rapid desensitization, resensitization and, potentially some aspects of opioid tolerance (54).

Agonist binding to the mu-receptor leads to its rapid phosphorylation thereby promoting binding of beta-arrestin-2 , which then directs the receptor to endocytotic pathways. Endocytosis of the receptor is followed by three potential fates, i.e., recycling back to the neuronal plasma membrane, retention in an intracellular compartment, or degradation. Removing the mu-opioid receptor from the plasma membrane is thought to disable its signaling abilities (53).

7.3. Tolerance, desensitization and the importance of operational definitions

A number of investigators have stressed the importance of carefully defining terms like tolerance and desensitization (which are not interchangeable) (52-54). Tolerance is best defined as being the result of long-term (several days to weeks) opioid exposure, whereas desensitization is a more rapid (seconds to minutes) loss of mu-opioid receptor coupling to effectors. Resensitization is the dephosphorylation of the mu-opioid receptor without a bound agonist: this can occur via recycling through endocytosis. Evidence also supports a role for mu-opioid receptor dephosphorylation and resensitization directly on the plasma membrane without endocytosis (54). While desensitization and resensitization are amenable to molecular definitions, tolerance and addiction are much more complex and thought to involve long-term changes in brain neurocircuitry (55) that start with changes in the mesolimbic dopamine pathway.

7.4. Dopamine and the euphoric/award effects of opioids

In addition to opioid receptors, a second interrelated mechanism for the euphoric/award effects of opioids is thought to involve the mesolimbic dopamine pathway. The mesolimbic pathway connects the neurons in the ventral tegmental area (VTA) of the midbrain to the nucleus accumbens (NAcc) brain region. The NAcc is part of the ventral striatum. Yoshida *et al.* (56), using a rat model, found that the opioid fentanyl increases dopamine levels in the NAcc. Dopamine is a major neurotransmitter modulating the sensation of pleasure and reward and is synthesized by the neurons of the VTA. It is now generally accepted that all substances of abuse (e.g., cocaine and alcohol) exert their reinforcing effects in humans by increasing dopamine levels in the NAcc (57).

In their animal model, Yoshida *et al.* (56) found that both the mu- and delta-opioid receptors were involved in the increased accumbal dopamine release caused by opioids. Although beyond the scope of this review, considerable evidence shows that opioid binding to the mu-opioid receptors in the NAcc is sufficient to produce a positive reward effect in a rat model (58, 59). Mu opioid receptor action in the VTA can exert a positively reinforcing effect on rat behavior (58). It is likely that genetic variants in proteins that modulate dopamine levels could be important determinants of OUD/NAS.

Catechol-O-methyltransferase (COMT) is a major enzyme responsible for the degradation of

catecholamines including the neurotransmitters dopamine, norepinephrine, and epinephrine (60). Genetic variants in the COMT gene are known to influence pain perception and modulate the effectiveness of opioid treatment for pain (60).

8. THE GENOMICS OF OPIOID DEPENDENCE IN ADULTS

Before focusing on the genomics of NAS, we will first address the question of whether there is a heritable component to opioid-dependence in adults (61). An affirmative answer here would lend credence to the notion that genomics could be at play in NAS.

8.1. Classic studies in adults strongly support a genetic component to OUD

Classical genetic studies using a USA Vietnam-Era Twin (VET) Registry cohort, composed of about 7000 identical and fraternal male twin pairs, found that about 31-34% of substance dependency (in general) could be attributed to genetic factors with variation depending upon the particular substance (62). Heroin, an opioid, showed the highest (54%) genetic contribution. In contrast, a study with 1198 white male-male twins by Kendler *et al.* showed that opioid heavy use, abuse, and dependence had a heritability ranging from 60% to 80% (63). A separate study by Kendler *et al.* (64) for a female twin population gave similar results, i.e., a heritability of 52% for opiate use. Collectively, these results suggest that OUD has a significant heritable component, but these studies did not identify the potential genes involved. To make more definitive genetic conclusions requires the acquisition of DNA samples followed by single polynucleotides polymorphism (SNP) association studies and/or genome-wide association studies (GWAS), as described below.

8.2. Allelic variation in cytochrome P450 can affect opioid metabolism

Hydrocodone, codeine, oxycodone, and propoxyphene are among the most often prescribed pain-relieving opioids used during pregnancy (www.asahq.org/about-asa/newsroom/news-releases/2014/02/opioids-in-pregnancy). Pharmacogenetic factors influencing the level of free opioids and their bioactive metabolites are likely to influence the incidence and severity of NAS (65). Agarwal *et al.* (66) have written an excellent review on opioid pain management and pharmacogenetics in adults. Genetic variants in cytochrome P450 (CYP) are a well-documented source of variability in drug responses and pharmacokinetics (67, 68). Hepatic CYP enzymes oxidize a wide variety of drugs and thereby facilitate their detoxification and elimination. However, some prodrugs, such as codeine, are bioactivated by CYPs. As shown in Figure 3, inactive codeine is converted to morphine by the action of CYP2D6, which is also involved in the metabolism of most opioids. There are at least 80 genetic variants (alleles) of CYP2D6 including variants that are “poor metabolizers” as well as variants that are “rapid metabolizers.” It is also possible for an individual to be an “ultra-rapid metabolizer” by having multiple copies of the wild-type CYP2D6. For an individual with the ultra-rapid metabolizer phenotype, a normal dose of codeine can result in enhanced sedation and an increased risk of OUD (www.ncbi.nlm.nih.gov/books/NBK100662/). In patients taking codeine, the area-under-the-curve for plasma morphine can vary by 30-fold between ultra-rapid and poor metabolizers (69).

Recent exploratory work by Dickerson *et al.* (70) suggest that SNPs in cytochrome P450s could be important factors in predicting NAS severity due to buprenorphine. It is known, for example that cytochrome P450 3A (CYP3A) converts buprenorphine into its active metabolite, norbuprenorphine. Using a relatively small study population (N=14), these investigators found that the concentration of buprenorphine in cord blood (more on this below) was associated with SNP rs3745274 ($p = 0.003$) in cytochrome P450 2B6 (CYP2B6). Moreover, statistically significant correlations were found between SNPs (rs2273697, rs9282861, and rs3745274) in uridine diphosphate-glucuronosyltransferase 1A1 (UGT-1A1) and buprenorphine-glucuronide: UGT1A1 converts buprenorphine into buprenorphine-glucuronide which is an inactive metabolite.

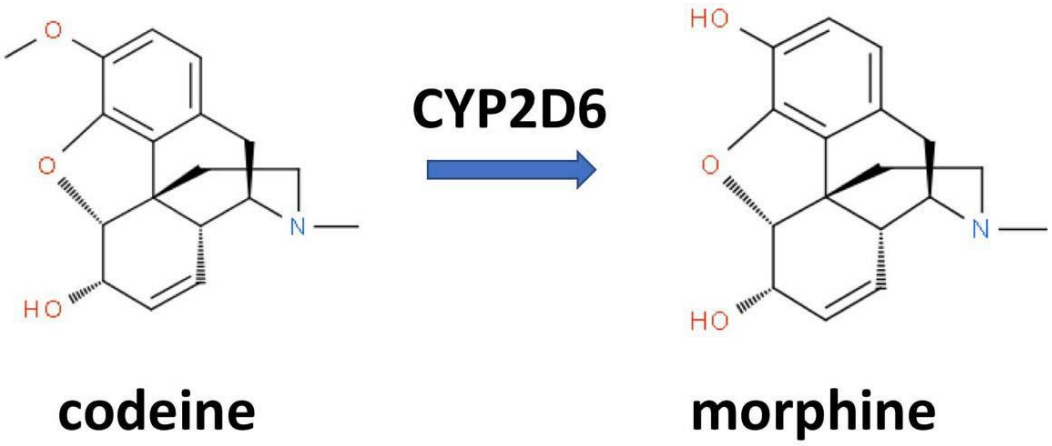

Figure 3. Codeine is prodrug that is bioactivated by cytochrome P450 2D6 (CYP2DG) to morphine. The primary analgesic effect of codeine is due to its conversion to morphine.

8.3. Breastfeeding mothers, CYP2D6 and NAS risk

In addition to codeine use during pregnancy, it has been estimated (in 2009) that about 40% of breastfeeding mothers may be prescribed codeine for pain associated with childbirth (71). An amazingly consistent finding in many clinical studies is the association of breastfeeding with a decreased NAS severity (72). It is important, therefore, to ascertain if there any circumstances in which codeine in human milk might exacerbate NAS severity. Madadi *et al.* (71) found that breastfeeding mothers who were CYP2D6 ultra-metabolizers had infants with CNS depressive symptoms. In 2018 the FDA issued a warning against the use of codeine in breastfeeding women (see www.fda.gov/-Drugs/DrugSafety/ucm549679.htm). Despite its clear relevance, there is very little information on CYP2D6 SNPs and the risk or incidence of NAS.

8.4. Single polynucleotides polymorphisms (SNPs) in the mu-opioid receptor (OPRM1 gene) can affect adult OUD

SNPs are a common type of genetic variation due to single nucleotide changes in a DNA sequence. SNPs occur at a frequency of about one out of every 300 nucleotides, and most have no health-related significance. SNPs in the gene region between the start and stop sites (i.e., the gene body), or the promoter region, can have functional significance. Many of the key molecular players described above are prime “candidate genes” for SNP -OUD/NAS association studies.

Both the *in vitro* and *in vivo* research on the opioid receptors presented above suggest that genetic variants in these receptors, particularly the mu-receptor, could modulate the behavioral effects observed in NAS and drug addiction in general. Support for these suggestions has recently been reviewed by the National Institute on Drug Abuse (73). Clarke *et al.* (74) sought to determine if two SNPs in the gene encoding OPRM1 (rs62638690 and rs17174801) would have a different frequency in a population with opioid and/or cocaine dependence compared to the general population, i.e., a case-controlled SNP association study design. Protective variants of OPRM1 are expected to be less prevalent in the opioid-dependent population. This study found a positive association between SNP rs62638690 and a decreased risk of opioid/cocaine-dependence in a European-American (EU) population, but not in an African American (AA) population. SNP rs62638690 occurs in a coding region of the OPRM1 and results in phenylalanine replacing a cysteine, which can decrease the sensitivity of OPRM1 to some opioids (75). Although the population size was adequate in the Clarke *et al.* (74) paper, the interpretation is complicated due to the investigators grouping heroin- and cocaine-dependent subjects into one “addicted”

population. Nevertheless, the finding that genetic variants of the mu-opioid receptor can affect the risk of opioid use disorder confirms the central role of this receptor. As detailed below, there is also evidence suggesting that genetic variants of OPRM1 are relevant to NAS.

8.5. Genome-wide association study (GWAS) of OUD in adults

The availability of inexpensive SNP genotyping has rapidly advanced the promise of using large-scale genomic data to help individualize medical treatment and help predict an individual's disease susceptibility. Genome-wide association studies (GWAS)(ghr.nlm.nih.gov/primer/genomic-research/gwastudies) utilize large-scale SNP genotyping data that span the entire genome and typically measure about 0.6 to 1.0 million SNPs per individual. Unlike the SNP-association studies mentioned above, GWAS do not target a particular small set of etiologically relevant SNPs but look at a very large SNP array with the goal of finding a small subset of SNPs providing molecular insights and/or clinically relevant information. The cost of SNP genotyping is in the $100-$200 range per sample.

Gelernter *et al.* (76) conducted a large scale GWAS study of opioid dependence (and/or other substance dependence) in both AA and EU adults diagnosed with OUD, and controls. In the AA subgroup, SNPs in both the potassium voltage-gated channel modifier subfamily G member 2 (KCNG2) and the potassium voltage-gated channel subfamily C member 1 (KCNC1) were found to be significantly associated with OUD. The KCNC1 protein is a member of a family of transmembrane proteins that modulate voltage-dependent potassium channels important in the rapid repolarization of fast-firing brain neurons. The KCNG2 protein is a gamma subunit of the voltage-gated potassium channel and is important in regulating neurotransmitter release.

9. THE GENOMICS OF NAS

Both SNP association studies or GWAS with NAS are complicated by the fact that genetic variants in both the maternal and the infant DNA could be important. The placenta is made up primarily of cells with fetal DNA, with a small contribution of maternal DNA from the decidual cells, which come from the lining of the uterus. An optimal experimental design would utilize DNA from mother-infant dyads.

9.1. Single polynucleotides polymorphisms (SNPs) and NAS severity

A very relevant study by Wachman *et al.* (77) in infants with NAS looked at the association of SNPs in OPRM1 and COMT with hospital LOS and the requirement for NAS treatments. In this multisite study, infants were eligible if they were exposed to maternal methadone or buprenorphine *in utero* for 30 days or longer. DNA obtained from 86 mother-infant dyads were genotyped for a small set of SNPs in relevant candidate genes. Infants with OPRM1 SNP rs1799971 G allele were found to require less medical treatment and also had a shorter LOS (17 days vs. 24 days). Similar results were found for NAS infants with COMT SNP rs4680 G allele, i.e., they required less medical treatment and had a shorter LOS. Nevertheless, these two SNPs explain only about 6% of the variability in LOS (51). After adjustments for infant OPRM1 genotype and breastfeeding, there was no association between maternal SNPs with NAS outcomes. These investigators also found a very robust association between breastfeeding and a decreased LOS.

Wachman *et al.* (51) extended their studies attempting to link relevant SNPs to NAS severity. They used the same infant and maternal DNA samples collected in the 2013 study but with a more extensive microarray looking at 80 SNPs located in 14 genes, including the prepronociceptin gene (PNOC), the opioid receptor kappa 1 gene (OPRK1), the opioid receptor delta 1 (OPRD1), OPRM1, and the COMT gene (and others). PNOC is an opioid neuropeptide precursor that is cleaved into nociception which is a ligand for the opioid receptor-like receptor OPRL1. In this study, infants with the PNOC SNP rs732636 A allele and/or the OPRM1 SNP rs702764 C allele had a more severe NAS outcome. For the mothers, OPRM1 SNP rs1799971 G allele was associated with a shorter LOS, while OPRD1 SNP rs204076 A allele was associated with a longer LOS. With the additional SNPs used in this study, Wachman *et al.* (51) were able to explain 15%

of the LOS variability. A follow-up study in a new independent cohort of 133 mother-infant dyads was conducted that focused only on SNPs in PNOC and COMT. In this new study, NAS infants whose mothers had the COMT rs4680 G allele were less likely to need treatment with two NAS medications (78). Using a combined cohort (2017 study plus 2015 study), Wachman *et al.* (78) found that infants with the PNOC rs47332636 A allele showed a decreased need for NAS medication, whereas infants whose mothers had the PNOC rs351776 allele showed an increased need for NAS medications and a longer LOS. Moreover, infants with mothers having the COMT rs740603 were treated less often with any medications. The pioneering studies by the Wachman group strongly suggest that SNP genotyping in mother-infant dyads could be useful in both identifying infants with severe NAS risk and for establishing individualized treatment regimens.

10. EPIGENETICS PLAYS A ROLE IN ADULT OUD

Genomics is the most static of the "omics" and, from the above discussion we know that OUD involves dynamic molecular and neurocircuitry processes. Epigenetics involves dynamic changes to DNA transcription not based on alterations in nucleotide sequence (79). The three major mechanisms for epigenetic regulation, i.e., DNA methylation, chromatin remodeling through histone modification, and microRNA are all at play in the dynamic regulation of the opioid system (80) and therefore relevant to NAS.

Direct evidence for the role of epigenetics in modulating opioid responses in humans has recently been published (81). These investigators studied gene dysregulation in postmortem human brain striatum biopsies from long-term heroin users. They found that heroin use produced impairments in the glutamatergic neurotransmission that were linked to excessive acetylation of lysine residues in histone protein H3 (one of five main histone proteins important in determining chromatin structure). Acetylation of histone proteins reduces their positive charge, thereby reducing their interaction with DNA, generally unblocking transcription by RNA polymerase II and enhancing transcription. The expression of glutamate ionotropic receptor AMPA type subunit 1 gene (GRIA1) was found to be modulated by histone acetylation, and this gene is known to play a role in drug use disorders (81). Using a rat heroin self-administration model, these investigators found that a histone acetylation inhibitor drug, JQ1, could reduce heroin self-administration as well as cue-induced drug seeking behavior. Although JQ1 is now in phase 1 cancer trials, there are no ongoing trials for NAS or OUD. The use of JQ1 in an animal model of NAS would be an important first step. Moreover, there is little information of any kind on the potential role of histone acetylation in NAS.

11. DNA METHYLATION IS AN IMPORTANT EPIGENETIC FACTOR INFLUENCING NAS SEVERITY

In pioneering work, Wachman *et al.* (82) found that DNA methylation in the promoter region of the mu-opioid receptor gene was an important epigenetic factor influencing NAS severity in infants exposed to methadone or buprenorphine. DNA methylation of cytosine nucleotides in a promotor generally blocks gene transcription. Wachman *et al.* (82) found that increased methylation in the mu-opioid receptor gene was associated with more serve NAS outcomes. Recent work with adult men having OUD also shows elevated DNA methylation in the mu-opioid promotor region (83).

12. PROTEOMICS AND NAS

Proteomics has been successfully applied to understanding the systems biology of alterations occurring in synaptic proteins as a result of morphine exposure (84). This research is almost exclusively in animal models. The potential of clinical proteomics in the area of pediatrics/neonatology has been recognized for almost a decade (85-87)}(85). Many diagnostic tests for neonatal diseases lack sensitivity and specificity and often rely on a morphological description of the damaged organ. In the case of NAS, the diagnostic criteria can be very dependent upon a constellation of nonspecific signs. Since proteins are the "nanomachines" performing most bodily functions, most disease states will be accompanied by alterations in the expression and post-translational modifications of proteins.

Proteomics holds the potential for discovering protein biomarkers for diagnosing NAS with high sensitivity and specificity, and for stratifying NAS severity (88). Moreover, once identified, protein biomarkers can provide insights into molecular mechanisms and novel therapies. Despite its potential, very little research has focused on the clinical proteomics of NAS.

While it would be optimal to perform proteomics analyses on a tissue or biofluid directly relevant to NAS, this is not a practical approach for NAS were the CNS is a key opioid target. A systemic biofluid such as plasma from a cord and/or maternal blood sample is a viable choice. Newborn urine samples are an excellent choice since they can be serially and non-invasively obtained from newborns and are initially sterile. The methodology for subjecting very small urine samples to high throughput proteomics has been well characterized (89).

13. METABOLOMICS AND NAS, AN INFORMATION GAP

In some respects, the prenatal and neonatal toxicology of opioids is a subset of metabolomics. While “opioid toxicology” focuses on opioid xenobiotics and their metabolites, “opioid metabolomics” broadens this focuses by including the set of all metabolites altered by opioids. As detailed above, NAS is very often entangled with maternal polydrug exposure and other maternal environmental factors, e.g., poor nutrition. Ideally, it would be optimal to quantify polydrug levels, their metabolites as well as the levels of all other relevant pain-modulating metabolites: this is the promise of metabolomics.

The exposome and exposomics are rapidly emerging concepts in systems medicine that are highly relevant to NAS (www.cdc.gov/niosh/topics-/exposome/default.html). As initially conceived, the exposome includes the measurement of all internal and external health-related exposures experienced by an individual. The exposome, for example, would include prenatal exposures, such as maternal polydrug use as well as postnatal exposures, such as opioid-replacement therapy. The “internal” exposome can be assessed by the omic technologies discussed above, i.e., genomics, epigenomics, proteomics, and metabolomics. Of these omics, it has been suggested that metabolomics should be the main emphasis of exposomics. A GC-TOF-MS metabolomic assay (with urine or plasma) covering exposome-type compounds along with over 200 metabolites now costs less than $100 (see metabolomics.ucdavis.edu/core-services/metabolomics-central-service-core). Ghanbari and Sumner (90) have written an excellent review on the power of metabolomics for investigating biomarker discovery in drug addiction. This review covers many “proof of concept” studies using well-controlled animal models. Unfortunately, metabolomics has not yet been applied to NAS studies.

Urine, as detailed above, is a very practical systemic body fluid for NAS proteomic studies and the same logic applies to metabolomic NAS studies. Moreover, since NMR spectroscopy is a non-destructive metabolomic assay, the same urine sample can be used for proteomic analysis. The human urine metabolome is amazingly well-characterized (91) and 2402 unique chemical species can be quantified by using multiple analytical platforms (www.hmdb.ca/). NMR alone can quantify at least 85 different chemical species.

14. SUMMARY AND PERSPECTIVE

A systems medicine approach to NAS holds much promise, and the available omics data strongly supports a continued emphasis on this integrative approach. Maternal and neonatal urine samples are particularly well-suited for proteomic and metabolomic analyses. During the biomarker discovery phase of a multi-omic NAS study, it is likely that the total cost for whole genome SNP (maternal and newborn) genotyping, DNA methylation analysis (maternal and newborn), urine proteomic and metabolomic analyses would be less than the cost of one day in a neonatal intensive care unit (about $3000). After the discovery and validation phases, the cost of testing a select set of multi-omic parameters should be considerably reduced. The ideal set(s) of multi-omic parameters should optimize NAS diagnosis, risk stratification, prognosis, reduce overall cost, and provide individualized treatment guidance.

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Abbreviations: AA, African-American; CNS, central nervous system; COMT, catechol-O-methyltransferase; CYP, cytochrome; ESC, "Eat, Sleep, Console"; EU, European American; GC-TOF-MS, gas-chromatography-mass spectrometry; GDP, guanosine diphosphate;

GIRK, G-protein-gated inwardly rectifying potassium channel; GPCR, G-protein-coupled receptor; GRIA1, glutamate ionotropic receptor AMPA type subunit 1; GTP, guanosine triphosphate; GWAS, genome-wide association study; KCNC1, potassium voltage-gated channel subfamily C member 1; KCNG2, potassium voltage-gated channel modifier subfamily G member 2; LBW, low birth weight; LC-MS, liquid chromatography-mass spectrometry; LOS, length of stay; MOR, mu-opioid receptor; MRI, nuclear magnetic resonance imaging; MS, mass spectrometry; NAABT, National Alliance of Advocates for Buprenorphine Treatment; NAcc, nucleus accumbens brain region; NAS, neonatal abstinence syndrome; NICU, neonatal intensive care unit; OPRM1 , opioid receptor mu 1; OPRD1, opioid receptor delta 1 gene; OPRK1, opioid receptor kappa 1 gene; ORL1, opioid-like-subtype-1; OUD, opioid use disorder; PNOC, prepronociceptin gene; SNP, single polynucleotides polymorphism; SSRI, serotonin reuptake inhibitor; UGT1A1, uridine diphosphate-glucuronosyltransferase 1A1; VET, USA Vietnam-Era Twin; VTA, ventral tegmental area

Key Words: Neonatal Abstinence Syndrome, Systems Medicine, Neonatology, Genomics, Metabolomics, Proteomics, Single Nucleotide Polymorphism, Opioids, Newborns, Opioid Use Disorder, Pregnancy, Review

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