Published in final edited form as:
J Perinatol. 2014 March ; 34(3): 169–173. doi:10.1038/jp.2013.170.

Great Expectorations: The Potential of Salivary "Omic" Approaches in Neonatal Intensive Care

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Abstract

Among those that require critical care, preterm neonates have the greatest limitations on available blood or body fluids for clinical or research-based assessments. Recent technological advancements have improved our ability to detect genetic, proteomic and microbial material at the nanoscale level, making analyte and biomarker assessment from even the smallest quantities possible. Saliva is a unique body fluid that not only may be noninvasively and repeatedly obtained, but also contains multiple serum components making it promising for noninvasive assessment of the newborn. The integration of high-throughput or 'omic' approaches on neonatal saliva holds great potential to improve diagnostic and prognostic accuracy for a wide range of developmental and pathological conditions affecting the vulnerable preterm neonatal population. Herein, we review the clinical applications and technical considerations regarding the integration of salivary 'omic' technology into the neonatal intensive care unit (NICU).

Search terms

Saliva; proteome; microbiome; genome; transcriptome; neonate

Introduction

Critically ill preterm neonates require frequent monitoring of the adequacy of ventilation and oxygenation, electrolytes, nutritional status, and infection risk. Blood sampling for their clinical assessment is paralleled with developmentally unique and sometimes severe procedural risks including apnea, bradycardia, and in severe cases, intraventricular hemorrhage. Combined with their small size and limited blood volumes, the extent of diagnostic, prognostic, and research assays that can be performed on the premature newborn is limited. While some adjunctive lab barriers can be circumvented through the application of microscale assays, frequent assessments or large-volume tests (e.g. coagulation studies or karyotyping) may significantly increase the need for subsequent blood transfusion. Saliva is a plentiful biofluid that is easier to obtain than blood without associated untoward side effects even in the most immature preterm infants[1]. Thus, neonatologists have long championed the use of saliva as a noninvasive biofluid that could be utilized to monitor the clinical status of their vulnerable patients[2].

Historically, neonatal salivary assays have been limited to single proteins or microbes[3,4]. Recently, technological advances have permitted the high-throughput analysis of saliva for

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All authors declare no conflict of interest. This work is not and has not been under consideration elsewhere.

thousands of genes, proteins, and metabolites from a single sample source[5–7]. Further, analysis and characterization of the salivary microbiome holds the unique potential for correlating the presence of pathological organisms and aberrant colonization patterns with disease[8–10]. Thus, saliva may enhance neonatal diagnostic and prognostic testing where blood-based assays might not reasonably be performed. In this review, we will highlight the current applications of salivary transcriptomic, proteomic and microbiomic analyses in the newborn and review the clinical applications and technical considerations regarding the integration of salivary 'omic' technology into the neonatal intensive care unit (NICU).

Saliva as a Biofluid

Saliva is composed of water, electrolytes, hormones, microorganisms, mucins, enzymes, proteins, immunoglobulins, and the nucleic acids, DNA and RNA[11]. In addition, toxins, drugs and metals are readily detectable[12–14]. Saliva is produced in the three major salivary glands, the parotid, submandibular and sublingual, as well as minor salivary glands found in the lower lip, tongue, palate, cheeks and pharynx. Salivary components arise through a combination of ultrafiltration, active transport, and diffusion within each salivary gland[15]. The distinction between major and minor salivary glands is based upon anatomical size and not salivary production[16]. All glands play a distinct role in salivary composition. The parotid produces a serous secretion, the minor glands generate mucous secretions, and a mixed product arises from the submandibular and sublingual glands. Combined, these fluids allow for appropriate digestion, antimicrobial protection and overall oral health[17]. Although beyond the scope of our review, salivary gland ontogeny, the role saliva plays in digestion and host immune defense, and the neuroendocrine regulation of inflammation by salivary polypeptides are central concepts and have been reviewed in excellent detail[18–22].

Collection, Storage and Technical Considerations

There are unique considerations for salivary analyses in the premature neonate. For example, the effect of the maturing salivary glands on the constituents in saliva in the preterm newborn remains largely unknown. Additionally, the effects of salivary flow rate and hydration status on salivary components, while well studied in adult populations, are poorly studied in the newborn and may ultimately affect target detection[23]. Although these knowledge gaps may not ultimately influence research findings, they should be considered when designing a clinical study.

Applying 'omic' technologies to neonatal salivary samples has challenges. Although salivary acquisition is benign, sufficient volume collection may limit down–stream analyses. Commercially available collection kits for transcriptomic and proteomic analyses, such as Qiagen™ RNAprotect Saliva Reagent or DNAGenotek™, recommend a minimum of 200 μL up to 2 mL of free flowing saliva, respectively, for proper analyses. This volume is difficult to obtain, not only in premature infants, but also in term newborns reaching the nadir of their post-natal diuresis. However, these technical challenges can be overcome as good quality and sufficient quantity of total RNA is available from as little as 5 μL of neonatal saliva[24]. Additionally, a recent report has shown that acquisition and processing protocols can be modified to address the unique challenges of salivary analysis in the newborn[24]. As newly emerging salivary collection systems, such as sponges and absorbent filter papers enter the market, investigators will have more options to consider for neonatal salivary collection and should not be deterred by either small sample size or modified use of commercially available extraction protocols.

Proper and timely sample processing of saliva may be essential for reliable downstream analysis. Prognostic and diagnostic sensitivity and specificity may be altered by salivary mucins (forms complexes with other proteins), the method of saliva processing (alter the

original proteomic composition)[25], or gender[26]. However, the salivary proteome has been well-studied in adults[23] and much of the challenges associated with its use as a diagnostic fluid are being addressed. For example, saliva proteins often have extensive post-translational modifications[27], which are genetically determined, and may be related to microbial colonization[23]. The single-stranded structure of RNA makes it inherently unstable and subject to rapid degradation from ubiquitous nucleases found in the oral cavity. For transcriptomic analysis, samples must be processed immediately upon collection in order to halt gene expression changes and inhibit RNases. Commercially available stabilizing agents (e.g. RNAprotect Saliva Reagent) have eliminated these concerns and most are stable at room temperature for an extended period of time, making them ideal for multicenter trials and even international studies. Conversely, salivary proteins are relatively more stable compared to gene transcripts[28]. Although, proline rich proteins and salivary histatins are known to be more vulnerable to degradation compared to salivary mucins and other highly glycosylated proteins[29]. Of note, saliva is a mixed flora source composed of both eukaryotic and prokaryotic genetic material. This heterogeneity makes saliva ideal for microbiome analyses, but technically challenging when analyzing at the level of transcriptome or proteome. Spielman *et al*., recently showed that the presence of bacteria interfered with RNA sequencing of adult salivary samples[30]. However, minimizing the contribution of prokaryotic genetic material through the use of the salivary supernatant improved yield on the RNASeq platform.

Salivary Transcriptomics: *Overview*

The transcriptome provides insight into the function and development of a cell, tissue, or organism[31]. While the genome remains constant in each nucleated cell, the transcriptome is dynamic, rapidly changing in response to internal and external stimuli, drug therapy and the environment. Thus, gene expression changes represent the first opportunity to analyze cellular responses at the molecular level. However, unlike the proteome that can have post-translational modifications and DNA-protein and protein-protein interactions, the transcriptome is more streamlined, serving as the bridge between the genetic code and the complexities of the proteome[31]. Further, when one considers the diagnostic potential of non-coding RNAs, the transcriptome can be viewed not only as the intermediary between genome and proteome, but also as a means to identify regulatory functions in the setting of health and disease. In the premature newborn, whose normal fetal development has been disrupted by early birth, relating aberrant gene expression changes to unique morbidities and phenotypes could prove to be a powerful and novel diagnostic tool for the development of new or personalized treatment strategies.

In recent years, detection of salivary mRNAs, exosomal RNAs, and non-coding microRNAs have been reported[32–35]. The biological source of these salivary transcripts is heterogeneous. Cellular mRNA is often derived from the buccal epithelium cells, while cell-free mRNA is derived from multiple systemic sources following hematological filtration through the salivary glands[15]. Dietz *et al*., recently compared the gene expression profiles of neonatal cellular whole saliva and cell-free salivary supernatant from the same newborns[24]. Results of this study showed a 92.5% concordance between gene expression profiles in both whole saliva and the cell-free salivary supernatant. These findings suggest that the contribution of genes originating from the buccal epithelium is only a small fraction of the overall salivary gene expression profile in the newborn and that saliva serves as a noninvasive biofluid capable of informing the clinician about systemic disease. This research is supported by comparative expression analyses between blood and saliva. Adult research has shown a 28% concordance between proteins found in saliva and plasma[36]. Similarly, there is a 38% concordance of gene transcripts detectable in whole saliva and whole blood in the

newborn[37]. Thus, saliva truly is a 'window into the body' and an ideal body fluid for analysis in the neonate.

Salivary Transcriptomics: *Applications* (table 1)

In 2010, Maron *et al.* were the first to describe the enormous amount of real-time developmental information available from the neonatal salivary transcriptome[38]. By performing whole transcriptome microarray analyses on saliva samples collected serially from preterm infants from birth to discharge, the authors showed that developmental information from nearly all organ systems were readily detectable in as little as 50 μL of saliva. Notably, as subjects matured in the NICU and progressed from nasogastric to full oral feeds, their salivary transcriptomes reflected a more mature oral feeding pattern. There were significant gene expression changes related to neurodevelopment, cranial nerve maturation, sensory integration and hypothalamic regulation of feeding behavior as infants achieved full oral feeds[39]. These findings suggested that the neonatal salivary transcriptome could serve as an objective indicator of readiness to orally feed in the newborn. Importantly, this proof-of-principle study laid the foundation for the assessment of other neonatal developmental milestones and/or morbidities through salivary transcriptomic analyses of well-designed cohort studies.

While broad-based 'omic' approaches are a necessary first-step in the identification of informative biomarkers, honing in on specific transcripts as they correlate to disease or development is required for their application and integration into clinical care. Bedside point-of-care (POC) diagnostic platforms are rapidly emerging for the detection and quantification of select nucleic acids associated with specific disease[5–7,40]. Adult salivary transcriptomic diagnostic platforms have been reported for oral[41], lung[42], pancreatic[43], breast[44] and ovarian cancers[45]. These platforms are composed of a discrete number of genes for rapid diagnosis or disease screening. While the challenge of identifying a signature nucleic acid platform for neonatal assessment may seem daunting, advances in bioinformatics is easing the road from high-throughput screening to high-yield gene target(s)[46]. For example, following identification through gene expression microarray analyses, the salivary biomarker neuropeptide Y2 receptor, (*NPY2R*), has been recently shown to have a 95% positive predictive value for an immature oral feeding pattern in the newborn[47]. Thus, utilizing emerging technologies with targeted bioinformatics may allow for the development of POC assays to inform personal care plans while improving clinical care and outcomes in the premature neonatal population.

Salivary Proteomics: Overview

Using the search terms "saliva" and "proteome" on PubMed returned over 50 manuscripts published since April 2012. The majority of these investigations have largely been pioneered in adults and include diagnosis of periodontal disease, dental caries, cancer, autoimmune disease, diabetes, as well as diagnosis of viral (including HHV/HIV) and bacterial infections[48,49]. Rapid oral fluid-based assays of HIV status, presence of drugs of abuse, hormone and growth factors are currently available. Multiple POC salivary applications using lateral flow, microbead-based, or nucleic acid detection assays are in development[5].

Although most of the presently available salivary assays target clinical conditions that have little applicability in neonatology, the same scientific methodology for salivary analysis can be applied to develop relevant assays for neonatal-specific disease states. These assays will likely exploit the unique proteome of preterm newborn saliva as compared to adults[50–52]. One clinically significant potential application of neonatal salivary proteomics is improving the poor diagnostic accuracy for infection.

Salivary Proteomics: *Applications* (table 1)

Early identification of potentially infected neonates has remained largely elusive despite multiple attempts to identify highly sensitive and specific biomarkers[53]. Many biomarkers tested are found in saliva including complement fragments (C3, C4), cytokines [TNF-α, interleukin (IL)-1 alpha/beta, IL-2, IL-6, IL-8], MMPs 1–3, 9, multiple antimicrobial proteins/peptides (histatin, lactoferrin, alpha and beta-defensins, cathelicidin, S100 proteins), acute phase reactants (C-reactive protein, haptoglobin, transferrin, fibronectin) and immunoglobulins (IgG, IgE, and IgM)[49,50]. The presence or abundance of these and yet undiscovered salivary proteins may yield important diagnostic or prognostic utility regarding infection development and or progression.

Another application of salivary proteomics may be to more accurately inform clinicians about developmental stage. Developmental stage drives multiple facets of neonatal intensive care including parent counseling of potential prognosis and outcomes, initiation of oral feeding, and the timing of interventions/assessments. Changes in the salivary proteome are present at distinct developmental stages and thus may enhance our understanding of developmental biology[54] and improve counseling accuracy regarding short and long-term risks associated with developmental age. Other potential applications include measurement of salivary hormones in the determination of growth[55] and the presence/extent of stress responses[56].

The Salivary Microbiome: Overview

The oral cavity, with its continuous environmental exposure, is a diverse assemblage of microorganisms (e.g. 700 species) and acts as a portal for commensal and pathogenic bacteria to gain access into both the respiratory and digestive tracts[57]. The many niches within the mouth (e.g., tongue, supra- and subgingival plaques, oral mucosa) harbor distinct bacterial communities[58]. However, saliva is considered to be an adequate proxy for the oral cavity's microbiota since it contains a fraction of microbes from each of these surfaces, as microbes colonizing oral surfaces routinely slough off the mucosal epithelium and accumulate within saliva[59]. Additionally, the relative stability of bacteria in saliva, despite constant exposure to a matrix of antimicrobial and bacterial-promoting agents, makes saliva a suitable surrogate[60]. In most individuals, oral homeostasis is achieved between the mucosal surfaces and the microbiota within this blend of antimicrobial peptides and bacteria, comparable to the homeostasis that exists at the interface between the intestinal epithelium and the gut microbiota[61].

The Salivary Microbiome: Applications (table 1)

The microbial composition of saliva has been studied for decades, perhaps most exhaustively in its role in the pathogenesis of dental caries[62] and periodontitis[63,64]. However, the advent of culture-independent techniques has generated a more comprehensive approach to identifying these organisms, the time frame in which they colonize the mouth, and their communication with the host's immune system[57,65]. Large scale initiatives applying these techniques to the oral microbiome have been incorporated into the NIH sponsored Human Microbiome Project in adult cohorts[66]. However, the incipient bacterial communities detected through neonatal saliva samples may be the earliest window into understanding microbial promoters of health. Conversely, infectious risks factors responsible for a myriad of childhood respiratory and gastrointestinal diseases are likely introduced during the early establishment of the oral microbiome.

Though the intra-uterine environment and neonatal intestinal tract at birth have historically been considered sterile, more recent studies, including those that incorporate non-culture

based techniques, have detected microbial DNA in amniotic fluid and meconium suggestive of a microbial colonization process that is initiated in utero[67,68]. This early microbial colonization continues during delivery itself and in the immediate postpartum period as feeds are initiated. Based on oral mucosa swabs collected from newborns within seconds of delivery and then compared with maternal samples, differences in microbial composition quickly emerge based on mode of delivery. Infants born vaginally have bacterial flora in their oral mucosa resembling maternal vaginal communities. Those born via Caesarean section, without the bolus of bacteria provided via passage through the vaginal canal, have oral cavity bacterial profiles comparable to those of maternal skin[69]. Additionally, aberrant bacterial colonization of the oral mucosa secondary to Caesarean delivery have been linked with permanent changes to the intestinal microbiota[70]. These changes to the intestinal microbiota, seeded by the altered flora of the oral cavity, have been linked with infant health outcomes, including atopy and body mass indices (BMI)[71–73].

Several routinely administered maternal antenatal therapies, including systemic steroids and antibiotics, have been shown to impact the oral microbiota acquired by newborn infants. Total bacterial density was markedly increased in infants exposed to either antenatal steroids or steroids and antibiotics, while those exposed to antenatal antibiotics alone had a reduction in bacterial density[74]. Alterations in the early oral microbiome likely impact subsequent mucosal immune development. Surveillance of fecal microbial composition and salivary IgA content during the newborn period (1 week to 5 years of age) shows enhancement of the mucosal salivary IgA system in those infants with an early, intense colonization by *Bacteroides fragilis*[75].

Few prospective studies explore the evolution of the neonatal salivary microbiome and subsequent acquisition of normal and aberrant gastrointestinal and respiratory floras. Prospective monitoring of the salivary microbiome of adolescent monozygotic and dizygotic twins over a 10 year period demonstrate how environmental factors, including household residence and caretakers, have a greater influence on microbial composition in these twin pairs than genetics[76]. No study has examined the long-term impact of neonatal hospitalization on the evolution of the oral microbiome and development of mucosal immune function. The cadre of factors affecting bacterial colonization of the oral cavity- including prolonged exposure to a hospital environment, nasogastric and endotracheal tubes, and caretakers outside of parents- could have permanent and seemingly detrimental effects on the oral microbiome.

Conclusions

Whether on a transcriptomic, proteomic, or microbiomic platform, noninvasive salivary ‘omic’ approaches to clinical care and assessment offer a window into neonatal development and pathophysiology not previously realized with traditional blood based assays. Continued development of tools to make data accessible and integration possible for researchers and clinicians alike will be needed to make the transition to bridge the gaps between results and translation for clinical use. As technology continues to emerge for rapid assessment of salivary biomarkers and microorganisms, integration of salivary diagnostics into the NICU holds great promise for improved surveillance and assessment of the premature newborn.

Abbreviations

NICU	neonatal intensive care unit
DNA	Deoxyribonucleic acid

RNA	Ribonucleic acid
NPY2R	neuropeptide Y2 receptor
HHV	human herpes virus
HIV	human immunodeficiency virus
TNF	tumor necrosis factor
MMP	matrix metalloproteinase
Ig	immunoglobulin
NIH	National Institutes of Health
BMI	body mass index

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Table 1

Neonatal applications of salivary “omics”

	Potential Applications	**Limitations**
Transcriptomics	• Monitoring of real-time gene expression changes as they relate to disrupted developmental patterns in the premature newborn • Identification of novel salivary biomarkers that correlate with neonatal sequelae (i.e. retinopathy of prematurity, necrotizing enterocolitis, bronchopulmonary dysplasia) • Development of point-of-care diagnostic platforms to alert the caregiver to infants at risk for such neonatal outcomes	• RNA quality may be degraded, limiting downstream analyses. • Mixed prokaryotic and eukaryotic source in saliva samples may interfere with sequencing (RNASeq).
Proteomics	• Early identification of infected neonates • Accurately inform clinicians about developmental stage • Measurement of salivary hormones in the determination of growth and the presence/extent of stress responses	• Proper and timely sample processing of saliva may be essential for reliable downstream analysis. • Sufficient volume collection may limit down–stream analyses.
Microbiomics	• Saliva is a mixed flora source composed of both eukaryotic and prokaryotic genetic material. This heterogeneity makes saliva ideal for microbiome analyses • Bacterial communities detected in neonatal saliva samples may be the earliest window into understanding microbial promoters of health • Alterations in the early oral microbiome likely impact subsequent mucosal immune development	• Neonatal saliva has low bacterial biomass, especially if collected early in life. Application of aggressive extraction protocols, comparable to those used with meconium samples, can optimize isolation of bacteria. • Microbial flora is not uniform throughout the mouth, making careful, consistent collection under sterile conditions critical.