Original Article

Salivary RNA sequencing highlights a sex-specific developmental time course towards oral feeding maturation in the newborn

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Background: Our ability to understand the molecular mechanisms responsible for delayed maturation of oral feeding in the preterm newborn is limited. Our objective was to perform RNA Sequencing (RNASeq) on salivary gene transcripts to advance our understanding of the developmental pathways, associated networks, and sex-specific differences associated with oral feeding success in the newborn.

Methods: This prospective, observational, single-center study was conducted in the Neonatal Intensive Care Unit at Tufts Medical Center (Boston, MA) from 2014 to 2017. Infants ranging from 34 to 39 weeks' post-menstrual age were recruited for this study (n=26). There was equal representation of successful (n=13) and unsuccessful (n=13) oral feeders. Differential gene expression profiles between successful (n=13) and unsuccessful oral feeders (n=13), matched for gestational age, post-menstrual age, and weight, as an entire cohort and separated by sex (males n=12; females n=14) were measured and analyzed.

Results: There was no statistically significant difference in gestational age, birth weight, or post-menstrual age between either successful and unsuccessful oral feeders or males and females enrolled in this study. Sixty-three genes were differentially expressed between the successful (n=13) and unsuccessful oral feeders (n=13), highlighting delayed maturation of the nervous system, tissue morphology, embryonic and hematologic development, and hematopoiesis amongst unsuccessful oral feeders. When separated by sex, females (n=88 genes) and males (n=78 genes) revealed distinct salivary profiles. Unsuccessful male oral feeders were found to have delayed maturation in neurodevelopment, memory and learning pathways, while unsuccessful female oral feeders had delayed maturation of facial, palate, and gastrointestinal development.

Conclusions: RNASeq analysis of genes present in neonatal saliva provides a near real-time window into ongoing development, identifies sex-specific pathways and biological networks associated with oral feeding success, and provides caregivers with important opportunities to personalize care and target treatment strategies based upon an infant's sex and individual gene expression profile.

Keywords: RNASeq; saliva; feeding; premature neonate

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Introduction

Attainment of oral feeding competency is a major determinant of length of stay in the Neonatal Intensive Care Unit (NICU) and represents a developmental challenge for the majority of the 15 million infants born prematurely (<37 weeks' gestational age) worldwide each year (1-3). Inappropriate feeding attempts can lead to acute and long-term morbidities, as well as prolonged hospitalizations

with associated healthcare costs (1-6). Furthermore, infants who fail to successfully orally feed by corrected term gestational age (GA) are at increased risk for developmental delays throughout infancy and childhood (4-6). Despite the prevalence of oral feeding morbidities and their long-term health consequences, our ability to assess oral feeding maturity, and more importantly determine the biological mechanisms limiting oral feeding success, remain a clinical challenge (7-10).

Successful oral feeding is dependent upon the simultaneous maturation and integration of the gut-brain axis, as well as sensorimotor, neurodevelopmental and gastrointestinal systems (3,11). Disruption in any one of these systems may vary among newborns, thus a 'one-size' fits all approach to treatment strategies and assessment tools to improve oral feeding outcomes is not effective. Cochrane Reviews conducted in both 2012 and 2016 confirmed the futility of available feeding assessment tools for use in the newborn, concluding both times that there is currently "no evidence to inform clinical practice" (8,9).

Variation in the maturation of biological systems involved in oral feeding is believed to be affected, in part, by sex and GA (12,13). Males typically learn to orally feed at older post-menstrual ages (PMAs) compared to females (12), and infants born at earlier GAs learn to orally feed at older PMAs compared to infants born later in gestation (13). In order to provide relevant information to caregivers to personalize treatment strategies and improve oral feeding outcomes, assessment tools must be able to simultaneously evaluate the diverse biological systems required for oral feeding competency and report on the real-time developmental status of an individual newborn.

This study utilized RNA sequencing (RNASeq) in order to advance our understanding of delayed developmental pathways limiting feeding success, identify relevant networks associated with oral feeding competency, and explore sex-specific differences involved in oral feeding maturation in the newborn. Sequencing was performed on whole saliva, a rich source of systemic gene expression (14,15), collected from sex- and age-matched infants who could and could not orally feed. Previously, our laboratory used saliva to discern between successful and unsuccessful neonatal oral feeders utilizing both gene expression microarrays (11), as well as high-throughput, multiplex reverse transcription-quantitative polymerase chain reaction (RT-qPCR) platforms (11,16). Here, we hypothesized that the RNASeq platform would provide a more comprehensive and unbiased analysis of neonatal development, as it relates to oral feeding, and significantly improve our clinical approach to oral feeding difficulties in the newborn. We present the following article in accordance with the STROBE reporting checklist (available at https://pm.amegroups.com/article/view/10.21037/pm-21-45/rc).

Methods

Subject selection and recruitment

This prospective, observational, single-center study was conducted from 2014 to 2017 in the Tufts Medical Center NICU with approval by the Tufts Medical Center Institutional Review Board. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from parents of infants ranging from 34 to 39 weeks' PMA. There was equal representation of successful and unsuccessful oral feeders. Race of subjects was defined by the parents and recorded in the medical record. The Tufts Medical Center NICU utilizes the cue-based feeding assessment protocol of Ludwig and Waitzman (17). In accordance with this protocol, no infant less than 32 weeks' PMA is offered oral feeding in the NICU. Infants were considered unsuccessful oral feeders if they took <50% of feeds by mouth (non-feeder); successful oral feeders took 100% of enteral nutrition by mouth and did not have a nasogastric tube in place (feeder) for at least 24 hours. For all demographic data, statistical significance was set at $P<0.05$.

Saliva collection & quality control

Saliva samples were obtained using previously described techniques (18). Briefly, saliva (~10 to 20 µL) was collected with a 1 mL syringe attached to low wall suction, placed immediately in 500 µL of RNAProtect Saliva (QIAGEN) at the bedside, vortexed, put on ice and stored at 4 ℃ for a minimum of 48 hours up to 4 weeks. Saliva was only collected from a single time point for each subject. Samples were collected during the day and prior to a feed to limit the impact of emerging circadian rhythms on gene expression as well as breast milk contamination. Total RNA was extracted with the RNAProtect Saliva Mini Kit (QIAGEN) per manufacturer's instructions. On column DNase treatment was performed for each sample to eliminate DNA contamination. Extracted total RNA was stored at –80 ℃ pending quality assessment analysis.

Prior to RNASeq, the quality and quantity of extracted total RNA was assessed on the Agilent Bioanalyzer 2100. Only those samples that met pre-established criteria designed specifically to target cell-free RNA in saliva were subsequently sequenced. Salivary cell-free RNA, as compared to cellular RNA that is largely derived from epithelial and hematopoietic cells within the oral cavity, is believed to be reflective of the systemic body (19), and thus was the target of this analysis. Quality assessment criteria included: (I) a minimum of 500 ng of total RNA per sample; and (II) a RNA Integrity Number (RIN) between 5 and 8, the expected range for cell-free RNA (20).

RNA sequencing

Samples that met qualitative criteria underwent next generation sequencing at the Tufts University Genomic Core facility on the Illumina HiSeq platform. Library preparation was performed with the Illumina TruSeq Stranded Total RNA kit. Ribosomal depletion occurred with the Ribo-Zero Globin kit. Following library preparation, the libraries were denatured, introduced into the flow cell, and subjected to bridge amplification in order to create clonal clusters of single stranded cDNA molecules (21,22). Libraries were sequenced using Rapid V2 chemistry via paired end 150 base format, targeting a sequencing depth of 25 million paired reads or more per sample.

Data analysis

Raw data were obtained in the form of FASTQ files (23). Bioinformatics analyses were performed on the Tufts University High Performance Cluster (Medford, MA). Sequencing quality was assessed with FASTQC (Babraham Bioinformatics, Babraham Institution, Cambridge, UK). Reads were truncated to remove base positions that scored less than a low median score. A median quality score of <20 was deemed unusable. Tuxedo Tools were used to analyze the RNASeq results (24,25). Briefly, reads were mapped to the UCSC hg19 human genome with Tophat 2/Bowtie 2. Normalization and differential expression analyses were performed with Cuffdiff.

Statistical and computational analyses

Data were analyzed in their entirety based upon feeding status, as well as separated by sex. All analyses were conducted using Qlucore with an adjusted false discovery rate (FDR) P value of 0.05. Genes that were differentially expressed between feeding stages were identified and further explored with the use of Ingenuity® Pathway Analysis (IPA) software (QIAGEN Inc, version: 463341M https://www.qiagenbionformatics.com/products/ingunuity-pathway-analysis) (26). IPA utilizes a non-topological based approach to identify over represented genes in a given pathway and was used to identify developmental pathways that were statistically significantly impacted based on feeding status (27). Qlucore was used to perform principle component and hierarchical cluster analyses and 3D Principal Component Analysis (PCAs) and heat map visualizations (28).

Results

Thirty-two infants were recruited for this study; 26 subjects met RNA sequencing quality criteria and ultimately were sequenced (81% success rate). Subject demographics of all subjects who underwent sequencing, separated by feeding status and sex, are summarized in *Table 1*. Mean RNA sequencing read alignment rates averaged 35% (Table S1). The inclusive analysis of all subjects identified 63 genes that were differentially expressed between the successful (n=13) and unsuccessful oral feeders (n=13). When separated by sex, 88 differentially expressed genes were identified among the female cohorts (n=14), 14 of which overlapped with the original 63 genes identified in the total cohort. Comparatively, 78 differentially expressed genes were identified among the male cohort (n=12), six of which overlapped with the original 63 genes. No overlap of differentially expressed genes was observed between females and males (*Figure 1*). Differentially expressed genes identified via all three evaluations are presented in *Figure 2*, while chromosomal location of each gene is provided in Table S2. Ten differentially expressed genes were located on the X chromosome; no genes were located on the Y chromosome. PCAs displayed distinct clustering of successful feeders *vs.* unsuccessful feeders (*Figure 3*), with corresponding heat map analyses and gene lists (*Figure 4A-4C*). The color-coded heat maps depict genes that were up-regulated (yellow) or down-regulated (blue) between unsuccessful and successful oral feeders. Of all three dataset comparisons (total, females, and males), the male subgroup showed the most distinctive clustering based upon feeding status.

Differentially expressed developmental pathways identified in all three cohort analyses, along with their significant P values and genes, are described in *Table 2*.

Table 1 Patient demographics based on feeding status and sex

Demographics based on feeding status	Successful feeders	Unsuccessful feeders	P value[a]
All Infants			
Mean gestational age (range), wk	34.71 (33.1–38.3)	33.67 (30.4–35.6)	0.05
Mean birth weight (range), g	2,373 (1,350–3,182)	2,022 (1,240–2,905)	0.09
Mean post-menstrual age (range), wk[b]	35.48 (34.3–39.1)	35.5 (34.1–39)	0.93
Mean weight at sample collection (range), g	2,290 (1,410–3,098)	2,070 (1,555–2,730)	0.19
Race, no. (%)			
• Caucasian	92	69	–
• Hispanic	8	15	–
• Asian	0	8	–
• Not reported	0	8	–
Males			
Mean gestational age (range), wk	34.78 (34.1–36.4)	33.71 (32.4–35.6)	0.12
Mean birth weight (range), g	2,496 (2,339–3,079)	2,122 (1,225–2,905)	0.27
Mean post-menstrual age (range), wk	34.45 (34.6–36.7)	35.55 (34.1–37)	0.88
Mean weight at sample collection (range), g	2,393 (2,049–2,968)	2,195 (1,617–2,730)	0.43
Ethnicity, no. (%)			
• Caucasian	100	67	–
• Hispanic	0	33	–
Females			
Mean gestational age (range), wk	34.6 (33.8–38.28)	33.60 (30.4–35.5)	0.21
Mean birth weight (range), g	2,250 (1,350–3,182)	1,924 (1,240–2,279)	0.20
Mean post-menstrual age (range), wk	35.5 (34.29–39.14)	35.45 (34.3–39)	1
Mean weight at sample collection (range), g	2,203 (1,410–3,098)	1,962 (1,555–2,453)	0.31
Ethnicity, no. (%)			
• Caucasian	83	72	–
• Asian	0	14	–
• Hispanic	17	0	–
• Not reported	0	14	–

[a], paired *t*-test; [b], PMA at sample collection.

The 63 genes that were differentially expressed between all successful and unsuccessful oral feeders, clustered into the following most statistically significant developmental networks: nervous system development and function, tissue morphology, embryonic development, hematologic development and function, and hematopoiesis. Systems biology analyses highlighted genes related to memory and learning, disruption in palatal shelf formation, maturation of circadian rhythms, abnormal morphology of hindgut and mesenchyme and development of the abdomen (29,30). However, when separated by sex, two distinct analyses emerged.

For the female cohort, the most statistically significant differentially expressed developmental networks included

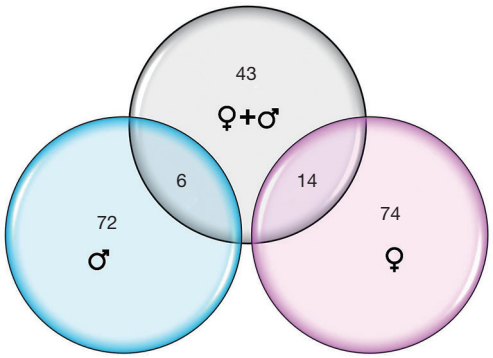

Figure 1 Venn diagram depicting differentially expressed genes between successful and unsuccessful oral feeders. When separated by sex, a small number of transcripts are shared between the entire cohort and females (n=14 genes) and males (n=6 genes). However, males and females of similar GAs, PMAs and birth weights have distinct, non-overlapping salivary profiles.

hematologic development and function, immune cell trafficking, lymphoid tissue structure and development, digestive system development, and humoral immune response. Genes within these pathways are associated with atypical neurogenesis of the intestine, tooth development (i.e., root development, incisor development), development of the secondary and hard palate and an increase of the intestinal villus (31,32).

For the male cohort, the most statistically significant differentially expressed developmental networks included nervous system development and function, cardiovascular system development and function, connective tissue development and function, embryonic development, and hair and skin development and function. Differentially-expressed genes within these networks have been associated with abnormal myelin sheath development, decreased size of the olfactory bulb, dentate gyrus, and the anterior commissure, as well as abnormal morphology of CA1 pyramidal neurons in the hippocampus, a key component in memory (33-35).

Discussion

To date, next-generation sequencing (NGS) platforms have largely been utilized in the neonatal population for either whole genome or whole exome sequencing in critically ill newborns or those with suspected monogenetic disorders (36-40). However, the vast majority of infants born prematurely are neither affected by genetic mutations nor syndromes. Rather, neonatal morbidities are largely a result of disrupted developmental pathways that are a direct consequence of preterm birth. Thus, applying NGS technology to explore real-time gene expression in these at-risk infants holds great potential for furthering our understanding of the molecular mechanisms of neonatal disease and personalizing treatment strategies geared to the individual. To our knowledge, this is the first study to perform high-throughput RNASeq on the premature newborn to better understand the biological mechanisms associated with oral feeding success. We have demonstrated that this approach provides a near real-time window into ongoing development, identifies sex-specific pathways and biological networks associated with impaired oral feeding, and provides caregivers with important opportunities to personalize care and target treatment strategies based upon an infant's sex and individual gene expression profile.

While infants in the study did have varying gestational ages, ultimately all will need to achieve oral feeding success prior to discharge home. Understanding specific pathways involved in that maturation, irrespective of gestational or post menstrual ages, will be essential in order to develop personalized care approaches to improve feeding outcomes and potentially reduce length of stay in the hospital. When performing a combined comparative systems biology analysis between all successful and unsuccessful oral feeders, developmental pathways involving the nervous, tissue and embryonic systems appear to play a key role in oral feeding. Specifically, pathways involved in cranial nerve (CN) development (CN I, III and IV), sensory integration, and facial development were all identified as being differentially expressed between successful and unsuccessful oral feeders. These pathways are not only biologically relevant but have been shown previously by our group to be essential for oral feeding (11,41). In our prior work, we demonstrated that expression profiles of genes involved in olfactory (*PLXNA1*) and vision (*NPHP4*), as well as facial development (*WNT3*) (11), and cranial nerves (41), predict oral feeding maturation in the newborn. The prospective validation of the importance of these developmental pathways further substantiates their critical role in oral feeding. However, when separated by sex, males and females of similar GAs, PMAs and weight revealed distinct salivary profiles, suggesting that neonates may follow a sex-specific, developmental time course towards oral feeding success.

Clinically, it is well established that female infants will achieve oral feeding competency prior to males of the same GAs and PMAs (12). In fact, sex-specific maturation of oral

Cohort	Differentially expressed genes
All infant	*ACP5*, *ARSD*, *BCYRN1*, *BLM*, *BRI3BP*, *C16orf93*, *C6orf226*, *C9orf93*, *CCR4*, *CENPL*, *CPA4*, *DBP*, *EFNB1*, *FAM83D*, *FOXO3*, *GIPR*, *GJA9*, *GPR22*, *HIST1H3H*, *IMPG1*, *JAKMIP1*, *KANK3*, *KDR*, *KRI1*, *LAMC1*, *LOC100130954*, *LOC100506321*, *LOC100506688*, *LOC100652999*, *LOC283404*, *LOC550112*, *LOC646278*, *MLXIPL*, *MMP17*, *MPI*, *MUC20*, *NAGPA*, *NR6A1*, *NUP35*, *OR8U1*, *PAQR4*, *PAQR6*, *PARP3*, *PLEKHA1*, *PPIL6*, *PSORS1C2*, *PVRL3*, *RASD1*, *RRP7B*, *SH3BP5L*, *SIRT2*, *SLC4A4*, *SMOX*, *SNORA6*, *SYNPO2*, *TCTN2*, *TIGIT*, *TMPRSS11BNL*, *VSIG4*, *ZNF324B*, *ZNF382*, *ZNF699*, *ZNF714*
Females	*ABHD12*, *ABTB2*, *ACD*, *ACP5*, *ALPPL2*, *ATG9B*, *BCL2*, *BIRC3*, *C14orf129*, *C19orf54*, *CD28*, *CENPL*, *CEP70*, *CLEC18B*, *CMKLR1*, *COMMD3-BMI1*, *CPA4*, *CTTNBP2NL*, *DNAH8*, *DNAJB7*, *EEPD1*, *EML3*, *EN1*, *FAM22G*, *FAM3D*, *FBXW4P1*, *FST*, *GJB4*, *HAP1*, *HIST1H2BF*, *HIST1H4J*, *HSD11B1L*, *HSF4*, *IFT140*, *KCNC2*, *KIAA1239*, *KLF8*, *LOC100287015*, *LOC100506136*, *LOC100652999*, *LOC401093*, *LOC441454*, *LOC550112*, *LOC645513*, *LOC728377*, *MAPK10*, *MKRN3*, *NAGPA*, *NCS1*, *NIPAL1*, *NUP35*, *OGDH*, *OLFML2B*, *OR8U1*, *PAQR6*, *PDCD1*, *PHF13*, *PHLPP2*, *PI4K2A*, *PLTP*, *PPIL6*, *PRKCH*, *PRSS8*, *PTK6*, *PVRL3*, *RAD52*, *RAD54L*, *RASD1*, *RBM43*, *SAPCD2*, *SLC35A2*, *SLCO2B1*, *STIL*, *SULT1A2*, *THOC3*, *TMEM102*, *TNFSF4*, *TP53INP2*, *VWA1*, *ZNF235*, *ZNF280C*, *ZNF382*, *ZNF414*, *ZNF594*, *ZNF714*, *ZNF827*, *ZNF83*, *ZNRF1*
Males	*AACS*, *ARHGEF12*, *BLM*, *BOLA3*, *C6orf132*, *CCBP2*, *CCDC137*, *CCDC14*, *CDH13*, *CHML*, *COQ4*, *CROCCP2*, *DBC1*, *DCLRE1B*, *EMG1*, *EML5*, *FAM83D*, *FAM84B*, *FOXO3*, *GLRX2*, *GPM6B*, *GPR125*, *GPR22*, *GSG1*, *HEATR3*, *HSH2D*, *KLF3*, *LOC100128590*, *LOC100130451*, *LOC100507299*, *LOC152217*, *LOC202181*, *LOC642236*, *LOC84989*, *MAP1S*, *METTL15*, *MOCS3*, *MRPL41*, *MUM1*, *MYL9*, *NACA*, *NDUFA12*, *NDUFA7*, *NDUFAB1*, *NGEF*, *NPIPL3*, *OPLAH*, *PAK1IP1*, *PLEKHA5*, *PNPLA7*, *POLR3E*, *PRICKLE3*, *RBM41*, *RGAG4*, *RNF26*, *RPP38*, *S100A13*, *SEPX1*, *SHQ1*, *SLC35E3*, *SLC39A13*, *SMOX*, *SNORD21*, *SORBS3*, *TGIF1*, *THYN1*, *TIGD7*, *TM6SF1*, *TMEM160*, *TMPRSS11BNL*, *TNFRSF21*, *TRMT2A*, *UBA7*, *XPC*, *ZFPL1*, *ZNF32*, *ZNF600*, *ZNF613*

Figure 2 List of differentially expressed genes identified between all successful oral feeders *vs.* unsuccessful oral feeders, females only, and males only. Shared genes between the entire cohort and females are highlighted in red (n=14); shared genes between the entire cohort and males are highlighted in green (n=6).

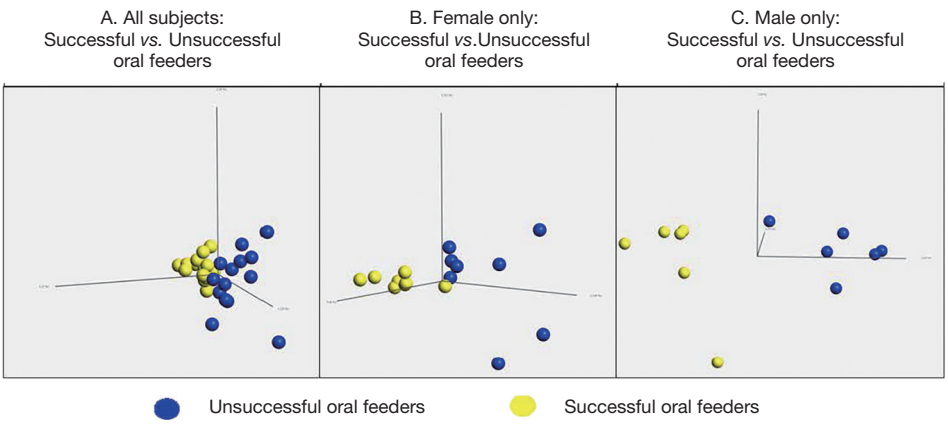

Figure 3 Principal component analysis (PCA) of successful feeders *vs.* unsuccessful feeders. (A) All infants recruited in the study; (B) females only; and (C) males only. Yellow dots represent successful oral feeders; blue dots represent unsuccessful oral feeders.

motor function and development has been seen as early as 15 weeks' gestation (42). However, the molecular mechanisms responsible for these findings are poorly understood. In the current study, male infants who could not successfully feed were more affected than their female counterparts by nervous system aberrations, particularly as it related to memory and learning. Conversely, unsuccessful female oral feeders were more affected by structural impairments involving intestinal, tooth and palate development compared to males of similar birth weights, GAs and PMAs.

Comparative analyses between male successful and unsuccessful oral feeders highlighted differential expression of genes involved in the neurogenesis of the hippocampus, the migration and morphology of the Cajal-Reelin neurons (43,44), and the morphology of the hippocampal CA1 regions. Interestingly, reelin secreting neurons are located

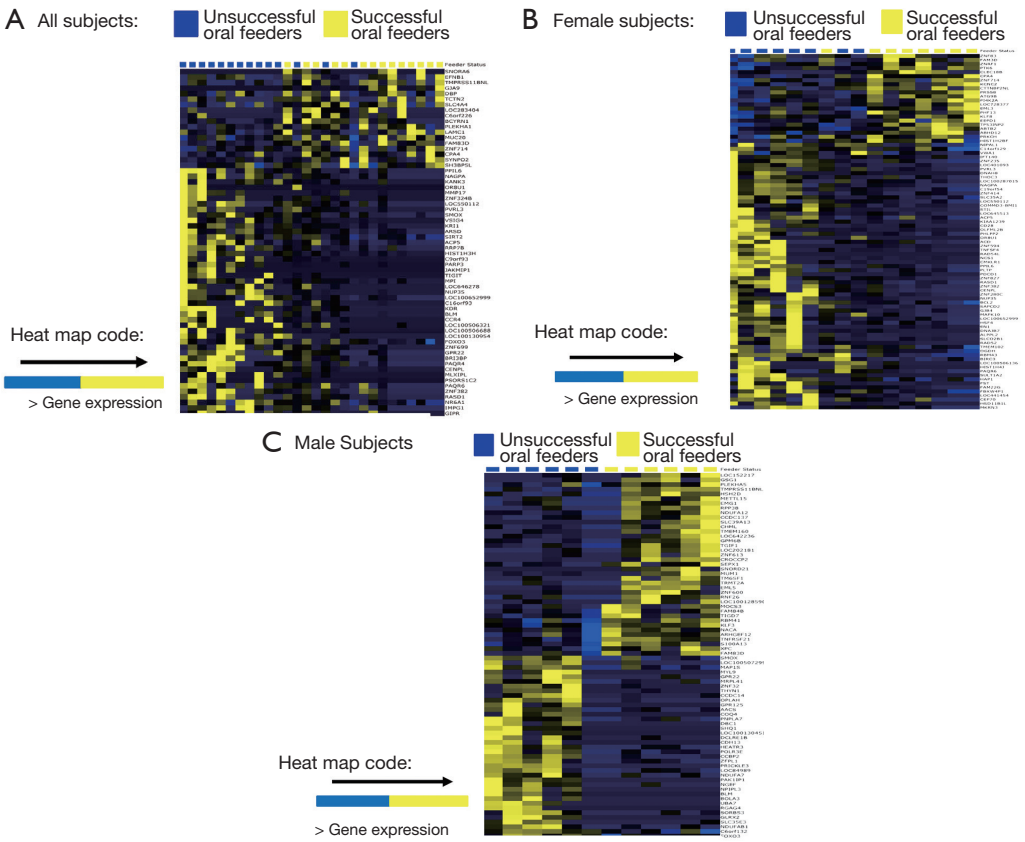

Figure 4 Heat maps of successful feeders *vs.* unsuccessful feeders. (A) All infants recruited in the study; (B) females only; and (C) males only. Gene lists are provided to the right of each heatmap. Yellow squares at top of heatmap correspond to successful oral feeders; blue squares at top of heat map correspond to unsuccessful oral feeders. Within the heatmap itself, yellow corresponds to increased gene expression, while blue represents decreased gene expression.

in the marginal zone of the neocortex and the hippocampus and have been an area of investigation in the setting of memory disorders, including Alzheimer's disease (45). In addition, CA1 regions of the hippocampus are required for contextual memory retrieval, detailed episodic memories, and normal myelination (46). Thus, these data suggest that memory and learning may be delayed or impaired in some male infants struggling to orally feed. Conversely, unsuccessful female oral feeders had gene expression profiles associated with abnormal hard and secondary palate development, as well as disrupted morphology and neurogenesis of the intestine. None of these infants displayed palate malformations. Rather, these data suggest a delay of infant palate maturation, believed to be essential for proper oral feeding, may be a limiting factor to oral feeding success (11). Thus, by applying the RNASeq platform to noninvasively obtained saliva samples, we are able to see clear sex-specific developmental aberrations, allowing us to move beyond merely reporting epidemiological associations and clinical findings, and delve much deeper into the biological mechanisms that are potentially responsible for them.

Clinical applications

Currently, there are several clinical tools available to improve feeding outcomes in the newborn (47-49). However, each device or intervention targets specific developmental pathways, albeit sensory integration or oral motor development. As such, these tools can only best be utilized when applied in a directed fashion to address developmental delays specific to the newborn. Applying high-throughput sequencing technology to explore gene expression allows us to circumvent this limitation. For example, in order to expedite oral feeding and/or treat developmental delays,

Table 2 List of differentially expressed developmental pathways identified between all three cohorts (all successful feeders *vs.* unsuccessful feeders, only the female cohort and only the male cohort) along with their significant P values and numbers of associated genes

Subjects	Developmental pathway	P values and numbers of associated genes within pathway
All infants	Nervous system development and function	P values: <0.006 to <0.0001, N=7 genes: *EFNB1, FOXO3, LAMC1, NECTIN3, NR6A1, RASD1, SIRT2*
	Tissue morphology	P values: <0.02 to <0.002, N=10 genes: *BLM, CCR4, EFNB1, FOXO3, KDR, LAMC1, NECTIN3, NR6A1, SLC4A4, TCTN2*
	Embryonic development	P values: <0.02 to <0.002, N=12 genes: *BLM, EFNB1, FOXO3, GIPR, KDR, LAMC1, MMP17, NECTIN3, NMP17, PLEXHA1, SIRT2, TCTN2*
	Hematologic development and function	P values: <0.02 to <0.002, N=9 genes: *BLM, CCR4, EFNB1, FOXO3, KDR, PLEKHA1, SLC4A4, TIGIT, VSIG4*
	Hematopoiesis	P values: <0.02 to <0.002, N=6 genes: *BLM, CCR4, EFNB1, FOXO3, KDR, SLCA4*
Females	Hematologic development and function	P values: <0.01 to <0.0001, N=12 genes: *BCL2, CD28, CMKLR1, COMMD3-BMI1, FST, MAPK10, PDCD1, PLTP, PRKCH, RAD52, TMEM102, TNFSF4*
	Immune cell trafficking	P values: <0.01 to <0.0001, N=8 genes: BCL2, CD28, CMKLR1, MAPK10, PDCD1, PLTP, TMEM102, TNFSF4,
	Lymphoid tissue structure and development	P values: <0.01 to <0.0001, N=7 genes: *BCL2, CD28, COMMD3-BMI1, FST, PDCD1, PRKCH, TNFSF4*
	Digestive system development and function	P values: <0.01 to <0.0002, N=7 genes: *BCL2, COMMD3-BMI1, FST, GSKIP, NECTIN3, PDCD1, PTK6*
	Humoral immune response	P values: <0.01 to <0.0002, N=4 genes: *BCL2, CD28, PDCD1, TNFSF4*
Males	Nervous system development and function	P values: <0.04 to <0.0008, N=6 genes: *BRINP1, FOXO3, GPM6B, SLC39A13, TNFRSF21, XPC*
	Cardiovascular system development and function	P values: <0.05 to <0.003, N=7 genes: *ACKR2, ARHGEF12, BLM, CDH13, FOXO3, MAP1S, MYL9*
	Connective tissue development and function	P values: <0.05 to <0.003, N=8 genes: *ARHGEF12, BLM, BRINP1, CDH13, FOXO3, NACA, SLC39A13, TGIF1*
	Embryonic development	P values: <0.05 to <0.003, N=6 genes: *BLM, KLF3, NDUFAB1, SLC39A13, TGIF1, ZNF613*
	Hair and skin development and function	P values: <0.05 to <0.003, N=4 genes: *ARHGEF12, CDH13, FOXO3, XPC*

increased kangaroo care with parents could be utilized in infants lagging in sensory integration (49), while the use of the FDA-approved NTrainer System®, designed to improve nutritive sucking, could be used in infants with delayed facial or neurodevelopment (47). Understanding these differences is essential to developing and implementing targeted and personalized therapies to improve feeding and associated long-term outcomes. Blindly providing treatment strategies to infants struggling to orally feed without a clear understanding of their developmental status, diminishes the effectiveness of the therapy. Rather, in order to truly inform care and improve outcomes, we must be able to successfully integrate near real-time monitoring of neonatal development into newborn care in order to provide targeted, sex-specific, and individualized therapies. While the feasibility of integrating RNASeq analysis of neonatal salivary transcripts into neonatal care may be limited by cost, time and the required expertise in downstream bioinformatic analyses, data generated by this approach may inform the design of a user friendly, cost-effective, targeted array or multiplex RT-qPCR platform for rapid assessment of the developmental feeding stage of the newborn.

Limitations

One of the most appealing aspects of this research is that the data were derived from approximately 10 µL to 20 µL of noninvasively collected whole saliva. However, given the low volume and starting total RNA concentrations in each sample, we could not perform confirmation testing of the RNASeq data with RT-qPCR. Emerging data suggests that this additional step may not be required (50), however, prospective confirmatory studies should be performed in larger cohorts to assess applicability of findings.

In addition, there are unique issues to consider when utilizing whole saliva on the RNASeq platform, including the impact of cellular material on sequence reads, as well as the effect of microbial debris (51). In the current study, there is a wide range of alignment rates between samples, most likely due to varying microbial genomic contamination that does not map to the human genome and or degraded RNA. Nevertheless, this pilot study serves as a proof of principle that neonatal saliva samples are amenable to the RNASeq platform for hypothesis discovery. In order to improve performance on the RNASeq plaform, our laboratory recently published on techniques to optimize output and alignment rates of neonatal saliva on the RNASeq platform, including the use of specific library preparation kits to minimize interference of microbes on the platform (52). Accounting for varying rates of alignment to the human genome by considering the degraded quality of RNA found in saliva, varying cellular contents, small sample volumes, and the bacterial sequences that comprise the oral microbiome will be essential to ensure the integrity of future studies.

Every attempt was made to match successful and unsuccessful oral feeders based upon their sex, gestational and post-menstrual ages. Although none of the demographic data achieved statistical significance, it is possible that differences in both gestational and postmenstrual ages may have skewed the data. In addition, female infants were also shown to have dysregulation of pathways involved in hematopoiesis and immune response. While it is possible that genes within these pathways play a role in oral feeding or gut development, it should be noted that the most common cell types in human saliva are epithelial cells, leukocytes and erythrocytes (53). While future research will need to be conducted to determine what roles, if any, these pathways may play in oral feeding maturity, it is possible that differential cell counts within whole saliva may have resulted in these findings. Finally, our limited sample size makes it unlikely that these data can be universally applied to all newborns across varying PMAs. Nevertheless, our findings continue to contribute to an expanding body of literature demonstrating the biological complexity, as well as sex-specific, time sensitive and distinct maturation, of developmental pathways involved in oral feeding competency in the neonate.

Conclusions

RNASeq of gene transcripts present in neonatal saliva to assess oral feeding competency is feasible, informative and provides near real-time information regarding ongoing development in the neonate. Results of this study reveal that unsuccessful male feeders have delayed nervous system and memory development, while unsuccessful female oral feeders of similar GAs, PMAs and birth weights were more affected by delayed facial structural and gastrointestinal development. While males and females are known to have distinct timelines for the development of oral feeding maturation, integrating gene expression sequencing platforms into neonatal care will allow us to better understand these differences at an organ system level. This approach to noninvasive assessment of the newborn may be used to develop targeted and personalized treatment strategies for the millions of infants affected by oral feeding difficulties born each year. Importantly, this proof of principle study lays the foundation for the assessment of a multitude of other morbidities affecting the preterm newborn and holds significant promise for improving care and outcomes for this vulnerable population.

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://pm.amegroups.com/article/view/10.21037/pm-21-45/coif). The series "Neonatal Feeding and Developmental Issues" was commissioned by the editorial office without any funding or sponsorship. JLM reports that she has received funding for research through Tufts Medical Center and NICHD helped to fund this study, but both Tufts Medical Center and NICHD had no role in the design and conduct of the study; collection management analysis and interpretation of the data; preparation, review or approval of the manuscript; and decision to submit the manuscript for publication. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This prospective, observational, single-center study was conducted from 2014 to 2017 in the Tufts Medical Center NICU with approval by the Tufts Medical Center Institutional Review Board. The two review panels registered under this IORG are associated with the Tufts Health Sciences Human. Research Protection Program: The IRB # for IRB-RED is IRB00000577, the IRB # for IRB-BLUE is IRB00001236 and the IRB study protocol is #8349. Informed consent was taken from all parents of participating infants.

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Supplementary

Table S1 Read alignment/mapping rates for all infants

Successful Female Feeders	Mapping Rate	Unsuccessful Female Feeders	Mapping Rate	Successful Male Feeders	Mapping Rate	Unsuccessful Male Feeders	Mapping Rate
1	55.40%	1	21.90%	9	42.90%	9	19.60%
2	20.20%	2	43.40%	10	42.60%	10	24.80%
3	5.90%	3	48.90%	11	41.40%	11	51.10%
4	16.10%	4	40.60%	12	16.30%	12	7.30%
5	28.00%	5	32.80%	13	34.90%	13	37.50%
6	93.60%	6	17.40%	14	4.50%	14	22.40%
7	43.70%	7	94.30%				

Table S2 Genes and chromosomal location for all cohorts

All Subjects		Males Only		Females Only	
Gene	Chromosomal Location	Gene	Chromosomal Location	Gene	Chromosomal Location
ACP5	19p13.2	*AACS*	12q24.31	*ABHD12*	20p11.21
ARSD	Xp22.33	*ARHGEF12*	11q23.3	*ABTB2*	11p13
BCYRN1	2p21	*BLM*	15q26.1	*ACD*	16q22.1
BLM	15q26.1	*BOLA3*	2p13.1	*ACP5*	2q37.1
BRI3BP	12q24.31	*C6orf132*	6p21.1	*ALPPL2*	2q37.1
C16orf93	16p11.2	*CCBP2*	3p22.1	*ATG9B*	7q36.1
C6orf226	6p21.1	*CCDC137*	17q25.3	*BCL2*	18q21.33
C9orf93	9p22.3	*CCDC14*	3q21.1	*BIRC3*	11q22.2
CCR4	3p22.3	*CDH13*	16q23.3	*C14orf129*	14q32.2
CENPL	1q25.1	*CHML*	1q43	*C19orf54*	19q13.2
CPA4	7q32.2	*COQ4*	9q34.11	*CD28*	2q33.2
DBP	19q13.33	*CROCCP2*	1p36.13	*CENPL*	1q25.1
EFNB1	Xq13.1	*DBC1*	9q33.1	*CEP70*	3q22.3
FAM83D	20q11.23	*DCLRE1B*	1p13.2	*CLEC18B*	16q23.1
FOXO3	6q21	*EMG1*	12p13.31	*CMKLR1*	12q23.3
GIPR	19q13.32	*EML5*	14q31.3	*COMMD3-BMI1*	10p12.2
GJA9	1p34.4	*FAM83D*	20q11.23	*CPA4*	7q32.2
GPR22	7q22.3	*FAM84B*	8q24.21	*CTTNBP2NL*	1p13.2
HIST1H3H	6p22.1	*FOXO3*	6q21	*DNAH8*	6p21.2
IMPG1	6q14.1	*GLRX2*	1q31.2	*DNAJB7*	22q13.2
JAKMIP1	4p16.1	*GPM6B*	Xp22.2	*EEPD1*	7p14.2
KANK3	19p13.2	*GPR125*	4p15.2	*EML3*	11q12.3
KDR	4q12	*GPR22*	7q22.3	*EN1*	2q14.2

Table S2 (*continued*)

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Table S2 (*continued*)

All Subjects		Males Only		Females Only	
Gene	Chromosomal Location	Gene	Chromosomal Location	Gene	Chromosomal Location
KRI1	19p13.2	*GSG1*	12p13.1	*FAM22G*	9q22.33
LAMC1	1q25.3	*HEATR3*	16q12.1	*FAM3D*	3p14.2
LOC100130954	9q34.3	*HSH2D*	19p13.11	*FBXW4P1*	22q11.23
LOC100506321	14q23.3	*KLF3*	4p14	*FST*	5q11.2
LOC100506688	5p15.33	*LOC100128590*	2p22.1	*GJB4*	1p34.3
LOC100652999	12q13.13	*LOC100130451*	2q34	*HAP1*	17q21.2
LOC283404	12q13.13	*LOC100507299*	9q21.12	*HIST1H2BF*	6p22.2
LOC550112	4q13.2	*LOC152217*	3q29	*HIST1H4J*	6p22.1
LOC646278	15q13.1	*LOC202181*	5q35.3	*HSD11B1L*	19p13.3
MLXIPL	7q11.23	*LOC642236*	9q13	*HSF4*	16q22.1
MMP17	12q24.33	*LOC84989*	10q21.3	*IFT140*	16p13.3
MPI	15q24.1	*MAP1S*	19p13.11	*KCNC2*	12q21.1
MUC20	3q29	*METTL15*	11p14.1	*KIAA1239*	4p14
NAGPA	16p13.3	*MOCS3*	20q13.13	*KLF8*	Xp11.21
NR6A1	9q33.3	*MRPL41*	9q34.3	*LOC100287015*	8p23.1
NUP35	2q32.1	*MUM1*	19p13.3	*LOC100506136*	7q21.3
OR8U1	11q12.1	*MYL9*	20q11.23	*LOC100652999*	12q13.13
PAQR4	16p13.3	*NACA*	12q13.3	*LOC401093*	3q25.1
PAQR6	1q22	*NDUFA12*	12q22	*LOC441454*	9q22.33
PARP3	3p21.2	*NDUFA7*	19p13.2	*LOC550112*	4q13.2
PLEKHA1	10q26.13	*NDUFAB1*	16p12.2	*LOC645513*	4q26
PPIL6	6q21	*NGEF*	2q37.1	*LOC728377*	7q35
PSORS1C2	6p21.33	*NPIPL3*	16p12.2	*MAPK10*	4q21.3
PVRL3	3q13.13	*OPLAH*	8q24.3	*MKRN3*	15q11.2
RASD1	17p11.2	*PAK1IP1*	6p24.2	*NAGPA*	16p13.3
RRP7B	22q13.2	*PLEKHA5*	12p12.3	*NCS1*	9q34.11
SH3BP5L	1q44	*PNPLA7*	9q34.3	*NIPAL1*	4p12
SIRT2	19q13.2	*POLR3E*	16p12.2	*NUP35*	2q32.1
SLC4A4	4q13.3	*PRICKLE3*	Xp11.23	*OGDH*	7p13
SMOX	20p13	*RBM41*	Xq22.3	*OLFML2B*	1q23.3
SNORA6	3p22.1	*RGAG4*	Xq13.1	*OR8U1*	11q12.1
SYNPO2	4q26	*RNF26*	11q23.3	*PAQR6*	1q22
TCTN2	12q24.31	*RPP38*	10p13	*PDCD1*	2q37.3

Table S2 (*continued*)

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Table S2 (*continued*)

All Subjects		Males Only		Females Only	
Gene	Chromosomal Location	Gene	Chromosomal Location	Gene	Chromosomal Location
TIGIT	3q13.31	*S100A13*	1q21.3	*PHF13*	1p36.31
TMPRSS11BNL	4q13.2	*SEPX1*	16p13.3	*PHLPP2*	16q22.2
VSIG4	Xq12	*SHQ1*	3p13	*PI4K2A*	10q24.2
ZNF324B	19q13.43	*SLC35E3*	12q15	*PLTP*	20q13.12
ZNF382	19q13.12	*SLC39A13*	11p11.2	*PPIL6*	6q21
ZNF699	19p13.2	*SMOX*	20p13	*PRKCH*	14q23.1
ZNF714	19p12	*SNORD21*	1p22.1	*PRSS8*	16p11.2
		SORBS3	8p21.3	*PTK6*	20q13.33
		TGIF1	18p11.31	*PVRL3*	3q13.13
		THYN1	11q25	*RAD52*	12p13.33
		TIGD7	16p13.3	*RAD54L*	1p34.1
		TM6SF1	15q25.2	*RASD1*	17p11.2
		TMEM160	19q13.32	*RBM43*	2q23.3
		TMPRSS11BNL	4q13.2	*SAPCD2*	9q34.3
		TNFRSF21	6p12.3	*SLC35A2*	Xp11.23
		TRMT2A	22q11.21	*SLCO2B1*	11q13.4
		UBA7	3p21.31	*STIL*	1p33
		XPC	3p25.1	*SULT1A2*	16p11.2
		ZFPL1	11q13.1	*THOC3*	5q35.2
		ZNF32	10q11.21	*TMEM102*	17p13.1
		ZNF600	19q13.41	*TNFSF4*	1q25.1
		ZNF613	19q13.41	*TP53INP2*	20q11.22
				VWA1	1p36.33
				ZNF235	19q13.31
				ZNF280C	Xq26.1
				ZNF382	19q13.12
				ZNF414	19p13.2
				ZNF594	17p13.2
				ZNF714	19p12
				ZNF827	4q31.21-q31.22
				ZNF83	19q13.41
				ZNRF1	16q23.1

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