Promise for Autism and Neurodevelopmental Disorders

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UPSTATE
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Disclosures

- Dr. Middleton is an unpaid member of the Scientific Advisory Board of Quadrant Biosciences, which has provided financial support for much of the work that is described
- Dr. Middleton is a co-owner of intellectual property rights for more than a dozen preliminary patents related to this work for identifying biomarkers of brain disorders
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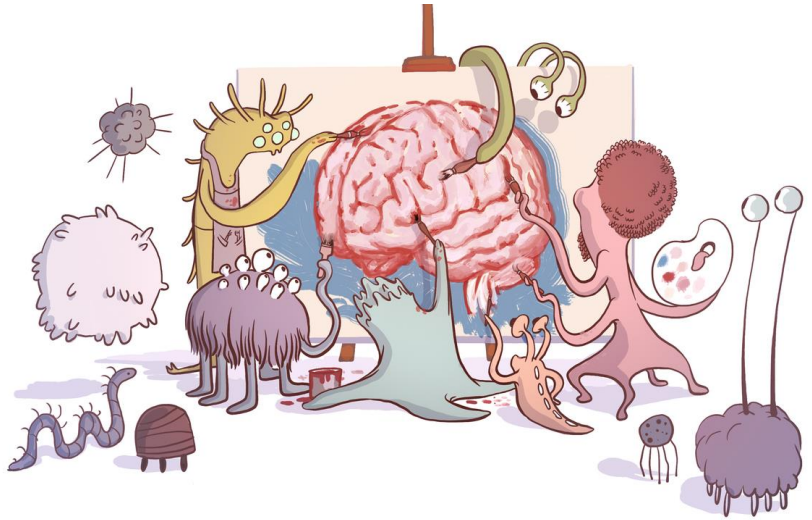
QUADRANT BIOSCIENCES

NIH National Institute of Mental Health

The Premise for Promise

Evidence shows that the interaction between the gut and brain can influence brain development and behavior. This interaction can be abnormal in children with autism spectrum disorder and neurodevelopmental disorders.

Let’s take a look at how we can measure and possibly correct abnormal gut-brain interactions to understand and improve health of subjects with autism spectrum disorder.

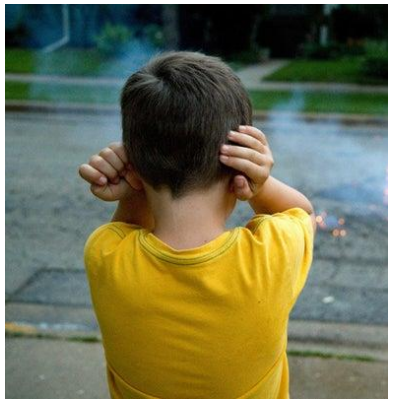


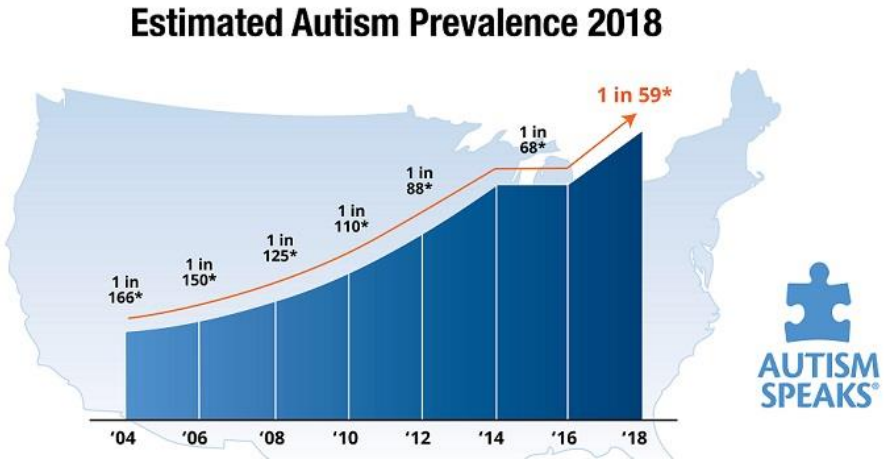
Objectives

- Brief overview of autism and diagnostic challenges
- An introduction to your “2nd brain” and “2nd genome”
- Highlight diagnostic potential of “profiling” saliva for biomarkers of autism spectrum disorder that sample gut-brain interactions
- Discuss biological basis and potential therapeutic implications of work

Autism Spectrum Disorder

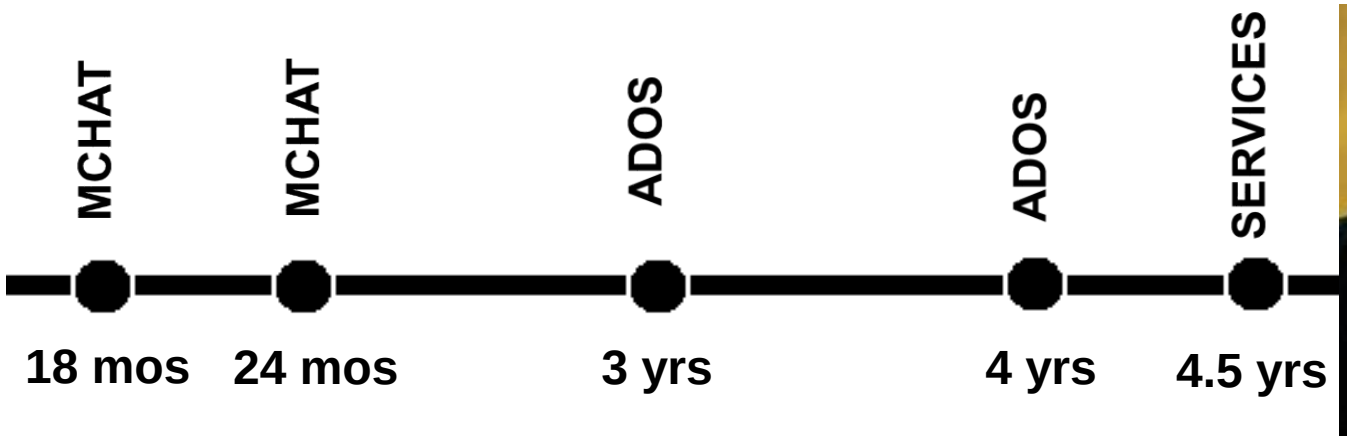
- Constellation of variable symptoms rather than a single disorder
- Now affects 1 in 59 children (1 in 45 children age 3-17)
- Occurs in all ethnic and SES groups, males 4x females





- Hallmark features of ASD are impaired social interaction and communication, particularly non-verbal communication, and restricted or repetitive behaviors

The Autism Diagnostic Odyssey



http://www.netwellness.org/healthtopics/autism/childwithblocks.jpg

*Modified Checklist for Autism in Toddlers (MCHAT)

*Autism Diagnostic Observation Schedule (ADOS)

- **MCHAT**:
  - 20 Yes/No questions (takes 15 min for parent to complete)
  - Very sensitive (>90%), but Positive Predictive Value = 14%
  - Not valid until 18+ months
- **ADOS**:
  - >2 hours for set-up, administration, and scoring
  - Requires training, long wait list for evaluations

Robins et al., 2014.

Searching for Causes of Autism

- Considered the most heritable neuropsychiatric condition, with concordance rates of 76% in identical twins, 34% fraternal male twins, 18% fraternal mixed twins, and 10% siblings
- Despite this, no single gene accounts for >1% of cases (apart from syndromic cases)
- Small % of cases have de novo genetic mutation – but at least 90% are idiopathic (unknown cause) – making genetic screening very low yield
- However, ASD is associated with several pre- and perinatal risk factors: stress, trauma, infection, nutritional excess, alcohol, seizure medications, vitamin D or folate deficiency, increased parental age (esp. fathers)
- And there are high rates of comorbid conditions:
  - 20-30% develop epilepsy
  - 25-30% diagnosed sleep disorders (half receive medication)
  - 30-40% have GI disturbances
- Collectively, these findings suggest possible epigenetic and microbial contributions to ASD risk (shared environment → similar microbiomes)

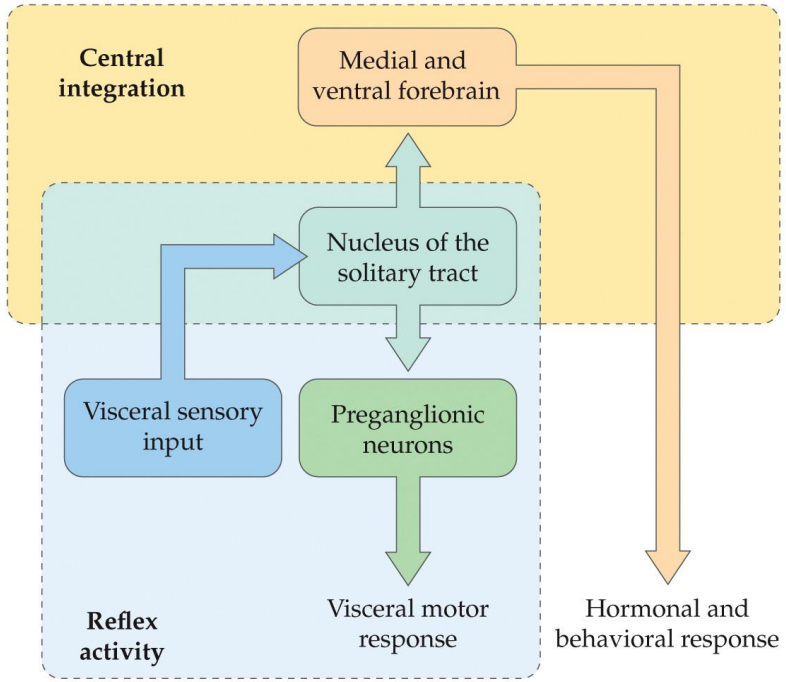
Two Important Terms

- **Microbiome**
  - *Consists of the 10-100 trillion symbiotic microbial cells harbored by each person, primarily bacteria in the upper and lower gut (Ursell, 2012)*
  - *Essentially, whatever lives in you or on you that is not you, as well as the RNA, protein and metabolic products they produce*
  - *Commonly now referred to as your "Second Genome"*
- **Microtranscriptome**
  - *Let's first define the term "transcriptome"*
    - *The "expressed" portion of the human genome that is converted to RNA and encodes all of the known protein-coding genes and regulatory RNA transcripts that do not encode protein*
  - *The microtranscriptome consists of the smallest classes of these RNAs, which are mostly non-coding and regulatory in nature (i.e., influence the expression of other genes)*
  - *Our focus is on micro-RNA (miRNA)*

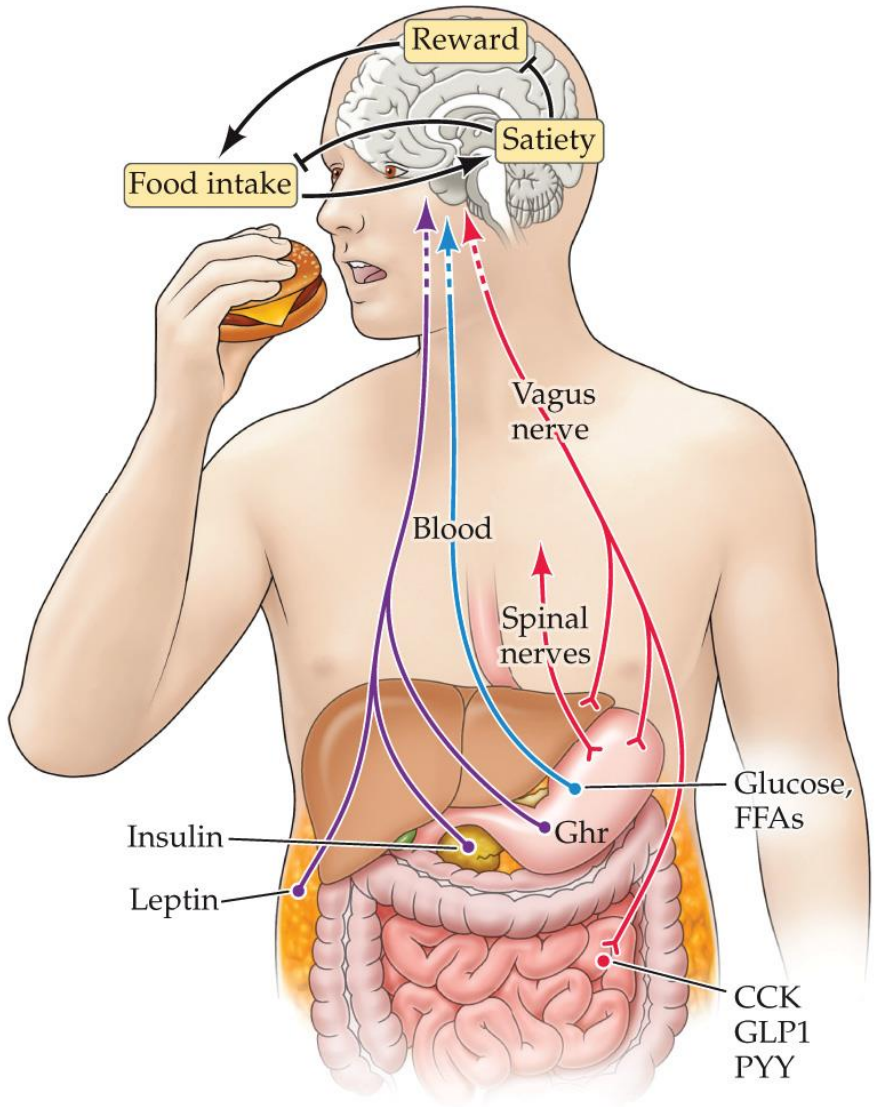
- *SALIVA IS A RICH SOURCE OF MICROBIOME AND MICROTRANSCRIPTOME INFORMATION*

Another important term: Gut-Brain Axis

- Two-way connections between brain-stem nuclei and end organs in our upper and lower gastrointestinal (GI) tract
- Strongly modulated by sympathetic ("fight or flight") and parasympathetic ("rest and digest") connections and factors driving their activity
- Such determinants can include adrenergic signals, stress hormones, serotonin, dopamine, as well as environmental and psychological factors such as mood, aromas, fear, memories
- <u>Major role of vagus nerve</u> on both directions of signals



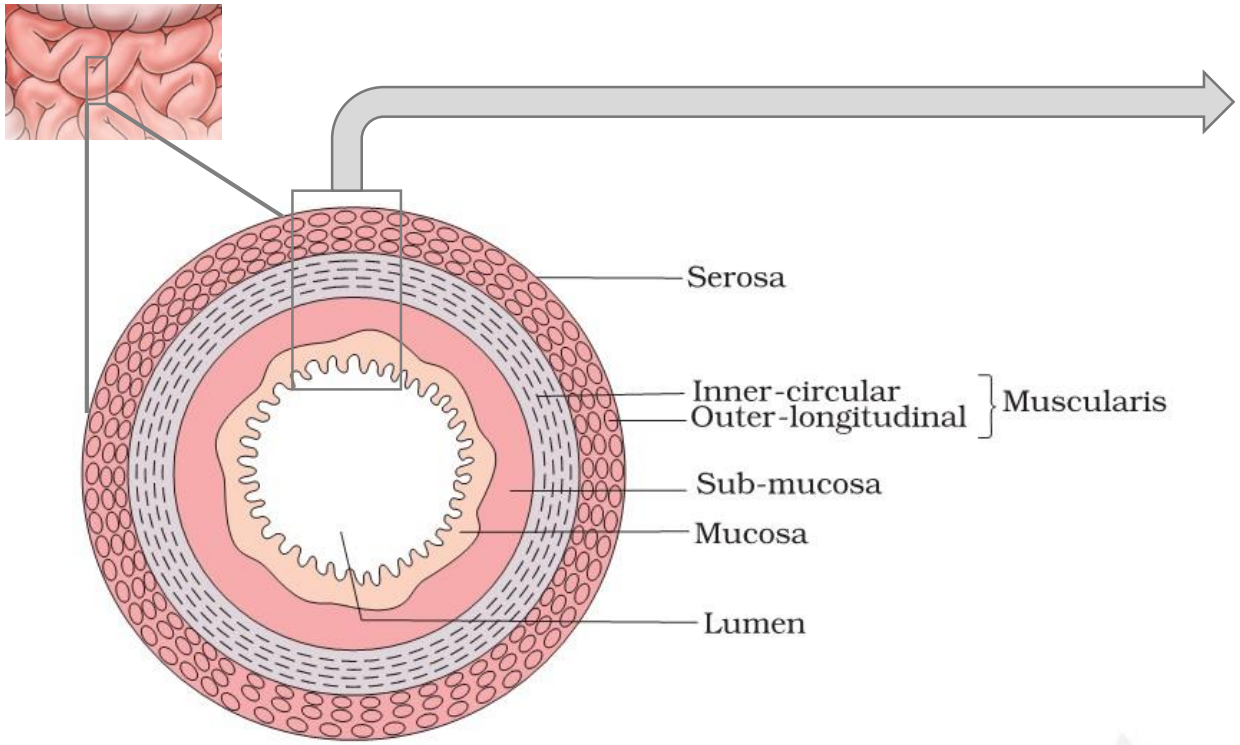
NEUROSCIENCE 6e, Figure 21.5

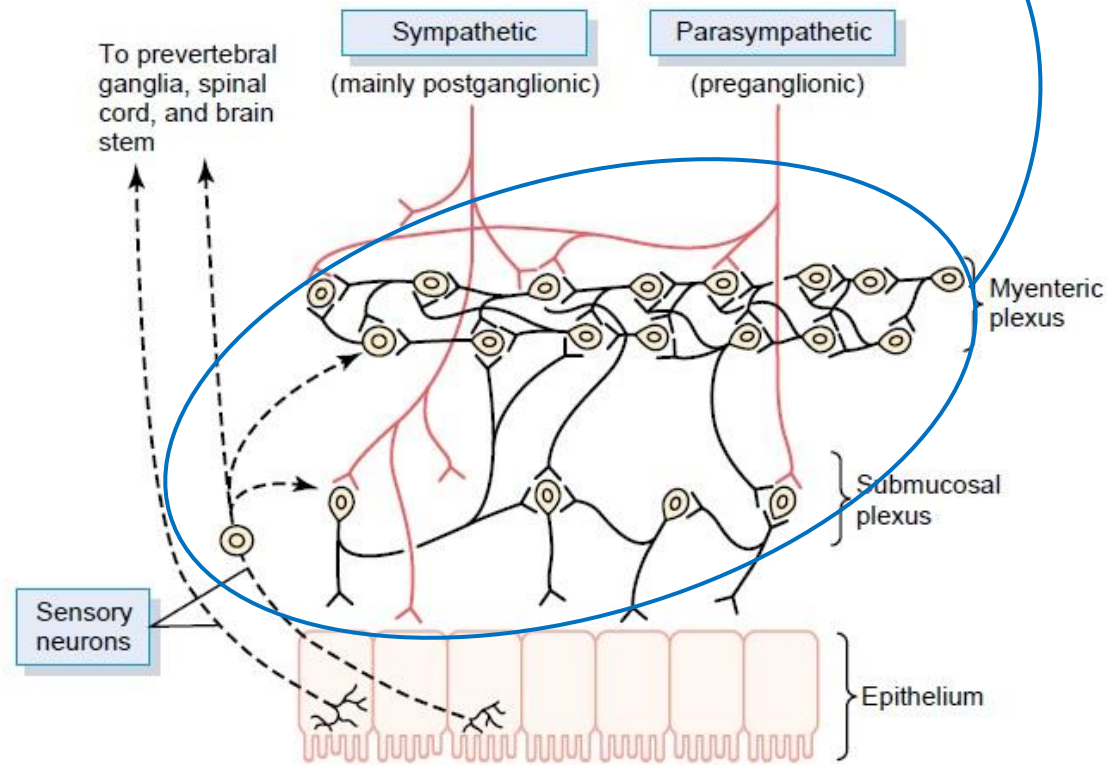


A Closer Look at Gut-Brain Interactions

Sensory neurons in the gut provide input to the brainstem and spinal cord (esp. Vagus). These same sites provide outputs back to the gut where they modulate muscle-activity (Myenteric plexus) as well as secretions (Submucosal plexus)

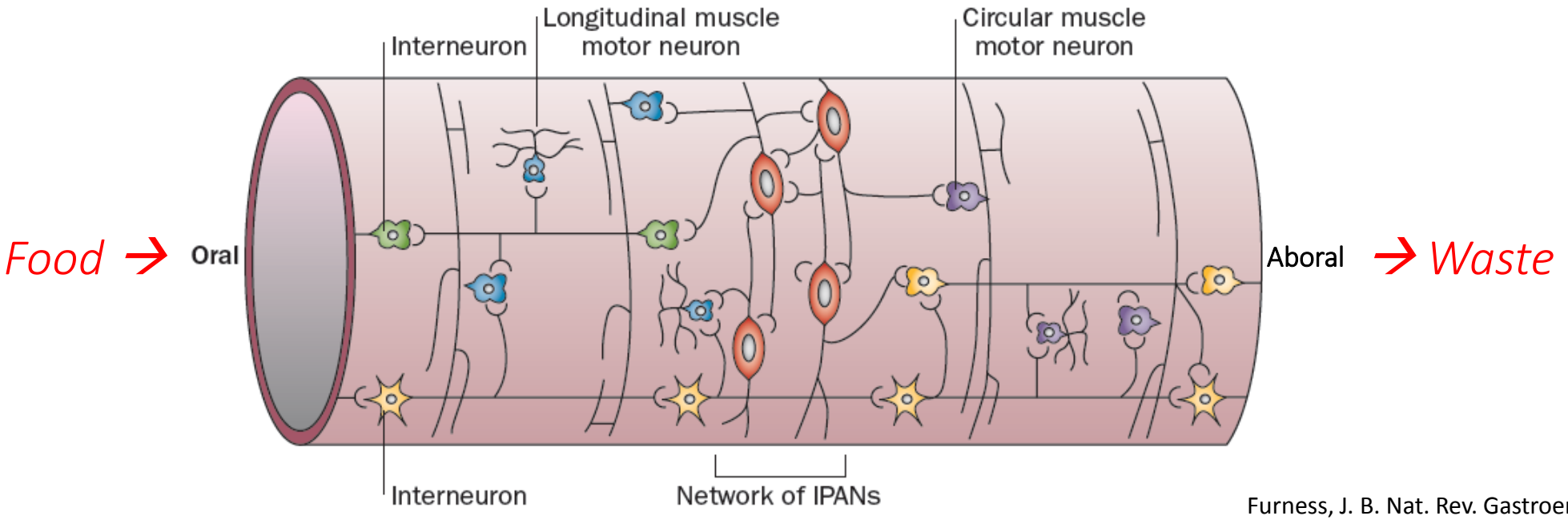
The collection of sensory, motor and secretory cells collectively comprise the "2nd Brain"





Guyton & Hall (2010)

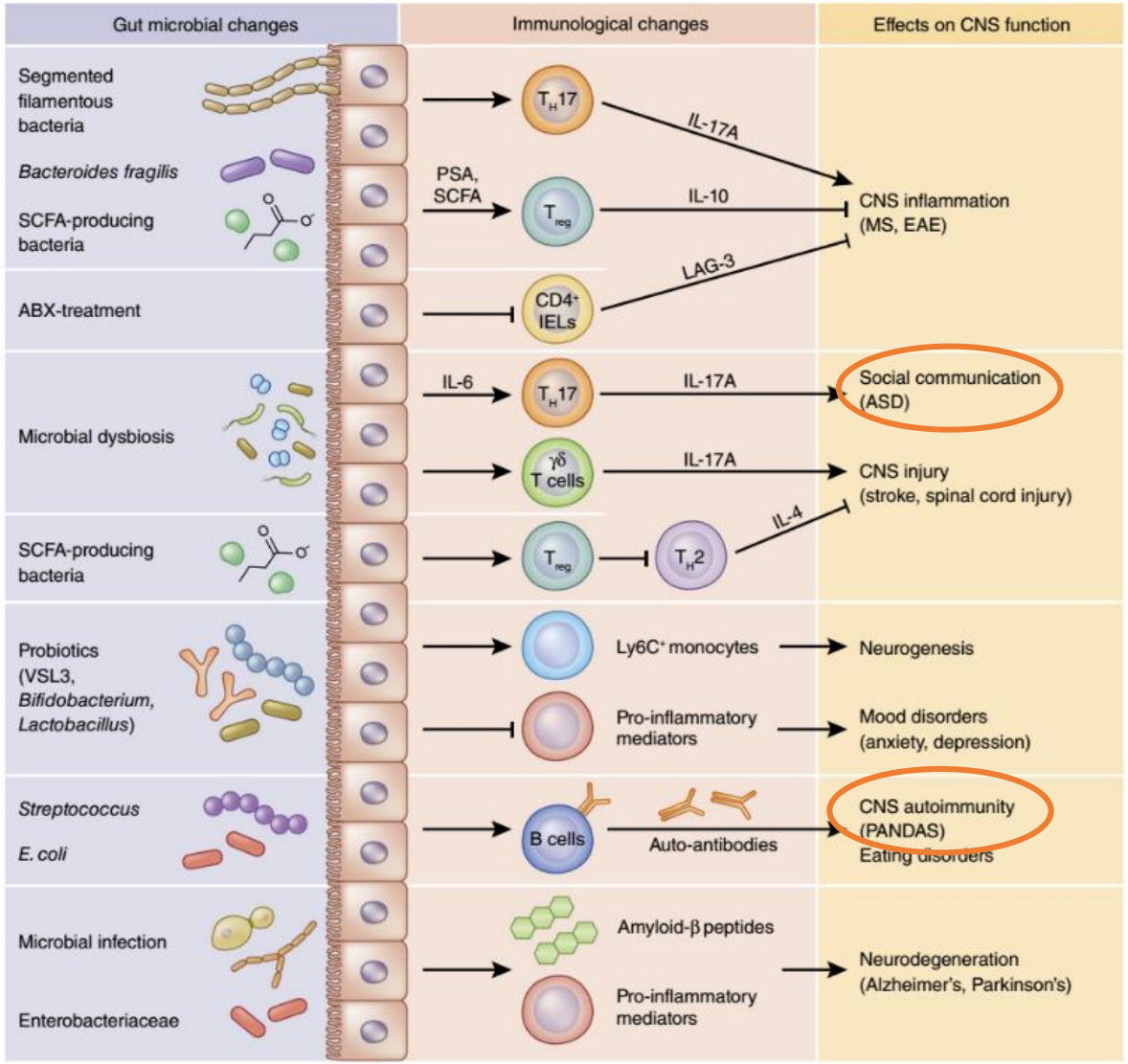
A rich network of neurons in your gut controls entire process of digestion



Furness, J. B. Nat. Rev. Gastroenterol.

- **The activity of all of these neurons is affected by signals in their microenvironment (which includes MANY compounds released by gut bacteria)**

Models of gut-brain-microbiome changes in different brain conditions



Fung et al., Nature Neuroscience 2017

Previous changes reported in ASD:

Increased: *Lactobacillus*, *Desulfovibrio*, *Clostridium*, cluster 1
Decreased: *Bacteroides/Firmicutes*, ratio

Increased: *Sutterella*

Increased: *Clostridium*, *Bacteroides*, *Porphyromonas*, *Prevotella*, *Pseudomonas*, *Aeromonas*, Enterobacteriaceae
Decreased: *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Staphylococcus*, Bifidobacteria

Increased: *Lactobacillus*
Decreased: *Prevotella*, *Coprococcus*, *Veillonellaceae*

Increased: *Sutterella*, *Lachnospiraceae*, *Ruminococcaceae*

Increased: *Lactobacillus*
Decreased: *Bifidobacterium*, *Enterococcus*

Increased: *Bacteroidetes*, *Proteobacterium*
Decreased: *Actinobacterium*, *Bifidobacterium*

Increased: *Clostridium* clusters I and II

But note inconsistent directions of some changes!

Other notable microbiome findings relevant to autism

- Normal delivery results in colonization of offspring with distinctly different microbiome than caesarean delivery, and the combined results of 61 studies conducted in 19 countries shows that the odds of autism is 33% more likely following cesarean delivery (JAMA, 2019)
- Mice raised in “germ free” conditions exhibit behavioral changes (less exploration, impaired learning, withdrawal from novel social situations) and reduced brain volume
- Social impairments and slowed brain development in mice can be rescued by adding as little as one probiotic commensal bacteria (e.g., *Lactobacillus ramnosus*), as long as the vagus nerve is intact
- Seizures are more common in ASD and anti-seizure effects of ketogenic diet depend on gut microbiome
- Antibiotics used for MRSA or intestinal colitis (vancomycin and minocycline) appear to mitigate behavioral symptoms in ASD subjects (and change composition as well as inflammatory state)
- Fecal transfer therapy in 18 children with ASD improved bacterial diversity and GI symptoms 8-weeks after treatment, along with improvement in social function

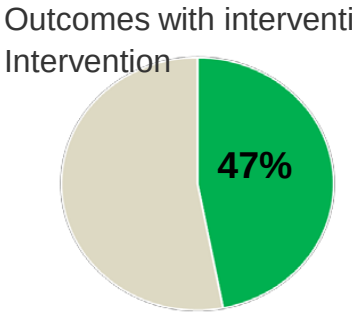
Lu et al., 2018; Buffington et al., 2016; Urbano et al., 2014; Kang et al., 2017

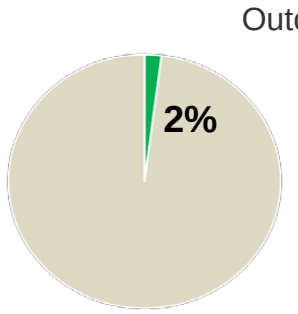
The Major Questions for our Autism Studies

- Can we improve the detection and diagnosis of children with ASD at an earlier age, using saliva-based epigenetic and microbial analyses?
- If so, what is the biological basis for this utility?
  - What cells, tissues, and bacteria contribute the most to the ‘saliva gene pool’?
  - Is this information relevant to neurodevelopmental disorders?
  - Do these markers change with treatment?

Why push for early diagnosis?

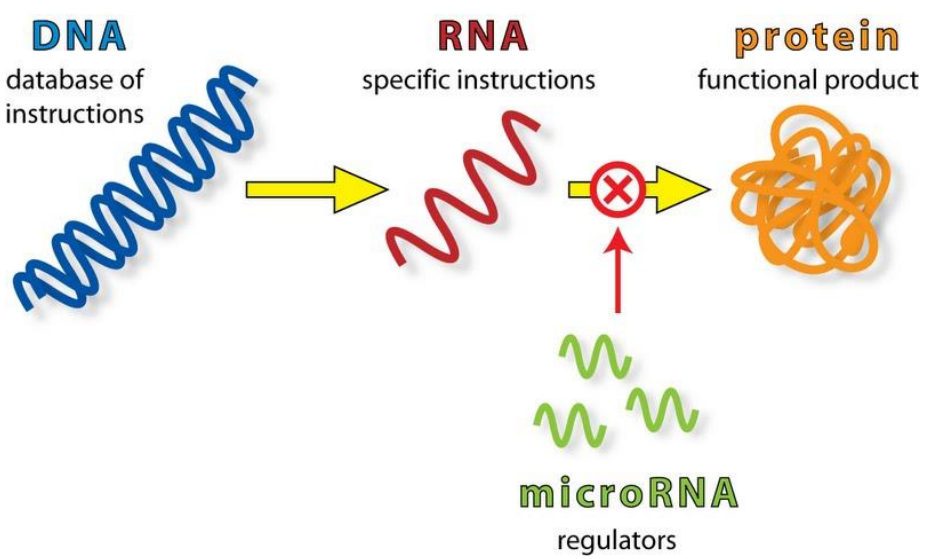
1) Current prognosis is poor: most children retain diagnosis and require support for life
   - 90% of adults with autism unable to live independently and have < 1 social interaction/month
2) Individuals with higher levels of adaptive function fare much better than those with intellectual disabilities and severe autistic symptoms
   - 50% of high-functioning autism spectrum adults have college degree, but still have significant social impairment and incomplete intellectual development
3) Significant change in outcome is linked early intervention
   - Lovaas and colleagues (1987): 47% of ASD children who received early intervention became nearly indistinguishable from their peers in both cognitive and educational functioning (e.g., achieved an average IQ score, joined a typical 1st grade classroom) compared to 2% of ASD children who did not.



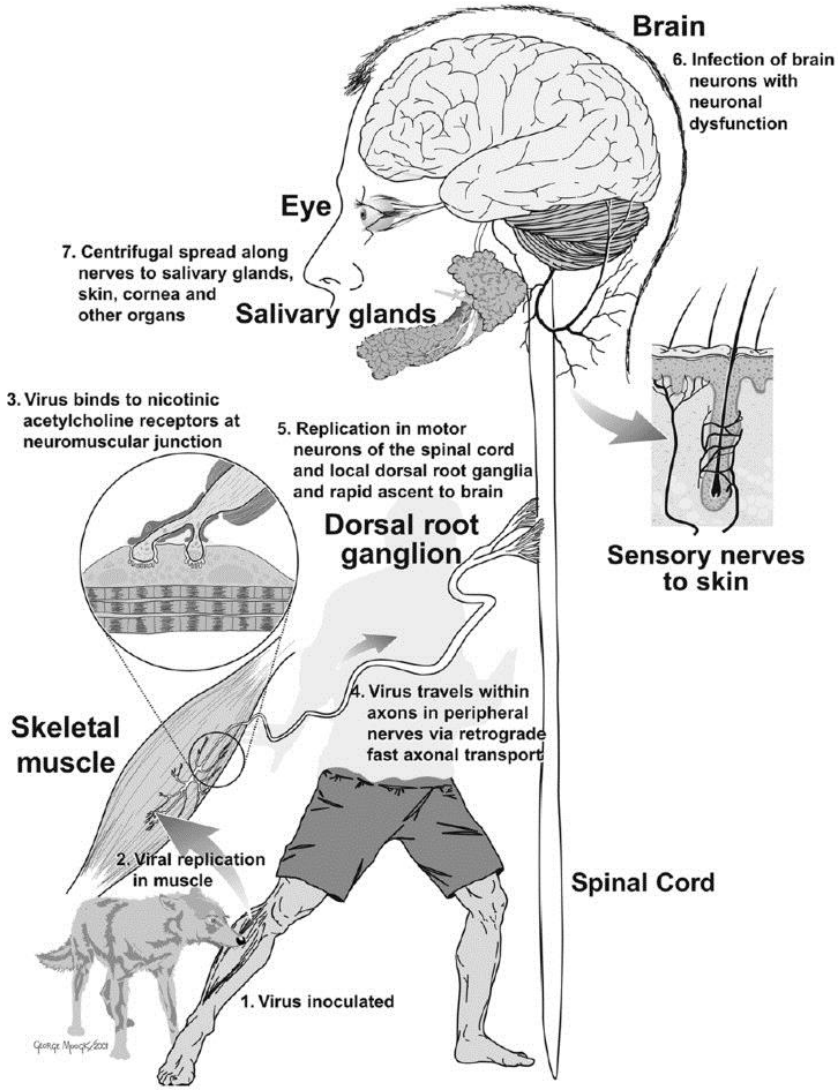


1st focus: MicroRNA (miRNA)

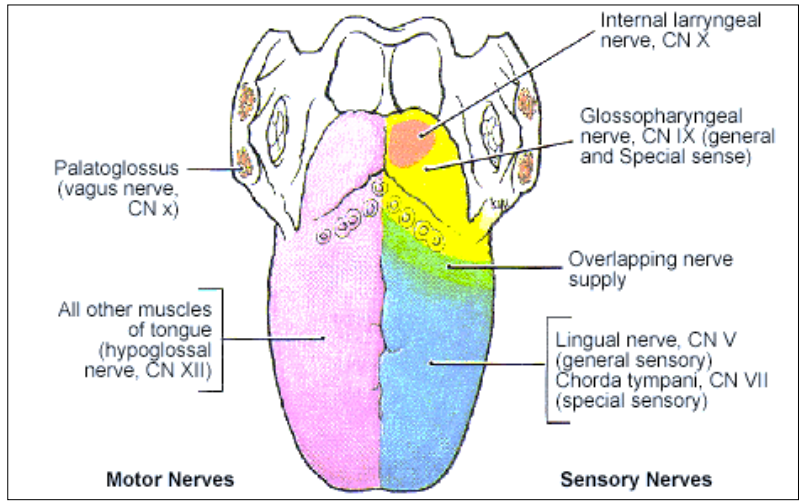
- Made by all cells in the body
- Degrades mRNA or blocks translation of mRNA into proteins
- Transported along axons from central to peripheral sites
- Extruded into extracellular space
- Can enter neighboring or distant cells in exosomes or on carrier proteins
- Easily isolated in extracellular fluids
- Critical to brain development, learning, and nearly every cellular process!



Brain miRNAs can follow same route as rabies RNA: Gut-Brain Connections

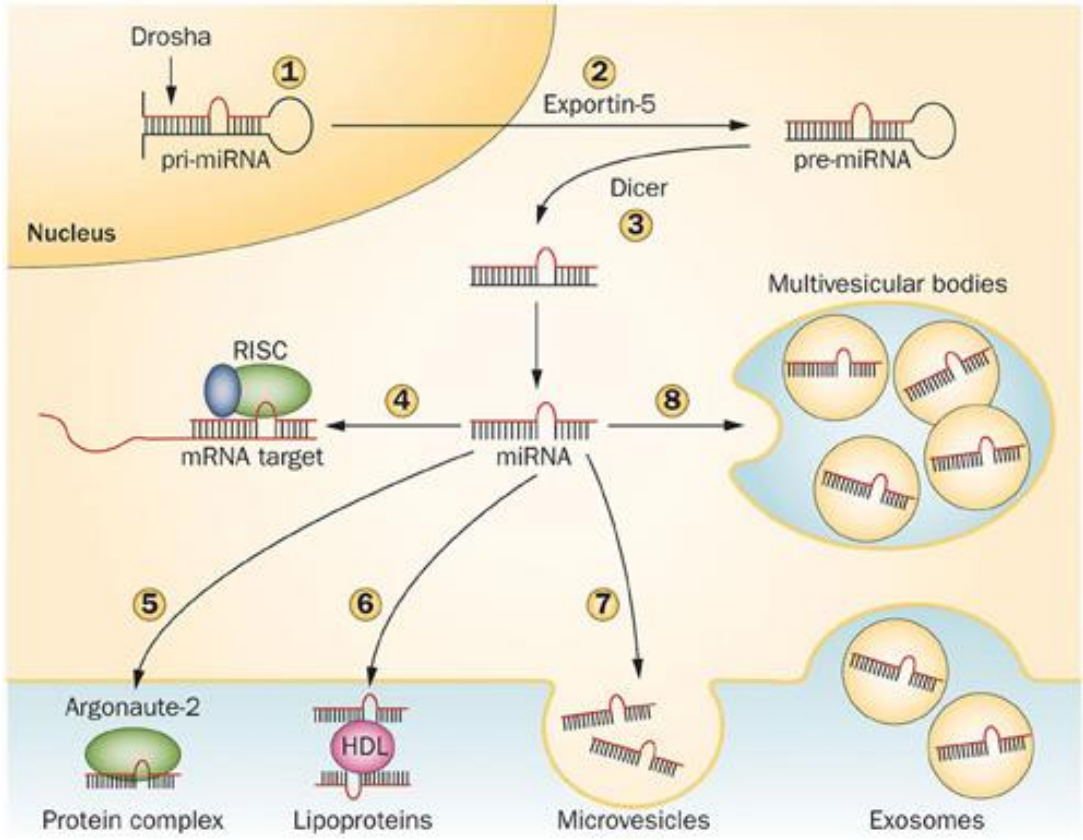


5 pairs of cranial nerves supply the oral cavity and continuously release miRNAs into mouth



Trigeminal, Facial, Glossopharyngeal, Vagus & Hypoglossal
Involved in taste, sensation, speaking, swallowing, salivation

miRNAs released in *exosomes* can alter protein expression in remote cells



Mafioletti et al. 2014 *Front Neurosci*

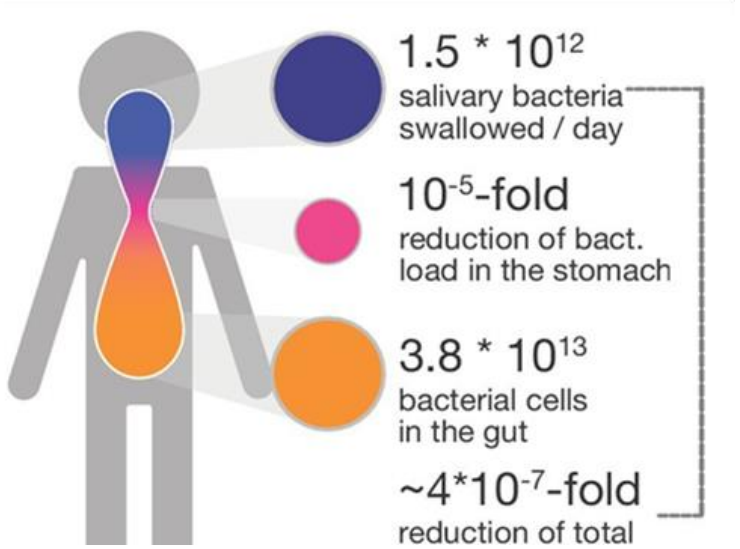
This may even be a way to influence gene expression in gut bacteria!

Pilot Study Methods

- SUNY Upstate Pediatrics Department funded recruitment of 50 children during well-child visits
- Ascertained ASD and typically developing children, ages 4-14
- Children expectorated saliva in a small vial that preserved miRNA and microbial content
- Comprehensive Next Generation Sequencing of all saliva content
  - SUNY Molecular Analysis Core, Upstate Medical University
- Mapped sequence data to human miRNAs
- Machine learning algorithms identify top features that distinguish ASD from non-ASD children
- Examined correlations with functional measures of ASD or adaptive behavior

Why Saliva?

It is a rich source of both human miRNA (that may arise in brain stem or salivary glands) and microbiome elements!



There is also extensive microbial transmission from oral to lower GI sites

Schmidt and colleagues (2019) determined that approximately 1.5 trillion bacteria are passed to the stomach each day, where there is a 500,000-fold reduction due to the local stomach environment, enabling 3,000,000 bacteria to be added to the lower GI tract each day.

Hicks et al. BMC Pediatrics (2016) 16:52
DOI 10.1186/s12887-016-0586-x

BMC Pediatrics

RESEARCH ARTICLE Open Access

Salivary miRNA profiles identify children with autism spectrum disorder, correlate with adaptive behavior, and implicate ASD candidate genes involved in neurodevelopment

Steven D. Hicks[1], Cherry Ignacio[2], Karen Gentile[3] and Frank A. Middleton[3,4,5*]

Abstract

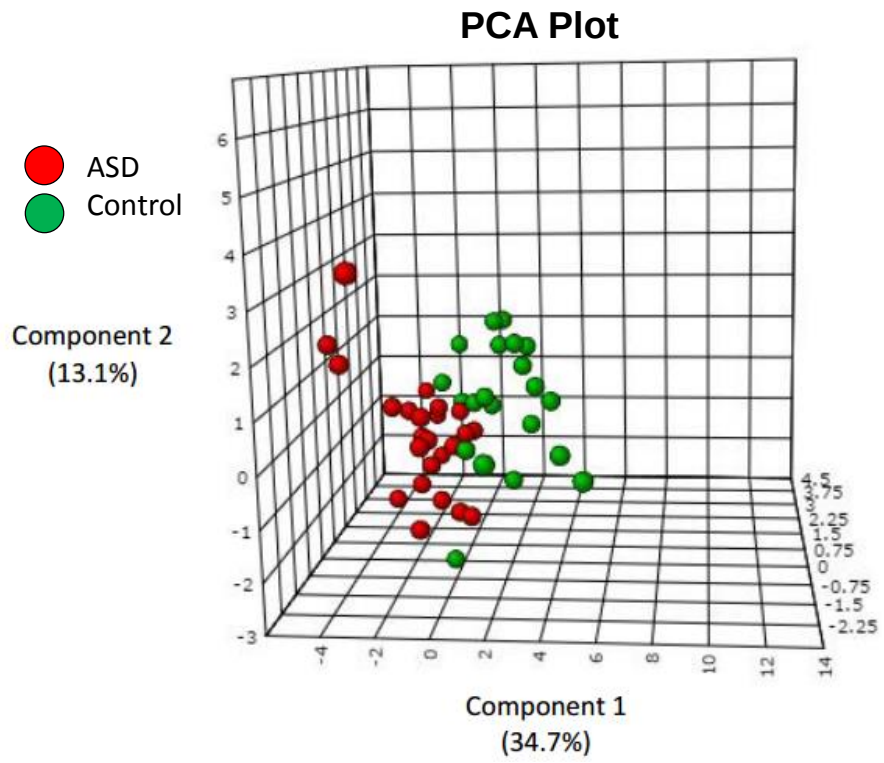
Background: Autism spectrum disorder (ASD) is a common neurodevelopmental disorder that lacks adequate screening tools, often delaying diagnosis and therapeutic interventions. Despite a substantial genetic component, no single gene variant accounts for >1 % of ASD incidence. Epigenetic mechanisms that include microRNAs (miRNAs) may contribute to the ASD phenotype by altering networks of neurodevelopmental genes. The

Characteristics of ASD pilot study cohort

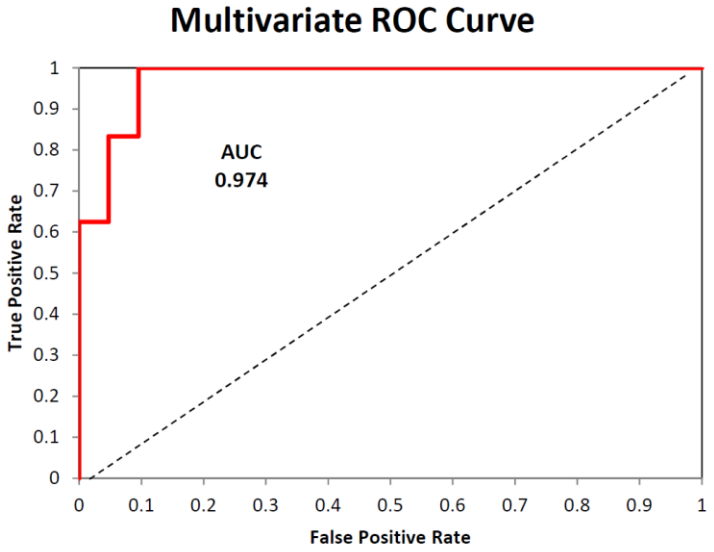
				Vineland Adaptive Behavior Scales						
Controls	Age (years)	Sex	ADOS Comp	Comm	Social	ADLs	Comp	Birth Age (weeks)	Weight (%ile)	Height (%ile)
Mean	9.2	16M, 5F		110.1	104.4	100.4	105.3	39.2	78.2	68.4
StDev	2.5			10.0	15.7	11.0	12.7	1.3	16.5	20.6
Range	4-13			88-127	81-146	85-124	87-132	36-42	50-100	33-97
ASD										
Mean	9.1	19M, 5F	10.6	76.0	77.8	73.6	70.7	38.3	64.7	59.5
StDev	2.4		4.1	15.3	14.3	10.9	10.2	2.5	29.6	25.7
Range	5-13		3-16	49-113	47-108	52-95	48-90	31-41	5-99	10-99
P value	0.182	0.816		0.001	0.001	0.000	0.000	0.294	0.915	0.848

ADLs, Activities of Daily Living; ADOS, Autism Diagnostic Observation Schedule; Comm, Vineland Communication score; Social, Vineland Socialization score; Comp, Composite score. There were no differences in age or gender composition, birth age, weight or height. Note, the highly significant differences in Vineland scores.

Findings: Diagnosis by saliva miRNA expression



Scree plot of multivariate loadings for 14 miRNAs in ASD and Control children generated by PCA



Multivariate Regression Classification

		Observed		
		Autism	Control	Total
Predicted	Autism	24	2	26
	Control	0	19	19
	Total	24	21	45
	Accuracy	100%	90.5%	95.6%

Overall, the set of miRNAs was more than 95% accurate (it only mis-classified 2 controls as ASD)

Statistical features of top miRNAs

Neurodevelopmental Correlations

miRNA	Age (Yrs)	ADOS Comm	ADOS Social	ADOS C+S	VABS Comm	VABS ADL	VABS Social	VABS Comp
miR-628-5p	-0.26	-0.04	-0.29	-0.21	**-0.35**	**-0.35**	**-0.38**	**-0.40**
miR-127-3p	-0.35	-0.25	-0.19	-0.15	**-0.36**	**-0.41**	**-0.46**	**-0.45**
miR-27a-3p	0.14	-0.04	-0.03	-0.04	**0.35**	**0.36**	**0.41**	**0.42**
miR-335-3p	-0.20	0.25	-0.07	0.02	**-0.46**	**-0.51**	**-0.49**	**-0.51**
miR-2467-5p	-0.02	-0.14	0.00	0.00	**-0.38**	**-0.37**	**-0.37**	**-0.40**
miR-30e-5p	0.19	0.08	-0.28	-0.26	**0.37**	**0.50**	**0.50**	**0.50**
miR-28-5p	0.19	0.05	0.38	0.33	**-0.40**	**-0.42**	**-0.42**	**-0.43**
miR-191-5p	-0.17	0.34	0.22	0.34	-0.27	-0.21	-0.29	**-0.30**
miR-23a-3p	0.15	-0.12	-0.27	-0.22	**0.42**	**0.49**	**0.46**	**0.49**
miR-3529-3p	-0.09	0.33	0.23	0.29	**-0.46**	**-0.35**	**-0.47**	**-0.46**
miR-218-5p	0.04	-0.06	0.06	0.06	-0.25	-0.26	**-0.31**	-0.30
miR-7-5p	0.01	-0.09	0.14	0.09	**-0.40**	**-0.39**	**-0.41**	**-0.45**
miR-32-5p	0.24	0.27	0.15	0.14	0.30	**0.36**	**0.39**	**0.36**
miR-140-3p	-0.05	-0.20	-0.16	-0.24	-0.15	-0.24	-0.22	-0.23

Domains & Index	Subdomain
Communication miR-335-3p miR-3529-3p	Receptive Expressive Written
Daily Living Skills miR-335-3p miR-30e-5p	Personal Domestic Community
Socialization miR-335-3p miR-30e-5p	Interpersonal Relationships Play and Leisure Time Coping Skills
Motor Skills	Fine Gross
Maladaptive Behavior Index (Optional)	Internalizing Externalizing Other

- Most miRNAs significantly correlate with functional measures (Vineland)
- A few miRNAs significantly correlate with multiple Vineland measures

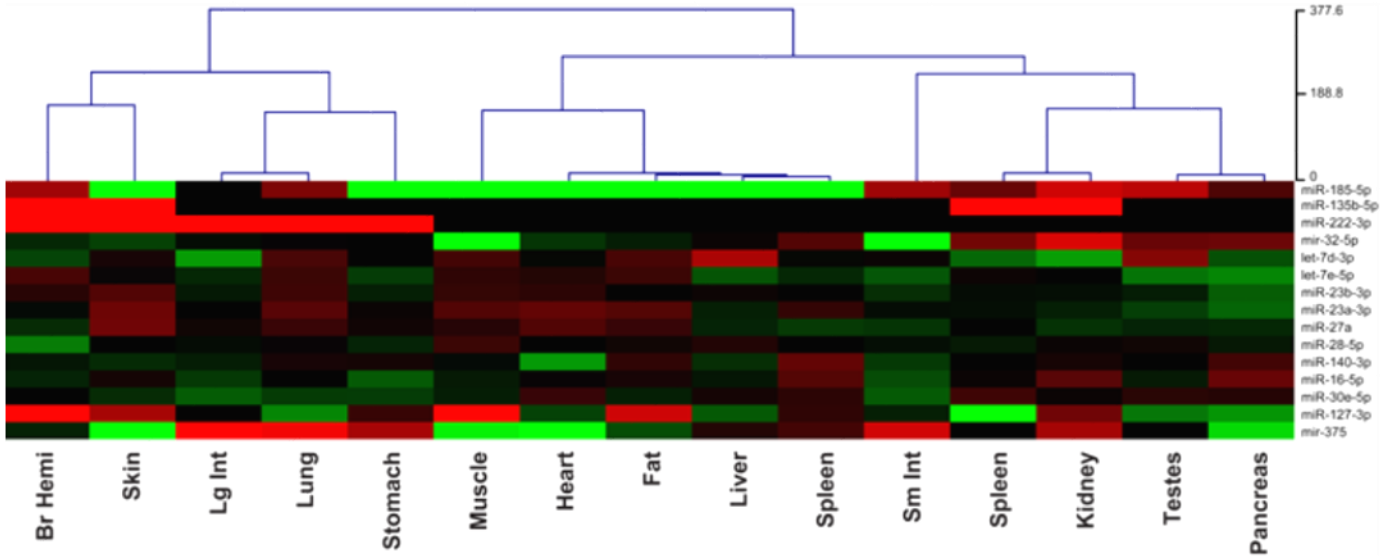
Neurodevelopment and ASD genes are targets of top miRNAs

Gene Ontology Term		Match Count	% overlap	Fold Enrichment	P Value	Bonferroni FDR	Match to AutismDB	% of Node in AutismDB
GO:0043005	**Neuron projection**	**51**	**4.1**	**2.28**	**5.86E-08**	**2.88E-05**	13	25.5
GO:0030424	**Axon**	**31**	**2.5**	**2.98**	**1.14E-07**	**5.61E-05**	9	29.0
GO:0042995	Cell projection	75	6.0	1.65	1.98E-05	0.0097	16	21.3
GO:0044463	Cell projection part	24	1.9	1.57	0.032549	1.0000	8	33.3

- The mRNA targets of the 14 miRNAs are 2-3X more likely than chance to be involved in neurodevelopment, and > 25% of these are previously-identified ASD candidate genes

Half of the top saliva miRNAs are present at high levels in brain

RED = HIGH
GREEN = LOW



Pilot Study Take-Home Messages and Questions

- **>95% accuracy in a pilot sample**
  - False positives < 10%; False negatives <5%
- Used only **14 miRNA** biomarkers
- The miRNAs show a direct relationship to measures of adaptive behavior, and association with genes that regulate brain development and function
- Many of the miRNAs are expressed at high levels in the brain (as well as other notable tissues)
- Would be nice to know if the same miRNAs change in other studies of ASD tissues
- Would be nice to know if the miRNA changes might be reversible with therapy

A true “real world” test… REPRODUCIBILITY

frontiers in Psychiatry

PERSPECTIVE
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A Comparative Review of microRNA Expression Patterns in Autism Spectrum Disorder

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Are saliva miRNA biomarkers seen in other studies from ASD subjects?

27 miRNAs changed in 2 or more of the 12 studies published to date

		DIRECTION OF CHANGE IN DIFFERENT BIOMATERIALS				
miRNA ID	Seed Sequence	CNS	Blood	Saliva	Lympho-blast	Olfactory
miR-7-5p	GGAAGAC	↑ ↓		↑		
miR-10a-5p	ACCCUGU	↑			↑	
miR-15a-5p	AGCAGCA	↓	↓			
miR-15b-5p	AGCAGCA	↓	↓			
miR-19b-3p	GUGCAAA	↑	↑ ↓			
miR-21-3p	AACACCA	↑ ↑				
miR-23a-3p	UCACAUU	↑ ↓		↓	↓ ↑	
miR-27a-3p	UCACAGU	↓	↑	↓		
miR-30e-5p	GUAAACA			↓	↓ ↑	
miR-92a-3p	AUUGCAC		↓		↓	
miR-92b-3p	AUUGCAC	↓			↓	
miR-93	AAAGUGC	↓			↓	
miR-103a-3p	GCAGCAU		↓		↓	
miR-106b-5p	AAAGUGC	↑	↑		↑	
miR-132	AACAGUC CCGUGGC				↑ ↑	
miR-140-3p	ACCACAG	↑		↑		
miR-146a-5p	GAGAACU	↑			↑	↑
miR-146b	GAGAACU	↑			↑	
miR-155-5p	UAAUGCU	↑ ↑				
miR-195-5p	AGCAGCA		↓ ↑		↓	
miR-199b-5p	CCAGUGU				↑ ↑	
miR-219-5p	GAUUGUC	↑			↑	
miR-320a	AAAGCUG	↑	↓		↓	
miR-335-3p	UUUUCAU	↑		↑		
miR-338-5p	ACAAUAU	↑			↑	
miR-451a	AACCGUU	↑	↓		↓	
miR-494	GAAACAU GGUUGUC	↑	↑			
		23.1	19.0	42.8	25.8	25.0

Saliva study shows most overlap (43%) compared to other tissues

Are brain miRNA changes related to ASD reversible?

Examined using Fetal Alcohol Exposure (FAE) Model in Rats

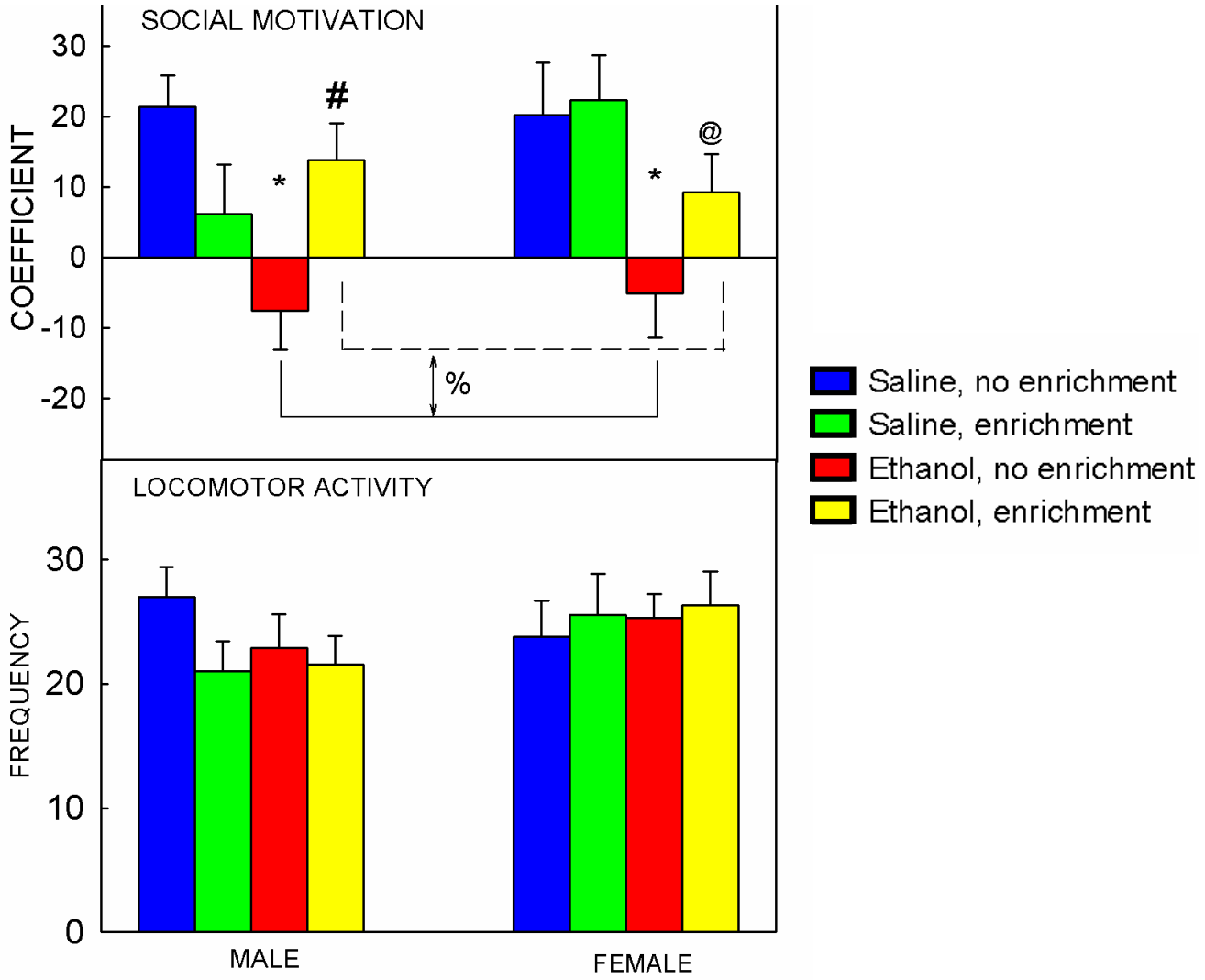
"Of those individuals with diagnosable Fetal Alcohol Syndrome, only 15% have an IQ under 70 that would qualify them for developmental disability services... However, the other 85% have symptoms that would qualify them under the category of Autism"

(Kellerman, 2007)

Notably, fetal alcohol exposure also produces cerebral folate deficiency (reduces expression of folate transporters) which is strongly associated with ASD

We modeled FAE using a single "binge" exposure on gestational day 12 (about halfway through pregnancy in rats), during a time when the amygdala is undergoing significant development, and then looked at social behavior and miRNA expression in amygdala in the offspring

Social Interaction Chamber



frontiers in PEDIATRICS

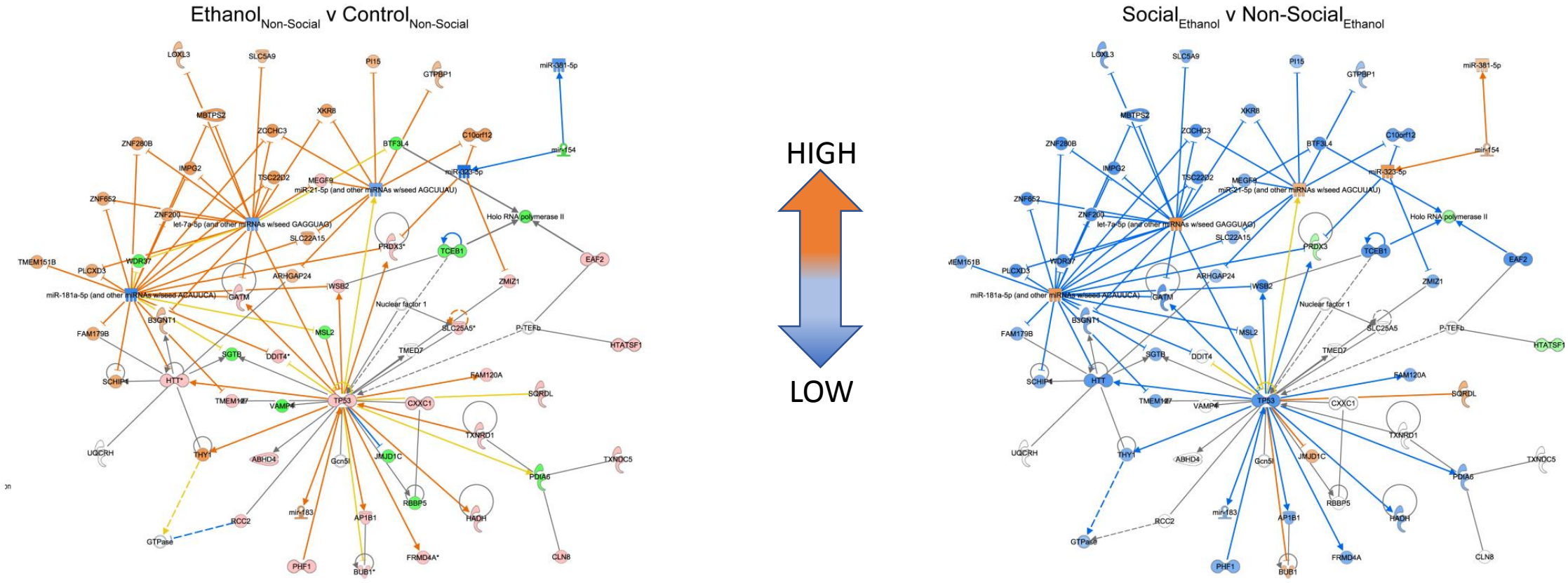
ORIGINAL RESEARCH ARTICLE
published: 24 September 2014
doi: 10.3389/fped.2014.00103

Effects of acute prenatal exposure to ethanol on microRNA expression are ameliorated by social enrichment

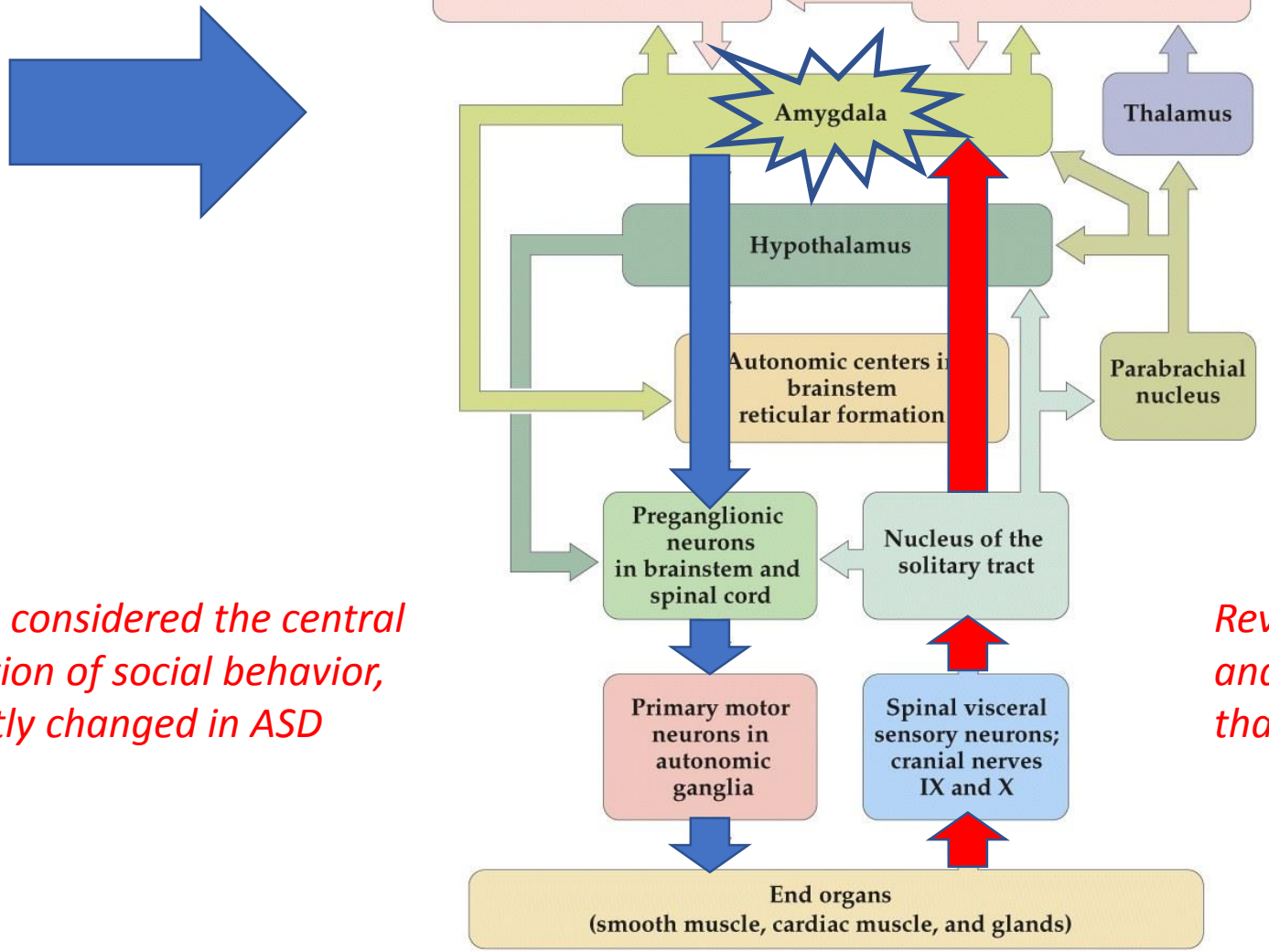
Cherry Ignacio[1,2,3], Sandra M. Mooney[3,4] and Frank A. Middleton[1,2,3]**

Cell Cycle regulatory gene targets of miRNAs are highly INCREASED in the amygdala after a single fetal exposure

This is completely REVERSED by postnatal social enrichment



Amygdala critical for bi-directional regulation of gut-brain-behavior



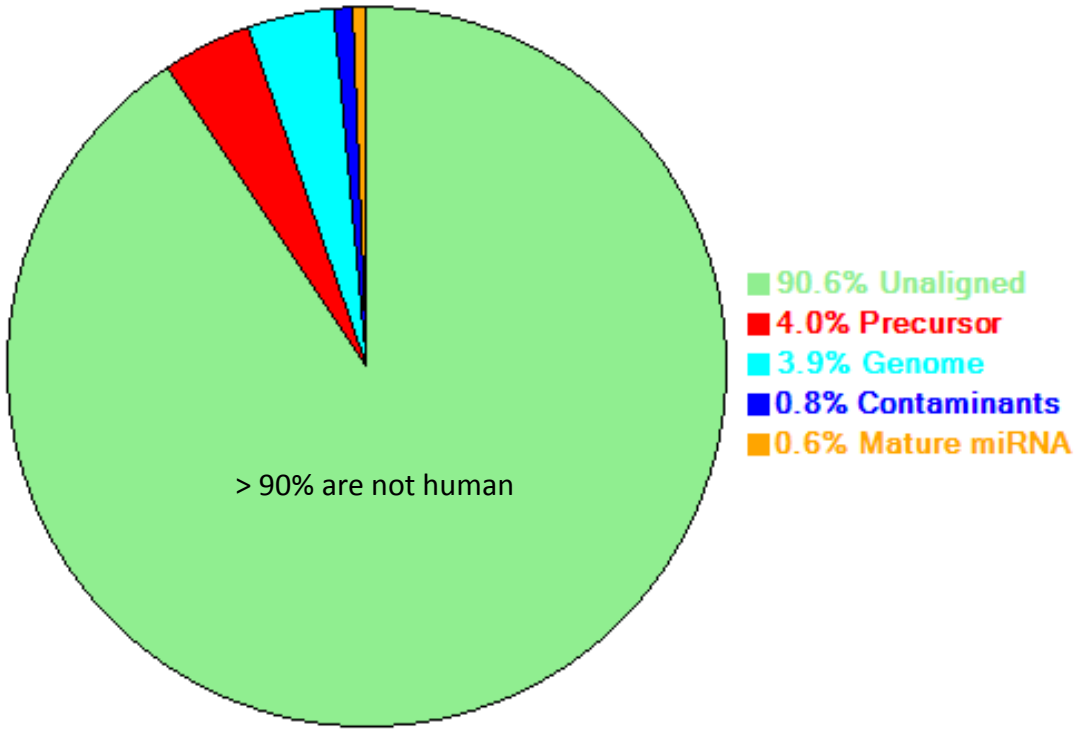
***NEUROSCIENCE 5e*, Figure 21.7**

The amygdala is considered the central 'hub' for regulation of social behavior, and is consistently changed in ASD

Reversibility of amygdala miRNA changes and behavioral deficits in rats suggest that brain-gut signaling could change, too

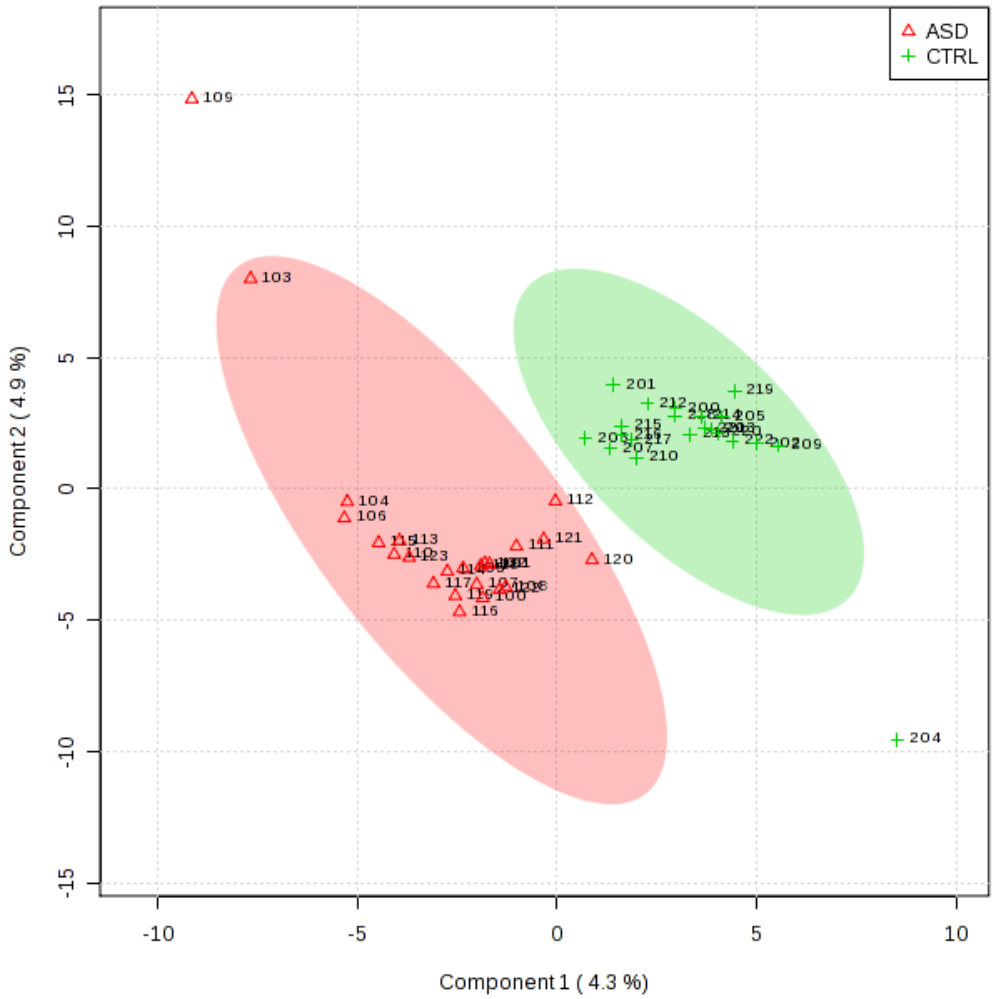
Was the oral microbiome changed in children with Autism Spectrum Disorder?

In our pilot study, the original goal was to identify and count miRNA…

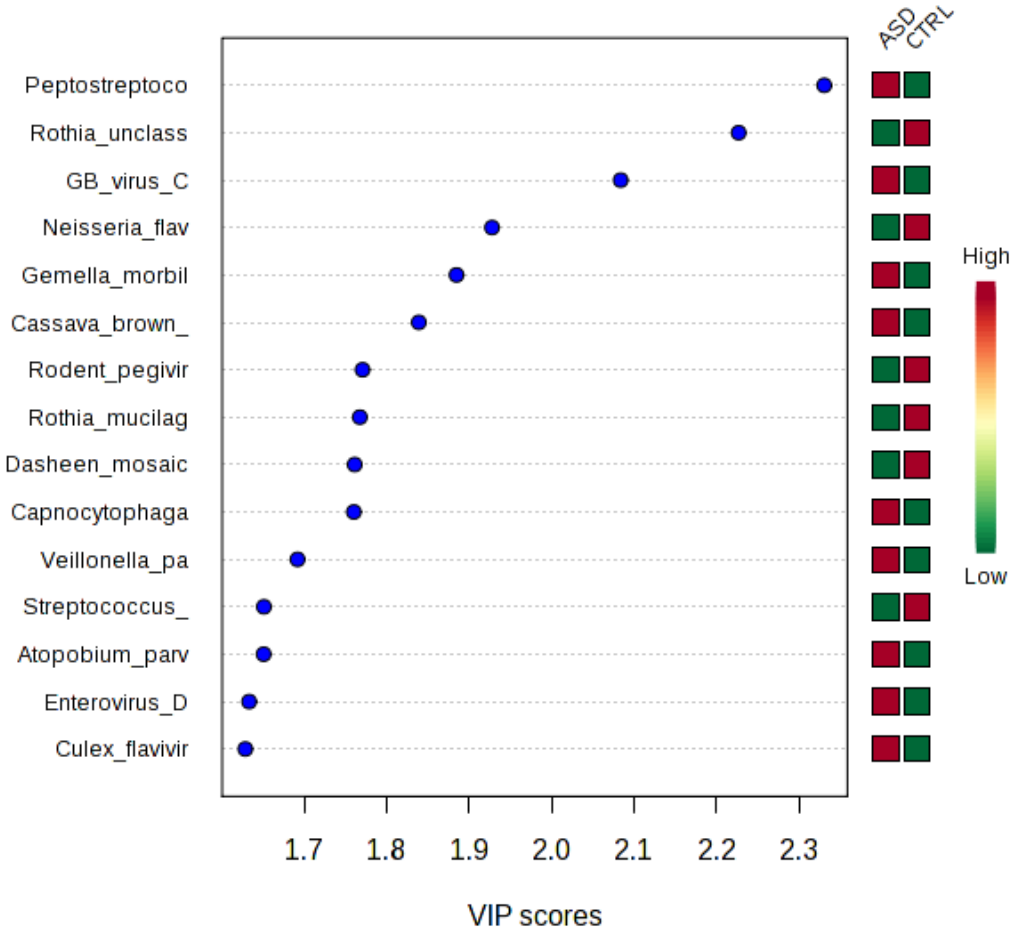


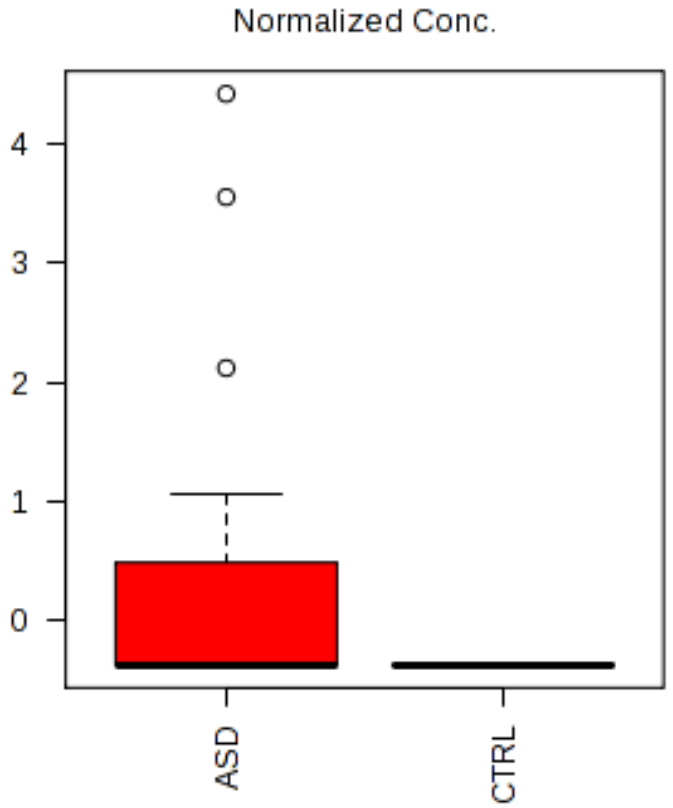
Then a funny thing happened on the way to the forum

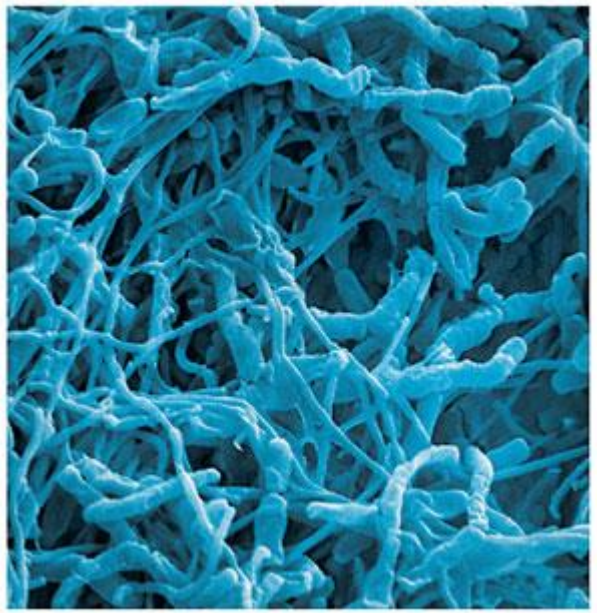
And microbiome data also separated subject groups



And the classifiers can be ranked by importance…







Note the top-ranked: *Peptostreptococcus*

Possible Relevance with PANDAS (Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococci)

Symptoms include: Compulsive behavior, tics, hyperactivity, inattention*, fidgetiness*, separation anxiety*, irritability*, sadness, emotional lability*, sleep disturbances*, night-time bed wetting, fine/gross motor changes, joint pain (source: NINDS)*

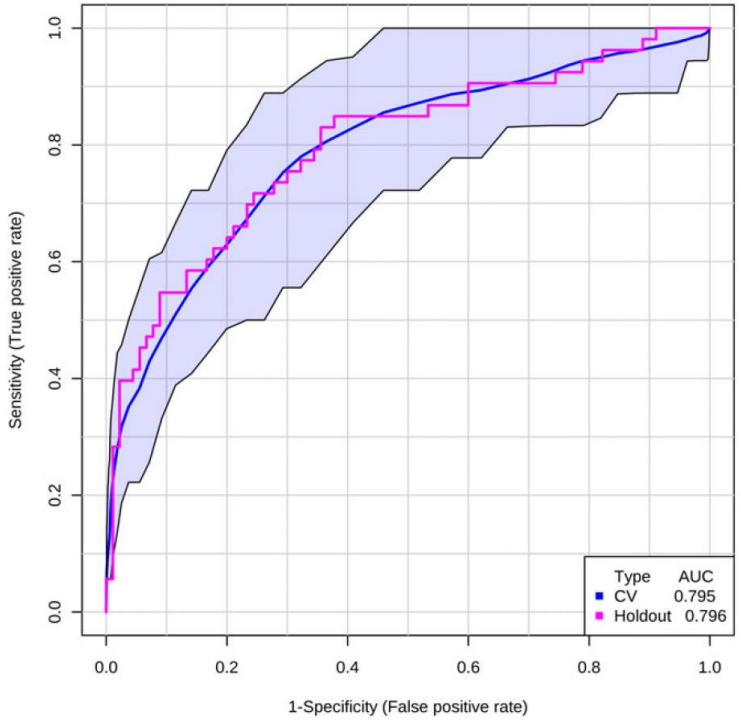
Next Step: Replication Study to Look at Oral Microbiome

- NIH funded recruitment of 346 children
- ASD, Typically Developing and Non-ASD Developmental Delay
- Focused on ages 18 months – 6 years of age
- Used swab instead of expectoration for saliva
- Same molecular methods as pilot study
- Two and three group comparisons of taxa, metabolic pathways and medical/demographic traits

Replication Study Participants and Performance

Clinical characteristics	ASD (n = 180)	TD (n = 106)	DD (n = 60)
Demographics			
Age, mean (SD), years	53 (16)	43 (16)*	50 (13)
Male (%), No.	154 (86)	64 (60)*	43 (70)*
Caucasian (%), No.	107 (59)	67 (63)	40 (67)
Body mass index (SD), kg/m^2	16.5 (2.8)	16.4 (2.0)	17.0 (3.1)
Oral/GI factors			
Time of collection (SD)	12:29 (2:48)	12:21 (2:43)	12:43 (2:38)
Time since last meal (SD), hr	3 (3)	3 (3)	2 (2)
Time of last tooth brush (SD), hr	8 (5)	5 (4)	5 (3)
Food/medical allergies (%), No.	38 (21)	9 (9)*	5 (8)*
Dietary restrictions (%), No.	25 (14)	8 (8)	11 (18)
Probiotic use (%), No.	5 (3)	0 (0)	2 (3)
GI disturbance (%), No.	39 (22)	3 (3)*	12 (20)
Medical characteristics			
Cesarean section (%), No.	35 (19)	9 (9)*	5 (8)*
Birth weight (SD), kg	3.3 (0.9)	3.2 (0.7)	3.2 (1.2)
Asthma (%), No.	18 (10)	8 (8)	10 (17)
Fully vaccinated (%), No.	169 (94)	97 (92)	58 (97)

5 ratios (of 8 different taxa)



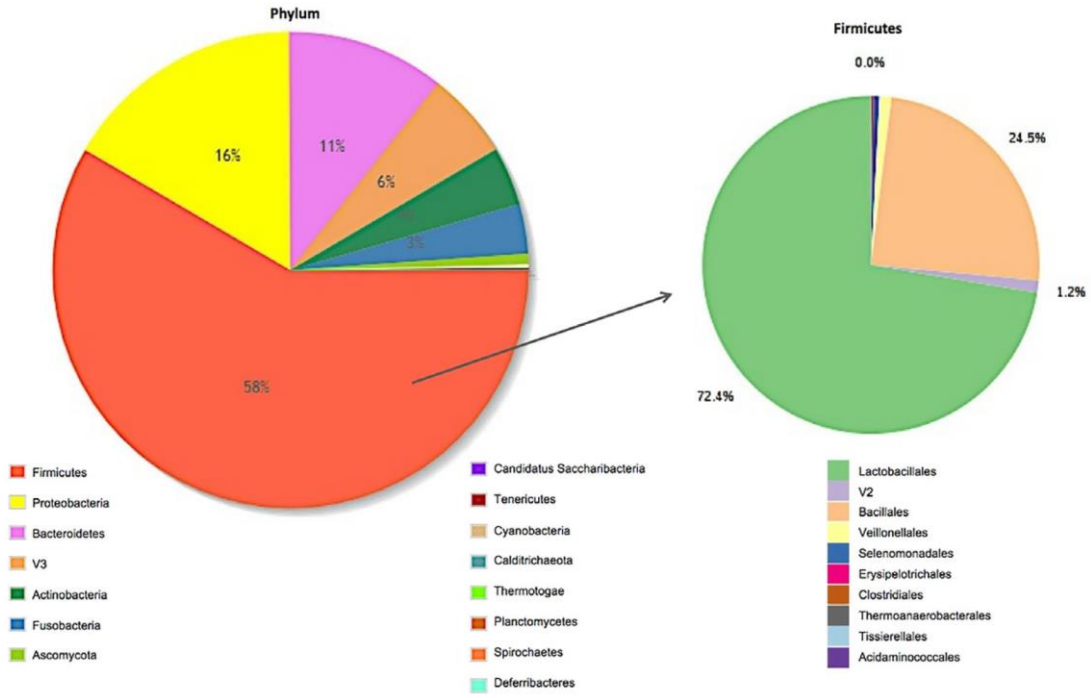
Correct Predictions:

139/180 ASD children

71/106 TD children

AUC = 0.80 in training and testing

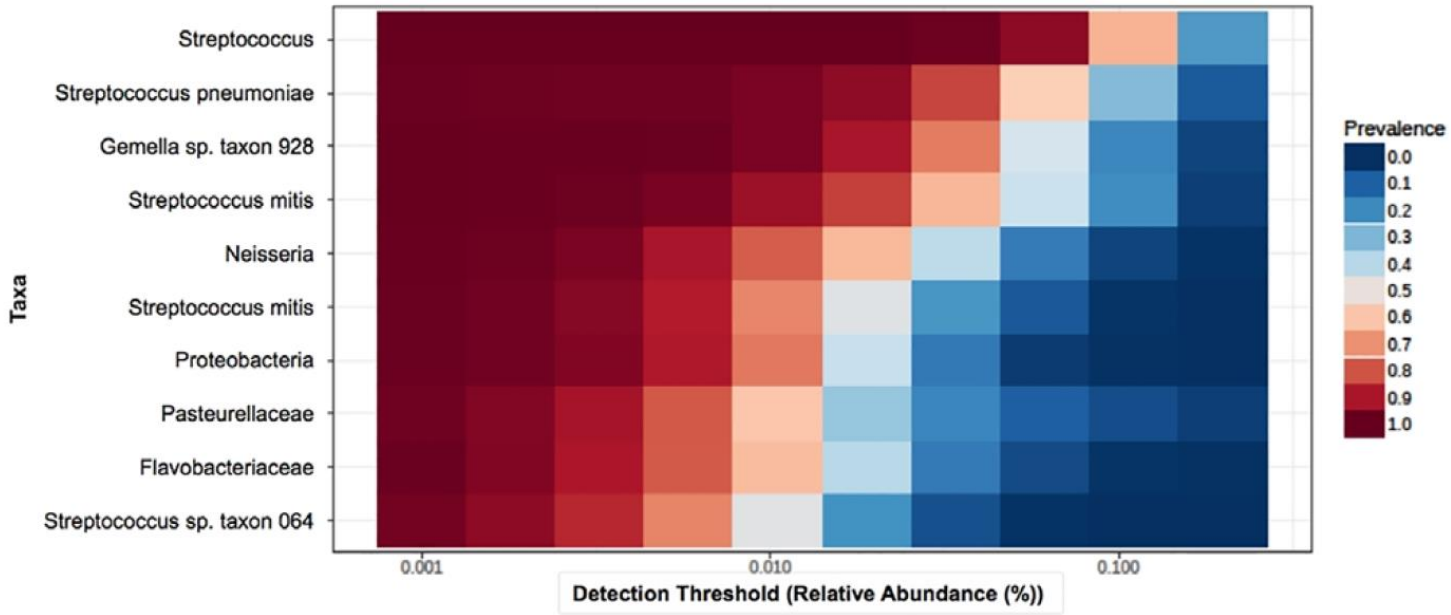
‘Core’ Oral Microbiome is Fairly Stable



Phyla Level:
Mostly Firmicutes

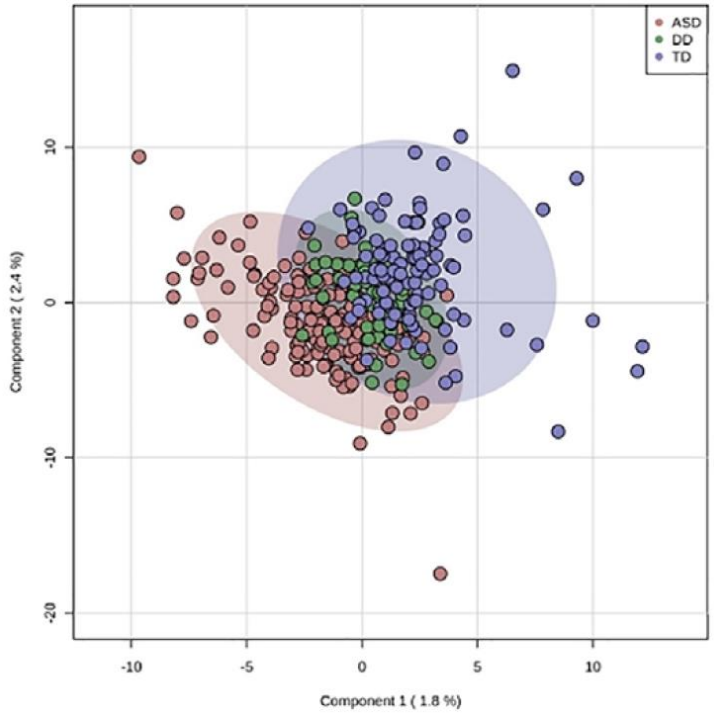
Class Level:
Mostly Lactobacilli

Species Level:
Mostly Streptococci



But Major Differences in Taxa and Bacterial Metabolism

Taxon	FC	FDR
ASD vs. TD		
Mucilaginibacter sp. PAMC 26640	0.17	0.001
R. tataouinensis TTB310	0.85	0.001
Limnohabitans sp. 63ED37-2	1.05	0.01
Planctomycetales	1.21	0.04
B. vulgatus	0.43	0.05
Gemmata sp. SH-PL17	0.86	0.05
Cyanobacteria	2.38	0.06
Bacteroides ovatus	0.23	0.07
Thiobacillus denitrificans ATCC 25259	0.53	0.10
Porphyromonas gingivalis TDC60	1.24	0.10
ASD vs. DD		
Brucella	2.79	0.05
Flavobacterium sp. PK15	0.41	0.05
E. faecalis OG1RF	2.27	0.05
C. minutus PCC 6605	0.62	0.11
Comamonas testosterone TK102	0.69	0.11
Pseudomonadaceae	0.77	0.11
Cellulomonas fimi ATCC 484	1.63	0.11
Flavobacterium psychrophilum	0.62	0.11
Flavobacterium crassostreae	0.74	0.11
M. luteus NCTC 2665	1.34	0.11



ASD
DD
TD

KEGG pathway	χ^2	FDR	Mann–Whitney
Energy metabolism	24.8	0.00047	ASD > TD ASD > DD
Translation ribosomal structure and biogenesis	18.2	0.0062	ASD > TD ASD > DD
Pyrimidine metabolism	15.8	0.013	ASD < TD ASD < DD
Lysine degradation	15.3	0.013	ASD > TD
Nucleotide metabolism	14.5	0.016	ASD < TD ASD < DD
Carbon metabolism	12.6	0.030	ASD > TD ASD > DD
Nucleotide transport and metabolism	12.6	0.030	ASD < TD ASD < DD

Also Differences Related to GI Disturbances in ASD Children

	Taxon	log2FC	P values
	Jonesia denitrificans	-1.01	0.000157
*	Fusobacterium nucleatum subsp. animalis	1.28	0.000138
	Negativicutes	0.83	0.000149
	Arthrobacter	1.46	0.000362
	Acidipropionibacterium acidipropionici	0.57	0.000235
	Tropheryma whipplei	0.76	0.000377
	Micrococcus luteus	0.50	0.000383
	Cellulomonas fimi	0.73	0.000445
	Riemerella anatipestifer	1.15	0.000835
	Arthrobacter sp. YC-RL1	0.57	0.000878
*	Campylobacter pinnipediorum subsp. pinnipediorum	1.05	0.000883
	Trueperella pyogenes	1.20	0.00096
	Gordonia	0.89	0.000717
	Veillonella parvula	0.41	0.000948
	Eubacterium eligens	0.74	0.000836
*	Campylobacter gracilis	1.50	0.000679

* Specifically increases proinflammatory cytokine expression and monocyte activation in colon

* Campylobacter is a very common cause of food-borne illness, GI distress, esp. in children

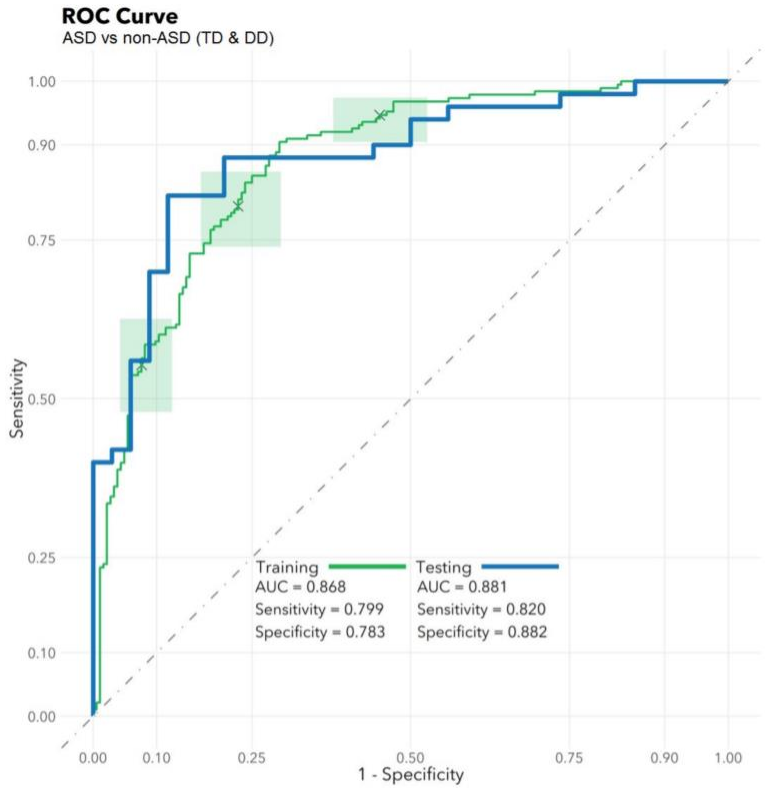
Conclusion: There is utility in profiling oral microbiome

Final Step: Large Replication Study Combining All Data Types

- 456 children
- ASD, Typically Developing and Non-ASD Developmental Delay
- Focused on ages 18 months – 6 years of age
- Used swab instead of expectoration for saliva
- Combined human RNA and microbial RNA and taxa in a robust Machine Learning Algorithm
  - Gradient Boosting Machine Learning with SVM
  - Training using 100-fold Monte Carlo Cross-Validation in 85% of Samples
  - True accuracy of classification model determined by testing in hold out set

Validation Study Participants and Performance

Clinical characteristics	All (*n* = 456)	Train set (*n* = 372)		Test set (*n* = 84)	
		ASD (188)	Non-ASD (184)	ASD (50)	Non-ASD (34)
Demographic					
Male sex, # (%)	337 (76)	156 (83)*	122 (66)	45 (90)	26 (76)
Mean age, mos (SD)	51 (16)	54 (15)*	49 (16)	53 (15)	46 (16)
White race, # (%)	296 (67)	122 (65)*	126 (69)	29 (58)	23 (68)
Medical					
BMI, kg/m^2 (SD)	16.7 (2.5)	16.6 (2.9)	16.8 (2.3)	16.6 (2.1)	16.7 (2.4)
Sleep disorder, # (%)	141 (32)	85 (45)*	31 (17)	21 (42)	8 (24)
ADHD, # (%)	63 (14)	41 (22)*	18 (10)	4 (8)*	0 (0)
GI diagnosis, # (%)	57 (13)	36 (19)*	16 (9)	7 (14)	1 (3)
Asthma, # (%)	43 (10)	16 (9)	18 (10)	6 (12)	2 (6)
Gestation, wks (SD)	38.6 (2.6)	39 (3)	39 (3)	38 (2)	39 (2)
fam hx, # (%)	172 (39)	93 (50)*	51 (28)	29 (58)	10 (29)
Behavioral					
VABS Comm (SD)	82.7 (22.8)	72.2 (20.1)*	93.5 (20.7)	73.5 (20.8)*	93.4 (18.2)
VABS Social (SD)	84.7 (22.7)	72.2 (16.4)*	97.4 (22.6)	73.5 (18.0)*	96.3 (15.0)
VABS Adaptive (SD)	84.8 (20.0)	74.9 (15.2)*	95.0 (20.0)	73.6 (18.9)*	96.6 (11.8)
ADOS, mean (SD)	6.1 (2.6)	6.7 (2.4)*	4.5 (2.9)	6.7 (1.6)*	3.2 (1.1)



Correct Predictions:

41/50 ASD children

18/21 TD children

12/13 DD children

This represents a positive predictive value of 91%

Sensitivity and specificity comparisons to other commonly used pediatric tests

		Sensitivity	Specificity	PPV
Diagnostic (at risk population)	**Saliva RNA**[1]	82%	88%	91%
	ADOS-II[2,3]	73%	71%	22%
	Rapid Influenza H1N1[6]	51%	98%	94%
	Rapid Antigen Group A Streptococcus[7]	85%	95%	N/A
Screening (general population)	**M-CHAT-R**[4]	91%	95%	14%
	Pure-Tone Hearing Screen[5]	50%	78%	8%

1. Hicks SD et al. *Front Genet.* 2018;9:534. **2.** Lord C et al. *J Psychoed Assess*. 2014;32(1):88-92. **3.** Maddox BB et al. *J Autism Dev Disord.* 2017 Sep; 47(9): 2703–2709. **4.** Robins DL et al. *Pediatrics.* 2014;133(1):37-45. **5.** Halloran DR et al. *Arch Pediatr Adolesc Med.* 2009. **6.** Chu et al. *Influenza Other Respir Viruses.* 2012. 6(2): 80–86. **7.** Stewart EH et al *Plos One.* 2014;9(11):e111727.

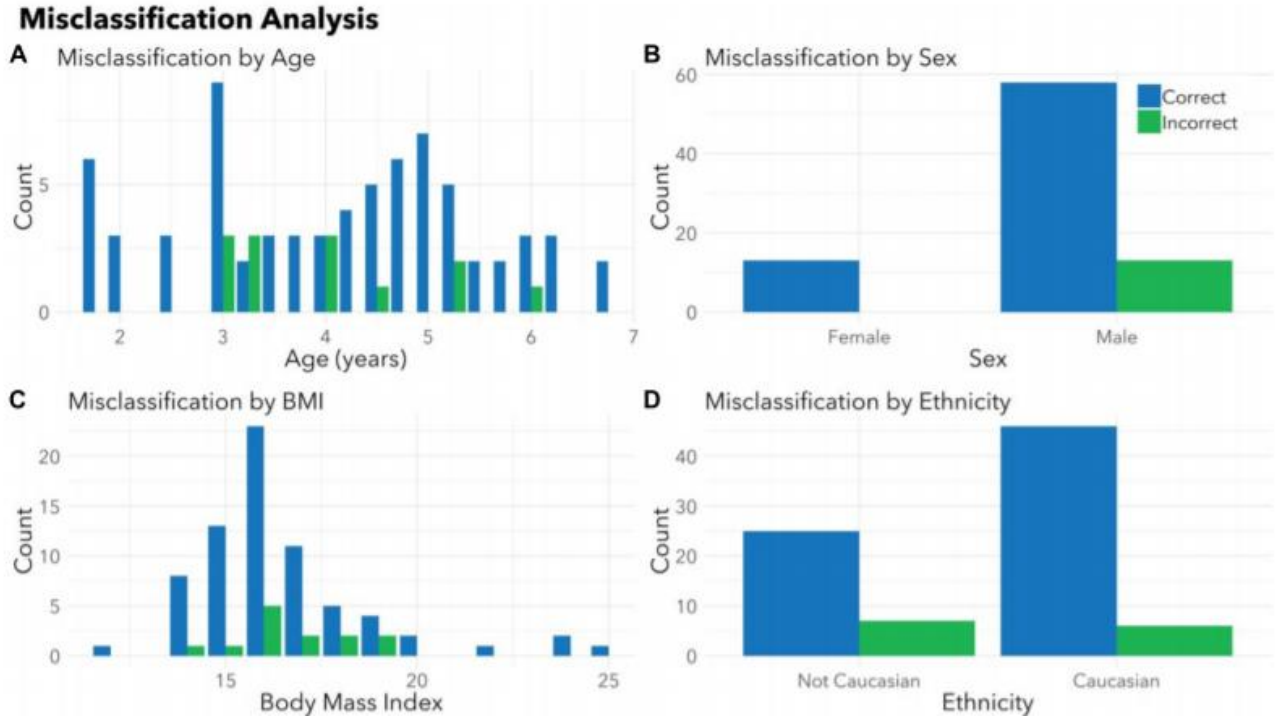
Validation Study Findings

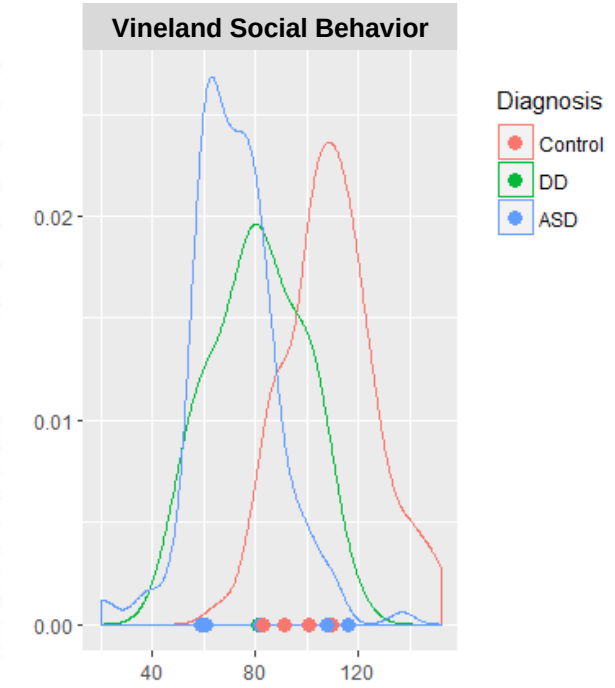
Incorrect Predictions:

9/50 ASD children

3/21 TD children

1/13 DD children





No discernible pattern related to Age, BMI, Sex, or Ethnicity

Conclusions

1) Saliva contains both miRNA and microbiome elements and sits at the interface of brain, gut, and immunefunctions
2) Salivary microbiome and miRNA profiles are altered in children with ASD and can be used to accurately differentiate children with ASD from their non-ASD peers (including both typically developing children and those with developmental delay)
3) miRNA and microbiome elements may have functional relationships with brain development pathways and other critical genetic and environmental variables
4) More work is needed to assess all factors that influence the salivary microenvironment, but it is a promising place to start

Ongoing Work

- Validate polyomic panel in additional 750 children ages 18 mos - 4 years with positive MChAT-R test from diverse areas (Syracuse, Pennsylvania, Ohio, Texas, Missouri, AND other sites – to confirm the findings)
- Confirm test-retest reliability after 12 months
- Develop measures of endophenotypes
- Combine with whole-exome sequencing
- Timeframe: August 2021
- Funded by NIH
  - $250K STTR Phase I award in 2016
  - $2.3M STTR Phase II award in 2018
  - $330K STTR supplement award in 2019

www.upstate.edu/autismstudy

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