Cerebral Palsy Research: On the Cutting Edge

CP Research Consortium of Michigan
3rd Biennial Conference

Sponsored by the Cerebral Palsy Research Consortium of Michigan, in collaboration with Michigan State University, University of Michigan, and Wayne State University.

USING GENE EXPRESSION IN ARCHIVED NEWBORN BLOOD SPOTS TO IDENTIFY ANTECEDENTS OF CEREBRAL PALSY

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QUICK CP EPIDEMIOLOGY: PREVALENCE

- Population-based studies based on registries in Europe and Asia find that CP prevalence at school age is between 1.5 – 2.5 cases per thousand live births
- This means that about one in 500 children has CP, or about 6,000 – 10,000 new cases each year in the US.
- No good population data in the US, as we have no population registries.

QUICK CP EPIDEMIOLOGY: TIME TRENDS

- Increased survival of very premature infants that began in the 1960's has not been matched by reductions in the survivor prevalence of CP.
- As a result a modest increase in the prevalence of CP was seen in most CP registries which may be leveling off now, as survival levels off, and possibly, as rates of CP drop in very premature survivors.
- One recent 4-state study suggests that US prevalence might be as high as 3.5/1,000

QUICK CP EPIDEMIOLOGY : TRADITIONAL RISK FACTORS

- PREMATURE BIRTH
  - 50 fold higher risk in infants < 28 wks.
- FETAL GROWTH
  - Moderate risk factor, especially at term. Not nearly as important as gestational age.
- BIRTH ASPHYXIA

  Not as important as once thought, because:
  - Some degree of birth asphyxia is very common, and most infants recover completely
  - Prenatally compromised infants often respond poorly to the stress of labor ; e.g. Down's syndrome babies have low Apgar scores.

QUICK CP EPIDEMIOLOGY : NEWER PUTATIVE RISK FACTORS

- COAGULATION
  - About 5-10% of CP is from perinatal stroke. It is plausible, but not proven, that some may have polymorphisms of the coagulation system
- THYROID HORMONES
  - Low thyroxine after birth a risk factor (not certain if causal) in preterm; possibly also at term. A syndrome which is a form of CP (neurologic cretinism) linked to iodine deficiency in endemic goiter areas.
- INFECTION/INFLAMMATION
  - Increasing evidence for a role of antepartum infection, especially in preterm birth (Fetal Inflammatory Response Syndrome - FIRS)

QUICK CP EPIDEMIOLOGY: PREVENTION

- HEAD, BODY COOLING
  - Head/body cooling for encephalopathic term newborns now established and is standard of care
- MAGNESIUM SULFATE
  - Several trials and observational studies show reduction in CP with use in premature labor
- CAFFEINE FOR APNEA IN PREMATURES
  - One trial showing halving of CP

R01 NS 055101: INTEGRATED MOLECULAR EPIDEMIOLOGY OF THE ORIGINS OF CEREBRAL PALSY (AKA THE OWL STUDY)

A NESTED CASE-CONTROL STUDY OF CEREBRAL PALSY

INITIATED IN SEPTEMBER 2009

Department of Epidemiology, Michigan State University

OWL

Origins, Wellness & Life-history in CP

Funded by National Institutes of Health

HYPOTHESIZED PATHWAYS STUDIED IN OWL AND CP SUB-TYPES

- Inflammatory
  - link to bilateral CP in prematures?
- Thyroid hormone
  - link to any CP in prematures?
- Hypoxic/ischemic
  - link to spastic quadriplegia?
- Coagulation
  - link to hemiplegia at term?

OWL DATABASE: PARTICIPANTS

- CP cases: Ages 2-16, born in MI, recruited from child neurology and CP clinics in Ann Arbor, Lansing and Grand Rapids
- Matched controls: birth year, gender, and gestational age (< 28;32-34;35-37;>37 weeks) principally from area primary care practices, and, for cases < 32 weeks, newborn follow-up programs.
- Siblings of cases: We recruit the nearest age sibling to cases for comparison.
- Twins: We make a special attempt to find twin sets discordant for CP.

OWL DATABASE: DATA COLLECTED

- For cases and controls, we get
  1. Maternal interview (pregnancy, reproduction)
  2. Permission to obtain birth certificates and maternal and infant hospital discharge abstracts from birth (available from Michigan Department of Community Health)
  3. Permission to obtain and study archived newborn blood spots from state.
  4. Permission to review medical records

SPECIAL RESOURCE FOR TWIN RECRUITMENT

- The Michigan Twin Registry
  - established in 2007
  - Supported by NIH grants to Department of Psychology at MSU (Kelly Klump PhD, PI)
  - Has surveyed > 5,000 twin births in Michigan from birth certificates
  - Intake questionnaire asks about CP

KEY RESOURCE

ARCHIVED BLOOD SPOTS LEFTOVER FROM NEWBORN SCREENING

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THE MICHIGAN BIOTRUST FOR HEALTH

- Since 1987, Michigan law has mandated storage of left-over blood from newborn genetic screening until age 21, and recently extended this requirement to permanent storage.
- Most spots are stored at ambient temperature.
- In 2008, this collection (N = 4 million specimens) was organized into the "Michigan Biotrust for Health", a research-usable archive, with photography and -80 freezing of all new specimens, and, eventually, all specimens.
- Consent for anonymous use has been requested since late 2010 from all mothers (70% consent).

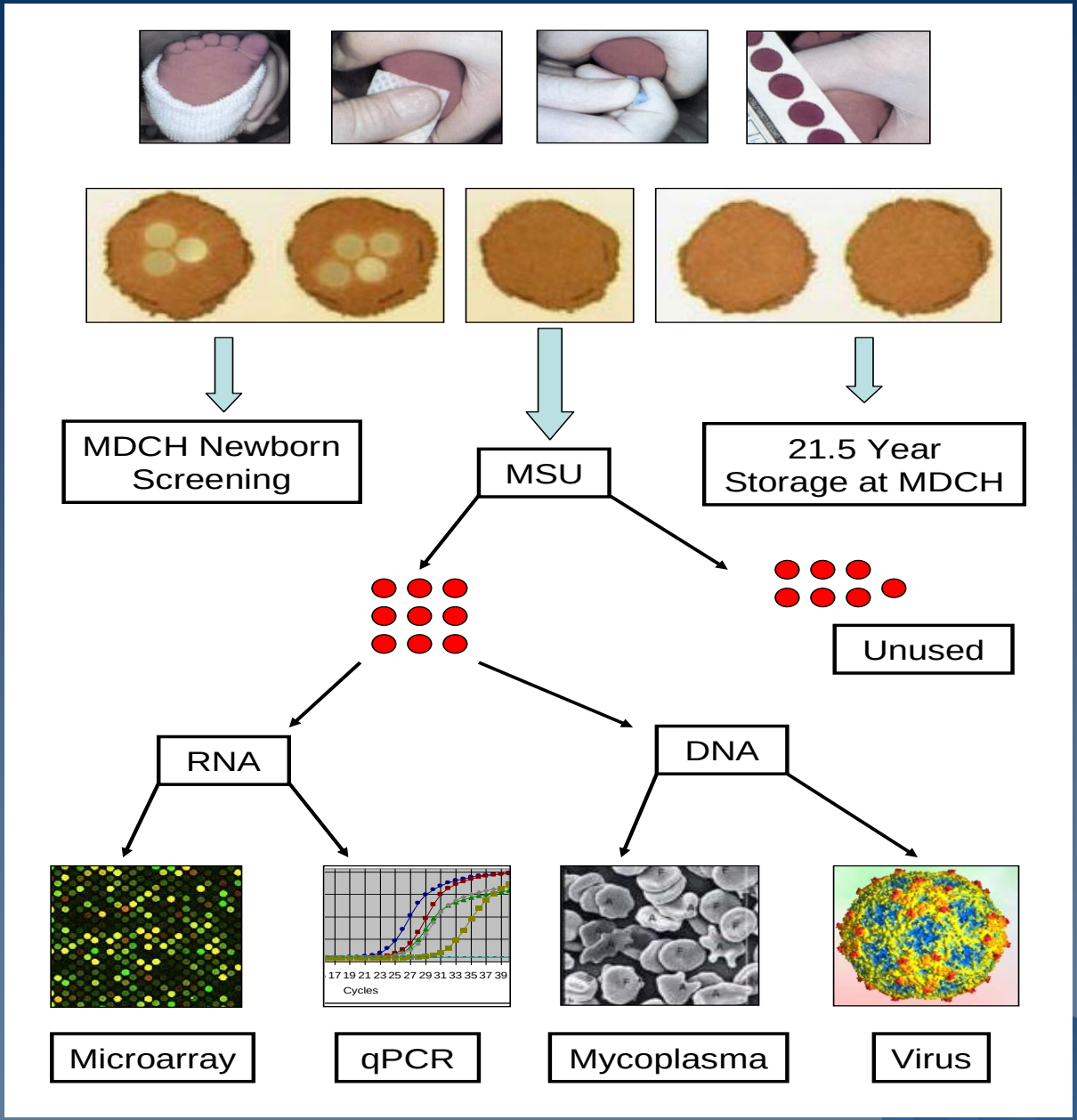
WHAT CAN BE STUDIED ON THESE ARCHIVED BLOOD SPOTS?

1. Proteins are not well maintained on unfrozen spots
2. Human DNA can be retrieved
3. Viral and bacterial DNA can be retrieved
4. Surprisingly, mRNA can also be retrieved, though with some degradation over time

WHAT ARE WE LOOKING FOR IN THE BLOOD SPOTS?

- Evidence of viral (CMV, HSV) DNA
- Evidence of mRNA expression profiles reflecting activation of four hypothesized pathways
- Other mRNA expression pathways that differ between cases and controls

What do we do with the archived newborn blood spot?



LABORATORIES INVOLVED

- MICROARRAY GENE EXPRESSION
  - Van Andel Research Institute, Grand Rapids, MI (James Resau PhD, Kyle Furge PhD, Sok-Kean Khoo PhD)
- qPCR mRNA VALIDATION
  - Department of Physiology, MSU (Julia Busik PhD)
- Viral DNA
  - Department of Pediatrics, University of Minnesota (Mark Schleiss MD, Yeon Choi PhD)

ALL SLIDES FROM THIS POINT ON ARE ABOUT NEWBORN BLOOD SPOTS

"THE VALUE OF THE TRANSCRIPTOME"

Department of Epidemiology, Michigan State University

BLOOD SPOT ANALYSIS IN OWL

- Our focus is on gene sets, or pathways, and not on individual gene analysis
- We also focus on pre-hypothesized pathways reflecting our ideas about what pathways are likely to be perturbed around birth in babies who later develop CP
- Available statistical algorithms for gene set analysis can be improved

NEW MICROARRAY TECHNOLOGY

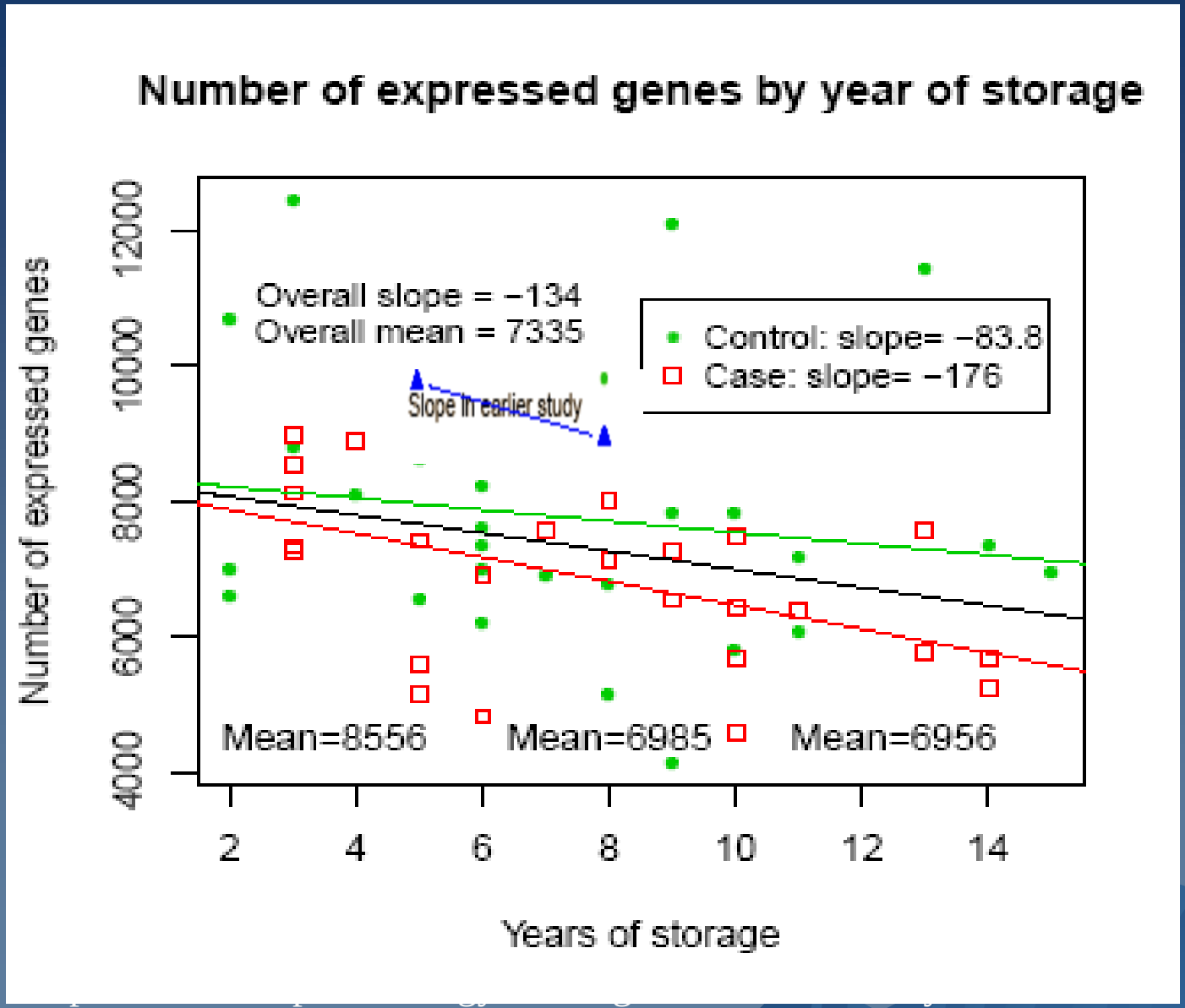
- Total RNA is extracted from three 3mm punches and concentrated using glass-fiber filter systems, and the WT-Ovation Pico RNA Amplification System (NuGEN Technologies) is used to generate single-stranded cDNA.
- Agilent Whole Human Genome Gene Expression 8x60K Microarray. This array has 60,000 oligonucleotide probes (60bp) covering 27,958 Entrez gene RNAs and 7,419 long intergenic non-coding RNAs.
- Amplification is initiated both at the 3’ end and randomly throughout the whole transcriptome.

VALIDATION OF MICROARRAY DATA

- Case and control spots always run together as pair without identification of status to lab.
- DNase treatment to eliminate DNA contamination
- After each 50 arrays, an additional assay is performed using commercial RNA samples specified in the MicroArray Quality Control Consortium (MAQC).
- RNA integrity number (RIN) must be > 2.0
- Correlation coefficient for VARI microarray analyses between technologists is 0.97.
- 1 µg cDNA is sent to Dr. Busik for qPCR analysis

IMPACT OF TIME

- The New Agilent technology in use in our study, but not in our pilot work, yields about twice as many genes, 7-8,000 compared to 3-4,000
- Gene expression was 18% lower in spots 6-10 years old than in spots 0-5 years old, with virtually no further decline in spots 10-14 years old.
- These results were slightly better, but not really different, from our pilot work


Number of expressed genes by year of storage
Number of expressed genes
4000
6000
8000
10000
12000
2
4
6
8
10
12
14
Years of storage
Overall slope = −134
Overall mean = 7335
Slope in earlier study
Control: slope= −83.8
Case: slope= −176
Mean=8556
Mean=6985
Mean=6956

GENE PATHWAYS

- Rather than study individual genes, we study pathways or gene sets representing families of genes related by functionality
- These pathways are derived either
  - Experimentally – gene sets that are expressed after exposure to an agent
  - Canonical or Curated – genes that are expected from prior knowledge to participate in similar functions
- We look for up or down regulation or bidirectional regulation of gene sets, summing activation of the individual genes in the set, compared to all genes expressed in the microarray

Seven Gene Sets Representing Four Hypothesized Pathways	
PATHWAYS (N OF GENES)	REPRESENTATIVE GENES
INFLAMMATORY	
• canonical (*n*=173) (0006954)	CCL2, IL-10, LY86, TLR2
• empirical (*n*=67) FIRS	LTB4R, ALOX5AP, CD11b
THYROID HORMONE	
• canonical (*n*=191) (V$T3R_Q6)	PAX1, RARG, HAS3, LMO4
• empirical (*n*=150)	PSMA4, REEP1, UTP3
ASPHYXIAL	
• canonical (*n*=38)	VEGFA, PDGFA, GLUT1, HK1
• empirical (*n*=59)	LOX, EGLN3, P4HA2, CNTNAP1
COAGULATIVE	
• canonical (*n*=43) (0007596)	F2, F4, PLG, CD36, CD59

SOME EARLY FINDINGS ON 53 PAIRS

Ho NT, Furge K, Fu W, Busik J, Khoo SK, Lu Q, Lenski M, Wirth J, Hurvitz E, Dodge N, Resau J, Paneth N: Gene expression in archived newborn blood spots distinguishes infants who will later develop cerebral palsy from matched controls. *Pediatric Research* 2012 Dec 26 (e-publication)

SOCIO-DEMOGRAPHIC CHARACTERISTICS OF 53 PAIRS OF CASES AND CONTROLS

	37 weeks and above		Under 37 weeks	
	Cases (n =33)	Controls (n =33)	Cases (n =20)	Controls (n = 20)
% Black	12%	3%	10%	20%
% Hispanic	0	4%	6%	0
% White or other	88%	93%	84%	80%
% Mother married	82%	82%	71%	82%
% Home owner	86%	68%	59%	71%
% Medicaid	21%	6%	25%	35%

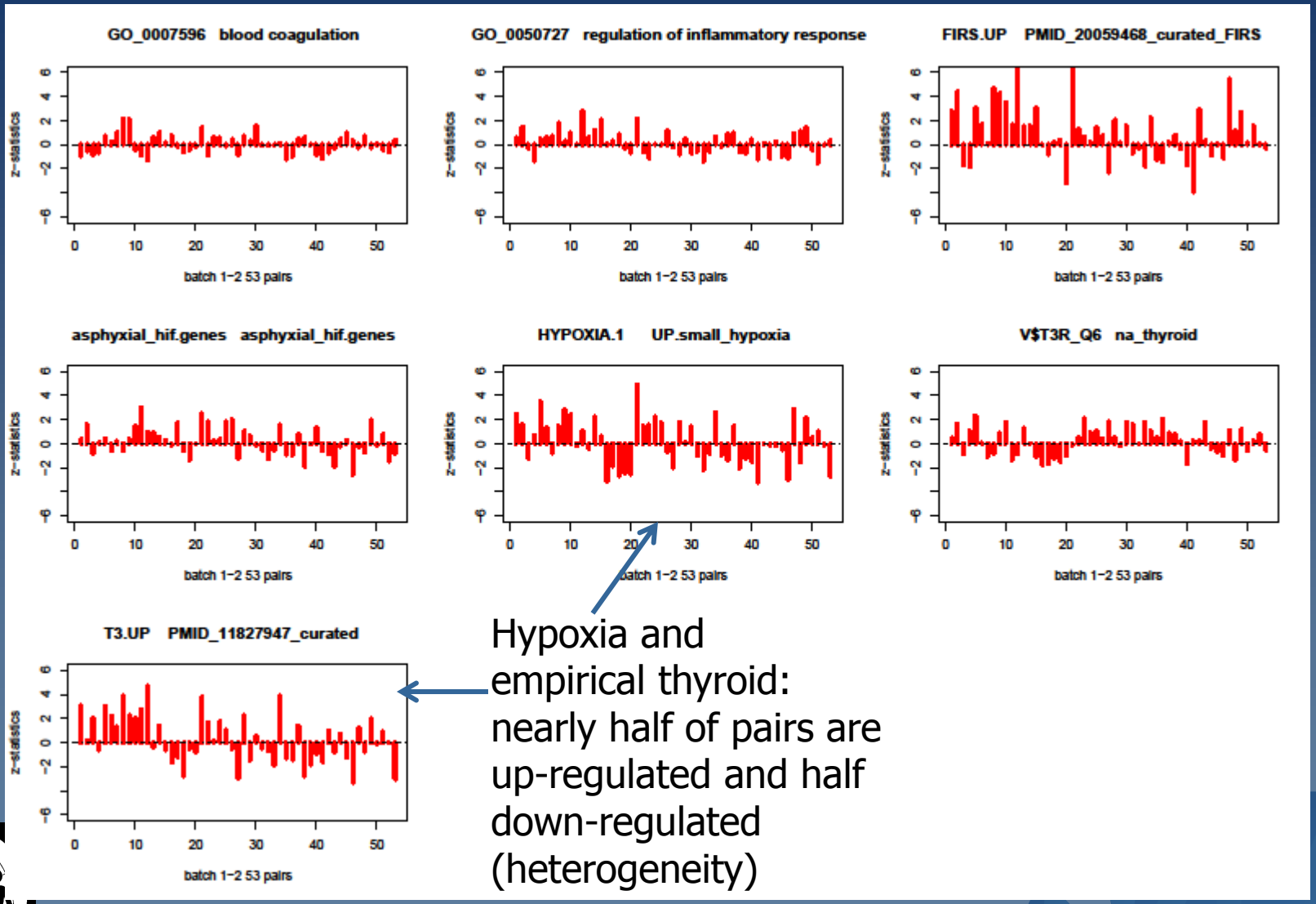
BIOLOGICAL/CLINICAL CHARACTERISTICS OF 53 PAIRS OF CASES AND CONTROLS

	37 weeks and above		Under 37 weeks	
	Cases (n =33)	Controls (n =33)	Cases (n =20)	Controls (n = 20)
Mean Gestational Age	39.5 wks	39.6 wks	30.2 wks	31.8wks
Mean Birthweight	3,372 g	3,562 g	1,884 g	2,106 g
Fetal Growth Ratio	0.99	1.04	0.95	1.01
Small for Gestation	9%	6%	10%	5%
5 min Apgar Score	**8.2**	**8.8**	**7.5**	**8.5**
% Admitted to NICU	**36%**	**9%**	95%	78%
Age blood spot obtained	1.7 days	1.3 days	1.5 days	1.2 days

Microarray Findings for Up, Down Regulation and Both for the Seven Hypothesized Pathways

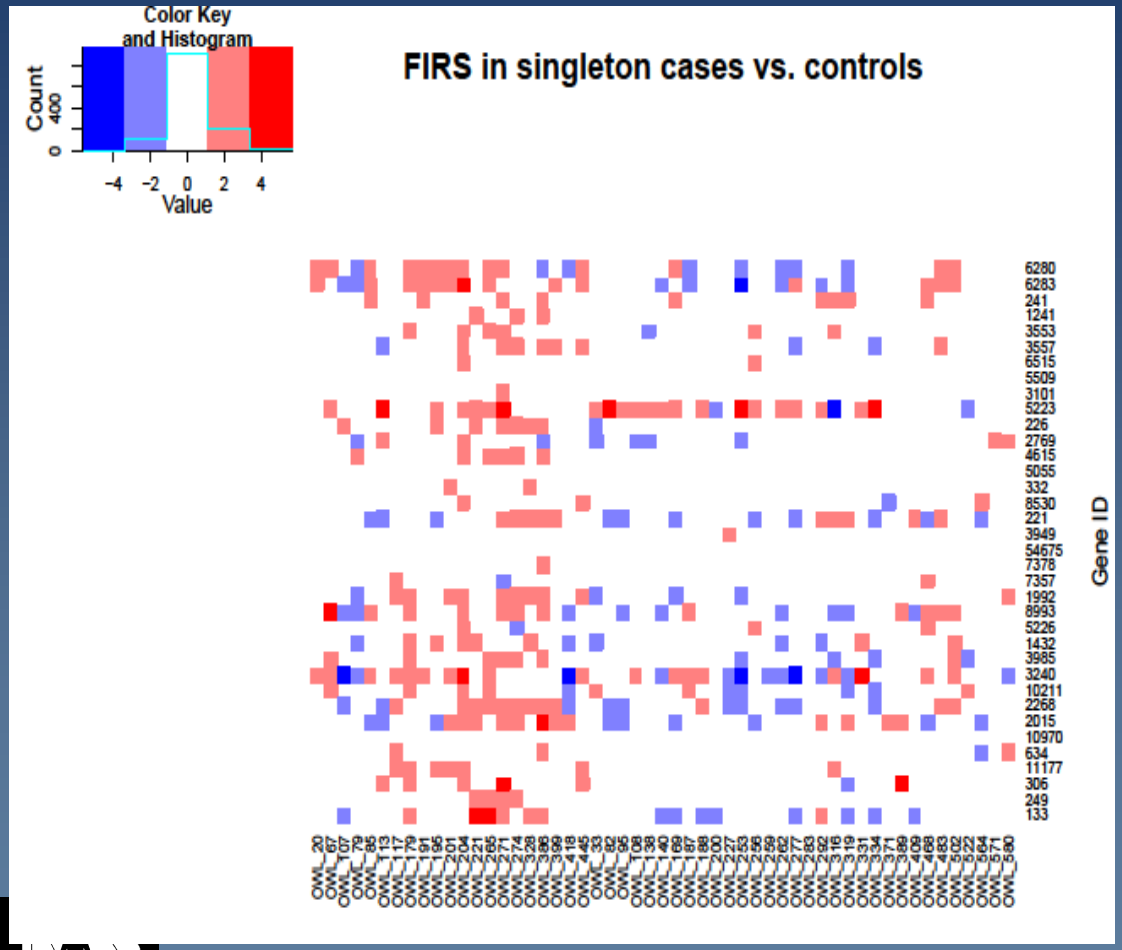
53 singleton case-control pairs				
PATHWAYS	Global Z	Effect size (Δ/σ)	UP	BOTH
Inflammatory				
Canonical	1.2	0.16	0.23	0.5
Empirical	6.71	0.92	<0.0001	<0.0001
Thyroidal				
Canonical	1.8	0.25	0.07	0.044
Empirical	2.2	0.3	0.027	<0.0001
Asphyxial				
Canonical	1.05	0.14	0.29	0.03
Empirical	0.77	0.1	0.44	<0.0001
Coagulative				
Canonical	0.36	.05	0.71	0.98

Z-STATISTICS OF RELATIVE MRNA EXPRESSION IN 53 CP CASE-CONTROL PAIRS: Seven pre-hypothesized gene sets reflecting four pathways.



HEAT MAP OF RELATIVE mRNA EXPRESSION IN 53 CP CASE-CONTROL PAIRS: Fetal Inflammatory Response Syndrome Gene set

(Madsen-Bouterse SA et al: Am J Reprod Immunol. 2010;63:73-92



Plots are log 2 fold changes between case and control for each gene for each pair:

Most FIRS genes are differentially up-regulated in singleton pairs.

The red zone correspond to the group of pairs with positive z-statistics.

Z-scores for the FIRS pathway under different conditions				
	N	Global Z-score	Effect size (Δ/σ)	GLOBAL P VALUE
LIMB INVOLVEMENT (N = 40)				
QUADRIPLEGIA	17	5.27	1.28	<0.0001
DIPLEGIA	13	4.03	1.12	<0.0001
HEMIPLEGIA	10	-0.88	-0.28	0.37
GESTATIONAL AGE (N = 53)				
< 32 WEEKS	12	1.52 (+0.44)	0.44	0.12
32 - 36 WEEKS	8	0.89 (+0.32)	0.32	0.37
≥37 WEEKS	33	7.1 (+1.24)	1.24	<0.0001

CONCLUSIONS

- The transcriptome is well preserved in filter paper blood spots, even after long periods of unfrozen storage, and can be used to assess perturbation of biological states of epidemiologic interest.
- Vast collections of archived newborn blood are kept by many states (Michigan – 4 million; California – 14 million frozen; NY ?) that can shed light on conditions with roots in pregnancy or the perinatal period
- We see strong evidence of enhanced perinatal inflammation in term-born symmetrical CP, and suggestions of disturbances of thyroid hormonal function and of asphyxial exposures.

THANKS FOR LISTENING

I'M HAPPY TO TAKE QUESTIONS

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