Biomarkers, environment and children's health

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Children are not “little adults”

Early Life Exposure to Pollutants in:

- Air
- Water
- Soil and Dust
- Breast Milk and Food
Disease Pathogenesis
- Genotoxicity
- Carcinogenesis
- Immunotoxicity
- Neurotoxicity

Environmental Exposures
- Air pollution
- Metals
- Pesticides
- Smoking/ETS
- Unhealthy Diet

Biomarkers

Cardiovascular Disease
- Asthma
- Respiratory Disease
- Cancer
- Adverse Birth Outcomes

Disease Outcomes
Biomarkers help link environmental exposures to disease outcomes

Types of Biomarkers

- Exposure
- Effect
- Susceptibility
Challenges of Biomarker Studies in Children

- Specific needs of study design
- Non-invasive sampling
- Biomarker validation for children
- Ethics beyond consent
- Translation of biomarker results into intervention strategy

Specimens need to be collected and processed in a variety of ways for biomarker studies:

- **Green Top (Heparin)**
  - 9 ml media
  - 1 ml WB
  - 0.5 ml x2 WB sta -80°C
  - 200 ul x6 Blood Smears 100ul, x2, -80°C
  - Remaining WB Ficoll Isolation 1340RPM, 30min

- **Red Top**
  - 3 ml
  - Centrifuge 1200RPM 10min

- **CA Harvest Protocol**
  - CA Tubes 1 ml 4°C
  - 1 ml Plasma -80°C
  - 1.8 ml x2 Lymph Cryo
  - 1.8 ml x2 Gran Cryo
  - 1 ml x2 RBC -80°C

- **CA Slides x10 -20°C**

- **MN Harvest Protocol**
  - MN Slides x10 -20°C
  - 1 ml
  - 9 ml media
  - 1 ml WB
  - 9 ml media
  - 1 ml WB
  - 1 ml WB sta -80°C
  - 200 ul

- **Comet Assay 100ul, x2, -80°C**

- **CA Tubes x1 4°C**

- **CA Slides x10 -20°C**

- **CA Harvest Protocol**

- **MN Harvest Protocol**

- **Red Top**
  - 3 ml
  - Centrifuge 1200RPM 10min
  - 0.5 ml x2 Serum -80°C
  - 0.5 ml x2 Clot -80°C

- **Blood Smears x6 -20°C**

- **CA Slides x10 -20°C**

- **Comet Assay 100ul, x2, -80°C**

- **CA Tubes x1 4°C**

- **CA Slides x10 -20°C**

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Specimens need to be collected and processed in a variety of ways for biomarker studies:
Biomarkers of exposure
Case study: Lead
Effects of lead poisoning in children (>10 μg/dl):

- learning disability
- lowered IQ
- mental retardation
- behavior problems
- heart disease

Symptoms:

- hyperactivity
- low attention span
- headaches
- irritability
- anemia
Decline in blood lead in Ontario children correlated with decreasing consumption of leaded gasoline, 1983-1992

Lead exposure in inner-city children

Melman et al, 1998 Env. Health Persp. 106; 655-657
Lead around the world

1. THAILAND
   - Bangkok
     - 5.6 ± 2.3 μg/dl (newborns)
     - 9.0 ± 3.6 μg/dl (secondary school)
   - Ruanphanchanas et al., 2002
   - Suepiantham, 2002

2. PHILIPPINES
   - Manilla
     - 11.8 – 49.9 μg/dl
     - 9.9 μg/dl (unexposed)
   - Suplido, Ong 2000

3. RUSSIA
   - Volgograd
     - 7.2 μg/dl
   - Ekaterinburg
   - Krasnouralsk
   - Rubinet et al. 2002

4. USA
   - National reference
     - 2.2 μg/dl
   - California (Latinos)
     - 0.92 μg/dl
   - CDC, 2002
   - Eskenazi et al. 2003
Runs better unleashed
Case Study: Pesticides

Does pesticide exposure affect children’s health?
What are Pesticides?

- substances used for *preventing, destroying, mitigating, or repelling* any pest.

- Includes:
  - Insecticides
  - Herbicides
  - Rodenticides
  - Fungicides

Environmental Protection Agency. http://www.epa.gov/pesticides/about/index.htm
Where are pesticides found?

- Agricultural Fields
- Pesticide Drift
- Fruits & Vegetables
- Household Products
- Public Housing
- Contaminated Water
- Schools

Children Often have Higher Pesticide Exposures

- Exploring environment
- Hand-to-mouth behavior
- Playing near the ground
- Children eat, drink, and breathe more
- Developing organs are more sensitive
- Children are smaller
- Children may be less able to detoxify pesticides

½ of lifetime pesticide exposure occurs in first 5 years of life

CHAMACOS Cohort (N=601)

- 96% within 200% poverty
- 96% Latina
- 44% worked in agriculture during pregnancy
- 84% have farmworker in household
- Children are followed from birth to age 9

http://ehs.sph.berkeley.edu/chamacos/
CHAMACOS Study Area

Agriculturally Applied Organophosphates
Average Annual Pounds
From 1999 through 2001

Pounds applied per PLS section (1 sq mi)
Zero
1 to 100 lbs
101 to 1000 lbs
1001 to 5000 + lbs

Major Highway
2000 ft concour

Source: California Dept. of Pesticide Regulation, the U.S. Geologic Survey and Geographic Data Technology Inc. April, 2003
Organophosphate Pesticide (OP) use in the Salinas Valley

500,000+ lbs used annually:

- Dimethyl (DM) phosphates
  ~220,000 lbs. (42%)
- Diethyl (DE) phosphates
  ~199,000 lbs. (38%)
- Other
  ~104,000 lbs. (20%)
Organophosphates (OP)

- Widely used in agriculture
- Degrade quickly (hrs-days)
- Acute neurotoxins
- Cholinesterase inhibition is the main mechanism of OP toxicity
Prenatal and Child OP Metabolites in CHAMACOS and National Reference*

Geometric Mean (95% CI) for Total OP Metabolites

- 1st pregnancy
- 2nd pregnancy
- NHANES* Women 18-40
- Child 6 month
- Child 12 month
- Child 24 month
- NHANES* Children 6-11

*National Health and Nutrition Examination Survey
CHAMACOS studies have shown:

- Substantially higher levels of pesticide metabolites in nearly all urine samples collected from low-income women in Salinas Valley, CA contained compared to national averages (Bradman et al., 2005, 2007)

- Decrease in gestational duration were associated with pesticide exposures (Eskenazi et al., 2004)

- Abnormal reflexes in infants (after first 3 days of life) were associated with prenatal organophosphate exposure (Young et al., 2005)

- Pervasive developmental disorder< autism-like complex of behavioral characteristics> (Eskenazi, 2009)
Biomarkers of effect
**CHOLINESTERASE**

- **Acetylcholinesterase (AChE):** AChE catalyses the rapid hydrolysis of acetylcholine (ACh) to acetate and choline.
- **Inhibited by OPs:** AChE may be inhibited by OP chemicals, resulting in acute neurotoxicity and death.
- **Widely used:** Widely used to monitor OP exposure in agricultural workers and other exposed groups.
- **Broad inter-individual variability:**
Association of Cord Blood Acetyl Cholinesterase and Length of Gestation

Low ChE correlates with:

- Low birthweight \((p=0.02)\)
- Premature birth \((p=0.007)\)

Eskenazi et al, 2004; EHP, 112, 10, 1116-1124
ChE levels in cord blood were significantly lower than levels in older children and levels in mothers (p<.001)

Small but significant differences in mothers levels at 26 wk versus delivery (p<.001)

No difference in ChE activity between children at 24 month and 60 month of age

In progress: nanosensors and ChE genomics
Case study: Cytogenetic damage
Cytogenetic Biomarkers:

- **Chromosome Aberrations**
  - Background Levels in Humans
  - 1% of cells with CA
  - 100 cells scored

- **Sister Chromatid Exchanges**
  - 5-8 SCE per cell
  - 25-50 cells scored

- **Micronuclei**
  - 5-8 MN per 1000 cells
  - 1000 binucleated cells scored
Cytogenetic Biomarkers: Second Generation

Interphase FISH

Metaphase FISH

Novel MN Analyses
Biomarkers of Effect: Chromosome Aberrations

From: Rossner 2002
Effect of Age on MN frequency in Lymphocytes

- Strong age effect in adults
- Effect in children unclear

Bonassi et al. EMM. 37: 31-45 (2001)
MN Frequency in Exfoliated Cells

- MN levels are similar in children and adults of inner city (p=0.4)
- Broad inter-individual variability, ~3-fold for children, and ~2-fold for adults

From: Huen et al, 2006
Inner City Oakland
Mothers and Children: Effects of air Pollution

Huen et al. 2006. EMM.
• Regional O₃ levels were highly correlated with season by month ($r^2=0.84$, $P=0.02$)

• O₃ levels were associated with MN frequency (FR=3.37) in both exfoliated and lymphocyte cells in children and adults.

Huen et al. 2006. EMM.
Cytogenetic Damage

**Increase**

- **Ozone levels**
  - Mothers: FR=3.37 (p=0.01)
  - Children: FR=13.50 (p=0.04)
- **Traffic-related air pollution in children:** FR= 3.33 (p=0.05)
- **Smoking in the household**
  - Mothers: FR= 1.05 (p=0.08)
  - Children: FR= 1.09 (p=0.05)

**Decrease**

- **Vitamin use in mothers:** FR=0.17 (p=0.10)
- **Gas appliances, mothers:** FR=0.40 (p=0.12)

*Huen et al. 2006. EMM.*
Biomarkers of Susceptibility
Paraoxonase (PON1)
Individual Susceptibility to OPs Varies by Paraoxonase (PON1) Activity

- PON1 enzyme detoxifies OPs in the body and protects AChE from inhibition
- Detoxification depends on quantity and efficiency of enzyme

Source: Chambers, PNAS 2008
**PON1 Activity is Determined by Genetic Make-up**

- *PON1* gene located on the long arm of chromosome 7
- Gene has multiple polymorphisms (SNPs)

*PON1*<sub>-108</sub>

- Total quantity of enzyme
- C allele > T allele

*PON1*<sub>192</sub>

- Catalytic efficiency of enzyme
- R allele > Q allele
Ethnic Differences in PON1 gene frequency

PON1<sub>Q</sub> allele frequency

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Value</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whites</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Hispanics</td>
<td>0.59</td>
<td>(Seattle)</td>
</tr>
<tr>
<td>Asians</td>
<td>0.4</td>
<td>(Salinas)</td>
</tr>
<tr>
<td>African-Americans</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>
Functional Genomics of Paraoxonase 1 (PON1)

Oxidative Stress
- cardiovascular disease
- diabetes
- rheumatoid arthritis

OP Sensitivity
- PON1 can hydrolyze oxon derivatives of OPs
- Animal models provide evidence of a protective role of PON1
PON and OP Pesticide Susceptibility

PON1 Findings:

Genetic variability (SNPs and haplotype) has greater effect in children than adults

Diazinon: 26-fold difference in susceptibility among newborns
Some newborns are up to 65 times more susceptible than adults with highest enzyme levels.

Chlorpyrifos: 50-fold difference in susceptibility among newborns
Some newborns are 130-164 times as susceptible as highest adults.

Current pesticide standards may not protect the most vulnerable.


Effects of Age

- PON1 levels in children up to 7 years of age were lower than those of adults (p<0.005).

Holland et al. 2006, Huen 2009
Genetic Influences on PON1 Ontogeny

- *PON1 192* genotype significantly modified the effect of age on POase activity ($p<0.0001$)
PON1 and Birth Outcomes
Health effects of PON1

• Association of low PON1 enzyme activities in mothers with small head circumference in neonates in New York (*Berkowitz et al.*, 2003; 2005)

• Low PON1 in children with autism (*Pasca et al.* 2006)

• PON1 involved in lipid peroxidation and related health outcomes such as cardio-vascular and neuro-degenerative diseases (*rev. in Costa and Furlong*, 2002)

• Associated with male infertility (*Padungtod et al.*, 1999)

• PON1\textsubscript{192} associated with complications and outcomes of pregnancy (*Chen et al.* 2004; *Lawlor et al.*, 2006)
Child PON1-108 Genotype and Fetal Growth (N=433)

Length of Gestation

Birth Weight

Length

Head Circumference

Child -108

<table>
<thead>
<tr>
<th>Length of Gestation</th>
<th>Birth Weight</th>
<th>Length</th>
<th>Head Circumference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>N=76</td>
<td>P &lt; 0.05</td>
<td>N=433</td>
</tr>
<tr>
<td>CT</td>
<td>N=236</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>N=121</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Child PON1\textsubscript{192} Genotype and Fetal Growth (N=437)

- **Gestational Age**:
  - QQ, QR, RR
  - N= 108, 222, 107

- **Birth Weight**: 3280, 3300, 3320, 3340, 3360, 3380, 3400, 3420, 3440, 3460, 3480, 3500 grams

- **Length**: 49.75, 50, 50.25, 50.5 cm

- **Head Circumference**: 33.75, 34 cm

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Variability and Vulnerability: Genetic Effects on Birth Outcomes

Effects of Single SNPs (child genotypes):
2 SNPs were associated with preterm birth (N=470):
- $PON1_{192}QQ$ vs $PON1_{192}RR$ (OR: 3.52, $p=0.03$)
- $PON1_{108}TT$ vs $PON1_{108}CC/CT$ (OR: 2.62, $p=0.02$)

Effects of 2 SNPs in combination (child genotypes):
$PON1_{192}QQ/PON1_{108}TT$ vs $PON1_{192}RR/PON1_{108}CC$
- Shorter gestational age ($\beta=-1.03$, $p=0.006$)
- Smaller head circumference ($\beta=-0.90$, $p=0.007$)
Genetic Effects beyond SNPs: Haplotypes Effects on Gestational Age

<table>
<thead>
<tr>
<th>Child PON1 Haplotype</th>
<th>Frequency</th>
<th>Beta Coefficient</th>
<th>95%CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference G G C T R</td>
<td>27.2%</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>7 G A T T Q</td>
<td>1.1%</td>
<td>-2.28</td>
<td>(-3.35,-1.21)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>5 G A C T Q</td>
<td>11.1%</td>
<td>-0.37</td>
<td>(-0.74,0.005)</td>
<td>0.053</td>
</tr>
<tr>
<td>3 C G T T Q</td>
<td>13.6%</td>
<td>-0.33</td>
<td>(-0.72,0.05)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

9 of the most common haplotypes (5 PON1 SNPs) were examined for associations with gestational age.

Compared to the most frequent haplotype (Reference), haplotype 7 was associated with shorter gestational duration.

N=440
CHAMACOS Findings and Conclusions

- Pesticide exposures are widespread in CHAMACOS agricultural minority cohort
- Newborn children have lower enzyme levels (PON1, ChE) than their mothers and are more vulnerable to exposures
- Broad age- and genotype-dependent variability in susceptibility to pesticide exposure in women and children of CHAMACOS cohort
- Pesticides are associated with adverse growth and neurodevelopment outcomes
Future Directions of Biomarker Studies in Children

- Diverse ethnic and age groups
- Sophisticated collection of biological and environmental samples
- Development and application of novel biomarkers
- Longitudinal multidisciplinary programs
- Comprehensive exposure assessment: from exposure to “exposone” (*C. Wild, 2005*)
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