ANALYSIS OF CRANIOFACIAL MORPHOLOGY IN A TNAP KNOCKOUT MOUSE MODEL

John DuRussel MS, DDS, MS

CRANIOSYNOSTOSIS
- Premature fusion of cranial bones
- 1/2500 live births
- May involve one or multiple sutures
- Isolated (85%) or Syndromic (15%)
- Biologic process unknown

Infant with craniosynostosis

- Elevated intracranial pressure
- Impaired cerebral blood flow
- Airway obstruction
- deafness, blindness, seizures
- Developmental delay and learning disabilities
- Esthetic compromises

CURRENT TREATMENT: CRANIAL VAULT REMODELING SURGERY
- Excise prematurely fused sutures and correct calvarial deformities
- Initial surgery at 3-6 months old to allow for exponential brain growth

HYPOPHOSPHATASIA (HPP)
- Deficiency of Tissue Non-specific Alkaline Phosphatase (TNAP)
- Disrupted mineralization of the skeleton and dentition (weak bones and teeth)
- Seizures due to poor Vitamin B metabolism
- Craniosynostosis in the context of low bone density (40% of patients)
- Death (respiratory dysfunction due to weak rib bones)

ENPP1 AND TNAP CONTROL MINERALIZATION
- Ectonucleotide pyrophosphatase/phosphodiesterase-1 (Enpp1) - the primary osteoblastic generator of pyrophosphate (PPi)

ENPP1 AND TNAP CONTROL MINERALIZATION
- Tissue non-specific alkaline phosphatase (TNP) hydrolyzes PPi to Pi, which is essential to the growth of hydroxyapatite crystals.
ENPP1 AND TNAP CONTROL MINERALIZATION

Deposition of calcium pyrophosphate dihydrate crystals

ATP → Enpp1 → PPi → TNAP → P1

Matrix

Osteoblast

ENPP1 AND TNAP CONTROL MINERALIZATION

Deposition of calcium pyrophosphate dihydrate crystals

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Matrix

Osteoblast

Qualitative Craniofacial Phenotype of the TNAP−/− Mouse Model of Infantile Hypophosphatasia

WT

TNAP−/−

Open cranial suture in P20 wild-type mouse

Fused/closed cranial suture in TNAP KO mouse

• shape abnormalities
• hypomineralization
• craniosynostosis

2D Shape Abnormalities in TNAP−/− Mouse Model of Infantile Hypophosphatasia

2D linear craniofacial measurements (digital calipers)

Age = P15

WT = white
KO = black

Age = P30

WT = white
KO = black

2D Shape Abnormalities in TNAP−/− Mouse Model of Infantile Hypophosphatasia

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TNAP Enzyme Replacement Therapy

Hypophosphatasia

Current regimen of TNAP enzyme replacement therapy

Revised earlier regimen of TNAP therapy

Survivals, normal becom strong bones, minimal effects

Surgeries to alleviate craniosynostosis

SPECIFIC AIMS

1. Determine if TNAP−/− mice exhibit craniofacial morphologic abnormalities similar to those seen in patients with infantile hypophosphatasia (TNAP deficiency)
   • Genotype comparison
   • HPP long bone phenotype subset comparison

2. Establish the timing of onset of craniosynostosis in the TNAP−/− mouse model of infantile hypophosphatasia

HYPOTHESES

• TNAP−/− mice have craniofacial skeletal shape abnormalities similar to those seen in human infants with hypophosphatasia

• TNAP−/− mice have an increased incidence of craniosynostosis

Whyte et. al., 2012

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Whyte et. al., 2012
METHODS

SAMPLE
- Mixed genetic background
- Age
  - 15 day old (P15)
  - 20 day old (P20)
- Genotype
  - TNAP<sup>−/−</sup>
  - Wild type
- TNAP<sup>−/−</sup> Clinical Phenotype
  - Normal, slight, moderate, severe

MICRO-COMPUTED TOMOGRAPHY (MICRO-CT)
- Whole dissected calvaria
- Skulls scanned at 18 micron resolution
- Three dimensional images reconstructed at 18 cubic micron effective voxel size
- Craniosynostosis assessment
- Craniofacial shape assessment

CORONAL SUTURE FUSION ASSESSMENT
- Coronal sutures viewed on two-dimensional slices of micro-CT scans
- Verified by visualization under a dissecting microscope
- Comparison between TNAP<sup>−/−</sup> and Wild Type mice

CORONAL SUTURE FUSION ASSESSMENT

3D MORPHOMETRIC CRANIOFACIAL ANALYSIS
- Pre-established 3D landmarks utilized to analyze differences in craniofacial form, shape, and growth with micro-CT imaging and Dolphin
MORPHOMETRIC CRANIOFACIAL ANALYSIS
BY EUCLIDEAN DISTANCE MATRIX ANALYSIS

- Allows for quantification and comparison of 3D form (size and shape), shape and growth differences between sample groups
- Uses x, y, z landmark coordinate data (Dolphin) for statistical comparison of linear distances between every landmark placed as ratios between groups

NORMALIZATION OF 3D LINEAR MEASUREMENTS

TNAP−/− AND TNAP+/− MICE GROUPING BY PHENOTYPE FOR 3D MORPHOLOGICAL ANALYSIS

STATISTICS
- Rater reliability tests for 3D landmark placement
- Fischer’s exact test—coronal suture fusion assessment
- EDMA – form, shape, growth
- Mixed model pairwise comparison with Tukey’s test—comparison between genotypes and across phenotypes with combined information from all linear distances (normalized measurements)
- Principle Component Analysis—summarize all linear measurements by the most contributory variables (normalized measurements)

RESULTS
Craniosynostosis Assessment with Micro-CT

Coronal suture synostosis in ~34% of 20 day TNAP<sup>−/−</sup> mice

**EUCLIDEAN DISTANCE MATRIX ANALYSIS: SIGNIFICANT FORM DIFFERENCES**

<table>
<thead>
<tr>
<th>Group Comparisons</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P15 Wild Type vs. TNAP&lt;sup&gt;−/−&lt;/sup&gt; Mice</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>P15 Wild Type vs. Severe TNAP&lt;sup&gt;−/−&lt;/sup&gt; Phenotype</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>P20 Wild Type vs. TNAP&lt;sup&gt;−/−&lt;/sup&gt; Mice</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>P20 Wild Type vs. Severe TNAP&lt;sup&gt;−/−&lt;/sup&gt; Phenotype</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>P15 Wild Type vs. Slight TNAP&lt;sup&gt;−/−&lt;/sup&gt; Phenotype</td>
<td>0.013</td>
</tr>
</tbody>
</table>

*Statistically significant if confidence intervals do not cross zero; α = 0.05*

- Significant form difference between genotypes at both 2 and 3 weeks old
- Significant form difference between phenotypes at both 2 and 3 weeks old
  - Severe phenotype vs. wild type - significant differences
  - Other phenotypes vs. wild type - not statistically different, other than that shown

**EUCLIDEAN DISTANCE MATRIX ANALYSIS: SIGNIFICANT SIZE AND SHAPE DIFFERENCES**

<table>
<thead>
<tr>
<th>Group Comparisons</th>
<th>Confidence Interval—Size</th>
<th>Confidence Interval—Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>P15 WT vs. TNAP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.488 - 0.190</td>
<td>0.300 - 0.089</td>
</tr>
<tr>
<td>P15 WT vs. Severe TNAP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1.155 - 0.801</td>
<td>0.671 - 0.483</td>
</tr>
<tr>
<td>P20 WT vs. TNAP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1.057 - 0.588</td>
<td>0.319 - 0.163</td>
</tr>
<tr>
<td>P20 WT vs. Severe TNAP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1.791 - 1.635</td>
<td>0.660 - 0.589</td>
</tr>
</tbody>
</table>

*Statistically significant if confidence intervals do not cross zero; α = 0.01 or the 99% confidence interval*

- Significant size and shape difference between genotypes at both 2 and 3 weeks
- Significant size and shape difference between wild type mice and the severe TNAP<sup>−/−</sup> phenotype at both 2 and 3 weeks old

**EUCLIDEAN DISTANCE MATRIX ANALYSIS—SIGNIFICANT GROWTH DIFFERENCES**

**MIXED MODEL PAIRWISE COMPARISON OF 3D CRANIOFACIAL LINEAR DISTANCES BY GENOTYPE**

15 day old Wild Type vs. TNAP<sup>−/−</sup>

<table>
<thead>
<tr>
<th>3D Linear Measure</th>
<th>Difference (mm)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal bone length</td>
<td>-0.19</td>
<td>0.04</td>
</tr>
<tr>
<td>Skull width</td>
<td>0.19</td>
<td>0.06</td>
</tr>
<tr>
<td>Skull height</td>
<td>0.20</td>
<td>0.02</td>
</tr>
</tbody>
</table>

α = 0.05

- Significantly shorter frontal bone length
- Significantly larger skull width and height

20 day old Wild Type vs. TNAP<sup>−/−</sup>

<table>
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<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal bone length</td>
<td>-0.40</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

α = 0.05

- Significantly shorter frontal bone length

**EDMA SUMMARY**

- Significant difference in form, size, and shape between genotypes at both two and three weeks of age
- Significant difference in form, size, and shape between Wild Type mice and the Severe TNAP<sup>−/−</sup> subset at both two and three weeks of age
- Significant difference in the growth pattern from two to three weeks of age between the genotypes
- Significant difference in the growth pattern from two to three weeks of age between the Wild Type mice and the Severe TNAP<sup>−/−</sup> subset
### MIXED MODEL PAIRWISE COMPARISON OF CRANIOFACIAL LINEAR DISTANCES BY PHENOTYPE — 15 DAYS OLD

15 day old Wild Type vs. Severe TNAP\(^{-/-}\) Subset

<table>
<thead>
<tr>
<th>3D Linear Measure</th>
<th>Difference</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasale-anterior frontal bone</td>
<td>0.67 mm</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Frontal bone length</td>
<td>-1.50 mm</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Skull height</td>
<td>0.31 mm</td>
<td>0.01</td>
</tr>
<tr>
<td>Skull height</td>
<td>0.31 mm</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Skull height</td>
<td>0.36 mm</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

- Significantly shorter frontal bone length
- Significantly larger skull height dimensions

### MIXED MODEL PAIRWISE COMPARISON OF CRANIOFACIAL LINEAR DISTANCES BY PHENOTYPE — 20 DAYS OLD

20 day old Wild Type vs. Severe TNAP\(^{-/-}\) Subset

<table>
<thead>
<tr>
<th>3D Linear Measure</th>
<th>Difference</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasale-anterior frontal bone</td>
<td>0.94 mm</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Frontal bone length</td>
<td>-1.34 mm</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Skull height</td>
<td>0.35 mm</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Skull height</td>
<td>0.38 mm</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Skull height</td>
<td>0.41 mm</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

- Significantly shorter frontal bone length
- Significantly larger skull height dimensions
- No significant differences found for other phenotypes

### MIXED MODEL COMPARISON OF 3D LINEAR DISTANCES SUMMARY

- Significantly shorter frontal bone length, larger skull height, and larger skull width between genotypes at two weeks of age
- Two-fold decrease in average frontal bone length difference between genotypes from two to three weeks of age
- Loss of significant difference for skull width and height measures between genotypes at three weeks of age
- Significantly shorter frontal bone length and larger skull height measures between Wild Type and Severe TNAP\(^{-/-}\) subset at two weeks of age
- Same results, with even greater differences at three weeks of age

### PRINCIPLE COMPONENT ANALYSIS:

- 2 principle components covered 77% of the variance
- Severe TNAP\(^{-/-}\) phenotype subset significantly different from other subsets and from wild type mice
20 DAY OLD MICE

- 2 principle components covered 81% of the variance
- Severe TNAP⁻/⁻ phenotype subset significantly different from other subsets and from wild type mice

Greatest component loading of the following measures:
- Frontal bone length
- Posterior skull width measures
- Posterior skull height measures

Suggests that these measures exhibit a greater contribution to the variance among all measures in the severe phenotype subset
- This is the dome-shaped appearance that is expected to occur with premature coronal suture fusion

DISCUSSION

STUDY LIMITATIONS
- Inability to verify landmarks on micro CT scans due to poor tissue mineralization
  - This limited the number of landmarks available for the study
- Limited ages of samples available for analysis
  - Analysis of different ages (prenatal, P1, P7, etc.) would allow for delineation of when the morphological changes seen in the mutant mice begin to occur
  - This would help to establish an altered treatment regimen with enzyme replacement
- Significant damage of samples during preparation
- Use of micro CT for coronal suture analysis
  - Histological analysis is the gold standard
- Could only analyze the coronal suture by micro-CT due to hypomineralization

CONCLUSIONS
- TNAP⁻/⁻ mouse model of infantile HPP does phenocopy the craniofacial morphology of infantile HPP in humans
- This study reports a 34% incidence of coronal craniosynostosis in TNAP⁻/⁻ mice at 3 weeks of age
- This study showed a difference in form, shape, and growth patterns between genotypes
- Long bone clinical severe TNAP⁻/⁻ phenotype displays significantly greater amount of abnormal craniofacial form, shape, and growth than other phenotypic subsets
- This study showed the timing of onset of craniosynostosis and described the morphology of the TNAP⁻/⁻ mouse model of human infantile HPP, which has yet to be done
FUTURE STUDIES

• Studies of younger post-natal and pre-natal mice would serve to further describe development at an earlier age
• Continue with my pilot study efforts to stain, visualize, and place landmarks on embryonic mice
• Optimize staining protocol
• Difficult to see tissues due to poor mineralization
• Histo-morphogenetic studies of both the endochondral and intramembranous bones in the infantile HPP mouse model
• Cell type specific TNAP knockout mice: osteoblasts, chondrocytes
• Double mutant mice identify additional contributors to abnormal growth and development of the craniofacial skeleton

REFERENCES


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  • Cassandra Campbell
  • Nicole Punta

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THANK YOU! QUESTIONS?

PARTING THOUGHTS...

• If diminished TNAP activity does have a role in the etiology of craniosynostosis, TNAP enzyme replacement therapy could be the first successful, non-surgical treatment for craniosynostosis
• If the timing of onset of the craniofacial abnormalities seen in infantile HPP patients is determined, the TNAP replacement regimen can be altered accordingly