Allele-Specific Treatments for Cystic Fibrosis

October 28th

← Ben Spink
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(Ribosome) (Turmeric Root)
Cystic Fibrosis:

-Cystic Fibrosis (CF): autosomal recessive, 1 in 3000 Caucasian births

-Genetic defect in CFTR: Cystic Fibrosis transmembrane conductance regulator
  - Integral membrane glycoprotein
  - \( \Delta F508 \) most common mutation, but >1000 other mutations represent ~2% of the total mutations
Cystic Fibrosis:

Pathogenesis:
- Tissue-specific abnormalities in ion transport caused faulty production, processing, transport, or conductance of CFTR
- In the lung, yields increased transepithelial potential difference (increase in Na\(^+\) transport, decrease in Cl\(^-\))
- Reduced salt likely increases mucus viscosity, stasis and low O\(_2\) contribute to bacterial infection
- Parallel mechanism occurs in GI lumen

Cystic Fibrosis:

Clinical Features

Lung:
- Upper respiratory tract: sinusitis/rhinorrhea, polyps
- Lower respiratory tract: Excessive mucus production, airway blockage, low $O_2$
  - Microbes in sputum: *H. influenza*, *S. aureus*, *P. Aeruginosa*, *Aspergillus* (biofilms)
  - Infection/Inflammation
- End-stage events: cor pulmonale, respiratory failure
Cystic Fibrosis:

Pathogenesis/Clinical Features outside the lung:
- meconium ileus in infants
  - pancreatic insufficiency, often prompts further diagnostic tests
- genitourinary defects (inadequate nutrition/O$_2$ affects fertility, blockage of vas deferens)
CFTR in mice:

Murine Pathogenesis/Clinical Features:
- CFTR ΔF508: No lung pathology!
- However, CFTR mice have extensive GI problems, cellular localization and potentiometric analysis still possible
- CCSP-driven ENaC overexpression leads to CF-like lung pathology

CF Therapies

- Many treatments are mechanical (chest percussion, breathing exercises/apparatus, saline wash)
- N-acetylcysteine for mucus clearance, _-adrenergics for airway constriction, anti-inflammatories
- Antibiotics for infection
- GI difficulties: lipase, supplemental vitamin E/K
Cystic Fibrosis: Gene Therapy

- Early clinical trials demonstrated proof of principle, but were insufficient for clinical benefit
  - Used cationic liposomes or adenovirus
  - More recent trials have used non-viral vectors with better success
- Barriers to Gene Therapy Success:
  - Mucus, (literal barrier)
  - Glycocalyx
  - Basolateral location of receptors
- These are lung-specific problems: mouse model consequences
Potentiometric methods of analysis

- Electrical studies first used in CF research to confirm plasma membrane impermeability to Cl⁻, now a powerful diagnostic/research tool

How it works:
- Differences in [salts] = voltage
- Voltmeter connected to two electrodes
  - One electrode placed against target epithelium
  - Another (reference electrode) placed subcutaneously
- Potential is negative to varying degrees
Potentiometric methods of analysis

- Probing electrode perfused with various solutions that affect membrane potential

Saline: Baseline (a negative potential)
Amiloride: Blocks Na+ absorption, reduces potential difference
Cl⁻ free solution: encourages Cl⁻ secretion, in non-CF epithelia results in hyperpolarization, this is likely Cl⁻ secretion due to prior treatment with amiloride
Isoproterenol: Augments Cl⁻ secretion (↑cAMP), even more polarization
Forskolin: Also increases cAMP, and is a bronchodilator
Potentiometric methods of analysis

An example with NPD from Egan et al

- **Saline**: Baseline (a negative potential)
- **Amiloride**: Blocks Na\(^+\) absorption, reduces potential difference
- **Cl\(^-\) free solution**: encourages Cl\(^-\) secretion, in non-CF epithelia results in hyperpolarization, this is likely Cl\(^-\) secretion due to prior treatment with amiloride
- **Isoproterenol**: Augments Cl\(^-\) secretion (↑ cAMP), even more polarization
- **Forskolin**: Also increases cAMP, and is a bronchodilator

Grey filled square: WT
Red filled circles: 45mg/kg curcumin
Grey filled circle: Untreated ΔF508
Orange filled circles: 15mg/kg curcumin
Endoplasmic Reticulum Quality Control:

- Protein biogenesis requires proper folding & modifications
- Misfolded proteins are recognized by calreticulin/calnexin/others, sent back to be refolded (ERQC), or are sent to be degraded (ERAD)
- Ca$^{2+}$-dependent ER chaperones are known to be responsible for CFTR folding and appropriate membrane translocation
- SERCA (Sarcoplasmic/Endoplasmic Reticulum Ca$^{2+}$)
- Curcumin is a safe, relatively mild SERCA pump inhibitor
  - High affinity pump inhibitors (thapsigargin) are lethal for some cells
Egan et al

-Hypothesis: Could the disruption of Ca\(^{2+}\) levels affect CFTR degradation/processing and allow for increased membrane translocation and a therapeutic effect?

-Egan et al use CFTR ΔF508 mice (transporter is functional)

-Previous data suggest curcumin has therapeutic potential due to decrease in membrane potential-Will there be a therapeutic effect? Is the mechanism of action known? Is knowledge of the mechanism important?
Egan et al

Fig. 1. Nasal potential difference measurements in curcumin-treated and untreated CF mice. (A) The mean NPD observed after treatment with oral curcumin. NPD was measured for untreated wild-type mice (gray filled squares, n = 7), untreated ΔF508 CFTR CF mice (gray filled circles, n = 11), ΔF508 CFTR CF mice administered curcumin (45 mg/kg) by oral gavage once a day for three consecutive days (red filled circles, n = 8), and ΔF508 CFTR CF mice administered curcumin (15 mg/kg) by oral gavage three times a day for three consecutive days (orange filled circles, n = 10). (B) NPD was measured in oral curcumin-treated and untreated CFTR knockout mice. Knockout mice were administered curcumin (15 mg/kg) by oral gavage three times a day for three consecutive days. Gray filled symbols represent untreated animals (n = 4), and orange filled symbols represent treated animals (n = 4). Standard error bars are indicated in all traces. Solution changes are indicated by the arrows.

- Saline: Baseline (a negative potential)
- Amiloride: Blocks Na+ absorption, reduces potential difference
- Cl- free solution: encourages Cl- secretion, in non-CF epithelia results in hyperpolarization, this is likely Cl- secretion due to prior treatment with amiloride
- Isoproterenol: Augments Cl- secretion (_cAMP), even more polarization
- Forskolin: Also increases cAMP, and is a bronchodilator
Saline: Baseline (a negative potential)

Amiloride: Blocks Na\(^+\) absorption, reduces potential difference

Cl\(-\) free solution: encourages Cl\(-\) secretion, in non-CF epithelia results in hyperpolarization, this is likely Cl\(-\) secretion due to prior treatment with amiloride

Isoproterenol: Augments Cl\(-\) secretion (_cAMP_), even more polarization
Egan et al

Fig. 2. Effects of curcumin treatment on response of rectal potential difference (RPD) to forskolin, on survival, and on weight gain. (A) RPD measurements were performed with Cl− free solution containing amiloride and then in a solution containing low chloride, amiloride, and forskolin. Solution changes are indicated by arrows. Circles represent ΔF508 CFTR mice, diamonds represent heterozygotes, and squares represent wild-type mice. Open symbols correspond to pretreatment animals, while closed symbols correspond to curcumin-treated animals (n = 7 for pretreatment animals and n = 6 for posttreatment animals for each group). Standard error bars are indicated in all traces. (B) Survival was documented for 10 weeks after weaning. Open squares represent Colyte-treated mice (n = 10), gray triangles represent curcumin-treated mice (n = 10), and black circles represent untreated mice (n = 10). Mortality in each case was due to intestinal obstruction. (C) Weight gain was monitored over the course of the 10-week survival study depicted in (B). The bars represent the average weekly weight gain in grams ± SEM, n = 10 mice for each group (6 mice died in the no-treatment group; therefore, the average weight gain reflects 3 to 10 mice in this group). Stars represent a significant difference from untreated animals (P ≤ 0.01) (ANOVA, Kruskal-Wallis).

- Saline: Baseline (a negative potential)
- Amiloride: Blocks Na+ absorption, reduces potential difference
- Cl− free solution: encourages Cl− secretion, in non-CF epithelia results in hyperpolarization, this is likely Cl− secretion due to prior treatment with amiloride
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- Forskolin: Also increases cAMP, and is a bronchodilator
Fig. 3. Curcumin promotes the accumulation of mature ΔF508 CFTR in BHK cells. (A) Detection of curcumin-induced accumulation of complex-glycosylated ΔF508 CFTR by immunoblotting. BHK cells expressing the HA-tagged ΔF508 CFTR were treated with the indicated concentrations of curcumin for 16 hours. Cells expressing wild-type CFTR and cells expressing ΔF508 CFTR that had been incubated at reduced temperature (26°C) for 16 hours were included as controls. Immunoblotting for Na,K-ATPase α subunit was performed to ensure equal loading. (B) Cell

<table>
<thead>
<tr>
<th></th>
<th>CFTR-HA</th>
<th>wt</th>
<th>ΔF508</th>
</tr>
</thead>
<tbody>
<tr>
<td>temperature (°C)</td>
<td>37</td>
<td>26</td>
<td>37</td>
</tr>
<tr>
<td>curcumin (μM)</td>
<td>–</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Glycosylation is initiated in the endoplasmic reticulum (ER) by the transfer of a core glycan, comprising two N-acetylglucosamine, nine mannose and three terminal glucose residues, while the polypeptide is still associated with the translocon. After initial folding events, the terminal glucose residues and one mannose residue are removed to generate a homogenous glycosylation pattern shared by all N-linked glycoproteins. Structural diversification is introduced in the Golgi compartment in a protein- as well as a cell-type-specific manner and is accompanied by a change in glycan function.

Traffic 4:5 Page 313  May 2003
More Figure 3:
What shows proper location?
What shows effective ion transport?

equal loading. (B) Cell surface density of ΔF508 CFTR. BHK cells expressing ΔF508 CFTR tagged with the 3HA epitope in its fourth extracellular loop were incubated in the presence of curcumin (5 μM) or DMSO (control) at 37°C or at 26°C for 16 hours. Cell surface density of CFTR was measured by antibody to HA and by 125I-labeled secondary antibody. Data are expressed as the percentage of specific antibody binding per milligram of protein measured at 37°C. Data are means ± SEM; n = 3. (C) Iodide conductance of ΔF508 CFTR-expressing BHK cells. BHK cells were incubated in the presence of curcumin (5 μM) or DMSO (control) at 37°C or at 26°C for 16 hours. Activation of the ΔF508 CFTR channels was achieved by the addition of the cAMP-agonist cocktail (+cAMP: 20 μM forskolin, 0.2 mM 3-isobutyl-1-methylxanthine (IBMX), and 0.5 mM chloroephylthio-cAMP) at the arrow to iodide-loaded cells. Iodide efflux was measured with an iodide-selective electrode and normalized for protein content. Data are means ± SEM from two to four independent experiments. The basal iodide efflux was depicted only for DMSO-treated cells, because similar results were obtained for all the other conditions. Stars indicate that the amount of iodide efflux for curcumin and 26°C treated cells is significantly (P < 0.05) greater than that measured in DMSO-treated cells. (D) Curcumin effect on ΔF508 CFTR-calnexin interactions. CHO cells expressing ΔF508 CFTR were incubated for 3 hours at 37°C with DMSO or with 50 μM curcumin, after which they were lysed and subjected to immunoprecipitation with antibodies to CFTR. The immunoprecipitated proteins (IP) and aliquots of the lysates (L) were analyzed by Western blotting using an antibody directed against calnexin.
Curcumin, human consumption

- Not surprisingly, curcumin is readily for sale
- Could you eat this much naturally?
  At 15 mg/kg, this is 1.5g (!) of curcumin/day
  - Turmeric root (uncated) is ~1% curcumin, so 150g of root/day
- But, piperine increases bioavailability by 2000%
- Which would be great, except the recommended piperine consumption is 20mg/kg…
- Actually, 8g curcumin/day well tolerated
Curcumin, Wonder Drug

4/5 Most recent Medline titles for “curcumin”:
- Curcumin treatment abrogates endoplasmic reticulum retention and aggregation-induced apoptosis associated with neuropathy-causing myelin protein zero-truncating mutants.
- Curcumin synergistically potentiates the growth-inhibitory and pro-apoptotic effects of celecoxib in osteoarthritis synovial adherent cells.
- Curcumin Suppresses the Paclitaxel-Induced Nuclear Factor-{kappa}B Pathway in Breast Cancer Cells and Inhibits Lung Metastasis of Human Breast Cancer in Nude Mice.
- Curcumin therapy in inflammatory bowel disease: a pilot study.

- Egan et al suggest that curcumin resembles an isoflavonoid, and may directly bind mutant CFTR. How might one distinguish between ER chaperone effects and direct binding? Genetic/Physical methods?
Where to go next?

- Duration/Start Time of treatment?
- Potential complications caused by lung disease?
- Known role of inflammation on Na\(^+\) channels, and also N-acetylcysteine/curcumin inhibit NF-\(\kappa\)B activity-could inhibition of inflammation be therapeutic in an alternate manner?
- Differences in human absorption/metabolism?
- Lessons for other misfolding diseases?
Extra Slides!
High Salt vs. Low Volume Models of CFTR

EPITHELIAL DYSFUNCTION:
The epithelia affected by CF exhibit different functions in their native state, i.e., some are volume-absorbing (airways and distal intestinal epithelia), some are salt-absorbing but not volume-absorbing (sweat duct), whereas others are volume-secretory (proximal intestine and pancreas). Given this diverse array of native activities, it should not be surprising that CF produces very different effects on patterns of electrolyte and water transport. However, the unifying concept is that all affected tissues express abnormal ion transport function.

Harrison’s Principles of Internal Medicine, Chapter 241
Soft, POM-style questions

- What would be the effect of this on patient morale?
  - (I attended a talk where someone accused this paper of giving false hope to children with cystic fibrosis)
- Frequency of self-prescription? Would this phenomenon relate to distrust of medicine/science/Pharma?
Gentamicin-Induced Correction of CFTR Function in Patients with Cystic Fibrosis and CFTR Stop Mutations

Two Primary Topics of Discussion

- Gentamicin induced read-through of stop codons in mutant CFTR alleles
  - The key biochemical issue in this therapy
- A double blind placebo controlled cross over trial
  - The type of trial structure used by the investigators
  - The statistical tests used by the investigators
Gentamicin is a Aminoglycoside Antibiotic

All family members contain a 2-deoxystreptamine ring with different substitutions.

Moieties on the ring determine the specificity of binding to the ribosome.

Gentamicin Disrupts the A Site of the Prokaryotic Ribosome

- tRNA’s enter the ribosome at the A site
- The ribosome confirms that the codon matches the anti-codon
- The amino acid is transferred from the P-Site tRNA to the N-terminus of the A-Site amino acid
- The ribosome translates to the next codon moving A-site tRNA to the P-Site and leaving the A-Site empty

Gentamicin prevents correct recognition of the correct codon in the A-site and translation along the mRNA
Aminoglycosides Also Impact the Eukaryotic Ribosome

- The aminoglycosides bind to a 7 nucleotide long loop structure in the 16S rRNA (U1406-A1408, A1492-U1495)
- Only one base differs between pro- and eukaryotes (A1408G) and this changes the $K_d \sim 40,000$ fold\(^1\)
- However this is still enough that aminoglycosides can impact translation in eukaryotes as well as prokaryotes
- Theoretically, one of these mistakes could be to miss a stop codon and continue translation

Which CF Patients Might Benefit From Missed Stop Codons?

- **Class I**
  - Any nonsense mutation that introduces an early stop codon
  - Certain splice variants that introduce a stop codon but do not disrupt the protein function
- < 4.3% of all CF alleles
Mechanism of Gentamicin Action Determines Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous Stop Codon</td>
<td>11</td>
<td>6 W1282X / W1282X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 W1282X / G542X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 W1282X/3849+10KbC-&gt;T</td>
</tr>
<tr>
<td>Heterozygous Stop Codon</td>
<td>8</td>
<td>6 ΔF508 / W1282X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 G85E / W1282X</td>
</tr>
<tr>
<td>ΔF508</td>
<td>5</td>
<td>5 ΔF508 / ΔF508</td>
</tr>
</tbody>
</table>

Note three different stop codon mutations to read through
What Type of Study Design is Used and Why?

- A double blind, placebo control, cross over trial design
- A cross over design was used as the severity of CF varies so it is preferable to have each patient act as their own control
- So we have six groups homozygous stop, heterozygous stop and ΔF508 in sequence AB and homozygous stop, heterozygous stop and ΔF508 in sequence BA

What Problems are Associated With Cross Over Trials?

- Cross over trials are only effective when
  - Carry over – a residual effect from the therapy in period 1 that is still present in period 2
  - Studying chronic diseases

- What Steps did Wilschanski et al take to limit this effect?
  - One month washout period prior to study
  - No washout in between periods
  - Tested for the effects of carry over

- Statisticians really dislike this trial design as the data analysis is easy to get wrong (more coming)
Gentamicin Has an Effect on Stop Mutation CF Patients

- Three patient responses were tested
  - Basel PD, Amiloride response, Isoproterenol response
- Qualitatively more normal behaviour in the stop mutation group than the ΔF508 group
- How did they get the quantitative results?
What Type of Statistical Test is Used to Determine Significance?

- For the homozygous stop mutation group
  - T-test
- For the heterozygous stop mutation group
  - Mann-Whitney U test
- How are the two tests different, and why use two different tests?

<table>
<thead>
<tr>
<th>Nasal Potential Difference</th>
<th>Homozygous for Stop Mutation (N=11)</th>
<th>Heterozygous for Stop Mutation (N=8)</th>
<th>Homozygous for ΔF508 Mutation (N=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>-48±10</td>
<td>-42±3</td>
<td>-45±11</td>
</tr>
<tr>
<td>Placebo</td>
<td>-46±10</td>
<td>-41±10</td>
<td>-41±14</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>-34±12</td>
<td>-34±9</td>
<td>-46±12</td>
</tr>
<tr>
<td>P value for treatment effect</td>
<td>0.008†</td>
<td>0.25‡</td>
<td></td>
</tr>
<tr>
<td>Response to amiloride</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base line</td>
<td>33±9</td>
<td>32±7</td>
<td>32±14</td>
</tr>
<tr>
<td>Placebo</td>
<td>34±11</td>
<td>27±10</td>
<td>26±10</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>24±12</td>
<td>25±7</td>
<td>33±9</td>
</tr>
<tr>
<td>P value for treatment effect</td>
<td>0.05†</td>
<td>0.79‡</td>
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<tr>
<td>Response to chloride-free isoproterenol</td>
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<td></td>
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<tr>
<td>Base line</td>
<td>0.4±4.6</td>
<td>-0.5±2</td>
<td>2.2±5</td>
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<tr>
<td>Placebo</td>
<td>-0.4±2.7</td>
<td>-0.5±2</td>
<td>1.2±2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>-5.5±2.8</td>
<td>-4.8±2.6</td>
<td>1.8±2.5</td>
</tr>
<tr>
<td>P value for treatment effect</td>
<td>0.001†</td>
<td>0.036‡</td>
<td></td>
</tr>
</tbody>
</table>

* Plus–minus values are means ±SD. For the comparison of placebo or gentamicin with base-line values, only the P values for the treatment effect are shown; the P values for carryover effect and period effect were not significant.
† A t-test was used.
‡ The Mann–Whitney U test was used.
Parametric Testing Versus Non-Parametric Testing

- **Parametric Tests**
  - Use actual numeric values
  - Requires an assumption about the distribution
  - More powerful
  - Example t-test

- **Non-Parametric Tests**
  - Use data ranks
  - Require no assumption about distribution
  - Less powerful
  - Example Rank-Sum Test / Mann-Whitney U Test
T-Test Versus a Rank Sum Test: When to Use Which

- T-test compares means – Rank sum compares sum of ranks
- Means are subject to outliers and require the assumption of normality in the data
- Tests with ranks are used on non-normally distributed data or data with outliers

\[ t_{stat} = \frac{\bar{y}_1 - \bar{y}_2}{s_p \sqrt{\frac{1}{n_1} \frac{1}{n_2}}} \]

\[ s_p^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \]

\[ Z = \frac{\sum_{j=1}^{n_1} r_{ij} - \mu_{R1}}{\sigma_{R1}} \]

\[ \mu_{R1} = \frac{n_1(N + 1)}{2} \]

\[ \sigma_{R1}^2 = \frac{n_1 n_2}{12} (N + 1) \]
Example of Outlier Impact on Hypothesis Tests

- **Parametric T-Test**
  - Group 1 mean = 10.4
  - Group 2 mean = 19.8
  - $t_{stat} = 5.00$
  - $p = <0.001$

- **Non-Parametric Rank Test**
  - Rank Sum 1 = 112
  - Rank Sum 2 = 98
  - $Z_{stat} = 0.50$
  - $p = 0.68$

- Different conclusions

<table>
<thead>
<tr>
<th>Group 1 ($x_i$)</th>
<th>Group 1 (rank)</th>
<th>Group 2 ($y_i$)</th>
<th>Group 2 (rank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7</td>
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<td>10.7</td>
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<td>11.7</td>
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<td>8</td>
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<td>9.0</td>
<td>9</td>
<td>8.1</td>
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<tr>
<td>8.8</td>
<td>6</td>
<td>114.5</td>
<td>20</td>
</tr>
</tbody>
</table>
Are There Any Apparent Problems with the Statistics?

- The use of two different tests looks inappropriate
  - Most likely due to the small sample size one group failed a test of normality and non-parametric tests were used, nothing insidious

- t-tests / Mann-Whitney U tests are not correct and p-values are wrong
  - This is due to the complex underlying data structure
  - However, the p-values are most likely not off by a lot

- These approaches do not fully utilize the paired nature of the data, the stated reason for the cross over design
Cross Over Trial Data is Complex due to Many Variables

- The expected values in each block are the sum of many random variables
- $\mu$ = mean of patient group
- $\pi$ = effect from the period
- $\tau$ = effect of treatment
- $\lambda$ = carry over from previous period

$D = \mu + \pi_1 + \tau_2 + \lambda_A$

<table>
<thead>
<tr>
<th></th>
<th>Period 1</th>
<th>Period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequence AB</strong></td>
<td>$A = \mu + \pi_1 + \tau_A$</td>
<td>$B = \mu + \pi_2 + \tau_B + \lambda_A$</td>
</tr>
<tr>
<td><strong>Sequence BA</strong></td>
<td>$C = \mu + \pi_1 + \tau_B$</td>
<td>$D = \mu + \pi_2 + \tau_A + \lambda_B$</td>
</tr>
</tbody>
</table>
Many Variables Mean Simple T-tests are Not Correct

- Standard CROS test compares
  - \((A-B + D-C)/2\) - significant if treatment difference OR carry over effect
  - A secondary test is needed to distinguish where the effect comes from
  - Correlations are generally present complicating the selection of a significance level
  - Performing multiple tests and the correlation makes determining type I and type II error calculations
Do These Tests Take Advantage of the Cross Over Design?

- Stated reason for cross over design
  - For each patient to act as their own control
- T-tests and Mann-Whitney U tests ignore this control structure
- Paired t-tests or Wilcoxon Signed Rank test include this control structure and provide a more robust test (Oops!)
Gentamicin Dose Impacts Effectiveness of Treatment

- Same three patient responses were tested
  - Basel PD, Amiloride response, Isoproterenol response
- Qualitatively the response seems to be changing with dosage
- Again, how did they get the quantitative results?
Repeated Measures MANOVA Tests Dose Response Data

- ANOVA tests for differences between different groups
  - E.g. weights of different bird species
  - Expansion of the t-test to more than two groups
- MANOVA tests for changes in one group over time or other variable (e.g. dose)
  - E.g. leg strength each month after surgery
  - Expansion of paired t-test
An Example of Paired Tests versus By Group Testing

- **T-Test**
  - Group 1 = 46.5
  - Group 2 = 51.5
  - \( t_{\text{stat}} = -1.88 \)
  - \( p = 0.076 \)
- **Paired Test clearly significant** (\( d=5 \))

<table>
<thead>
<tr>
<th>Group 1 ( (x_i) )</th>
<th>Group 2 ( (y_i) )</th>
<th>Difference ( (d_i) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>45.5</td>
<td>50.5</td>
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<tr>
<td>44.9</td>
<td>49.9</td>
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<tr>
<td>41.9</td>
<td>46.9</td>
<td>5</td>
</tr>
<tr>
<td>47.3</td>
<td>52.3</td>
<td>5</td>
</tr>
<tr>
<td>44.5</td>
<td>49.5</td>
<td>5</td>
</tr>
</tbody>
</table>
Does Figure 3 Make Sense Given a Repeated Measures Design?

- The data is presented as the mean response for all patients at each dose.
- Repeated measures design uses relative comparisons to a single patient's past results.
- To match the statistics, the graphs should display the mean of the change for each patient from the previous dose.
Hotteling’s Trace Statistic is the Output of a MANOVA

- The equation below produces a testable $F_{\text{stat}}$
  - $N = \text{number of subjects}$
  - $T = \text{number of measurements}$
  - $Y'_d = \text{row vector of differences}$
  - $Y_d = \text{column vector for differences}$
  - $S^2_d = \text{covariance matrix of differences}$

- Determines if a subject has experience a change in any period – only one needs to be different to get significance

$$F_{\text{stat}} = \left( \frac{N - T - 1}{(N - 1)(T - 1)} \right) \left( \frac{N y'_d y_d}{S^2_d} \right)$$
Immunofluorescence Confirms Mechanism of Action

- **A** – Indicates antibody does not stain CFTR deficient cells
- **B** – Indicates a low level of CFTR receptor prior to treatment
- **C** – Indicates a higher level of CFTR after treatment
Summary

- The data presented supports gentamicin therapy for treatment of CF nonsense mutations.
- The statistics are not precisely correct but mostly like do not lead to incorrect conclusions.
Eukaryotic Translation Errors are Sequence Specific

Table 2
Effect of paromomycin on misreading during translation elongation.

<table>
<thead>
<tr>
<th>Firefly Mutation</th>
<th>Percent Misreading</th>
<th>Paromomycin</th>
<th>+ Paromomycin</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- Paromycin</td>
<td>+ Paromycin</td>
<td></td>
</tr>
<tr>
<td>245 CAC</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>245 AAC</td>
<td>0.007 +/- 0.001</td>
<td>0.02 +/- 0.003</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>245 GAC</td>
<td>1.4 +/- 0.16</td>
<td>1.0 +/- 0.09</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>245 GGC</td>
<td>0.6 +/- 0.1</td>
<td>0.7 +/- 0.1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>245 GGC</td>
<td>0.2 +/- 0.05</td>
<td>2.3 +/- 0.26</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>245 CUC</td>
<td>0.12 +/- 0.012</td>
<td>0.27 +/- 0.025</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>245 CAA</td>
<td>0.15 +/- 0.017</td>
<td>0.17 +/- 0.008</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>245 CAG</td>
<td>0.16 +/- 0.02</td>
<td>0.7 +/- 0.2</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>529 AAA</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>529 CAA</td>
<td>0.05 +/- 0.006</td>
<td>0.06 +/- 0.01</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>529 CAA</td>
<td>0.012 +/- 0.001</td>
<td>0.023 +/- 0.005</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>529 ACA</td>
<td>0.045 +/- 0.005</td>
<td>0.05 +/- 0.01</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>529 AGA</td>
<td>0.032 +/- 0.006</td>
<td>0.04 +/- 0.006</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>529 AUA</td>
<td>0.05 +/- 0.008</td>
<td>0.06 +/- 0.01</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>529 AAC</td>
<td>0.035 +/- 0.003</td>
<td>0.05 +/- 0.008</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>529 AUA</td>
<td>0.023 +/- 0.002</td>
<td>0.1 +/- 0.017</td>
<td>4.3</td>
<td></td>
</tr>
</tbody>
</table>

*Percent misreading is expressed as mean +/- standard deviation.
#Mutated nucleotides are underlined.
*Paromomycin concentration is 200 μg/ml.
**Changes ≥ 2-fold that yielded a statistically significant P value (< 0.05) using the Mann-Whitney test are underlined.