

# GENETIC AND PHYSIOLOGIC CORRELATES OF LONGITUDINAL IMMUNOREACTIVE TRYPSINOGEN DECLINE IN INFANTS WITH CYSTIC FIBROSIS IDENTIFIED THROUGH NEWBORN SCREENING

MARCI K. SONTAG, PHD, MARY COREY, PHD, JOHN E. HOKANSON, PHD, JULIE A. MARSHALL, PHD, STEVE S. SOMMER, MD, PHD, GARY O. ZERBE, PHD, FRANK J. ACCURSO, MD

**Objectives** To characterize the time course and physiologic significance of decline in serum immunoreactive trypsinogen (IRT) levels in infants with cystic fibrosis (CF) by mode of diagnosis and genotype, and to examine IRT heritability.

**Study design** We studied longitudinal IRT measurements in 317 children with CF. We developed statistical models to describe IRT decline. Pancreatic disease severity (Mild or Severe) was assigned using CF genotype and was confirmed in 47 infants through fat malabsorption studies.

**Results** Infants with severe disease exhibited IRT decline with non-detectable levels typically seen by 5 years of age. Infants with mild disease exhibited a decline in the first 2 years, asymptotically approaching a level greater than published norms. IRT and fecal fat were inversely correlated. IRT values in infants with meconium ileus (MI) were significantly lower than newborn-screened infants at birth. The high proportion of shared variation in predicted IRT values among sibling pairs with severe disease suggests that IRT is heritable.

**Conclusions** IRT declines characteristically in infants with CF. Lower IRT values in newborns with MI suggest increased pancreatic injury. Furthermore, IRT is heritable among patients with severe disease suggesting genetic modifiers of early CF pancreatic injury. This study demonstrates heritability of a statistically modeled quantitative phenotype. (*J Pediatr* 2006;149:650-7)

**S**erum immunoreactive trypsinogen (IRT), a pancreatic enzyme precursor, is elevated in the blood of infants with cystic fibrosis (CF),<sup>1</sup> leading to its widespread use in CF newborn screening (NBS) programs. IRT has been studied in conventionally diagnosed children and adults with CF,<sup>2</sup> but it has never been studied in a newborn-screened population using longitudinal data from birth through childhood. Longitudinal description of IRT levels in early CF may provide insight into the evolution of pancreatic injury in CF in a variety of clinical settings. For example, the rate of decline of IRT may relate to the severity of pancreatic disease. In addition, demonstration that IRT decline is a heritable trait would suggest the existence of gene modifiers of exocrine pancreatic injury in CF. Our primary objective was to characterize the time course and physiologic significance of IRT decline in infants with CF, and to determine if IRT elevation or decline is heritable. To explore the relationship between early pancreatic disease in CF and IRT time course, we examined serial IRT determinations in a large group of infants with CF, by mode of diagnosis (NBS vs meconium ileus [MI] vs false-negatives [FNs]), by CF genotype, and by pancreatic disease severity. The physiologic significance of elevated IRT was examined through fat malabsorption studies. Potential predictors of IRT decline were examined including sex, birth weight, gestational age, feeding method in infancy (breast vs formula), and pancreatic enzyme use. Finally, we assessed heritability, the

From the Department of Preventive Medicine and Biometrics, University of Colorado Health Sciences Center, Denver, Colorado; the Hospital for Sick Children and Department of Public Health Sciences, University of Toronto, Toronto, Ontario, Canada; Molecular Genetics, Beckman Research Institute, City of Hope, Duarte, California; the Department of Pediatrics, University of Colorado Health Sciences Center and The Children's Hospital, The Mike Morris Cystic Fibrosis Research and Treatment Center, Denver, Colorado.

Supported by Grant Number RO1 DK61886-02, National Institute of Diabetes and Digestive and Kidney Diseases; NIH, Grant Number MO1 RR00069, General Clinical Research Centers Program, National Center for Research Resources, NIH; Grant Number U01 HL081335, National Institute of Heart Lung and Blood Disease, NIH; and the Cystic Fibrosis Foundation.

Submitted for publication Nov 3, 2005; last revision received May 23, 2006; accepted July 12, 2006.

Reprint requests: Marci K. Sontag, PhD, The Children's Hospital, 1056 E. 19<sup>th</sup> Ave. B395, Denver, CO 80218. E-Mail: [sontag.marci@tchden.org](mailto:sontag.marci@tchden.org).

0022-3476/\$ - see front matter

Copyright © 2006 Mosby Inc. All rights reserved.

10.1016/j.jpeds.2006.07.026

CF	Cystic fibrosis	MI	Meconium ileus
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator	NBS	Newborn screening
FN	False-negative	PI	Pancreatic insufficient
HAZ	Height for age Z score	PS	Pancreatic sufficient
IRT	Immunoreactive trypsinogen	WAZ	Weight for age Z score

proportion of the total variation of IRT levels that could be explained by genetics, through examination of sibling pairs.

## METHODS

### Study Population

Children born in Colorado between 1982 and 2003 and diagnosed with CF through a statewide NBS program were eligible for this study. Each infant with two elevated IRTs was identified, and appropriate diagnostic testing was performed. If the infant had a positive sweat test, care in the CF Center was initiated; these children were diagnosed by NBS. In addition, infants who were FN on the newborn-screen and children with MI born in Colorado in the same time period were also eligible for the study. The MI and FN groups were mutually exclusive. NBS for CF began as a research program in 1982 and was added to the state panel for screening in 1987. This longitudinal study was performed under the approval of the Institutional Review Board, starting in 1982 and continuing through 2003. Consent was obtained from parents or legal guardians of each participating child at the child's annual CF examination.

### IRT Determination

As one component of the state-mandated newborn-screen in Colorado, all infants are screened for CF, using a two-tiered IRT-based screen. The first IRT level is collected before discharge from the hospital (currently 2-3 days of life, historically 1-2 days of life), and the IRT level is collected at the 2-week well-baby check recommended for all babies. In 1982 when CF NBS was first introduced in Colorado as a research program,<sup>3</sup> a modified radioimmunoassay (Trypsik, Sorin Biomedica, Saluggia, Italy) was used to determine IRT levels. When the State Laboratory assumed responsibility of CF screening in 1985, the assay changed to a time-resolved fluorimetric immunoassay (DELFLIA, Neometrics Inc., East Northport, NY) and in 1994 the assay changed again to another manufactured kit (DELFLIA, Perkin Elmer Inc., Boston, Mass). The NBS cutoff values have been described elsewhere.<sup>3,4</sup>

Children diagnosed with CF were offered the opportunity to enroll into a longitudinal study of disease progression in CF. As a part of that study, IRT determinations were collected at 2, 6, and 12 months, and yearly thereafter. IRT levels were determined by a modified radioimmunoassay (Trypsik, Diasorin Biomedica, Saluggia, Italy), and the manufacturer's reference ranges were used.

The Trypsik and DELFLIA assays measure different components of trypsinogen. The DELFLIA method measures both cationic and anionic forms of trypsinogen. The Trypsik method measures only the cationic form of trypsinogen. This difference required separate statistical models for the newborn-screen and research IRTs. The earliest points (from the NBS lab) were not included in the overall model because of the assay differences between the two laboratories. Unless otherwise stated, all IRT models represent the research IRTs

(Trypsik, 2 months of age to 21 years of age). The earliest IRT determinations do not influence the overall IRT decline models.

### Sweat Chloride

Sweat tests performed according to the Gibson-Cooke quantitative pilocarpine iontophoresis method were utilized for the analysis. If more than one sweat chloride was available, the highest sweat chloride was chosen.

### Anthropometric and Serum Vitamin Assessments

Annual height, weight, and laboratory values including vitamin levels were collected on each child. Height for age Z scores (HAZ) and weight for age Z scores (WAZ) were calculated using the 2000 CDC growth charts,<sup>5</sup> and the measurement of serum vitamin levels have been previously described.<sup>6</sup>

### DNA Analysis

Molecular analysis of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene was initially performed through commercial labs (Genzyme Genetics, Ambry Genetics, University of Colorado Health Sciences Center Molecular Genetics Lab). Broader genetic screening was performed on some children. The whole coding region and intronic boundaries of the CFTR gene were analyzed using DOVAM-S (Detection of Virtually all Mutations – SSCP), a robotically enhanced highly multiplexed form of SSCP that detects virtually all mutations, as demonstrated in multiple blinded analyses.<sup>7,8</sup>

### Fecal Fat Determination

A subset of children underwent fecal fat measurements at approximately 2, 6, 12, and 24 months of age at the same time as their IRT determinations. Percent fat excreted was determined through a 72-hour stool collection at the inpatient Pediatric General Clinical Research Center as previously described.<sup>9</sup> Fat intake was carefully monitored and fat excretion was analyzed using the methods of Van de Kamer et al<sup>10</sup> and was expressed as a percentage of fat malabsorption (grams of fecal fat/grams of fat intake). Stool was analyzed for total fat, medium chain fatty acids, and long chain fatty acids by a modified method of Jeejeebhoy et al.<sup>11</sup>

### Severity of Disease

Persons with CF are classified as pancreatic sufficient (PS) or pancreatic insufficient (PI), based on fecal fat. This classification has been shown to typically be determined by CFTR genotype.<sup>12</sup> Patients with at least one mutation from Class IV or V mutation typically have "mild" pancreatic disease and typically have PS. Patients with two mutations from Class I, II, or III typically have "severe" pancreatic disease and are classified as PI.<sup>13</sup> Pancreatic insufficiency develops over time, typically resulting in pancreatic insuffi-

ciency in the first 2 years of life.<sup>9</sup> Persons with PS initially may develop PI with age; however, children who develop PI have similar IRT decline patterns as those who have PI from early infancy.<sup>2</sup> We have assigned a mild or severe classification based on a literature review and commonly reported phenotypes in the literature. This assignment correlates well with pancreatic status, but it is not exact. We are using this method to divide subjects based on pancreatic disease severity, recognizing the limitations. Disease severity was assigned based on clinical presentation (evidence of malabsorption, stool patterns, malnutrition, and albumin) for patients who had a mutation that has not been classified into one of the above classes.

### Statistical Analysis

The initial elevated IRT at screening and the change (decline) in IRT values with age in persons with CF were the focus of the analysis. In addition, we tested the following variables as potential covariates in the analysis: presence of MI at birth, sex, sweat chloride, birth weight, gestational age, feeding method, and pancreatic enzyme use in infancy. T tests were used to determine differences between diagnostic categories at each age. To determine variability of IRT in the newborn period, we employed a linear test for trend assessing the first IRT determination from infants in the first 4 days of life before hospital discharge. SAS version 8.02 (SAS Institute, Carey, NC) was used for all analyses.

A longitudinal mixed effects approach was used to model IRT decline with age, accounting for repeated measures on the same subject, and allowing for subject-specific slopes and intercepts. We defined the quantitative phenotype as the patient-specific lines calculated through the model. An unstructured covariance structure was implemented, assuming a bivariate normal distribution for the random intercept and slope.

Longitudinal IRT was best modeled using a log<sub>10</sub> transformation of IRT and hyperbolic transformation of age. Following Couper et al,<sup>2</sup> we tested the basic mixed model with the form:  $\log_{10} IRT_{ij} = A_i + B_i W_{ij} + e_{ij}$ , where  $W_{ij} = \frac{1}{Age_{ij} + \theta}$ ,  $IRT_{ij}$  and  $Age_{ij}$  are the IRT and Age for subject  $i$  at the  $j_{th}$  time of measurement and  $A_i$  and  $B_i$  are random effects, patient-specific intercepts, and slopes for subject  $i$ , and  $\theta$  is a shift on the age axis that optimizes the fit. The slope from this model is in terms of  $W$ , rather than age. The slope varies with age and can be calculated as follows:  $\beta / (age + \theta)^2$ . Some of the IRT values in the newborn period >400 ng/mL were reported as 400ng/mL, and values from children that were below the limit of detection were reported as <5ng/mL. The value of 400ng/mL is sufficient information for the purposes of NBS where the goal is to identify infants with elevated IRT values. In older children with CF, IRT levels are frequently below the lower limit of detection of the assay (5ng/mL), likely because there is little functioning exocrine pancreas that can produce IRT.

To use the information from these censored values, we maximized the likelihood modified for the censored data, using the cumulative density function similar to typical methods used in survival analysis.<sup>14,15</sup> For each subject, the contribution to the likelihood is a product of probability density functions for observed outcomes and cumulative density functions for censored outcomes, given the observed outcomes. The appropriate likelihood is:

$$l_{ij} = \begin{cases} F(IRT_{ij} | a_i, b_i) & \text{if IRT is left censored} \\ f(IRT_{ij} | a_i, b_i) & \text{if IRT is not censored} \\ 1 - F(IRT_{ij} | a_i, b_i) & \text{if IRT is right censored} \end{cases}$$

where  $f$  is the normal probability density function and  $F$  is the normal cumulative density function of IRT, conditional on the random effects,  $a_i$  and  $b_i$ , which are assumed to have a bivariate normal distribution.

The IRT decline models were calculated separately for specific groups, based on disease severity determined by CFTR genotype and presence of MI at birth. The overall model for children with severe disease was determined with all children with a severe genotype (Table I; available at [www.jpeds.com](http://www.jpeds.com)). All of the children with unknown genotype (33 with one unidentified mutation and 39 without genotyping) were excluded from the disease severity model. Covariates were tested individually in the model using a likelihood ratio test ( $\chi^2$  test,  $\alpha = 0.05$ ). Both the main effect and an interaction term were tested in the model, allowing tests of modifier effects on both the slope and the intercept. If interaction terms were not significant in the model, the term was removed and only the main effect remained in the model.

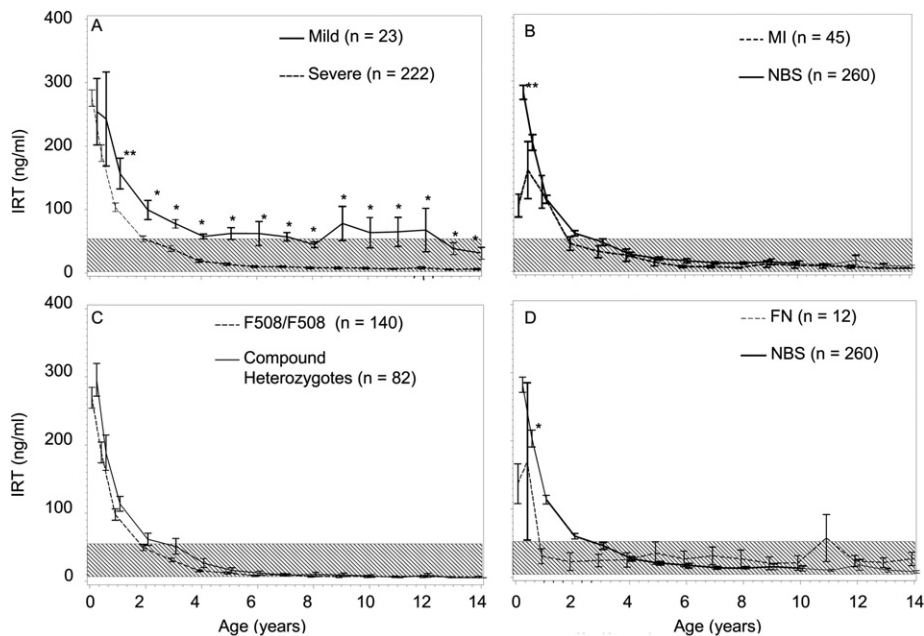
Heritability, the proportion of variation that can be attributed to shared genetics in predicted IRT values among sibling pairs, was approximated through calculation of the intraclass correlation for sibling pairs. Heritability was defined by the ratio of the variance attributable to genetic variation to the overall variance as follows:

$$\rho = \frac{\sigma_s^2}{\sigma_s^2 + \sigma_p^2},$$

where  $\sigma_s^2$  is the calculated variance between siblings and  $\sigma_p^2$  is the calculated variance between subjects within the same sibling pair. Approximate 95% confidence intervals were calculated using the delta method. The quantitative phenotype for each patient was extracted from the mixed modeling described above. Specifically, we used the patient specific slope on age, and the predicted IRT values at 2 months, 6 months, and 12 months, and yearly thereafter for each person. Heritability was calculated for siblings with severe disease; not enough siblings with mild disease were available to calculate heritability.

## RESULTS

IRT measurements were available for 317 children with CF who were born in Colorado since the inception of NBS (April 1, 1982) through January 1, 2003, with a total of 2264



**Figure 1.** Mean IRT determinations ( $\pm$  standard error) presented at each year of age (original data, not predicted values). **A**, disease severity, mild (by genotype) compared with severe. **B**, meconium ileus (MI) versus newborn-screened diagnosis. **C**, children with severe mutations: homozygous  $\Delta F508$  children versus compound heterozygotes. **D**, Infants who were false-negative (FN) on the newborn-screen (non-MI) versus those identified by newborn screening (NBS). \* $P < .05$ ; \*\* $P < .01$ .

observations (median 6, range: 1-16 per child). Length of follow-up varied from 2 days to 21 years, with a mean follow-up of 7.1 years. IRT measurements were determined at each annual visit, on or around the child's birthday. The mean values (original IRT value, not predicted values) at each year of age within different diagnostic and disease severity categories are presented in Figure 1. Diagnostic information, disease severity status, and total number of observations for the children are shown in Table II. The CFTR mutations found in our patient population and their assignment as severe or mild are presented in Table I. Three children died before completion of the study; all three of the children had IRT levels below the lower limit of detection before their deaths.

We investigated markers of malnutrition to support our assignment of children into mild and severe categories. At 6 years of age both HAZ and WAZ were significantly higher ( $P < .03$ ) in the group with mild disease (HAZ  $0.17 \pm 0.09$ , WAZ  $0.00 \pm 0.25$ ) than in the group with severe disease (HAZ  $-0.549 \pm 0.09$ , WAZ  $-0.65 \pm 0.08$ ). In addition, at 6 years 25-hydroxyvitamin D was significantly higher in the children with mild disease ( $46.0 \pm 3.1$  ng/mL) compared with the children with severe disease ( $35.0 \pm 1.9$  ng/mL). We did not find differences in serum retinol or the ratio of  $\alpha$ -tocopherol to total lipids.

### Disease Severity and Meconium Ileus

IRT decline is more rapid in patients with severe disease compared with patients with mild disease. The IRT decline models are displayed in Figure 2 (available at [www.jpeds.com](http://www.jpeds.com)). The modeled intercept was different between infants with MI and severe disease. Infants with MI had lower IRT levels in each

of the first 4 days of life than the NBS infants ( $P < .01$ ) (Figure 3; available at [www.jpeds.com](http://www.jpeds.com)). The differences between each day of collection (Day 0 vs Day 1, Day 1 vs Day 2, etc.) declined slightly but were not statistically significant (NBS  $P = .9$ , MI  $P = .51$ ). No statistical difference was detectable between the IRT decline model in the groups with severe disease and MI. The groups with MI and severe disease were combined for the overall model.

The final models of IRT decline were:

$$\text{Severe: } \log_{10}(\text{IRT}) = -2.35 + 46.8/(9.38 + \text{age}), \text{ and}$$

$$\text{Mild: } \log_{10}(\text{IRT}) = 1.45 + 2.23/(2.29 + \text{age}).$$

Comparison of the final model in children with PI from our data set to the final model presented by Couper et al<sup>2</sup> with the Toronto data are also presented in Figure 2, A.

### Infants Who Were False-Negative on the Newborn Screen

IRT values were lower in the newborn period in infants with a FN, and the overall pattern of IRT decline reflected lower IRT levels throughout the first 2 years of life than in infants who were identified by the screen (Figure 1, D).

### Potential Predictors of IRT Decline

Covariates suspected to be predictors of IRT initial elevation and subsequent decline (sex, birth weight, sweat chloride, birth length, gestational age, breast-feeding vs formula, and age of pancreatic enzyme therapy initiation) were tested in the final models of IRT decline. Only the timing of pancreatic enzyme therapy initiation made a significant contribution in the longitudinal model using the IRTs from 2

**Table II. Patient diagnostic categories and characteristics.**

Patient categories	Patients (measurements)
Total number of children studied	317 (2264)
Meconium ileus	45 (258)
False-negatives	11 (90)
Genotype known	
2 Severe mutations	222 (1668)
≥1 Mild mutation	21 (200)
Genotype unknown	
≥1 Unidentified mutation	33 (245)
Never genotyped	39 (141)
Clinically determined pancreatic sufficiency	2 (10)

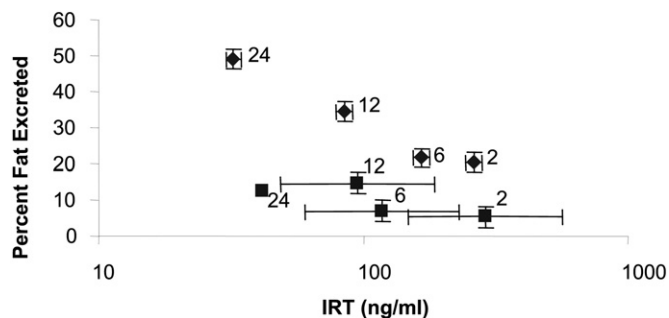
Patient characteristics	Outcome (n = number of patients with characteristic recorded)
Sex (male, %)	162 (51%)
Gestational age, (wk)	38.7 ± 2.6 (n = 250)
Birth weight (kg)	3.0 ± 0.3 (n = 265)
Breast-fed after birth (yes, %)	145; 79% (n = 183)
Months breast-fed (any)	6.7 ± 0.53 (n = 144)
Age formula started (wk)	1.9 ± 0.4 (n = 112)
Age at pancreatic enzyme initiation (d)	3.7 ± 1.4 (n = 176)
Age at introduction of solid foods (mo)	6.0 ± 0.3 (n = 175)

For modeling purposes, two children with unknown genotype but with known pancreatic sufficiency, demonstrated clinically, were included in the group with mild disease. Mean ± standard error, unless otherwise indicated.

months to 21 years of age, such that earlier enzyme therapy was associated with higher IRT (main effect  $P < .032$ ), although not associated with a more rapid decline (interaction term not-significant). To further investigate this association, we tested the timing of pancreatic enzyme therapy initiation with IRT level using only values from the neonatal-screen (before 2 months of age). The IRT values in the neonatal-screen were collected as a part of the diagnostic process, and they were collected before the initiation of pancreatic enzyme therapy. We again found a significant negative association between IRT values in the neonatal period and the age of pancreatic enzyme therapy initiation (main effect  $P < .01$ ), although the age of pancreatic enzyme therapy initiation was not associated with a more rapid decline (interaction term not-significant).

### Heritability

To determine the proportion of variability in IRT elevation and decline that might be caused by shared genetics, we calculated heritability of the subject-specific slope and predicted values at specific ages and the overall slope in 23 sibling pairs with severe genotypes. The highest heritability coefficient for the predicted values was observed at 2 months of age (51%, 95% CI 13%-90%), with significant heritability



**Figure 4.** Mean and standard error for IRT and percent fat excreted are inversely correlated in both subjects with pancreatic sufficiency and pancreatic insufficiency. Values are plotted for 2-, 6-, 12-, and 24-month visits, corresponding to the child's age (numbers next to points represent months of age at time of measurement). Only one patient with mild disease was observed at 24 months, therefore standard errors were not calculated.

also observed at 6 months of age (45%, 95% CI 3%-87%). Heritability of predicted values at older ages and the overall slope were not significant. Two families with multiple children were not included in this analysis because they had mild disease.

### Relationship of IRT to Fat Malabsorption

IRT levels inversely correlate with degree of malabsorption in initially hypertrypsinogenemic pancreatic insufficient children with CF. Results from 117 fecal fat balance tests and concurrent IRT determinations were available for 46 subjects, and 42 of those children also had genotype information available. Figure 4 shows the inverse correlation of IRT and fecal fat, the increase of mean percent fat excretion, and the decrease of mean IRT levels with age over the first 2 years of life in children with severe and mild disease, although the patterns in children with severe disease are more pronounced. IRT levels between subjects with mild and severe disease are not distinguishable in the first year of life, whereas the significant difference in fecal fat excretion increases with age.

## DISCUSSION

This study represents a longitudinal model of IRT decline in children with CF in a newborn-screened population, reflecting progressive pancreatic damage throughout childhood. Using a large collection of longitudinal IRT data in children, we were able to very carefully characterize the rate of decline in children with CF starting in the newborn period and continuing throughout childhood. The interpretation of IRT values and decline provides insight into the degree of pancreatic damage and remaining functioning exocrine pancreatic tissue in young children with CF. IRT is useful clinically in NBS. Beyond the neonatal period, serial measurements of IRT may be useful to corroborate clinical impressions of pancreatic sufficiency or insufficiency. More importantly, however, IRT may be useful as a surrogate outcome measure for clinical trials aimed at CFTR potentiation or correction performed early in life. We defined pa-

tient-specific rates of decline, which may be more meaningful clinically than instantaneous IRT determinations, and which may provide a model for comparisons between patients. A faster rate of decline may reflect more severe pancreatic damage. We found different rates of decline in children with mild and severe disease and confirmed our previously published report of differences in the initial elevation of IRT between infants with PI and infants with MI in a larger population.<sup>16</sup>

The differences in modeled IRT decline between the patients with mild and severe disease has been seen by other investigators in PS and PI.<sup>2</sup> Children who have PS are known to have IRT levels that are elevated or within the normal range throughout childhood, corresponding to some functional pancreatic tissue.<sup>2,17,18</sup> Children who have severe disease have rapidly declining IRT levels, compared with the children with mild disease, and on average have IRT levels less than the lower limit of detection by 5 years of age, reflecting complete loss of pancreatic function. Our results confirm previous findings, and they show a different IRT decline model in infancy and childhood for the groups with mild and severe disease. Although we defined disease severity based on CFTR genotype, which correlates well with pancreatic status, it is not exact. To confirm these assignments, we were able to show clinically important correlations between disease severity assignment and vitamin D levels, HAZ, WAZ, and fecal fat studies. Furthermore, fecal fat excretion correlated well with disease severity. The relationship between IRT and percent of fat excreted demonstrates the clinical relevance of IRTs in infants and toddlers with CF.

Two important findings are presented here. First, IRT determinations are not sufficient to identify differences between infants with mild and severe disease through the first year of life. Pancreatic damage is occurring in both groups, leading to elevated IRTs in both children with mild and severe disease. This finding supports the use of IRT as a screening tool in neonatal screening programs and confirms that both infants with PS and PI can be identified through IRT determinations in infancy. The identification of infants with mild disease, typically pancreatic sufficient, through the newborn-screen has been the topic of much debate. We very strongly support the identification and early treatment of these infants. Children classified with PS initially may develop pancreatic insufficiency,<sup>2</sup> and they should be monitored for signs of malnutrition. In addition, all infants (both with mild and severe disease) are at risk for hyponatremia and require salt supplementation to avoid complications of severe dehydration.<sup>19</sup> Within the past decade we have followed two infants with mild genotypes, and clinically confirmed pancreatic sufficiency, who suffered from severe hyponatremia. Without the careful attention provided by the CF Center these infants could have developed severe complications.

Second, the percent of fat excreted increases in both infants with mild and severe disease with increasing age; however, patients with severe disease have significantly higher percent of fat excreted than patients with mild disease at all ages. Infants who have severe disease (as defined by genotype)

may not require pancreatic enzyme supplementation at diagnosis through newborn-screen, but they may develop pancreatic insufficiency as they progress through infancy.<sup>9,20</sup>

Children with MI are known to have lower IRT levels in infancy, when compared with infants with CF without MI.<sup>16</sup> Our models show a higher intercept for the infants without MI; however, the subsequent rates of decline are similar. This may reflect more severe fetal pancreatic damage in infants with MI, resulting in more severe gastrointestinal manifestations at birth. Conversely this could reflect *less* pancreatic injury in infants with MI. However, given the lower intercept and no subsequent spikes of IRT in patients with MI, we hypothesize the pancreatic damage occurred in utero and is more severe in infants with MI at birth. The progression of pancreatic disease through childhood is similar between infants with MI and NBS as reflected by parallel rates of IRT decline between the two groups.

The other “negative” findings within this study are equally important. We have demonstrated that IRT initial elevation and decline is not predicted by sex, birth weight, sweat chloride, gestational age, or feeding method. Our data show that early pancreatic damage is not affected by sex, a well-described predictor of survival in CF, that is thought to be associated with microbiologic colonization, pulmonary function, and nutritional status.<sup>21</sup> The lack of association between IRT and birth weight and gestational age suggests that IRT values may be determined more by CFTR mutations and other genetic modifiers than by perinatal factors. Sweat chloride values are associated with CFTR mutation class<sup>22</sup>; however, we were not able to demonstrate an association between sweat chloride and early pancreatic injury as measured through IRT levels. This is consistent with the inability to detect a difference in IRT levels in the first 2 years between patients mild and severe forms of the disease, although sweat chloride concentrations are often lower in patients with mild disease than in patients with severe disease. Finally, the early nutrition of infants with CF is critically important, and we have found that the choice to breast or bottle feed does not influence the rate of pancreatic damage, as measured by IRT levels.

We had hypothesized that earlier treatment with pancreatic enzyme therapy might affect IRT levels either through a feedback mechanism to the pancreas, or because assays may not measure IRT accurately in the presence of enzyme supplementation. The assay used by the research lab measured only cationic trypsinogen, which does not appear to be affected by enzyme supplementation,<sup>23</sup> and the measurements in the newborn period were taken before the initiation of pancreatic enzymes, therefore avoiding the interaction problem. We found higher IRTs were associated with earlier enzyme introduction, in both modeled IRTs from the newborn period and from the older child. We conclude from this that initial IRT elevation must reflect more severe pancreatic disease. Infants with higher IRTs may have presented with more signs and symptoms of malabsorption, which may have prompted the clinicians to initiate earlier pancreatic enzyme

supplementation. This finding provides new insight into the significance of IRT as a biochemical marker of pancreatic disease in infancy and supports our conclusion that the IRT determinations are not subject to the environmental effect of enzyme supplementation. This knowledge may provide targets for therapeutic intervention with agents that have CFTR activity, and a specific population in which to initiate the intervention, which may help to slow pancreatic destruction and may even help to preserve pancreatic function. Small molecules that potentiate CFTR activity at the surface of cells or that correct  $\Delta F508$  trafficking through the endoplasmic reticulum are being developed. These molecules, if applied early, may preserve pancreatic function.<sup>24,25</sup>

The similarity of pancreatic disease among siblings with CF has long been known,<sup>26</sup> and has been believed to be the underlying CF genotype. We identified similarities in IRT decline between individual siblings that have not previously been described that point to additional genetic factors, specifically modifier genes. If modifier genes that affect IRT decline can be identified, they may provide better understanding of the underlying pathophysiology of CF pancreatic disease and may point to modifiers of CF lung disease. Because siblings also have a shared environment, it is possible this higher heritability may also be because of environmental effects. However, the pancreas is not susceptible to many environmental factors, and the heritability was seen in infancy, so there is a small window of time for environmental factors to have affected the pancreas.

Comparison of our model to the model of IRT decline developed by Couper et al<sup>2</sup> raises several interesting points (Figure 2, A). The intercept in our model is higher than Couper et al's intercept. Couper et al's data are from children and adults with CF who presented clinically; our data are from children who were identified in infancy by NBS or MI. Couper et al's model is, therefore, unable to accurately describe the initial IRT elevation in infancy, the time when IRT is most dramatically elevated. Couper et al's data also has IRT values well into adulthood, with actual values reported  $<5\text{ng/mL}$ ; our data were censored and all values  $<5\text{ng/mL}$  were reported as  $5\text{ng/mL}$ . This has two important implications. First, Couper et al's data may more accurately describe the actual rate of decline at the low range in adolescents and young adults because IRT determinations were available on many more persons. However, the values reported by Couper et al  $<5\text{ng/mL}$  may test the accuracy of the assay in the extreme low values. Second, the IRT determinations from the persons  $>18$  years of age in Couper et al's data may be influenced by a survivor effect. Although the relationship between severity of lung disease and pancreatic disease is not well understood, it can be hypothesized that the adults in Couper et al's data may have survived because of milder lung disease, and the milder lung disease may also reflect milder pancreatic disease. This survivor effect may explain the slower decline and higher value at 15 years of age compared with our model.

We used longitudinal data collected over the lifetime of

the child to characterize IRT decline, and we used the modeled slopes and intercepts as a quantitative phenotype. This study demonstrates heritability of a statistically modeled quantitative phenotype. Modeling quantitative phenotypes in this manner may help to define phenotypes in other chronic diseases and may point to phenotypes that are subject to modifier effects. Describing the rate of decline in IRT in children with CF, and that it is heritable, have important implications for future research and may provide an opportunity for therapy to prevent or halt future damage. The slower decline in some children may reflect other biologic or environmental processes that may affect the rate of decline and the rate of complete pancreatic destruction. Understanding the determinants of pancreatic destruction through our biochemical marker of IRT decline may provide opportunities for new therapies. We have shown that several suspected risk factors including sex, birth weight, gestational age, and feeding method in infancy did not influence IRT decline in our population. In addition, among siblings with CF, we have shown that IRT slope and the predicted value of IRT at specific ages are heritable. These findings point to a potential role for gene modifiers in early CF-related pancreatic disease.

*The authors would like to thank Ms Laura Taylor and Mr Daniel Wright from the Colorado Department of Public Health and Environment for their assistance in data collection and clarification, and Ms Iris Osberg and Mr Keith Hammond for assistance in interpretation. These studies were carried out as part of a PhD in Analytic Health Sciences – Epidemiology, at the University of Colorado Health Sciences Center.*

## REFERENCES

1. Crossley JR, Elliott RB, Smith PA. Dried-blood spot screening for cystic fibrosis in the newborn. *Lancet* 1979;1:472-4.
2. Couper RT, Corey M, Durie PR, Forstner GG, Moore DJ. Longitudinal evaluation of serum trypsinogen measurement in pancreatic-insufficient and pancreatic-sufficient patients with cystic fibrosis. *J Pediatr* 1995;127:408-13.
3. Hammond KB, Abman SH, Sokol RJ, Accurso FJ. Efficacy of state-wide neonatal screening for cystic fibrosis by assay of trypsinogen concentrations. *N Engl J Med* 1991;325:769-74.
4. Sontag MK, Hammond KB, Zielenski J, Wagener JS, Accurso FJ. Two-tiered immunoreactive trypsinogen-based newborn screening for cystic fibrosis in Colorado: screening efficacy and diagnostic outcomes. *J Pediatr* 2005;147:S83-S88.
5. Kuczmarski RJ, Ogden CL, Guo SS, Grummer-Strawn LM, Flegal KM, Mei Z, et al. 2000 CDC Growth Charts for the United States: methods and development. *Vital Health Stat* 11 2002;1-190.
6. Feranchak AP, Sontag MK, Wagener JS, Hammond KB, Accurso FJ, Sokol RJ. Prospective, long-term study of fat-soluble vitamin status in children with cystic fibrosis identified by newborn screen. *J Pediatr* 1999;135:601-10.
7. Buzin CH, Wen CY, Nguyen VQ, Nozari G, Mengos A, Li X, et al. Scanning by DOVAM-S detects all unique sequence changes in blinded analyses: evidence that the scanning conditions are generic. *Biotechniques* 2000;28:746-3.
8. Cohn JA, Neoptolemos JP, Feng J, Yan J, Jiang Z, Greenhalf W, et al. Increased risk of idiopathic chronic pancreatitis in cystic fibrosis carriers. *Hum Mutat* 2005;26:303-7.
9. Bronstein MN, Sokol RJ, Abman SH, Chatfield BA, Hammond KB, Hambidge KM, et al. Pancreatic insufficiency, growth, and nutrition in infants identified by newborn screening as having cystic fibrosis. *J Pediatr* 1992;120:533-40.

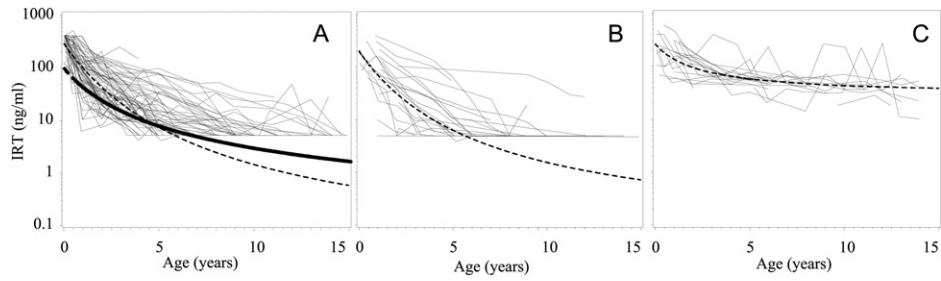
10. Van de Kamer J, ten Bokkel Huinink H, Wegers H. Rapid method for determination of fat in feces. *J Biol Chem* 1949;177:347-55.
11. Jeejeebhoy KN, Ahmad S, Kozak G. Determination of fecal fats containing both medium and long chain triglycerides and fatty acids. *Clin Biochem* 1970;3:157-63.
12. Kristidis P, Bozon D, Corey M, Markiewicz D, Rommens J, Tsui LC, et al. Genetic determination of exocrine pancreatic function in cystic fibrosis. *Am J Hum Genet* 1992;50:1178-84.
13. Tsui LC. The spectrum of cystic fibrosis mutations. *Trends Genet* 1992; 8:392-8.
14. Jacqmin-Gadda H, Thiebaut R, Chene G, Commenges D. Analysis of left-censored longitudinal data with application to viral load in HIV infection. *Biostatistics* 2000;1:355-68.
15. Thiebaut R, Jacqmin-Gadda H, Lepout C, Katlama C, Costagliola D, Le Moing V, et al. Bivariate longitudinal model for the analysis of the evolution of HIV RNA and CD4 cell count in HIV infection taking into account left censoring of HIV RNA measures. *J Biopharm Stat* 2003; 13:271-82.
16. Rusakow LS, Abman SH, Sokol RJ, Seltzer WK, Hammond KB, Accurso FJ. Immunoreactive trypsinogen levels in infants with cystic fibrosis complicated by meconium ileus. *Screening* 1993;2:13-7.
17. Cleghorn G, Benjamin L, Corey M, Forstner G, Dati F, Durie P. Serum immunoreactive pancreatic lipase and cationic trypsinogen for the assessment of exocrine pancreatic function in older patients with cystic fibrosis. *Pediatrics* 1986;77:301-6.
18. Durie PR, Largman C, Brodrick JW, Johnson JH, Gaskin KJ, Forstner GG, et al. Plasma immunoreactive pancreatic cationic trypsinogen in cystic fibrosis: a sensitive indicator of exocrine pancreatic dysfunction. *Pediatr Res* 1981;15:1351-5.
19. Whitehead FJ, Couper RT, Moore L, Bourne AJ, Byard RW. Dehydration deaths in infants and young children. *Am J Forensic Med Pathol* 1996;17:73-8.
20. Waters DL, Dorney SF, Gaskin KJ, Gruca MA, O'Halloran M, Wilcken B. Pancreatic function in infants identified as having cystic fibrosis in a neonatal screening program [see comments]. *N Engl J Med* 1990;322:303-8.
21. Davis PB. The gender gap in cystic fibrosis survival. *J Gend Specif Med* 1999;2:47-51.
22. Wilschanski M, Zielenski J, Markiewicz D, Tsui LC, Corey M, Levison H, et al. Correlation of sweat chloride concentration with classes of the cystic fibrosis transmembrane conductance regulator gene mutations. *J Pediatr* 1995;127:705-10.
23. Durie PR, Gaskin KJ, Geokas MC, O'Rourke M, Largman C. Plasma immunoreactive anionic pancreatic trypsin in cystic fibrosis. *J Pediatr Gastroenterol Nutr* 1982;1:337-43.
24. Pedemonte N, Lukacs GL, Du K, Caci E, Zegarra-Moran O, Galiotta LJ, et al. Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. *J Clin Invest* 2005;115:2564-71.
25. Suen YF, Robins L, Yang B, Verkman AS, Nantz MH, Kurth MJ. Sulfamoyl-4-oxoquinoline-3-carboxamides: novel potentiators of defective DeltaF508-cystic fibrosis transmembrane conductance regulator chloride channel gating. *Bioorg Med Chem Lett* 2006;16:537-40.
26. Corey M, Durie P, Moore D, Forstner G, Levison H. Familial concordance of pancreatic function in cystic fibrosis. *J Pediatr* 1989;115:274-7.
27. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, et al. Identification of the cystic fibrosis gene: genetic analysis. *Science* 1989;245:1073-80.
28. Borgo G, Mastella G, Gasparini P, Zorzanello A, Doro R, Pignatti PF. Pancreatic function and gene deletion F508 in cystic fibrosis. *J Med Genet* 1990;27:665-9.
29. Liechti-Gallati S, Bonsall I, Malik N, Schneider V, Kraemer LG, Ruedeberg A, et al. Genotype/phenotype association in cystic fibrosis: analyses of the delta F508, R553X, and 3905insT mutations. *Pediatr Res* 1992;32:175-8.
30. Highsmith WE Jr, Burch LH, Zhou Z, Olsen JC, Strong TV, Smith T, et al. Identification of a splice site mutation (2789 +5 G > A) associated with small amounts of normal CFTR mRNA and mild cystic fibrosis. *Hum Mutat* 1997;9:332-8.
31. Vazquez C, Antinolo G, Casals T, Dapena J, Elorz J, Seculi JL, et al. Thirteen cystic fibrosis patients, 12 compound heterozygous and one homozygous for the missense mutation G85E: a pancreatic sufficiency/insufficiency mutation with variable clinical presentation. *J Med Genet* 1996; 33:820-2.
32. Borrego S, Casals T, Dapena J, Fernandez E, Gimenez J, Cabeza JC, et al. Molecular and clinical analyses of cystic fibrosis in the south of Spain. *Clin Genet* 1994;46:287-90.
33. Weller F, Wiebicke W, Tummler B. [Turkish infant with hypoelectrolytemia and metabolic alkalosis as the sole manifestations of a mild form of cystic fibrosis (mutation D110H)]. *Klin Padiatr* 2000;212:41-3.
34. Oates RD, Amos JA. Congenital bilateral absence of the vas deferens and cystic fibrosis. A genetic commonality. *World J Urol* 1993;11:82-8.
35. Orozco L, Lezana JL, Villarreal MT, Chavez M, Carnevale A. Mild cystic fibrosis disease in three Mexican delta-F508/G551S compound heterozygous siblings. *Clin Genet* 1995;47:96-8.
36. Ferec C, Verlingue C, Guillermit H, Quere I, Raguene O, Feigelson J, et al. Genotype analysis of adult cystic fibrosis patients. *Hum Mol Genet* 1993; 2:1557-60.
37. Cystic Fibrosis Mutation Database [updated 7-13-2005]. Cystic Fibrosis Mutation Consortium. Available online at: <http://www.genet.sickkids.on.ca/cftr/>.
38. Castaldo G, Fuccio A, Cazeneuve C, Picci L, Salvatore D, Raia V, et al. Detection of five rare cystic fibrosis mutations peculiar to Southern Italy: implications in screening for the disease and phenotype characterization for patients with homozygote mutations. *Clin Chem* 1999;45:957-62.
39. Ronchetto P, Telleria Orriols JJ, Fanen P, Cremonesi L, Ferrari M, Magnani C, et al. A nonsense mutation (R1158X) and a splicing mutation (3849 + 4A---G) in exon 19 of the cystic fibrosis transmembrane conductance regulator gene. *Genomics* 1992;12:417-8.
40. Schwartz M, Anvret M, Claustres M, Eiken HG, Eiklid K, Schaedel C, et al. 394delTT: a Nordic cystic fibrosis mutation. *Hum Genet* 1994;93:157-61.
41. Wang J, Bowman CM, Wong LJ. A novel CFTR frame-shift mutation, 935delA, in two Hispanic cystic fibrosis patients. *Mol Genet Metab* 2000;70:316-21.
42. Dork T, Kalin N, Stuhmann M, Schmidtke J, Tummler B. A termination mutation (2143delT) in the CFTR gene of German cystic fibrosis patients. *Hum Genet* 1992;90:279-84.
43. Zielenski J, Bozon D, Kerem B, Markiewicz D, Durie P, Rommens JM, et al. Identification of mutations in exons 1 through 8 of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics* 1991;10:229-35.
44. Shoshani T, Augarten A, Yahav J, Gazit E, Kerem B. Two novel mutations in the CFTR gene: W1089X in exon 17B and 4010delTATT in exon 21. *Hum Mol Genet* 1994;3:657-8.
45. Romey MC, Tuffery S, Desgeorges M, Bienvenu T, Demaille J, Claustres M. Transcript analysis of CFTR frameshift mutations in lymphocytes using the reverse transcription-polymerase chain reaction technique and the protein truncation test. *Hum Genet* 1996;98:328-32.
46. Wong LJ, Wang J, Zhang YH, Hsu E, Heim RA, Bowman CM, et al. Improved detection of CFTR mutations in Southern California Hispanic CF patients. *Hum Mutat* 2001;18:296-307.
47. Alper OM, Wong LJ, Hostetter G, Cook J, Tenenholz B, Hsu E, et al. 1154insTC is not a rare CFTR mutation. *Am J Med Genet A* 2003;120:294-5.
48. Walkowiak J, Herzig KH, Witt M, Pogorzelski A, Piotrowski R, Barra E, et al. Analysis of exocrine pancreatic function in cystic fibrosis: one mild CFTR mutation does not exclude pancreatic insufficiency. *Eur J Clin Invest* 2001; 31:796-801.

**Table I. Classification of CFTR mutations as typically severe or mild in the patient population. Patients were considered to have mild disease with one or more mild mutations. References suggesting severity of mutation are presented.**

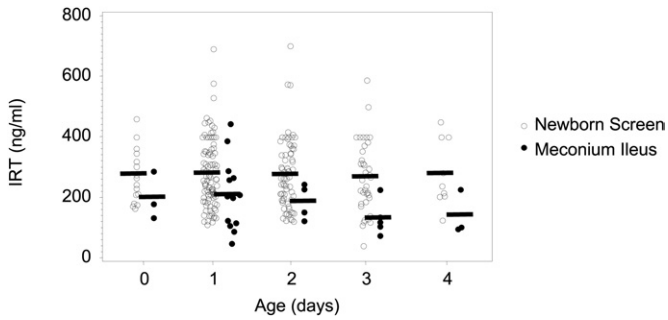
Severe			Mild		
Mutation	Allele Frequency	Percent	Mutation	Allele Frequency	Percent
ΔF508 <sup>27,28</sup>	397	71.27	R117H <sup>12</sup>	4	0.72
G542X <sup>12</sup>	21	3.77	R347P <sup>12</sup>	4	0.72
R553X <sup>12,29</sup>	10	1.80	2789+5G>A <sup>30</sup>	3	0.54
621+1G>T <sup>12</sup>	9	1.62	A455E <sup>12</sup>	2	0.36
G551D <sup>12</sup>	8	1.44	G85E <sup>31</sup>	2	0.36
N1303K <sup>32</sup>	6	1.08	D110H <sup>33</sup>	1	0.18
W1282X <sup>12</sup>	6	1.08	D1270N <sup>34</sup>	1	0.18
ΔI507 <sup>12</sup>	4	0.72	G551S <sup>35</sup>	1	0.18
R1162X <sup>32</sup>	4	0.72	I336K <sup>36</sup>	1	0.18
1898+1G>A <sup>37*</sup>	3	0.54	P67L <sup>37</sup>	1	0.18
3905insT <sup>29</sup>	3	0.54	R117C <sup>37</sup>	1	0.18
Q493X <sup>12</sup>	2	0.36	R334W <sup>12</sup>	1	0.18
R1158X <sup>38,39</sup>	2	0.36			
394delTT <sup>40</sup>	2	0.36			
663delT <sup>41</sup>	2	0.36			
1717-1G>A <sup>12</sup>	2	0.36			
2143delT <sup>42</sup>	2	0.36			
G178R <sup>43</sup>	1	0.18			
Q1382X <sup>37*</sup>	1	0.18			
Q2X <sup>37*</sup>	1	0.18			
R560T <sup>12</sup>	1	0.18			
V520F <sup>12</sup>	1	0.18			
W1089X <sup>44</sup>	1	0.18			
394delTT <sup>37,45</sup>	1	0.18			
406-1G->A <sup>37,46</sup>	1	0.18			
I154InsTC <sup>47</sup>	1	0.18			
I213delT*	1	0.18			
1898+5G>T <sup>37*</sup>	1	0.18			
2183AA-G <sup>48</sup>	1	0.18			
2183delAA* <sup>37</sup>	1	0.18			
2184insA <sup>48</sup>	1	0.18			
3349insT*‡	1	0.18			
3659delC <sup>12</sup>	1	0.18			
Not identified	36	6.46			

\*Clinically determined pancreatic status.

‡Novel mutation.



**Figure 2.** Log IRT declines in a predictable manner in children with CF. Longitudinal IRT levels on individual children are represented and overall predicted model for IRT decline. **A**, Subjects with severe disease (dashed line), with Couper et al's model overlaid (solid line). **B**, Infants with meconium ileus (MI). **C**, Subjects with pancreatic sufficiency. Longitudinal mixed effects modeling with likelihood modified for censored values was used to develop the statistical decline models.



**Figure 3.** IRT levels are lower in infants with meconium ileus (MI) but do not show significant variability day to day. Measurements on different infants with CF on each of the first 4 days of life demonstrate that infants with MI have lower IRT levels than infants without MI ( $P < .01$ ).