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Evidence for a Causal Relationship Between Early Exocrine Pancreatic Disease and Cystic Fibrosis–Related Diabetes: A Mendelian Randomization Study

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PATHOPHYSIOLOGY

Circulating immunoreactive trypsinogen (IRT), a biomarker of exocrine pancreatic disease in cystic fibrosis (CF), is elevated in most CF newborns. In those with severe CF transmembrane conductance regulator (CFTR) genotypes, IRT declines rapidly in the first years of life, reflecting progressive pancreatic damage. Consistent with this progression, a less elevated newborn IRT measure would reflect more severe pancreatic disease, including compromised islet compartments, and potentially increased risk of CF-related diabetes (CFRD). We show in two independent CF populations that a lower newborn IRT estimate is associated with higher CFRD risk among individuals with severe *CFTR* genotypes, and we provide evidence to support a causal relationship. Increased $\log_e$(IRT) at birth was associated with decreased CFRD risk in Canadian and Colorado samples (hazard ratio 0.30 [95% CI 0.15–0.61] and 0.39 [0.18–0.81], respectively). Using Mendelian randomization with the *SLC26A9* rs7512462 genotype as an instrumental variable since it is known to be associated with IRT birth levels in the CF population, we provide evidence to support a causal contribution of exocrine pancreatic status on CFRD risk. Our findings suggest CFRD risk could be predicted in early life and that maintained ductal fluid flow in the exocrine pancreas could delay the onset of CFRD.

Cystic fibrosis (CF), a recessive disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, affects organs including the lungs and pancreas. CF severity is partly determined by *CFTR*; for example, exocrine pancreatic insufficiency (PI) occurs primarily in individuals with two severe CFTR mutations, although the degree of pancreatic damage (PD) at birth and the age at which clinically defined PI develops are variable among patients with the same *CFTR* genotypes (1,2). CF-related diabetes (CFRD) develops later in life in a subset of these individuals who are PI but not in those who are pancreatic-sufficient (3). The relationship between PI and CFRD is not well-understood.

Prenatally, the healthy exocrine pancreas shows considerable growth and maturation during the third trimester. At birth, secretion of most proteases (e.g., trypsinogen) are

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almost at adult levels (4). In CF, pancreatic development is markedly compromised beginning in utero. Obstruction of small pancreatic ducts by precipitated proteinaceous acinar secretions and mucus lead to dilation of proximal ductal and acinar lumina that compromise the acini (5). This process continues postnatally, where it has been observed that the exocrine pancreas undergoes destruction and is replaced with fat and/or fibrotic tissue in patients with severe CFTR genotypes (6,7).

In many countries, increased circulating immunoreactive trypsinogen (IRT) levels at birth are used as a newborn screening (NBS) test for CF in which IRT values above the normal population level are a biomarker of CF. Given that the elevated serum IRT exhibits rapid decline in the first year of life in CF patients with severe *CFTR* genotypes (8), we presumed that a relatively less elevated IRT value (lower IRT) at birth reflected more severe early disease with extensive PD, reduction of acinar tissue, and less capacity to produce digestive enzymes.

CFRD prevalence increases with age, and by the fourth decade of life >40% of PI patients have CFRD (9). CFRD is associated with lower BMI, female sex, pulmonary exacerbations, and type 2 diabetes family history (10,11). Since CFRD is limited to PI individuals and primarily characterized by impaired secretion of insulin, it is hypothesized that progressive damage to the exocrine pancreas by inflammation and fibrosis may also compromise islet cells. In this regard, single nucleotide polymorphism rs7512462 in the solute carrier family gene *SLC26A9* has been shown to associate with both IRT birth measurements and the risk of developing CFRD, but not with CF lung disease (11–13). *SLC26A9,* an apical epithelial cell transporter that interacts with CFTR (14,15) and is expressed in the lung and at lower levels in the pancreas and prostate (16), is hypothesized to influence ion transport (chloride and bicarbonate) in pancreatic ducts prior to destruction of the epithelial exocrine tissue. Electrophysiological studies further support epithelial expression of SLC26A9 (17); hence, we suggest that loss of acinar epithelial tissue is concordant with loss of SLC26A9. In contrast to the exocrine pancreas, epithelial solute carriers such as SLC26A9 are unlikely to have a direct role in endocrine compartments, as the endocrine pancreas delivers its protein load to the bloodstream and surrounding cells without a specialized ductal system.

In two independent samples, we show that IRT levels at birth and their rate of decline, which reflect loss of pancreatic acinar tissue, are associated with CFRD among CF patients with severe *CFTR* genotypes. However, given the observational design, this association may be due to other unmeasured factors (i.e., confounding). A clinical trial that randomizes patients to a more or less severe degree of early exocrine PD could eliminate unmeasured confounding and assess the causal relationship with CFRD but is not possible; instead, we use an application of instrumental variable analysis known as Mendelian randomization (MR) (18). Assuming rs7512462 is unrelated to confounding factors that may influence the relationship between IRT and CFRD, MR compares the relationship between rs7512462 and IRT at birth with the relationship between rs7512462 and CFRD to estimate the causal effect of exocrine PD on the risk of CFRD (18).

RESEARCH DESIGN AND METHODS

In the Canadian Cystic Fibrosis Gene Modifier Study (CGS), DNA from 1,661 PI CF individuals (with known CFRD status) was genotyped on the Illumina 610-Quad array (Illumina). All CGS participants were diagnosed by characteristic clinical manifestations of CF. IRT measurements, including a baseline measure within 24 months of age, were available on 126 of these 1,661 individuals. The additional 1,535 CGS participants were genotyped at rs7512462 but were without IRT measurements.

A replication sample, without genotypes, included 260 CF PI patients from Colorado diagnosed through a statewide NBS program and voluntarily enrolled in a longitudinal study (8). Institutional review committees at all participating Canadian CF clinics and at the Children's Hospital Colorado approved this study.

Data collection, IRT protocols, genotyping, and quality control procedures are reported elsewhere (8,19,20).

CFRD status was determined on chart review. Individuals required a physician's diagnosis, no type 1 or type 2 diabetes, and one of the following: *1*) daily treatment with insulin or oral diabetes medication; *2*) abnormal 2-h glucose level (>11.1 mmol/L or >200 mg/dL) during modified oral glucose tolerance test; or *3*) $HbA_{1c} \geq 7\%$.

Statistical Methodology

In the CGS, a linear mixed-effects regression model, accounting for the limits of detection of the IRT assay (<3 ng/mL) by the statistical model (21), was used to estimate patient-specific IRT measurements at birth and rates of decline. IRT and age (days) were natural log ($\log_e$) transformed. Meconium ileus status was included in the regression model as a covariate since individuals with meconium ileus, on average, had IRT measurements taken at younger ages.

For the Colorado sample, we used linear mixed-effects modeling with $\log_e$ transformations of IRT and age, accounting for values >400 and <5 ng/mL beyond the limits of detection of the assay (21). Meconium ileus was not included in the model because individuals were ascertained at birth.

The patient-specific IRT birth and rates of decline estimates from these models were used as individual measures of prenatal and early postnatal PD.

To assess association between exocrine PD and CFRD, age at CFRD diagnosis (or age at last clinic visit without CFRD) was regressed on estimated values of $\log_e$(IRT) at birth and rate of decline using sex-adjusted Cox proportional hazard models for the Canadian and Colorado samples with the R software package (22).

To determine if exocrine PD contributes to the cause of CFRD, we estimated the causal effect using MR in the

Canadian sample. We compared it to the (possibly confounded) direct estimate obtained by regressing age of CFRD diagnosis on estimated values of $\log_e$(IRT) at birth in sex-adjusted Cox models. MR requires one exposure variable per instrumental variable. In this study, we use estimated $\log_e$(IRT) at birth, as opposed to both birth and rate of decline measures, to reflect the exocrine PD exposure. The causal effect on CFRD risk was estimated using the two-sample MR method with rs7512462 coded additively as the instrumental variable (18,23). The two-sample method uses the full CGS-genotyped sample (n = 1,661) in the MR analysis (23). Dividing the association estimate from the Cox regression of CFRD on rs7512462 by the average increase in $\log_e$(IRT) at birth per additional rs7512462 risk allele gives the $\log_e$(hazard ratio [HR]) that represents the association of genetically determined $\log_e$(IRT) levels at birth with CFRD risk (18). SEs for the two-sample causal HR were estimated using the delta method (24).

RESULTS

Is Early Exocrine PD Associated With CFRD?

IRT measurements decline with advancing age (Fig. 1 and Table 1). After adjustment for rate of $\log_e$(IRT) decline and sex, $\log_e$(IRT) at birth was associated with CFRD risk in the Canadian (HR 0.30; P = 0.001) and Colorado samples (HR 0.39; P = 0.012); we observed a 70 and 61% decrease, respectively, in the rate of CFRD for every one-unit increase in $\log_e$(IRT) at birth. Rate of decline of $\log_e$(IRT) was also associated with CFRD in the Canadian (P = 0.007) and Colorado (P = 0.01) samples; a sharper decline in IRT, reflective of a more rapid depletion of acinar cell mass, predicted greater CFRD risk (Table 2).

Is the Exocrine PD–CFRD Relationship Causal?

Using estimated $\log_e$(IRT) at birth (see RESEARCH DESIGN AND METHODS), we assessed whether PD causally contributes to CFRD by applying MR with rs7512462 as an instrumental variable. We then compared this causal estimate to the risk estimate obtained directly from regressing CFRD on $\log_e$(IRT) at birth (Fig. 2 and Table 2).

In the Canadian sample, rs7512462 was associated with estimated $\log_e$(IRT) at birth ($\beta = -0.53$; P = 0.003; $F_{1,124}$ = 8.99) in a linear regression and explained 7% of its variance; individuals with the T allele have lower levels of IRT at birth. This coefficient was the denominator for the MR estimate.

In the larger genotyped CGS sample, the rs7512462 T allele conferred increased CFRD risk (sex-adjusted HR 1.19; P = 0.04). The $\log_e$(HR) from this analysis was the numerator in the MR. These 1,661 individuals are a subset from a previously reported *SLC26A9*–CFRD association (11).

The two-sample causal estimate of the HR for CFRD was 0.72 (95% CI 0.49–1.05) (Fig. 2 and Table 2), corresponding to a 28% decrease in the rate of CFRD for every one-unit increase in $\log_e$(IRT) at birth. Although the MR–HR CI slightly overlaps 1, its width is overestimated (18,24). This MR estimate is equivalent to the HR estimated directly from a regression of CFRD on $\log_e$(IRT) at birth, without adjustment for rate of $\log_e$(IRT) decline (sex-adjusted HR 0.72; P = 0.017). This agreement between the causal and the direct HR estimate, along with an absence of association between rs7512462 and other CFRD correlates (12), suggests that the direct measure of association between exocrine PD and CFRD is largely free of confounding.

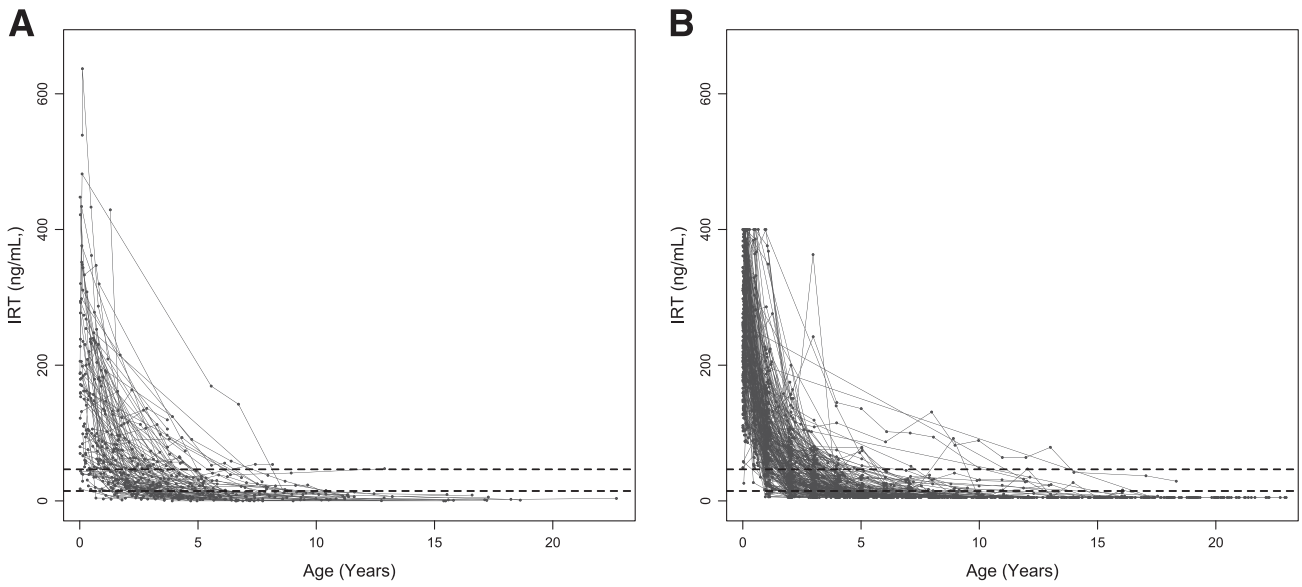

Figure 1—Longitudinal IRT trajectories for each participant in the Canadian (*A*) (n = 126) and Colorado (*B*) (n = 260) samples. Dotted lines represent the normal range of IRT measurement in the non-CF population (14.6–46.5 ng/mL).

Table 1—Characteristics of study participants

	Canadian with IRT	Canadian without IRT	Colorado with IRT
Sample size (*N*)	126	1,535	260
Female (%)	64 (51)	697 (45)	132 (51)
CFRD diagnosis	25	219	35
Median years at last study visit (or diabetes)	11.8 (16.6)	16.7 (23.8)	10.1 (16.5)
IRT measurements per patient (range)	1–13	—	1–22
IQR of $\log_e$(IRT) (ng/mL)	2.28–4.37	—	1.61–4.26
Median days at first IRT (range)	130 (3–718)	—	14 (1–718)
MI (%)	33 (26)	219 (14)	37 (19)*
Homozygous delF508 (%)	76 (60)	916 (60)	168 (65)

delF508, *CFTR* gene mutation Phe508del; IQR, interquartile range. *Meconium ileus (MI) status available on 197 participants from Colorado.

DISCUSSION

We demonstrate a robust, replicated association between exocrine PD and CFRD among CF patients with severe CFTR genotypes, providing evidence that lower early IRT is a biomarker for CFRD risk. Using the *SLC26A9* CF modifier (12,20) in an MR framework, we provide evidence that this relationship is causal. CFRD is also associated with type 2 diabetes family history and susceptibility genes (11). Therefore, severe injury to the pancreatic islets by exocrine pancreatic inflammation and fibrosis, perhaps acting in concert with a genetic background that confers increased susceptibility to insulin resistance, may contribute to greater risk of CFRD.

Among other assumptions (Fig. 2), MR requires that rs7512462 is unrelated to CFRD other than through the effect of exocrine PD. SLC26A9 is expressed in the pancreas, lungs, and prostate (16), but replicated evidence suggests SLC26A9 is not associated with CF lung disease (12,13). This assumption further requires that rs7512462 is not pleiotropic for CFRD, but the relationship is mediated by proximity of the endocrine compartment [only 15% of the pancreas in neonates (25)] to damaged exocrine pancreas. The co-occurrence of SLC26A9 and CFTR in exocrine ducts is consistent with recognized direct (14) and functional (15) interactions and with the early evolving CF exocrine disease (26). As both SLC26A9 and CFTR proteins provide ion transport (chloride and bicarbonate), a straightforward interpretation is that SLC26A9 may augment ion transport when CFTR function is impaired, explaining its modifying feature. However, epithelial solute carriers such as SLC26A9 would not appear to have a direct role in endocrine compartments. Animal models can be insightful, provided cross-species distinctions in disease processes are known. A chronological picture of disease in the CF pig model is consistent with human observations, with exocrine disease initiating prior to birth (27). The pig pancreas is noted for similarity in a number of respects to the human pancreas and provides the source of digestive enzyme-replacement therapy

Table 2—Cox proportional hazards model for effect of early exocrine PD [measured by $\log_e$(IRT) at birth and rate of decline] on CFRD risk adjusted for sex (other risk factors such as BMI and type 2 diabetes family history were not available) and the effect of prenatal exocrine PD [measured by $\log_e$(IRT) at birth] on CFRD risk (comparison between direct association by Cox proportional hazards modeling and two-sample causal estimation by MR; HR <1 implies greater CFRD risk with greater PD [lower IRT])

	HR	95% CI
Is early exocrine PD associated with CFRD?		
Canadian (*n* = 126; CFRD event = 25)		
$\log_e$(IRT) at birth	0.30	0.15–0.61
$\log_e$(IRT) rate of decline	0.004	1e-4–0.23
Colorado (*n* = 260; CFRD event = 35)		
$\log_e$(IRT) at birth	0.39	0.18–0.81
$\log_e$(IRT) rate of decline	0.002	2e-5–0.24
	Effect size (HR or mean difference)	**95% CI**
Is the exocrine PD-CFRD relationship causal?		
Effect of IRT on CFRD by direct association		
HR of CFRD for a unit increase of $\log_e$(IRT) (*n* = 126)	0.72	0.55–0.94
Causal effect of IRT on CFRD by MR		
Mean difference in $\log_e$(IRT) per additional rs7512462 risk allele (*n* = 126)	−0.53	−0.89 to −0.18
HR of CFRD for an additional rs7512462 risk allele (*n* = 1,661)	1.19	1.01–1.41
Two-sample causal estimate for HR of CFRD for a unit increase of $\log_e$(IRT)	$e^{[\log(1.19)/-0.53]} = 0.72$	0.49–1.05

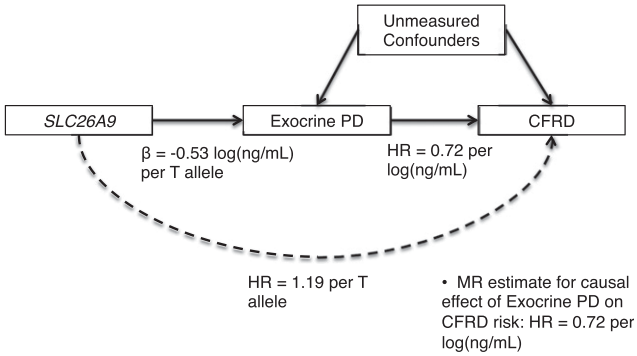

Figure 2—MR framework. MR estimate of causal effect of prenatal exocrine PD on CFRD risk was obtained by dividing the coefficient from the regression of CFRD diagnosis age on rs7512462 genotype, $\log_e$(HR 1.19), by the average increase in $\log_e$(IRT) at birth per additional *SLC26A9* rs7512462 risk allele, $\beta = -0.53$, yielding: $\log_e(HR_{causal}) = \log_e(1.19)/(-0.53) = -0.33$. Exponentiation provided an HR_{causal} of 0.72. This causal effect estimate of the HR was equivalent to the estimate obtained by directly regressing CFRD diagnosis age on $\log_e$(IRT) at birth (HR 0.72). Application of MR required that the *SLC26A9* instrument be robustly associated with the exposure and independent of confounding factors; these were satisfied by our *F* statistic of 8.99 and Mendel's law of independent assortment, respectively. Additionally, it was assumed that the relationship between rs7512462 and CFRD was mediated by prenatal exocrine PD (see DISCUSSION).

for CF patients. In the less-studied CF ferret model, there are indications of abnormalities in blood glucose and insulin regulation shortly after birth, but dilated pancreatic ducts, intraluminal material, and apoptotic cells of exocrine tissue are present, also supporting exocrine disease prior to birth (28).

Untreated CFRD negatively affects nutritional status and pulmonary function and is associated with increased mortality (29). Our findings highlight the possibility of predicting CFRD susceptibility in early life using, among other factors, the widely available NBS IRT measurement. The added value of including IRT rate of decline (i.e., decline in pancreatic reserve) in assessing CFRD risk (R^2 = 10.4 vs. 4.6%) suggests that CFRD is not determined by prenatal damage alone, and collecting additional measurements would improve prediction. The causal evidence between exocrine and endocrine pancreatic disease indicates that therapeutic interventions to promote ductal fluid flow in the exocrine pancreas could slow the progression of exocrine PD and perhaps delay onset of CFRD.

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