Pancreatic phenotype in infants with cystic fibrosis identified by mutation screening

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Objective: To determine the pancreatic phenotype of infants with cystic fibrosis (CF) diagnosed in the first week of life by a combined immunoreactive trypsin/mutation screening program.

Design: A prospective evaluation of pancreatic function in infants with CF at the time of neonatal diagnosis and up to the age of 12.

Setting: Two different centres (Verona, Italy and Westmead, Australia) to enable comparison of results between two regions where <60% or ≥90% of patients, respectively, have at least one single $\Delta F508$ mutation.

Patients: 315 children with CF including 149 at Verona and 166 at Westmead.

Interventions: Fat balance studies over 3–5 days and pancreatic stimulation tests with main outcome measures being faecal fat or pancreatic colipase secretion. Patients with malabsorption are pancreatic insufficient (PI) or with normal absorption and pancreatic sufficient (PS).

Results: 34 infants (23%) at Verona and 46 (28%) at Westmead were PS at diagnosis. 15% of those with two class I, II or III "severe" mutations and 26/28 (93%) of those with class IV or V mutations were PS at this early age. Of the 80 infants with PS, 20 became PI before the age of 12. All 20 had two severe mutations.

Conclusion: Neonatal mutational screening programs for CF are less likely to detect PS patients with non- $\Delta F508$ mutations. Of PS patients who are detected, those with two severe class I, II or III mutations are at particularly high risk of becoming PI during early childhood.

Neonatal screening for cystic fibrosis (CF) began in the early 1980s with measurement of immunoreactive trypsin (IRT) assays on dried blood spots obtained from infants in the first 4–6 weeks of life.^{1,2} Questions were raised about the reliability of these programs as they had not been validated for the 10%–15% of patients with normal fat absorption (pancreatic sufficient or PS).³ Subsequently however, 37% of screened infants were found to be PS,^{4,5} that is 2–3 times the 10%–15% observed in older patients.³ Furthermore, follow-up studies demonstrated that nearly 50% of the infants with PS near birth developed fat malabsorption (pancreatic insufficient or PI) in later childhood, thus accounting for the lower proportion of PS patients at an older age. It is significant that all those who transitioned from being PS to PI had two "severe" class I, II or III cystic fibrosis transmembrane conductance regulator (CFTR) mutations,⁶ as defined previously,^{7,8} whereas those with persistent PS had at least one class IV or V "mild" mutation.

Following the discovery of the CFTR gene,⁹ most screening programs have modified their screening strategy.¹⁰ In regions where more than 90% of patients with CF have at least one $\Delta F508$ mutation, analysis for $\Delta F508$ is performed on week 1 neonatal blood spots with an elevated IRT level.¹¹ This strategy has been further modified in regions (particularly southern Europe) where $\Delta F508$ is only present in 40%–60% of patients, by screening for an extended panel of non- $\Delta F508$ mutations (usually severe class I, II or III) common to that region.^{12,13} These IRT/DNA screening strategies are, however, not without problems. For instance, in regions where $\Delta F508$ is responsible for CF in over 90% of cases, up to 10% of patients have non- $\Delta F508$ mutations with a preponderance of PS patients¹⁴ and therefore screening for $\Delta F508$ alone would not detect such patients.

Currently, only limited data are available on the pancreatic phenotype of infants with CF diagnosed by IRT/DNA screening from a study¹⁵ restricted to 27 infants with only class I or II mutations. The present study was thus undertaken, firstly to determine the pancreatic phenotype of infants in either a $\Delta F508$ predominant or non-dominant region, and secondly, to determine what proportion of infants initially with PS later transitioned to being PI and whether this was determined by the presence of two severe mutations.

METHODS

All infants diagnosed with CF by neonatal screening programs at the Verona CF Centre (Verona), Italy and The Children's Hospital at Westmead (Westmead), Sydney, Australia from January 1993 to December 2001 were included in the study.

The basic strategy of the two screening programs was similar: 1) an IRT measurement was taken on dried blood spots obtained from infants in the first week of life; 2) if the IRT was elevated (at Verona >100 µg/l until May 1995 and >95 µg/l thereafter, and at Westmead >99th percentile), a mutation analysis was performed; and 3) the diagnosis was confirmed by sweat chloride estimation. The major difference between the two programs pertained to the mutational analysis: at Verona, a region where at least one copy of $\Delta F508$ was present in only 40%–60% of patients, the blood spots were analysed for $\Delta F508$, R1162X and N1303K prior to March 1995 and ten additional mutations (2183AA→G, 3849+10KbC→T, G542X, 1717-1G→A, R553X, Q552X, G85E, 711+5G→A, 3132delTG, 2789+5G→A)¹⁶

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; IRT, immunoreactive trypsin; MI, meconium ileus; PI, pancreatic insufficient; PS, pancreatic sufficient; PST, pancreatic stimulation test

thereafter; at Westmead, only $\Delta F508$ was screened for. The other minor difference in the screening strategy was that patients at Verona had a meconium lactase test performed and if either a CF mutation was detected and/or the lactase level was >0.5 U/l, a sweat chloride test was carried out. It is noteworthy that nine of the 13 mutations screened at Verona were severe mutations.^{7 17 18} At both centres, elevated IRT, the presence of at least one CF mutation (or elevated meconium lactase in infants from Verona) and an elevated sweat chloride ≥ 60 mmol/l were diagnostic of CF. Infants at both centres with borderline sweat chloride values of 30–59 mmol/l underwent repeat sweat testing and a more extensive panel of mutations was analysed. At Verona this included denaturing gradient gel electrophoresis of coding regions and at Westmead 16 non- $\Delta F508$ mutations: $\Delta 1507$, R117H, G551D, A455E, G542X, N1303K, W128X, 1717-1G $\rightarrow$ A, R560T, R347P, R334W, R553X, R1162X, S549N, 3849+10KbC $\rightarrow$ T and 621+1G $\rightarrow$ T.¹⁹ Excluding $\Delta F508$, six of the other mutations were common to both centres.

At Verona, IRT was assayed by the Pharmacia (Turku, Finland) standardised Delfia Neonatal IRT kit,²⁰ and a variation was used at Westmead.²¹ Sweat chloride testing was performed by the Gibson and Cooke method²² at both centres and meconium lactase testing as described previously.²³ The procedure used to prepare DNA for PCR from the dried blood spots on Guthrie cards was similar at both centres.^{24 25} Between January 1993 and February 1995, mutation analysis was performed by restriction enzymes²⁶ or heteroduplex analysis²⁷ and thereafter by a reverse dot blot assay.²⁸ At Westmead, DNA for mutation analysis was extracted from blood spots by isopropanol fractionation.²⁹

Assessment of pancreatic function in both centres

Fat balance studies

In formula-fed infants, dietary fat intake was recorded for 5 days, and stool was collected in the last 3 days of the 5-day balance study. In breastfed infants, where dietary fat intake was not estimated, stool was collected over 3 days.

Daily faecal fat content was determined by van de Kamer's technique³⁰ and modified by the Jeejeebhoy method for medium chain triglycerides.³¹ If the infant was already receiving oral pancreatic enzyme supplements, these were stopped 48 h prior to the fat balance study. Fat malabsorption was defined as a faecal fat loss $\geq 10\%$ of fat intake in formula-fed infants and faecal fat of ≥ 2 g/day in breastfed infants.⁴

Pancreatic stimulation test (PST)

The PST was performed only at Westmead using a quantitative marker perfusion technique.^{4 32} Pancreatic lipase and colipase were analysed by titrimetric assay³³ and infants with colipase secretion rates $\geq 1\%$ of mean control values were assessed as PS and those with rates $<1\%$ as PI.^{4 32}

Infants designated as PS at diagnosis were evaluated prospectively. Symptoms suggestive of malabsorption, such as

oily/fatty stools, weight loss and/or a decrease in weight percentile and/or a change in normal serum fat soluble vitamin levels to subnormal levels (serum vitamin A <0.6 μ mol/l, vitamin E <7.0 μ g/100 ml), and at Verona faecal chymotrypsin <5 U/g stool, provided indirect evidence of the development of PI which was confirmed by fat balance studies. Patients without a history of malabsorption and/or normal fat balances, normal stool microscopy and pancreatic isoamylase >14 U/l were designated as PS.

Sample size

The sample size was based on the hypothesis that there would be a 10%–15% reduction in the proportion of PS patients among those diagnosed by the IRT mutation strategy versus the older repeat IRT screening strategy (25% vs 37%, respectively). Therefore, at least 300 patients would be required in the current study group to detect this difference with a power of 80% at a significance of 5%. Thus, the study was conducted over 9 years to meet this requirement.³⁴

Data analysis

Data were analysed both cumulatively and separately for the two centres. The χ^2 test was performed to analyse the frequency of PS and PI patients and genotypes between the two phenotypes.

Ethics

The study was approved by the Ethics Committee at The Children's Hospital at Westmead and the Verona Hospital Ethics Committee.

RESULTS

Over the 9-year study interval, 315 neonates were diagnosed as having CF (149 patients at Verona and 166 at Westmead) by newborn screening or by the presence of meconium ileus (MI) (table 1). Gender distributions and the proportions diagnosed by newborn screening (83%, 85%) or because of MI (17%, 15%) (at Verona and Westmead, respectively) were virtually identical. The proportions of patients with PI and PS were also similar with 77% and 72% being PI and 23% and 28% being PS at Verona and Westmead, respectively. Although there was a slight preponderance of PS patients at Westmead, the difference was not significant. The proportion of PS patients of 26% from the combined clinic data was significantly lower than the 37% observed from a non-mutational screening program⁴ ($p<0.05$).

The 3–5-day fat balance results are shown for each centre in fig 1, and according to whether the infant was bottle or breast fed (panels A and B, respectively). Faecal fat varied widely among the PI patients, ranging from 11% to 70% of fat intake in the bottle-fed group and from 2.2 to 19 g/day in the breastfed group. All infants at Verona had fat balance studies. At Westmead 124 (75%) patients had their pancreatic status at neonatal diagnosis determined by fat balance studies (113 infants) or PST (11 infants). Of the 11 who had a PST, 10 had colipase secretion values $>10\%$ of average normal values and were PS and one had a value $<1\%$ of average normal colipase secretion and therefore was PI.⁴ There was a delay in evaluating pancreatic function in an additional 13 patients at Westmead due to parent preference. In 10 of these 13 patients at an average age of 2 years, faecal fats ranged from 11% to 58%, and in the remaining three patients faecal fats were 7.9%, 5.1% and 5.6% at 6 months, 1 and 2 years, respectively. A group of 29 (18%) patients at Westmead did not undergo pancreatic testing. They included 12 with MI, 16 with symptoms of malabsorption and one without symptoms of malabsorption who was a sibling

Table 1 Characteristics of Verona and Westmead patients with cystic fibrosis at diagnosis

	Verona	Westmead	Total
Number of patients	149	166	315
Male (%)	72 (48)	89 (53)	160 (51)
Newborn screening (%)	123 (83)	141 (85)	264 (84)
Meconium ileus (%)	26 (17)	25 (15)	51 (16)
PI (%)	115 (77)	120 (72)	235 (74)
PS (%)	34 (23)	46 (28)	80 (26)

All comparisons between Verona and Westmead were not significant by χ^2 analysis. PI, pancreatic insufficient; PS, pancreatic sufficient.

Figure 1 Feeding type and faecal fat output. (A) Three-day faecal fat output expressed as per cent of fat intake for formula-fed infants (crosses) (actual faecal fat ranged from 0.8 to 28.3 g/day). (B) Fat output in g/day in breastfed infants (diamonds) for both the Westmead and Verona clinics. The horizontal dotted line separates PI patients above the line from PS patients below the line. CHW, The Children's Hospital at Westmead; VCFC Cystic Fibrosis Centre, Verona.

of a PS patient. As those with MI or malabsorption symptoms had severe mutations, we have considered them as PI patients. The genotype/pancreatic phenotype relationships were evaluated for the three general genotypic groups: $\Delta F508$ homozygotes, $\Delta F508$ compound heterozygotes and non- $\Delta F508$ compound heterozygotes as shown in table 2. The compound heterozygote groups were further subdivided into subgroups according to whether mutations were type I–III severe (designated as F/x or x/x), type IV and V mild (F/m or x/m) or unknown (F/uk or x/uk). For both clinics and the combined data, two horizontal rows of data are given. The first row provides the absolute numbers of patients with that specific genotype, noting the bracketed numbers associated with the subtotals represent the subtotal number as a percentage of the total number (n) of patients at that clinic. For example, under the column for $\Delta F508$ homozygotes for Verona 37/149 (25%) and for Westmead 97/166 (58%) were $\Delta F508$ homozygotes. The second row provides the absolute number of PS patients for that genotype and in brackets the percentage of the subtotal for that clinic; for example, for $\Delta F508$ homozygotes 4/37 (11%) at Verona and 16/97 (16%) at Westmead were PS. As expected, Westmead had a significantly greater number of $\Delta F508$

homozygotes than Verona (97 vs 37, $p<0.001$ by χ^2 test). There were similar numbers of $\Delta F508$ compound heterozygotes (67 vs 64), whereas Verona had a significantly greater number of non- $\Delta F508$ patients (45 vs 5, Verona vs Westmead, respectively, $p<0.001$). Among the three general genotypic groups, $\Delta F508/\Delta F508$, $\Delta F508$ compound heterozygotes and non- $\Delta F508$ patients, in the combined clinics the proportions with PS were 15%, 34% and 32%, respectively. Notably, from the severe mutation groups $\Delta F508/\Delta F508$, $\Delta F508/x$ and x/x , 34 of 231 (15%) infants at this early age were PS. Not unexpectedly, of those with the mild mutations F/m and x/m, 26 of 28 (93%) were PS. The two exceptions had the $\Delta F508/3849+10\text{ Kbc}\rightarrow\text{T}$ genotype. During the 9-year study interval at both centres, patients were reviewed according to clinic policy every 3 to 12 months. Of the original 80 PS infants, 20 (14 from Westmead and six from Verona) developed positive screening tests for PI. Of these 20 patients, 15 had steatorrhoea (faecal fat $>10\%$ of fat intake) and an additional patient had colipase secretion $<1\%$, thus confirming the development of PI in 16 patients. Parents of the four remaining patients (all at Westmead) declined the offer of formal testing for their children. As shown in table 3, all 20 of

Table 2 Genotype/pancreatic phenotype relationships at diagnosis in the two centres

	n	$\Delta F508$ homozygotes	$\Delta F508$ compound heterozygotes				Non- $\Delta F508$ compound heterozygotes			
		Subtotal	F/x	F/m	F/uk	Subtotal	x/x	x/m	x/uk uk/uk	Subtotal
Verona	149	37 (25)	45	6	16	67 (45)	20	12	13	45 (30)
Westmead	166	PS 4 (11)	7	6	3	16 (24)	0	10	4	14 (31)
		97 (58)	29	12	23	64 (39)	3	0	2	5 (3)
Verona+Westmead	315	PS 16 (16)	6	10	12	28 (44)	1	0	1	2 (40)
		134 (43)	74	18	39	131 (41)	23	12	15	50 (16)
		PS 20 (15)	13	16	15	44 (34)	1	10	5	16 (32)

n, total number of patients. F: $\Delta F508$; m: gene mutations with mild phenotype (2789+5G→A, 3849+10 Kbc→T, D1152H, 1898+3A→G, R117H, R1066H, 2789+56→A, Q1476X); uk: unknown mutations (not identified with the panel used in the screening system); x: gene mutations with severe phenotype (N1303K; R1162X, G542X, 1717-1G→A, 3132delTG, R553X, 2183AA→G, Q552X, D1507, W1282X, E585X, 1078delT, G551D, R560TS549N; 711+5G→A, G85E, 621+1G→T).

For the three genotype columns, numbers are absolute with per cent in brackets. PS subtotals (%) are expressed as a per cent of the subtotals for that genotype at each of the clinics and for combined data, for example under $\Delta F508/\Delta F508$ at Westmead 16/97 (16%) were PS.

Table 3 Deterioration of pancreatic function: age and genotype

Centre	PS at diagnosis	Persistent PS patients	PS→PI*	Age (months) of PS→PI, median (range)	F/F	F/x
Verona	34	28	6	9 (2–34)	4	2
Westmead	46	32	14	15 (5–56)	8	4
Total	80	60 (75%)	20 (25%)	12 (2–56)	12	6

*Number of PS patients who became PI.

F/F: $\Delta F508$ homozygotes; F/x: $\Delta F508$ /mutation with severe phenotype; PI, pancreatic insufficient; PS, pancreatic sufficient.

these PS→PI infants had two severe mutations: 14 were $\Delta F508$ homozygotes and six were $\Delta F508$ compound heterozygotes. We did not observe this phenomenon in any of the patients who were originally PS with a $\Delta F508$ /m or x/m genotype.

DISCUSSION

The current study was designed to evaluate the pancreatic phenotype of a group of infants with CF diagnosed by two different neonatal mutation screening programs, one in NSW, Australia and the other in north east Italy. Despite the marked differences in genotype patterns between the two regions, the populations studied were remarkably similar with nearly identical rates of MI, gender distribution and the presence of pancreatic sufficiency as shown in table 1. The 23% and 28% rates of PS observed in the two clinics were nearly twice the 10%–15% rates reported from older non-screened clinics,^[35] thus confirming the previous report of a significantly increased proportion of infants with CF with PS in the first 6 weeks of life.⁴ The results, however, also demonstrated that the average proportion of patients with PS of 26% from the mutational screening strategy in the current study was significantly less than the 37% observed in the population screened by the older repeat IRT strategy.⁴ If the latter proportion was applied to the current population, one would anticipate in absolute terms that up to 120 infants would be PS, representing a 50% increase in the actual number of 80 identified.

The observed reduction in the proportion of PS patients in the current study was not unexpected. At Westmead only $\Delta F508$ is initially screened for and thus infants without at least one $\Delta F508$ mutation would not be detected except for those with either MI or a family history of CF, as shown by the Westmead data in table 2. Previously, older $\Delta F508$ predominant populations have demonstrated up to 10% of patients without $\Delta F508$ mutations and 66% of this subgroup were PS.¹⁴ Missing these patients in addition to another subgroup of $\Delta F508$ compound heterozygotes with low or borderline sweat chlorides as described previously¹⁹ would account for the decrease in absolute PS numbers in the current study. Although as yet we are uncertain as to whether a similar phenomenon exists in Verona, the data should alert community physicians/paediatricians that regardless of the mutation screening strategy, children presenting de novo at an older age with recurrent or persistent chest symptoms and infections, bronchiectasis and/or malabsorption, should be investigated for CF despite negative neonatal screening.

It is of interest in the current study that at the time of neonatal diagnosis, 34 of 231 infants with two severe PI-associated mutations ($\Delta F508$ homozygotes, $\Delta F508$ /x and x/x) were PS. This is an unusual finding in older patients with CF,^{7 14} but previously we have demonstrated that this subgroup loses their pancreatic function with age.⁶ Walkowiak *et al* have demonstrated a similar phenomenon in a small group of 27 infants with class I or II mutations, noting that all eight with PS at diagnosis had become PI within the first year of life.¹⁵ In the current study, 20 of the 34 PS patients with class I, II or III mutations at neonatal diagnosis developed PI and as shown in

table 3, the age of onset of PI varied considerably, ranging from 2 months to 11 years of age. In contrast, to date none of the 26 patients with mild class IV or V mutations (designated as $\Delta F508$ /m and x/m in table 2) have developed PI in their follow-up investigations, a finding consistent with the data from an older population where only one of 49 patients with class IV or V mutations had developed PI.⁷ These findings are important for clinical management and clearly indicate that one should not assign the PI or PS phenotype to infants with CF based on the results of phenotype/genotype studies in older patients. Ideally, patients should be assessed with fat balance studies, as in the current study, or with other appropriate indirect tests to determine the necessity for enzyme therapy.

In summary, the present study demonstrates that infants with CF diagnosed by two different IRT/mutation screening strategies have a comparably higher proportion of PS patients at the time of newborn diagnosis than older populations with CF. The proportion is, however, approximately 33% lower than in populations screened by the older repeat IRT non-mutation strategy. At least in programs where only $\Delta F508$ is screened for, the lower proportion is in part related to the non-detection of PS patients without a $\Delta F508$ mutation and physicians in regions with such a population should be aware that these patients remain undiagnosed in their community. The current findings also demonstrate that 34 (42%) of the PS patients at

What is already known on this topic

- Non-mutational CF neonatal screening programmes detect a high proportion of pancreatic sufficient infants with CF in the first month of life.
- Very limited data, restricted to patients with only class I or II mutations, are available on the pancreatic status of infants with CF diagnosed by an IRT/DNA screening programme.

What this study adds

- IRT/mutation screening strategies in either $\Delta F508$ dominant or non-dominant geographical regions have comparable results in terms of pancreatic phenotype but do not detect up to a third of the expected number of PS patients.
- At birth there are two subgroups of PS patients: (i) those who maintain their PS status, and (ii) those who lose their pancreatic function and become PI anytime from the first year of life up to late childhood.
- Those who transition from PS to PI have two severe mutations, thus emphasising that this subgroup requires careful follow-up of their pancreatic status.

the time of neonatal diagnosis had severe class I, II or III mutations, and nearly half of this subgroup suffered deterioration of their pancreatic function and became PI before 5 years of age. Clearly, this subgroup with severe mutations requires careful follow-up with regard to their pancreatic function.

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