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Original Article

Neonatal screening for cystic fibrosis: Comparing the performances of IRT/DNA and IRT/PAP☆

Jacques Sarles [f,i,*], Roch Giorgi [a,b], Patrice Berthézène [c,d], Anne Munck [f,g], David Cheillan [f,h], Jean-Charles Dagorn [c,d], Michel Roussey [e,f]

[a] INSERM, UMR912 "Economics and Social Sciences Applied to Health & Analysis of Medical Information" (SESSTIM), 13006 Marseille, France
[b] Aix Marseille University, UMR_S912, IRD, 13006 Marseille, France
[c] INSERM, U1068 "Centre de Recherche en Cancérologie de Marseille" (CRCM), 13009 Marseille, France
[d] Aix-Marseille University, UMR 1068, 13009 Marseille, France
[e] Hôpital sud CHU Université de Rennes I, 35203 Rennes, France
[f] AFDPHE, 75015 Paris, France
[g] CRCM Pédiatrique, Assistance Publique-Hopitaux de Paris, Université Paris 7, Hopital Robert Debré, Paris, France
[h] Service Maladies Héréditaires du Métabolisme, Groupement Hospitalier Est, INSERM U1060/Université Lyon 1/Hospices Civils de Lyon, Lyon, France
[i] Aix-Marseille University, Hôpital d'Enfants de la Timone, 13005 Marseille, France

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Abstract

Background: French health authorities promoted a study on 553,167 newborns comparing the performances of IRT/DNA and IRT/PAP for CF newborn screening.
Methods: In parallel to IRT/DNA, PAP was assayed in newborns with IRT > 50 μg/L. Provisional PAP cutoffs at 3.0 μg/L when 50 < IRT < 100 μg/L and 1.7 μg/L when IRT > 100 were used. Positive newborns were subjected to sweat test. Optimal cutoffs were established by a non-inferiority method.
Results: 95 CF newborns were identified (83 classical forms (ClF), including 9 meconium ileus (MI), and 12 atypical (mild) forms (AF) Of them, IRT/DNA identified 85 (73 ClF including 5 MI and 12 AF). PAP cutoffs at 1.8 μg/L when 50< IRT<100 μg/L and 0.6 μg/L when IRT>100 μg/L would identify 82 CF: 77 ClF, including 8 MI, and 5 AF. The number of sweat tests was 314 and 1039 in the IRT/DNA and IRT/PAP strategies, respectively.
Conclusions: Using the optimal cutoffs, the sensitivity of the IRT/PAP strategy would not be inferior to that of IRT/DNA if identification of MF is not required.

Keywords: Neonatal screening; Cystic fibrosis; PAP; IRT; CFTR; Non-inferiority

1. Introduction

Nationwide newborn screening (NBS) of cystic fibrosis (CF) was implemented in France in 2002 [1]. The screening strategy involved immunoreactive trypsinogen (IRT) as first tier, followed by analysis of a panel of CFTR mutations [2]. Such IRT/DNA strategy was already in use in several countries [3–5] with good performances. In 2009, the French "Haute Autorité de Santé" (HAS) published an audit of the first five years of CF screening in the country and concluded that performances were indeed good but that the DNA analysis tier raised some concern. Three potential problems were underscored: i/informed written consent, required by French bioethics laws before genetic analysis is performed, cannot be completely fulfilled in daily practice of a NBS program, ii/identification of healthy heterozygotes and atypical (mild) forms of the disease, due in part to the choice of

Abbreviations: CF-NBS, cystic fibrosis newborn screening; IRT, immunoreactive trypsinogen; PAP, pancreatitis-associated protein

☆ Previous communication: Part of this work was presented in abstract form at the 2011 ISNS Meeting in Geneva.

* Corresponding author at: Service de Pédiatrie Multidisciplinaire, Hôpital d'Enfants de la Timone, F-13385 Marseille Cedex 05, France. Tel.: +33 491 386736.
E-mail address: jacques.sarles@ap-hm.fr (J. Sarles).

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including R117H in the mutation panel, implies their management, which goes beyond the goal of newborn screening and iii/the panel of mutations is based on frequencies observed in the general population, which is unfair for ethnic minorities. Based on results from our team [6,7] and others [8–10] on the use of PAP assay instead of DNA analysis as second tier in CF screening, the HAS recommended that a large-scale (>500,000 newborns) study is conducted to investigate whether the performances of an IRT/PAP strategy could be optimized to match the performances of IRT/DNA, without the drawbacks generated by genetic analyses. We were contacted by the CNAMTS (French National Health Insurance Fund) to conduct such study, whose results are presented here. In parallel, another team was in charge of comparing the cost-effectiveness of the two strategies. Their results will be published elsewhere.

2. Methods

Organization, follow-up and analysis of the results of the study were placed under the supervision of a steering committee led by the Association Française pour le Dépistage et la Prévention des Handicaps de l'Enfant (AFDPHE, the French organization in charge of newborn screening).

2.1. Study sample

The study was conducted between February 1st., 2010 and January 31st., 2011 and involved 11 of the 23 French NBS centers. The centers were located in 8 regions (Bretagne, Ile-de-France, Lorraine, Midi-Pyrénées, Nord-Pas-de-Calais, Pays de Loire, Provence-Alpes-Côte d'Azur and Rhône-Alpes) chosen to be representative of the French population. This study was exempted from approval by the French National Committee on Informatics and Freedom (CNIL) since it involved anonymous data.

2.2. Screening strategies

Blood was collected on cards by heel pricking on day 3, as part of the NBS screening program. Newborns on whom the IRT/DNA or IRT/PAP protocols could not be performed because the parents refused the CFTR mutation analysis or because of lack of blood for PAP assays were excluded from the study.

During the 4-month training period prior to the beginning of the study, the participating centers introduced the PAP assay into their practice without significant problem and all laboratories were fully operative before inclusion started. Quality control was monitored by pooling results from identical samples assayed in all laboratories. No significant differences were observed among laboratories.

2.2.1. The IRT/PAP strategy

When blood IRT concentration at day 3 was ≥50 μg/L, PAP was assayed in duplicate on eluates from the same screening card, using an ELISA MucoPAP® kit (Dynabio, France). A 3 mm diameter punch of Whatman #903 paper was initially supposed to contain 5 μL blood. The punch being eluted in 150 μL PBS, the dilution factor of blood was taken as 1/30 and PAP concentrations were initially calculated using this factor. In fact, the actual blood volume is 3 μL (Dr G Loeber, personal communication), which means that the dilution factor is 1/50. A statement describing the conversion was published on the ISNS website (http://www.isnsneoscreening.org March 2011). All PAP concentrations were eventually corrected (multiplication factor = 1.66) and the values presented here are the corrected ones. Newborns were referred for sweat testing when PAP ≥ 3 μg/L if 50 μg/L < IRT < 100 μg/L and PAP ≥ 1.7 μg/L if IRT ≥ 100 μg/L. These cutoffs had been established in a previous study [7].

Raw data were collected monthly in each center and sent simultaneously to us and to an independent referee appointed by the CNAMTS, who performed the statistical analysis (Pr R Giorgi, Biostatistic team of INSERM UMR 912).

2.2.2. Determination of optimal cutoffs

The first criterion to calculate optimal cutoffs for the IRT/PAP strategy was the detection rate of CF, the goal being that it should not be lower than in the IRT/DNA strategy ("non-inferiority") [11,12]. This was done considering only the diagnosis of classical forms of CF, since the presence of meconium ileus at birth makes the diagnosis of CF, and atypical forms can mostly be identified by the IRT/DNA strategy. The 95% confidence interval (CI) of the detection rate obtained with the IRT/DNA strategy was calculated using the exact method (two-sided, with a 0.05 level of significance). In comparison with IRT/DNA, the IRT/PAP strategy was considered as non-inferior when the lower limit of the 95% CI of the estimated detection rate was above the non-inferior limit. As several PAP cutoffs were likely to result in non-inferiority of the IRT/PAP strategy, the number of sweat tests performed and the number of false positives obtained were used as additional criteria for defining optimum cutoff values for the IRT/PAP strategy.

2.2.3. The IRT/DNA strategy

This strategy (Fig. 1) was in use in France before the study started. IRT was assayed on blood eluted from screening cards using a DELFIA assay (PerkinElmer) in 8 centers or a radioimmunoassay (CisBio) in 3 centers. When IRT concentrations ≥ 55 μg/L, the same sample was assayed again in duplicate. When the mean IRT concentration of the duplicate was ≥65 μg/L, DNA analysis was performed. A kit for detecting 30 mutations in the CFTR was used (Elucigene™ CF30). When at least one mutation was detected, a sweat test was performed. When no mutation was detected, a failsafe procedure was applied to newborns with IRT ≥ 100 μg/L: a new blood sample was collected on day 21 for IRT assay, and a sweat test was performed if IRT_{21} ≥ 40 μg/L. Sweat tests were conducted according to the procedure used in each center. When quantitative pilocarpine iontophoresis was used the threshold was 40 mEq/L for babies less than 3 months old, and was 60 mEq/L when conductivity was used. CF newborns were classified in three categories: CF with meconium ileus, borderline (mild) forms (normal or borderline sweat test and atypical mutations) and classical forms.

Statistical analyses were performed using the R software, version 2.14.0 (R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; 2011. ISBN 3-900051-07-0, URL:

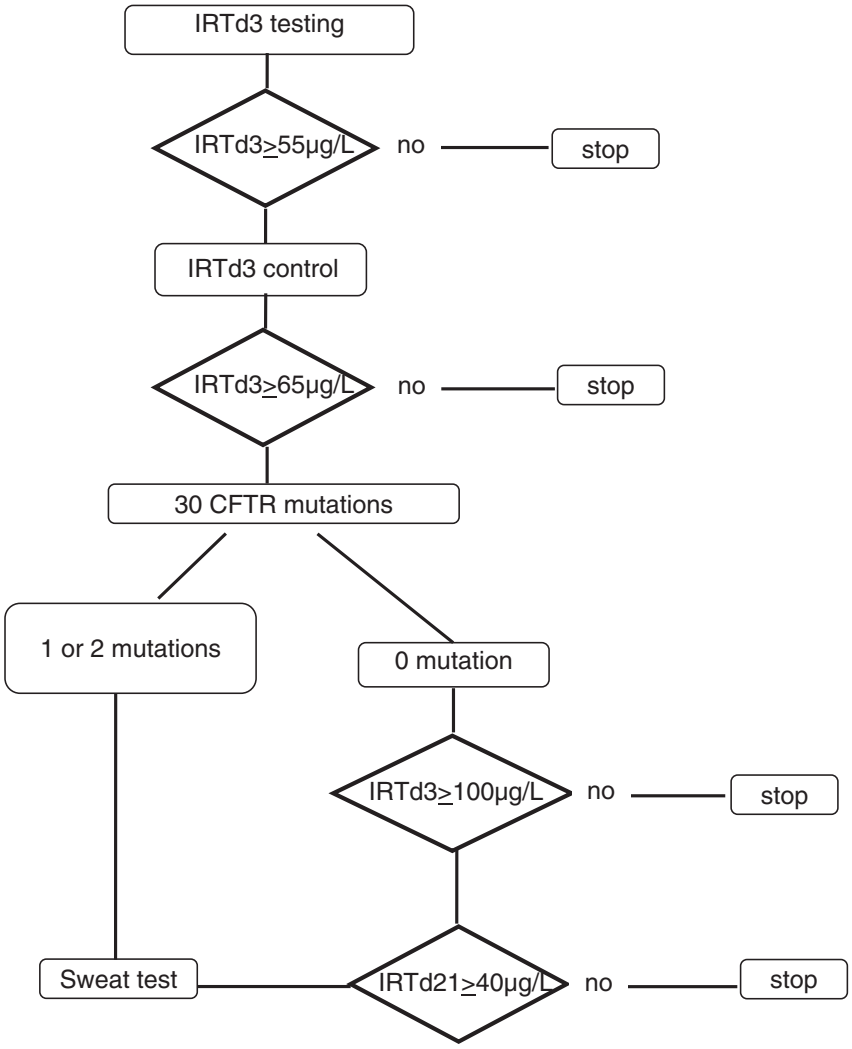

Fig. 1. French IRT/DNA strategy. d3 IRT: immunoreactive trypsinogen assay on a blood sample taken at day 3. d21: blood sample taken around day 21.

http://www.R-project.org/). The epiR R package was used for effectiveness analyses.

3. Results

During the 12 months of the study, 553,167 newborns were screened, among which 252 were eventually excluded. As a result, the analysis was conducted on data from 552,915 newborns including 8487 newborns with IRT ≥ 50 µg/L. Among these newborns, CF was confirmed in 95, corresponding to 74 classical forms, 12 atypical forms, and 9 meconium ileus. These numbers, obtained by combining both strategies, have been updated 24 months after the end of the study, making the risk of further false negatives very low. The incidence of the disease was 1/5950, lower than the incidence observed in previous years in France. However, the incidence reported for the whole country in the same period was similar (1/6168, unpublished data), showing that the sample studied, corresponding to 2/3 of the whole population of newborns in France, was representative and allowed valid comparison of the two strategies.

3.1. The IRT/PAP strategy

3.1.1. Determination of optimal cutoffs

3.1.1.1. IRT cutoffs. Values of IRT cutoffs used in the study were based on results of our previous study [7]. Our commitment being to evaluate the simplest possible screening procedure, implying that the initial IRT is not controlled before proceeding to PAP assay (contrary to the French IRT/DNA protocol) we decided to use an initial cutoff of 50 µg/L which should miss few classical forms of CF if any, without altering too much specificity. Another observation from our previous study was that CF newborns with highest IRT values tended to show lower PAP values than CF newborns with lower IRT values. A 100 µg/L cutoff discriminated

correctly the two populations. The aim of this study was therefore to select two optimal PAP cutoffs, one for IRT between 50 and 100 μg/L and one for IRT > 100 μg/L.

3.1.1.2. PAP cutoffs. Table 1 shows the results obtained for the IRT/PAP strategy when combining the two selected IRT cutoffs (50 and 100 μg/L) with different PAP cutoff values. As a result of the non-inferiority analysis, optimal PAP cutoffs were identified at 1.8 μg/L for IRT values between 50 μg/L and 100 μg/L and at 0.6 μg/L for IRT values above 100 μg/L. PAP cutoff values higher than 0.6 were not considered since two CF newborns with IRT > 100 μg/L had a PAP concentration at 0.7 μg/L. One can see in Table 1 that raising from 1.8 to 2.0 μg/L the PAP cutoff for newborns with IRT between 50 and 100 μg/L would increase specificity by decreasing the number of false positive cases, but it would also increase the risk of missing a CF case because two of the CF newborns identified in this study had a borderline PAP value at 1.9 μg/L.

3.1.2. The IRT/DNA strategy

The IRT/DNA strategy identified 2435 newborns (0.44% of the whole population) eligible for DNA testing (Table 2). Out of them, 2185 (89.7%) had 0 mutation, 165 (6.8%) had one mutation, and 85 (3.5%) had two mutations. Altogether, the IRT/DNA strategy led to the identification of 68 newborns with classical forms of CF and 12 with atypical forms. One of them, with a classical form of CF, was identified through the failsafe procedure applied to newborns with 0 mutations and IRT ≥ 100 μg/L. Five of the 9 newborns with meconium ileus were positive in screening. Taking into account all forms of CF, the IRT/DNA strategy led to a detection rate of 89.5% (95% CI: 81.5%–94.8%). When considering only newborns with classical forms of CF, the detection rate was 91.9% (95% CI: 83.2%–97.0%). The lower limits of the 95% CIs were used as limits in analyzing the performances of the IRT/PAP strategy. Six classical forms of CF were missed with the IRT/DNA strategy (Table 2). If positive cases in the calculation of PPV are newborns referred for sweat testing, then sweat tests that follow the molecular biology tier (n = 250) and the fail-safe procedure (n = 64) have to be taken into account. With these criteria, the PPV was 27.1% (95% CI: 22.3%–32.3%).

Table 2
Results of the two screening protocols.

Screening protocol	IRT/DNA	IRT/PAP
IRT positive	2441	8487
Screening test positive, recalled for ST	313	951
Classical CF	68	69
Classical CF with MI	5	8
Atypical CF	12	5
False positives (negative ST)	228	869
HZ	165	
Negative ST after failsafe procedure	63	
Test negative	552,602	551,964
Classical CF	6	5
Classical CF with MI	4	1
Atypical CF	0	7
Detection rate (%)	91.9	93.2
PPV (%)	27.1	8.6

Newborns included in the study: n = 552,915.
IRT positive: IRTd3 ≥ 55 μg/L and Ctrl IRT ≥ 65 μg/L (IRT/DNA) or IRTd3 ≥ 50 μg/L (IRT/PAP).
Screening test positive: *IRT/DNA*: At least one mutation of the 30 mutation panel or 0 mutation and IRT d3 ≥ 100 μg/L; *IRT/PAP*: PAP ≥ 1.8 μg/L if 50 ≤ IRTd3 ≤ 100 μg/L or PAP ≥ 0.6 μg/L if IRTd3 ≥ 100 μg/L (IRT/PAP).
ST = sweat-test; CF = Cystic fibrosis: MI = meconium ileus; PPV = Positive predictive value.

3.1.3. Performances of the IRT/PAP strategy

The established cutoffs would generate 951 sweat tests (0.17% of newborns) and identify 869 false positive cases (false positive rate among newborns submitted to sweat

Table 1
Determination of optimal PAP cutoffs: the selected cutoffs are highlighted.

IRT >50 and mean PAP >	IRT>100 and mean PAP >	Detection rate* (%)	Sweat tests n	% Suspects	TP				FP	FN			
					n	Classical forms**	AF§	MI$		n	Classical forms**	AF	MI
1.2	0.6	86.7	1706	0.308	84	70	6	8	1622	11	4	6	1
1.3	0.6	86.7	1502	0.271	84	70	6	8	1418	11	4	6	1
1.4	0.6	86.7	1344	0.243	84	70	6	8	1260	11	4	6	1
1.5	0.6	84.9	1210	0.219	83	69	6	8	1127	12	5	6	1
1.6	0.6	84.9	1111	0.201	83	69	6	8	1028	12	5	6	1
1.7	0.6	84.9	1021	0.184	82	69	5	8	939	13	5	7	1
1.8	0.6	84.9	951	0.172	82	69	5	8	869	13	5	7	1
1.9	0.6	84.9	904	0.163	82	69	5	8	822	13	5	7	1
2.0	0.6	83.2	845	0.152	81	68	5	8	764	14	6	7	1

* Lower limits of the 95% confidence interval of the detection rate for newborns with classical forms of CF. Values greater than 83.2% (the non-inferior limit) indicate that, with the corresponding cutoffs, the IRT/PAP strategy is statistically non-inferior to the current IRT/DNA strategy.
** Classical forms of CF without meconium ileus.
§ Atypical (mild) forms of CF.
$ Classical forms of CF with meconium ileus.

Table 3
False negatives identified in the study.

	IRT d3	Ctrl IRT	PAP	Cl^-	Mut 1	Mut 2
1	66	68	0.4	80	ΔF508del	ΔF508del
2	87.8	106.5	0.5	137	E1104X	E1104X
3	93.2	105.8	0.8	82	G91R	ΔF508del
4	71.1	56.7	0.3	80.0	ΔF508del	ΔF508del
5	67.9	54.4	1.5	99.0	ΔF508del	ΔF508del
6	87.1	82.9	4.5	70.0	E1104X	D110H
7	61.5	62	5.0	88.0	R553X	A455E
8	62.4	63.0	14.6	110.0	2183AA>G	907delCins11
9	117.0	81.5	15.6	130.0	S466X	S466X

Lines 1–3: false negatives in the IRT/PAP strategy, 6–9: false negatives in the IRT/DNA strategy, due to mutations not detected by the Elucigene™ CF30, 4–5: false negatives in both strategies. Ctrl IRT is the mean value of the duplicate IRT assay run if IRTd3 > 55 μg/L. A Ctrl IRT > 65 μg/L triggers DNA analysis. IRT and PAP concentrations in μg/L, Cl^- in mEq/L.

test = 91.4%, 95% CI: 89.4%–93.1%). The IRT/PAP strategy using these cutoffs is statistically non-inferior to the current IRT/DNA strategy and would lead to the identification of 69 newborns with classical forms of CF and 5 newborns with atypical forms. Eight of the 9 newborns with meconium ileus would be positive (Table 2). Such IRT/PAP strategy would also generate 12 false negative tests results, among which 7 newborns with atypical forms of CF (Table 3). When considering all forms of CF, the detection rate would be 86.3% (95% CI: 77.7%–92.5%). Considering only newborns with classical forms of CF the detection rate would be 93.2% (95% CI: 84.9%–97.8%). With the same criteria as for IRT/DNA, the PPV of IRT/PAP would be 8.6% (95% CI: 6.9%–10.6%).

Details on the newborns with classical forms of CF missed by either strategy are given in Table 2. Two newborns would be missed by both strategies.

It is noteworthy that raising the lower IRT cutoff from 50 to 55 μg/L would decrease the number of sweat tests from 951 to 797. The number of false positives would decrease to 715. The detection rate of classical forms of CF would be identical. However, one of the classical forms of CF was quite borderline (IRT = 55 μg/L) suggesting that such cutoff is not totally safe.

4. Discussion

Since CF newborn screening became available with the introduction of IRT assay on Guthrie cards, the most important improvement has been the introduction of CFTR mutation analysis, made possible by the transfer to the screening laboratories of molecular biology techniques. Many countries in which the disease is frequent, such as Australia [3], the USA [4] and France [2] have implemented the IRT/DNA strategy with very good performances. Commercial kits presently available allow detection of about 30 mutations, selected as the most frequent ones in the Western countries that initiated CF screening. As a result, lower detection rates are expected in non-European-derived populations [13]. To improve the strategy, mutation frequencies in each ethnic group must be determined beforehand to complete the mutation panel. However, increasing the number of investigated mutations will increase the number of identified heterozygotes. Such drawbacks curb the expanding of CF newborn screening. Furthermore, the use of genetic markers is prohibited in some countries. In other countries, there are ethical concerns about detection of atypical forms of CF [14] and the relevance of informing parents about carrier status [15] has resulted in encouraging research on non-genetic neonatal markers. This is the case in France. Previous studies have shown that PAP assay might replace the DNA tier of CF screening while keeping similar detection rates and a manageable recall of 0.25% of newborns for sweat testing [7]. However, switching from IRT/DNA, a strategy in practice since 2002, to IRT/PAP required that unquestionable evidence is produced. In order to gather the required information, French Health authorities promoted this study comparing IRT/DNA and IRT/PAP strategies on more than 500,000 newborns.

A mere prospective study with cutoff values decided in advance was not possible because, due to an evolution in the PAP assay kit involving a change from polyclonal to monoclonal antibodies for antigen capture and revelation, it was known that the PAP cutoffs previously established [7] would have to be adjusted. Hence, the first step of the study was to determine optimal PAP cutoffs, with sensitivity as first criterion, using a statistical approach based on non-inferiority, i.e. looking for a performance of IRT/PAP non-inferior to that of the current IRT/DNA strategy. To our knowledge, the present study is the first that used a statistical approach based on non-inferiority [11,12] to determine optimal cutoffs for CF-NBS.

Based on PAP cutoffs of 1.8 μg/L for 50 < IRT < 100 μg/L and 0.6 μg/L for IRT > 100 μg/l, sensitivities of both strategies are similar and the question is whether the specificity of IRT/PAP is acceptable. Percentage of newborns recalled for sweat testing was 0.17%, lower than the 0.25% expected from our previous study but about three times higher than in the IRT/DNA protocol. This percentage would trigger less than 1 sweat testing a week for each CF pediatric center in France. However, one must underscore that acceptability is a relative concept, highly dependent on practical, ethical and economic criteria. This is why the meaning of comparing the performances of the IRT/PAP strategy to those published with other strategies is limited. For instance, one of the advantages of PAP is the suppression of carrier detection and the much smaller number of mild forms identified; how would IRT/PAP compare with the strategy of the Massachusetts screening program [16] whose results, on more than 300,000 births, show very good sensitivity of IRT/DNA (2 CF missed among 112) but perform mutation analysis in 5% of newborns (compared to ~0.5% in the French algorithm) and sweat testing in all babies with at least one mutation, plus those with 0 mutation but IRT in the upper 0.02 percentile? Clearly, the IRT/PAP protocol is efficient when certain criteria are retained, and its implementation should be of interest in certain countries, not all. A recent report [17] compared three IRT/PAP protocols tested in Germany, Poland and Czech Republic. Their conclusion was that using IRT/PAP for CF screening is a good choice. They recommended using an IRT cutoff at the 99th percentile and a failsafe at the 99.9th percentile.

The present study involved a comparison of IRT/DNA- and IRT/PAP-based CF-NBS, but IRT/PAP/DNA strategies have been recently proposed [9,17], in order to find the best compromise

between the number of detected carriers or mild forms of the disease and the number of sweat tests, two large scale studies conducted in the Netherlands and the Czech Republic [9,17,18] compared IRT/PAP- and IRT/PAP/DNA-based CF-NBS. Results show that the two strategies perform equally well, although the Czech study reported a slightly lower detection rate for IRT/PAP/DNA [18]. Both studies clearly showed that using the 30 mutation panel in newborns with high IRT and PAP identified most but not all CF babies and that a further screening step had to be considered. The Czech chose sweat testing of newborns with 1 mutation and the Dutch chose as failsafe to sequence all exons of the CFTR gene in newborns with 1 mutation or 0 mutation if IRT > 100 μg/L. With the incidence of CF observed in France, applying the Dutch protocol to our study would add 1 false negative corresponding to an affected newborn with none of the mutations included in the kit Elucigene™ CF30 but with an IRT < 100 μg/L. Clearly, each screening center that starts an IRT/PAP strategy uses at the beginning published cutoffs, then adapts the strategy to the local situation after sufficient data is analyzed.

In conclusion, compared to IRT/DNA, the IRT/PAP strategy can have a similar detection rate of classical forms of CF while detecting a smaller number of mild forms. Switching from IRT/DNA to IRT/PAP should not raise any significant technical or practical problem in the laboratories in charge with PAP. The IRT/PAP strategy could be the best choice in countries with important ethnic diversity. In countries already using the IRT/DNA strategy, switching to IRT/PAP/DNA would maintain the detection of carriers of common CF mutations at some level, but the number of sweat tests would be greatly diminished. In France, the IRT/PAP/DNA strategy would only slightly differ from the IRT/DNA strategy. The only change would be that the control IRT assay, run in duplicate in newborns with an initial IRT > 55 μg/L, on which is decided mutation analysis if its mean value is > 65 μg/L is replaced by a PAP assay in duplicate. After mutation analysis, an alternative to the failsafe chosen in the Netherlands (sequencing) would be sweat testing, on the same criteria.

Conflicts of interest

Jean-Charles Dagorn is a co-inventor in the INSERM patent on the use of PAP assays in CF newborn screening and a consultant for DYNABIO SA. Other authors have no conflicts of interest to disclose.

Contributors' statement

Roch Giorgi controlled the data and conducted the statistical analyses.

Patrice Berthézène was in charge of data management for the INSERM.

Michel Roussey chaired the steering committee of the study.

Anne Munck controlled the data on CF newborns.

David Cheillan controlled the technical aspects of the study.

Jacques Sarles and Jean-Charles Dagorn coordinated and supervised data collection and wrote the draft of the manuscript.

All authors critically reviewed the manuscript and approved the submitted version.

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