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ORIGINAL ARTICLE

Screening in Cystic Fibrosis in the Chilean population. Pilot project for screening in newborns

Tamizaje de Fibrosis Quística en la población chilena. Proyecto piloto de pesquisa en recién nacidos

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What do we know about the subject matter of this study?

First 7-year Chilean pilot study of neonatal screening for cystic fibrosis by Immunoreactive Trypsinogen and Pancreatitis-Associated Protein (IRT/PAP) assay that is cost-effective, with lower predictive value than those using genetic determination and IRT as a third tier.

What does this study contribute to what is already known?

In this population, applying the IRT/PAP and IRT x PAP methodology, an incidence of 1/7,109 newborns has been determined, establishing cut-off values and percentiles of IRT, PAP, and IRT x PAP applicable to Chilean children that should be used in the future. With adequate state funding, we would be able to support an early diagnosis of cystic fibrosis with neonatal screening, assuring the population of timely treatment and higher survival rates.

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Abstract

Neonatal screening has been implemented internationally with different protocols and has become the routine method in the preclinical stage. Late diagnosis is associated with more severe symptoms with decreased survival and higher treatment costs. **Objectives**: To estimate the incidence of cystic fibrosis; to evaluate the performance of the screening algorithms Immunoreactive Trypsinogen and Pancreatitis-Associated Protein (IRT/PAP) and the IRTxPAP product; to analyze the cut-off value for IRT, PAP, and IRTxPAP, and to establish a methodology for its ongoing evaluation; finally, to evaluate the quality of IRT and PAP measurements. **Patients and Method**: a neonatal screening protocol was implemented using the IRT/PAP assays plus IRTXPAP product in a 7-year pilot study. Between 2015 and 2021, a total of 371,724 heel dried blood spot samples were collected in maternity and neonatology units from the public healthcare network in 17 hospitals in the Metropolitan Region (RM) and 15 in the Valparaíso Region (RV). 277,245 newborns met the inclusion criteria. **Results**: with IRT/PAP plus IRT x PAP the incidence was 1/7,109 NB. The cut-off value and percentiles were established for IRT, PAP, IRT x PAP. The best sensitivity and specificity obtained by ROC analysis gave an IRT value of 48,142 ng/mL (98.8th percentile), PAP of 1.68 ug/L and IRT x PAP of 140ug2/L. The performance of the IRT/PAP detection algorithms, the IRT x PAP product, and the quality of measurements were evaluated. **Conclusion**: The results allow us to report that the IRT/PAP plus IRTxPAP protocol can be implemented in Chile, complying with international guidelines, with adequate government funding.

Keywords:
Neonatal Screening;
Incidence;
IRT Cut-Off Values and Percentiles;
IRT/PAP and IRT x PAP Algorithms;
Cystic Fibrosis

Introduction

Cystic fibrosis (CF) is a rare, hereditary, autosomal recessive genetic disease in which the coding of the transmembrane conductance regulator protein (CFTR) is reduced or absent, resulting in multisystem involvement, mainly pulmonary and digestive[1]. Chile does not have CF screening, so the diagnosis is based on clinical suspicion with a median of 2.3 years and a median survival of 27 years (2019), far from the international experience[2]. In the United States and Europe, with excellent records, CF is diagnosed before 3 months with a current median survival of 47 years and 53 years in Canada, with worldwide agreement on its benefits. By 2021, 25 countries in Europe have already implemented this screening, in addition to two pilot countries, and in Latin America with 20 countries, where 12 of them have CF screening with variable newborn (NB) coverage[3,4]. In 1979, it was found that Immunoreactive Trypsinogen (IRT) could be a valid strategy for CF screening in newborns, so it was progressively implemented in most developed countries, and for the last three decades, it has become the routine method for diagnosis in the preclinical stage.

The IRT measurement as a first tier is more effective than other methods previously used, such as meconium albumin or meconium lactase levels, therefore, it has been chosen for diagnosis in all current protocols[5]. The value set as the IRT cut-off point in the first sample is the most important for the operation of the programs used worldwide, however, due to the drawback of a high false positive rate, a second tier is necessary with several alternatives such as a second IRT, Pancreatitis-Associated Protein (PAP), genetic study, next generation sequencing (NGS) or combinations of them, which has improved the diagnosis, quality of life and survival in patients with this potentially fatal disorder[6,7].

There is no single model to be implemented in each country as there may be many variables (economic, ethnic, geographic, methodological), therefore those that can be performed with good levels of sensitivity, specificity, and positive predictive value should be chosen[8]. This research uses PAP as a second line, following the experience of Sarles published in 2005, which is a protein absent in healthy individuals synthesized in high concentrations in the blood of NBs with CF[9]. IRT/PAP has important advantages since it does not require a second sample with loss of patients at the second appointment, has good sensitivity, does not identify healthy carriers, low number of inconclusive diagnoses, has a simpler implementation, is economically accessible, and has similar performance compared with genetic tests[10]. The use of the IRT x PAP product published by Wiedler et al.[10] proposes it as a second filter when compared with IRT/PAP with excellent discrimination at the cut-off value of 165 ug^2/L[2,11].

The success of neonatal screening is determined by three factors. First, the analytical performance of the selected techniques, which can be measured by internal and external quality controls to determine the precision and accuracy of the methodologies; second, and very importantly, the performance of the algorithms, where it is relevant to evaluate the sensitivity and positive predictive value (PPV); and third, the quality of the confirmatory method.

The objectives of this study are to estimate the CF incidence, to evaluate the performance of the IRT/PAP detection algorithms and the IRT x PAP product, to analyze the cut-off value for IRT, PAP, and IRT x PAP, and to establish a methodology for their ongoing evaluation; and to evaluate the quality of IRT and PAP measurements.

Patients and Method

Prospective study with IRT/PAP and IRT x PAP assays in the blood of newborns (NBs) processed at *Hospital San Juan de Dios*, a reference screening center in Chile for congenital hypothyroidism (CH) and phenylketonuria (PKU).

NBs recruitment and sample handling

371,724 blood samples were collected in the maternity wards and neonatology units of the public healthcare network, 17 hospitals in the Metropolitan Region, and 15 in the Valparaiso Region, after information and signature of informed consent, which was obtained in 80% of the NB screened for CH and PKU. In the Guthrie card (Ahlstrom Grade 226), the same used for the diagnosis of PKU and CH, 5 total drops from the newborn's puncture were placed, which allowed the processing of the 2 additional markers, IRT and PAP.

Inclusion criteria

Term newborns ≥ 37 weeks of gestation, with blood collected between 40 to 48 hours of life, and preterm newborns with 36 and < 37 weeks of gestation with samples collected at 7 days and 15 days of birth. The sample must be sufficient as determined by screening standards and with a transfer time of ≤ 7 days[12,13].

Exclusion criteria

Clinically, preterm newborns < 36 weeks, those with history of transfusion, sepsis, genopathies, deceased, meconium ileus, and hospitalized were excluded. From a preanalytical perspective, NBs from 36 to 37 weeks with samples collected before 40 hours or after 7 days of life, insufficient and early samples, incomplete data, and incorrect record entries were also excluded. A cohort of 277,245 NBs remained for analysis.

Laboratory search strategy

The samples before IRT analysis were stored refrigerated at 4°C, and those with positive IRT were stored at -20°C until the PAP assay was performed.

In the first stage, the determination of the IRT biomarker was performed through time-resolved fluorometry on GSP (PerkinElmer processor), and 2 values were used: analytical cut-off value ≥ 36 ng/mL (95.5 percentile) and action cut-off value which was set by researchers at ≥ 45 ng /mL (98.4 percentile), based on the experience reported by Sarles[9]. The analytical cut-off value corresponds to < 20% of the action cut-off value, considering 10% of coefficient variation. IRT determination was performed in duplicate on the same sample, but on different blood spots to minimize the effect of volumetric variability of dried blood[6]. For PAP measurement, the average of three IRTs was considered and, if the result was equal to or higher than the action cut-off value, PAP was processed in duplicate on the same sample, through time-resolved fluorometry. The PAP cut-off values depended on the IRT value and were set at ≥ 2.5 ug/mL for IRT between 45 and 99.9 ng/mL and > 1.6 ug/mL for IRT ≥ 100 ng/mL which were sent in parallel for diagnostic safety-netting with sweat test[14,15]. At the same time as the IRT/PAP assay, the calculation of the product of results (IRT x PAP) was performed as a second filter to reinforce the prediction of the disease, established at 165 ug/^{2}L[11,12] (figure 1).

Newborns with positive IRT/PAP and IRT x PAP results were contacted by phone and referred to the reference center to confirm the diagnosis of CF using the sweat test (Gold Standard) with Gibson-Cooke quantitative pilocarpine iontophoresis or chloridometry. Normal sweat chloride value is ≤ 29 meq/l on the third day after birth, any value between 30 and 59 meq/l (undetermined) should be repeated within 1 to 2 months. A value ≥ 60 meq/l is diagnostic confirmation.

False negative detection method: Participating hospitals were trained and instructed to monitor patients with negative screening results who present clinical symptoms and refer them accordingly.

Statistical methods used: SPSS Statistics V.24 software was used with the cleaned Excel database for descriptive statistics, calculation, and testing of cut-off values, ROC curves, percentiles calculation, sensitivity, specificity, positive predictive value (PPV), and p-values by Fisher and Chi^2 tests. With methodology published in 2016[11], the IRT x PAP cut-off value was calculated.

Evaluation of cut-off values: A sufficient database was available to determine our own cut-off values and percentiles. The IRT frequency distribution was calculated, and ROC analyses were performed for IRT, PAP, and modified ROC IRT x PAP according to Weidler's proposal[11].

Quality control: Precision, accuracy, and validation.

1. Precision control: IRT and PAP.
   1a. IRT control levels: Two first opinion controls provided by the manufacturer ranging from 30 ng/mL to 110 ng/mL and one-third opinion control (CDC, USA) ranging from 16.6 to 231.3 ng/mL.

2a. PAP control levels: Two sets of 3 controls analyzed in duplicate provided by the manufacturer with a range between 0.6 µg/L and 3.0 µg/L.

2. Accuracy control: Three external controls.
FBA, *Fundación Bioquímica Argentina*, every two months.
CDC (USA), every three months QUARTER 1-2-3.
RfB (Germany) *Referenzinstitut für Bioanalytik*, every four months.

3. Validation of IRT and PAP results: Levey-Jennings chart and combination of 6 Westgard rules[17] were used.

Results

Figure 2 shows how the final result of 38 CF patients was reached, starting from a screening population of 465,725 for CH and PKU, where 80% of parents signed informed consent, and 75% were ultimately included.

1. Incidence: In the overall population it was 1/7,109 with differences between regions, namely Metropolitan Region (1/7,966 NB) and Valparaiso Region (1/4,622 NB) with a non-significant p (Chi^2) of 0.01370 (99%CI) (table 1).
2. Evaluation of algorithms performance: IRT/PAP and IRT x PAP combined and separately encompass sensitivity, specificity, and PPV.

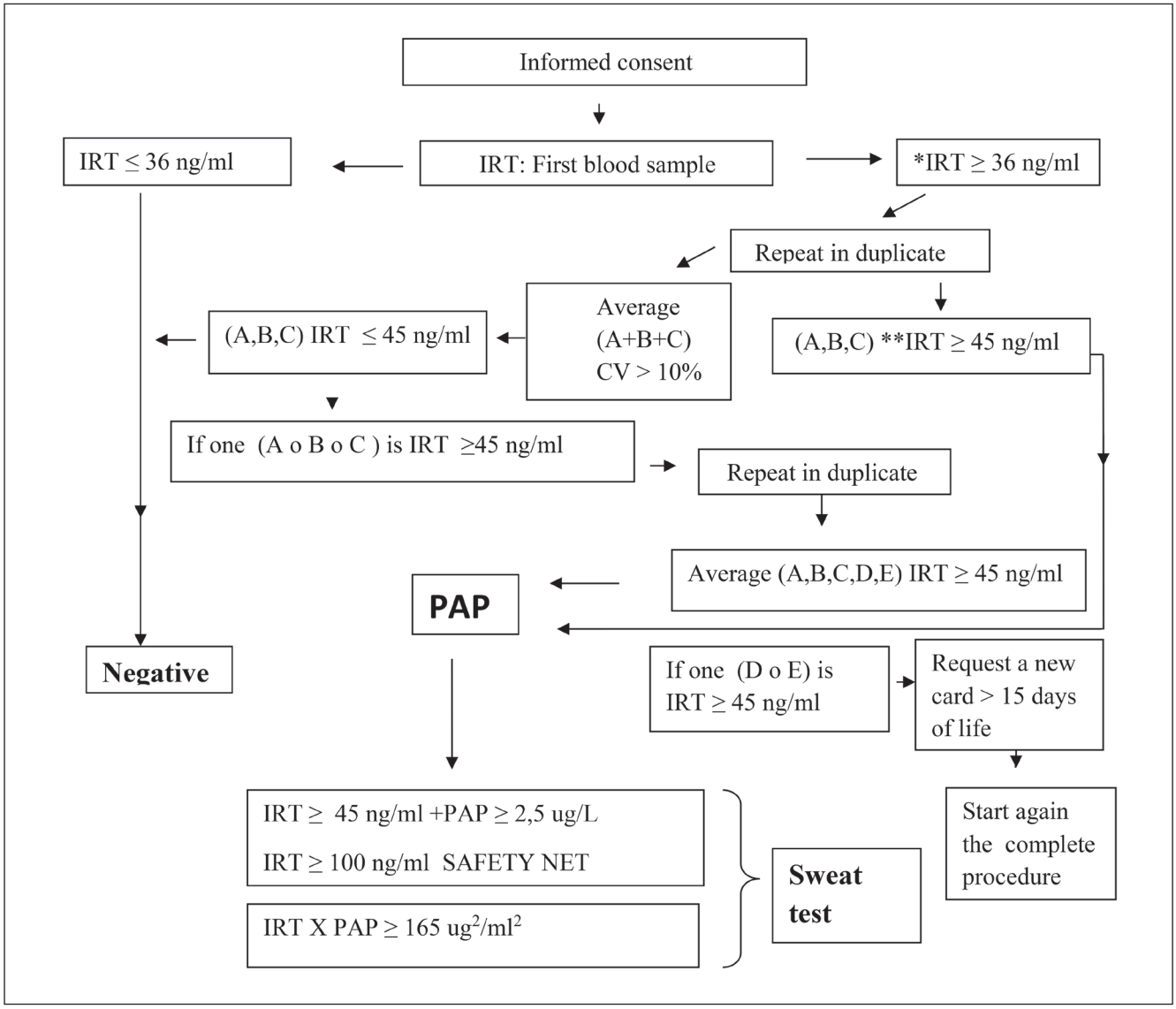

Figure 1. Global Strategy IRT/ PAP and IRT x PAP. IRT: Immune reactive Trypsinogen, PAP: Pancreatitis-Associated Protein, A: IRT determination, B: IRT first repetition, C: IRT second repetition: IRT third repetition, E: IRT fourth repetition. *analytical cut-off value. **action cut-off value . CV: coefficient variation.

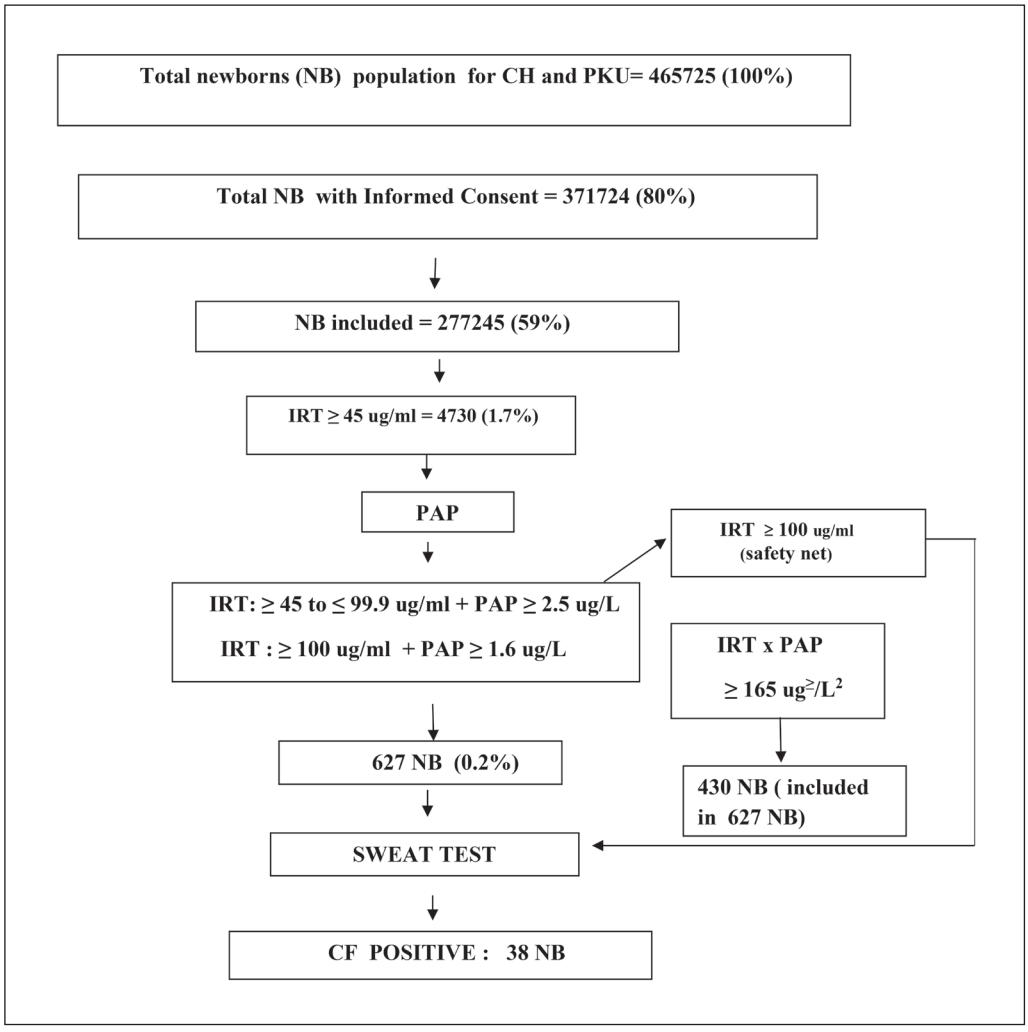

Figure 2. Algorithm applied to Metropolitan and Valparaíso regions. CF: Cystic fibrosis; HC: Congenital hypothyroidism, PKU: Phenylketonuria; IRT: Immune reactive Trypsinogen; PAP: Pancreatitis-Associated Protein.

- Concordance: When applying both strategies, 35 cases were detected. Each strategy missed one case that the other detected, and an additional case was sent directly to the sweat test (safety-netting IRT > 100 ng/mL), resulting in a total of 38 cases (table 2).

- False negative: A patient with clinical symptoms and negative screening was confirmed by a genetic study at 6 months of life.

- Doubtful sweat test: In 9 NBs, sweat chloride results ranged from 30 to 59 meq/l; in 7 of them, CF was confirmed through genetic testing, paid for by the parents and 2 are in follow-up with inconclusive diagnosis (Cystic Fibrosis Screen Positive Inconclusive Diagnosis - CFSPID)[18].

Table 1. IRT/PAP and IRTxPAP performances separated by Regions

	Metropolitan Region		Valparaíso Region	
NB Population	231016		46229	
Stategy	IRT/PAP	IRTxPAP	IRT/PAP	IRTxPAP
CF positive	27	28	9	9
CF negative	230457	230629	46158	46184
False positive	530	358	61	35
False negative	-	-	1	1
Non detected CF	2	1	-	-
Sensitivity %	93.1	96.5	90.0	90.0
Specificity %	99.8	99.8	99.9	99.9
PPV%	4.8	7.5	14.1	22.2
Incidence	1/7966[1]		1/4622[1]	

[1]p > 0.05. NB Newborns, CF Cystic Fibrosis, IRT: Immune Reactive Trypsinogen; PAP: Pancreatitis-Associated Protein. VPP: Predictive Positive Value.

Table 2. IRT/PAP e IRTxPAP Strategies in total population: Combined and separated

	Combined	Separated	
	IRT/PAP + IRTx PAP	IRT/PAP	IRTx PAP
NB population	277245	277245	277245
CF Positive	38	36	37
CF Negative	276839	276615	276813
False Positive	368	591	393
False Negative	1	1	1
Non detected CF	-	2	1
Sensibility %	97.4	92.3	94.9
Specificity %	99.8	99.8	99.8
PPV %	9.4	6.2	9.0
Incidence	1/7109	1/7109	

NB Newborns, CF: Cystic Fibrosis, IRT: Immune Reactive Trypsinogen; PAP: Pancreatitis-Associated Protein. PPV Predictive Positive Value.

3. IRT, PAP, IRT x PAP: When recalculating the cut-off values, the best sensitivity and specificity obtained by ROC analysis gave an IRT value of 48.142 ng/mL (98.8 percentile), PAP of 1.68 ug/L and IRT x PAP of 140ug^2/L^2. Figure 3 shows the percentile values and frequency distribution of IRT values in the population studied, obtaining an average value of 17.92 ng/ml. The 95th percentile value was 35.1 ng/ml and the 99th percentile was 50.3 ng/ml.
4. Evaluation of the IRT and PAP measurements quality: The results obtained in accuracy with the coefficient of variation for IRT was 5% and for PAP between 10.83 and 15.26%.

Accuracy assessment: the average relative error of IRT was -10.78 (target value - 4.7 and -16.86 L) and for PAP the average D/Dmax was -0.09.

Discussion

Neonatal CF screening is an undoubted necessity given the significant difference in survival, this being the most important parameter and therefore it is necessary to test, validate, and implement it in our population with ethnic diversity and genetic variables causing the disease[19]. Since 2014, the Ministry of Health has financed the pilot plan for the Metropolitan Region and later the Valparaiso Region in 50% of the NBs screened for CH and PKU users from the public health system[20]. This activity is carried out in the neonatal screening laboratory of the *Hospital San Juan de Dios* in the Metropolitan Region where the national screening program (CH) has been implemented since 1992 with all the installed capacity and experience for the best operation of a new protocol with internal and external international quality control for both markers.

As a first objective in our results, we report a CF incidence of 1/7,109 NBs, calculated in a cohort with informed consent signed by the mother, a figure that could change when having an evaluation of the whole country. The incidence in Europe varies from 1/1,353 to 1/25,000 NBs depending on geographic location and from 1/4,500 to 1/6,000 NBs in Eastern and Central-Western Europe, respectively[3]. In Latin America with a lack of registries, ethnically mixed population, and scarcity of screening programs, it is estimated between 1/8,000 to 1/10,000 NBs[3].

P (%)	IRT ng /mL
95	35.1
95.5	35.9
96	36.9
96.5	38.0
97	39.1
97.5	40.7
98	43.2
98.5	46.3
98.7	47.6
98.8	48.4
98.9	49.4
99	50.3
99.5	57.5
99.9	77.8

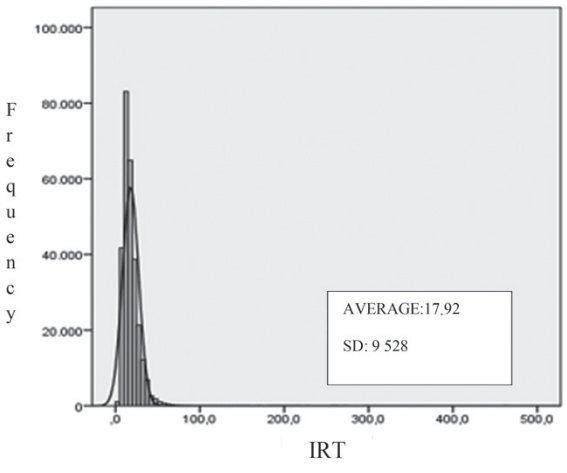

Figure 3. IRT: Frequency distribution and percentiles.

IRT cut-off values and percentiles: In 2019, the USA recommends a value between the 95th and 97th percentile and the 99th percentile in Europe[21,22]. At the beginning of the study, the investigators decided to set a cut-off value of IRT action of 45 ng/mL and, upon recalculation in the database by ROC analysis, this value results in 48.12 ng/mL (98.8th percentile) and 50.3 ng/mL (99th percentile), and lower when compared to other countries which range from 63.6 ng/mL (South America) to 94.8 ng/mL (Africa). This is probably due to the ethnic diversity, ambient temperature, and methodology employed in the countries[4,9]. (Supplementary table 1, online version available).

The use of percentiles allows international comparisons, and therefore the investigators propose an analytical cut-off value for IRT at the 98th percentile, an action one at the 99th percentile, and safety-netting at the 99.9th percentile, which should be used in every assay and together with dynamic cut-off values[23]. The measurements quality of precision and accuracy values obtained are acceptable and reliable.

The IRT/PAP strategy in international studies has been successful with a specificity and sensitivity close to 100% which agrees with this study with figures of 99.8% and 97.4%, respectively. In agreement with publications evaluating the performance of the IRT/PAP and IRT x PAP methodology (biochemical protocol), the PPV is in low limits similar to the published range with values between 7.8% and 15.3%, which gives us confidence in the methodology used[23].

In 2016, the usefulness of calculating the product of IRT x PAP was reported[11]. The measurement should always be performed in relation to previously measured IRT, which improves the sensitivity of PAP. The use of the product can be considered a safety strategy for PAP when associated with the IRT/PAP algorithm, however, no improvement in PPV is obtained. The combined use of both strategies favors increased sensitivity which is corroborated in this study (Supplementary table 2, available online version).

To increase the PPV some developed countries have incorporated a third level with genetic analysis or IRT, but the cost is much higher. The alteration of IRT and PAP proteins is a consequence of the pancreatic damage of the disease which can be variable, as opposed to the genetic variants (mutations) that are the cause.

The cut-off value of IRT x PAP that we obtained according to ROC analysis was 140 ug^2/L^2, lower than the published value of 165ug^2/L^2 which could be due to the lower IRT values in this study. The application of this formula decreases parental distress, cost savings in confirmation, and the burden of care for healthcare personnel. It is suggested to continue with this application using both IRT/PAP and IRT x PAP methodologies in order not to eventually lose patients as demonstrated in the concordance of this study.

CF neonatal screening is of variable cost with the highest value being the one that contemplates genetic study; all screening methods are cost-effective with IRT/PAP, being the most cost-effective in terms of case detected and life years gained which, in our case, amounts to 3 US dollars, well below the cost of using genetics[24].

The evaluation of the neonatal screening survival is difficult because the follow-up must be long-term. The Sydney cohort with a follow-up of 30 years establishes a difference between patients with or without screening in survival and lung transplantation[25]. When screening is implemented throughout Chile, the impact on the population can be evaluated every 5 years.

The results presented allow us to establish the feasibility of implementing the design following the 2018 European guidelines suggestions that raise the important points to be fulfilled and the essential elements to make the screening useful[3,26].

Since 2007, CF has been included in the system of explicit health guarantees (GES) once diagnosed, however, screening is not included before diagnosis, and it should be noted that state financial support is currently under study to make it a free and mandatory service for all NBs[27].

Recommendations for implementation indicate that it should be done in countries with a CF incidence < 1:25,000 using IRT/DNA protocol unless not available or not feasible, with a minimum sensitivity of 95%, diagnostic confirmation in sweat should be within 4 weeks of age, have a program of test evaluation, including follow-up plans, updating, and availability of a complete CF specialist team[5]. Following these recommendations, the organization of a national program should be gradual, with the creation of new reference centers trained in the care of this disease, multi-professional care for patient follow-up, provision of equipment for reliable determination of chloride in sweat with quality control of measurements and processes, a fundamental tool for diagnosis, and clinical guidelines reviewed by specialists that are already published[2,28].

Neonatal screening should not only be considered from the point of view of sensitivity, specificity, and cost-effectiveness but also as a right of the NB. The emergence of treatment with modulators will change many aspects of the disease including survival in which early diagnosis is even more relevant[29,30].

Limitations of the study: This is a pilot study that did not include all Chilean NBs, so the results are partial. At the beginning of the protocol, there were preanalytical errors and, during these 7 years, 49,000 samples were not incorporated into the data analysis. In 1.1%, there was an error in sample collection, 2.5% unsat-

isfactory samples, 27.1% of blood collected before 48 hours or after 5 days, and 69.3% more than 7 days of card transfer. This demonstrates the need for permanent training for the maternity staff in charge, which, although improving over time, is still insufficient and should be modified with more frequent training and supervision.

In relation to the diagnosis confirmation, the result of the sweat test was on average 20 days, however, children with insufficient sweat sample (7%) delayed 60 days on average for the repetition, this can be modified by increasing the frequency of sweat collection which currently has a restricted schedule.

Conclusions

With state financial protection, Chile can implement neonatal screening for all NBs with the known benefits.

The overall CF incidence in NBs with informed consent and included in the study was 1/7,019 which is within the expected range whose figure could be modified by implementing screening for the whole country.

The combination of IRT/PAP and IRT x PAP strategy has good sensitivity and specificity, but with a low PPV limit, described with this technique, and it is possible to improve with a third tier: IRT or genetic study. The investigators suggest continuing with these 2 applications because of the good results obtained using percentile cut-off values for analytical IRT (98th percentile), action (99th percentile), and safety-netting (99.9th percentile).

Ethical Responsibilities

Human Beings and animals protection: Disclosure the authors state that the procedures were followed according to the Declaration of Helsinki and the World Medical Association regarding human experimentation developed for the medical community.

Data confidentiality: The authors state that they have followed the protocols of their Center and Local regulations on the publication of patient data.

Rights to privacy and informed consent: The authors have obtained the informed consent of the patients and/or subjects referred to in the article. This document is in the possession of the correspondence author.

Conflicts of Interest

Authors declare no conflict of interest regarding the present study.

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