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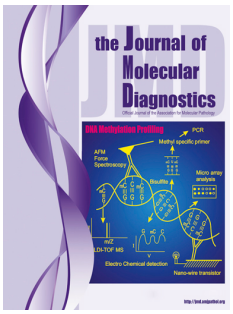

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PII: S1525-1578(21)00175-6

DOI: https://doi.org/10.1016/j.jmoldx.2021.05.018

Reference: JMDI 1094

To appear in: *The Journal of Molecular Diagnostics*

Received Date: 5 November 2019

Revised Date: 8 May 2021

Accepted Date: 27 May 2021

Please cite this article as: Sepúlveda-Hermosilla G, Freire M, Blanco A, Cáceres J, Lizana R, Ramos L, Assar R, Ampuero D, Aren O, Chernilo S, Spencer ML, Bernal G, Flores J, Rasse G, Sánchez C, Marcelain K, Rivas S, Branco GP, Galli de Amorim M, Nunes DN, Dias-Neto E, Freitas HC, Fernández C, Pérez P, Armisén R, NIRVANA team, Concordance analysis of ALK gene fusion detection methods in patients with Non–Small-Cell Lung Cancer from Chile, Brazil, and Peru, *The Journal of Molecular Diagnostics* (2021), doi: https://doi.org/10.1016/j.jmoldx.2021.05.018.

Concordance analysis of ALK gene fusion detection methods in patients with Non–Small-Cell Lung Cancer from Chile, Brazil, and Peru

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Short running head: Analysis of ALK gene fusion detection

FUNDING

This work received support from Chilean Government CORFO-International Center of Excellence Program (Grant # 13CEE2-21602) and Thermo Fisher Scientific. The funding sources were not involved in the study design, the collection, analysis, and interpretation of data; in the writing of the report, neither in the decision to submit the article for publication.

CONFLICT OF INTEREST STATEMENT

G.S., M.F., R.A., R.A.C., A.B., L.R., D.A., R.L., J.C. and P.P. were CEMP Pfizer Chile employees. H.F., E.D.N., D.N.N., G.P.B., M.G.A., C.F., G.B., J.F., S.C., O.A., M.L.S., G.R., C.S., K.M. and S.R. received a grant and non-financial support to perform this work for CEMP Pfizer Chile. Outside this work, H.F. discloses personal fees and non-financial support from Pfizer and BMS and non-financial support from AstraZeneca and Roche.

ABSTRACT

About 4 to 7 % of the Non-small cell lung cancer patients have ALK rearrangements and specific target therapies improve patients' outcomes significantly. ALK gene fusions are detected by immunohistochemistry (IHC) or Fluorescent *in situ* Hybridization (FISH) as gold standards in South America. Next Generation Sequencing (NGS) based assays are a reliable alternative, able to perform simultaneous detection of multiple events from a single sample. We analyzed 4,240 Non-small cell lung cancer samples collected in 37 hospitals from Chile, Brazil, and Peru; where ALK rearrangements were determined as part of their standard of care (SofC) using either IHC or FISH. A subset of 1450 samples was sequenced with the Oncomine Focus Assay (OFA), and the concordance with the SofC tests was measured. An orthogonal analysis was performed using a qPCR EML4-ALK fusion detection kit. ALK fusion prevalence is very similar for Chile (3.67%, N=2142), Brazil (4.05%, N=1013) and Peru (4.59%, N=675). Whereas a comparison between OFA and SofC assays showed similar sensitivity, OFA had significantly higher specificity and higher positive predictive value, which opens new opportunities for a more specific determination of ALK gene rearrangements.

INTRODUCTION

Precision medicine is revolutionizing the diagnosis and treatment of cancer patients. In recent years, the availability of drugs tailored to cancer driver alterations has changed the clinical practice and will continue to impact personalized care [1–5]. Lung adenocarcinoma is one of the most notorious examples of molecular guided cancer treatment [6]. In the last decade, the scenario evolved from no molecular tests to single-gene testing for EGFR sensitizing mutations to sequential tests for multiple genes, including *EGFR*, *ALK*, *ROS1*, among others. Recently, the Lung Cancer Mutation Consortium [7] reported that 64% of the 1007 NSCLC patient-derived samples harbor an oncogenic actionable driver alteration (25% on *KRAS*, 17% on *EGFR*, and 8% on *ALK* [8–13]), showing that multiplexed testing is capable of assisting physicians in the clinical decision, allowing the selection of the most accurate and personalized therapy [7]. The "*Updated Molecular Testing Guideline for the Selection of Lung Cancer Patients for Treatment With Targeted Tyrosine Kinase Inhibitors*", a combined effort from the International Association for the Study of Lung Cancer (IASLC), the College of American Pathologists (CAP), and the Association for Molecular Pathology (AMP), recommends as minimum testing for EGFR, ALK, and ROS1 in lung adenocarcinomas [14]. ALK rearrangements are present in 4-7% of lung adenocarcinomas. Numerous ALK inhibitors are approved to treat such tumors in many countries, including Latin America. ALK inhibitors have shown superiority over chemotherapy in first and second-line treatment [15,16], and second-generation ALK inhibitors increased progression-free survival compared to the first-generation inhibitors [17].

In clinical practice, fluorescence in situ hybridization (FISH) or Immuno-histochemistry (IHC) tests are the standard methods for testing ALK in many countries. While IHC is a reliable and fast methodology, using different tests for EGFR, ALK, and ROS1 sequentially or in parallel is time-consuming, requires more biological material from tumors, and can be more expensive than performing multiplexed testing.

Nowadays tissue collection for diagnosis has been extensively optimized reducing the invasiveness of the procedures and the amount of resected tissues. Precision medicine approaches should include the analysis in samples that are routinely preserved FFPE or a small needle using multiplexed assays, capable of detecting all sort of genomic alterations such as single-nucleotide variations, indels, gene-fusions and copy-number-alterations among others [5]. Next-Generation Sequencing (NGS) of solid tumor tissues fulfills this need and harbors the promise to be the gold standard in molecular diagnostics. Currently, in-house NGS testing services and "off the shelf" kits are obtaining regulatory approval on a gene-by-gene basis. Independent initiatives to assess the accuracy of molecular panels in real-world conditions can help to speed up the adoption of NGS in clinical practice.

Here, we present the results of the NIRVANA project, an international, multicentric study aimed to validate molecular diagnostic technologies in more than 4,000 lung cancer patients from Chile, Brazil, and Peru. We present the prevalence of ALK gene fusion events in these three countries, as well as the concordance between the results of standard-of-care ALK tests and the NGS-based Oncomine Focus Assay. To our knowledge, this is the largest study to assess the prevalence of ALK-fusion in South American countries so far.

MATERIALS AND METHODS

Ethics statement. The NIRVANA protocol (Validation of Molecular Diagnostic Technologies for Lung Cancer Patients) was registered with the identifier NCT03220230 at https://clinicaltrials.gov (last accessed 5/272021) and approved by local ethics committees (LECs) for each recruiting hospital in Chile, Brazil, and Peru. In Brazil, the National Ethics Committee (CONEP) also approved and provided oversight of the study. All patients provided informed consent for access to clinical, demographic, and pathology information as well as to available FFPE embedded tumor tissue. Strategies to protect their identity and privacy included anonymization procedures and a unique 8-digit identifier. Patients received no treatment as part of this study.

Patients and samples. Eligible patients were older than 18 years, with a histologically or cytologically proven diagnosis of NSCLC. Thirty-seven sites participated in this protocol: 9 in Chile, 19 in Brazil and 9 in Peru. All data was electronically captured and subjected to a 100% on-site source data verification, following Good Clinical Practice (GCP) guidelines. Prospective and retrospective samples were accepted, either from the primary tumor or metastatic sites, and most patients were treatment-naïve at the time of tissue biopsy. FFPE sections (5 µm of at least 5% cellularity [18,19]) were obtained for H-E staining and pathology verification (1 slide), ALK IHC (2 slides, 1 for the test and one for a negative control), also DNA and RNA purification (2-8 slides, see below) at each hospital pathology services, following a validated tissue sectioning protocol compatible with downstream assays. Supplementary Figure 1 provides a general layout of the study processes.

ALK rearrangement assay analyses (SofC ALK assay). ALK IHC tests were performed using the Ventana anti-ALK (D5F3) Rabbit monoclonal primary antibody (Roche, CE-IVD) in combination with the OptiView DAB IHC Detection kit and OptiView Amplification Kit in a fully automated immunohistochemistry assay on the Ventana BenchMark XT and Ventana BenchMark GX slide stainer. ALK FISH was performed according to Abbott Vysis recommendation. Ventana and Vysis Break Part tests were aggregated as SofC ALK tests. The results of the SofC ALK tests were reported to attending physicians.

NGS targeted Oncomine Focus Assay. DNA & RNA extraction from FFPE samples and sequencing were performed in five central laboratories that were trained in the procedures for sample handling, nucleic acids extraction, and sequencing to harmonize the technical procedures and assure the quality and integrity of the data, supported also by regular proficiency tests with certified controls (Horizon HD736, HD783, and HD784). OFA (Thermo Fisher Scientific, Carlsbad, USA) is a panel of 52 target genes designed for the analysis of DNA and RNA/cDNA derived from FFPE tissue. We isolated and quantified DNA and RNA using the RecoverAll and Qubit dsDNA/RNA HS kits (Thermo Fisher Scientific). We prepared DNA and RNA libraries according to the OFA library guideline and sequenced them in the Ion Personal Genome Machine System (PGM, Thermo Fisher Scientific, PN: 4462921). As a quality metrics threshold, we aimed for 200,000 reads for DNA (80% on-Target) and 5,000 reads for RNA, requiring the presence of at least 3 out of 5 control amplicons. ALK fusions were identified with the breakpoint assay method from the OFA panel, when a minimum of 20 reads at the breakpoint region was observed. All analyses have followed OFA's Analysis Workflow (v2.1) operating in the Torrent Browser and Ion Reporter server (Thermo Fisher Scientific).

Sample size and concordance analysis. Considering an ALK prevalence of 3-5% in reference populations, we estimated a sample size to reach levels of sensitivity and specificity with an error interval (95% CI) equal or narrower than 0.05 points [20,21]. We assumed that both SofC ALK and OFA were binary tests that yield either a positive or negative result (excluding other results from the analyses). Therefore, we estimate that 120 SofC ALK-positive cases were needed. The efficiency of the tests against the gold standards was measured with indexes like sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy. The general agreement of the pair was determined with McNemar's χ^2 and Cohen's Kappa methods [22].

Orthogonal validation. To estimate the analytical performance for the detection of ALK fusions by SofC and OFA, we used an EML4-ALK Fusion gene detection kit as a qPCR-based validated benchmark assay (AmoyDx, Xiamen, China, PN: 8.01.22001X024H), which detects the twenty-one most common fusion transcripts of the chimeric EML4-ALK gene. Fifty samples were analyzed following the manufacturer's instructions. We estimated the same statistical metrics for the concordance analysis. A post hoc statistical power analysis of the concordance metrics for the sample size was performed following the Bland-Altman Method [20].

Regression Analysis. We evaluated the relationship of clinical and demographic covariates over the results of the SofC ALK and OFA assays. We used univariate and multivariate regression analyses using the testing result of each method as a response variable. We evaluated the following covariates: sex, age at diagnostics, histology, cancer stage, sample strata (retrospective or prospective), the clinical procedure to obtain the biopsy, and the country and city of subject's recruitment. Covariates were considered statistically significant at p-value < 0.05. These analyses were implemented with the python package Statsmodels v0.10.1.

RESULTS

Study cohort description. This study recruited and tested 4,240 lung cancer patients (52.6% male, 47.4%

female) with ages ranging from 23 to 94 years (mean 66 years). Chilean subjects contributed half of the whole cohort, followed by Brazil (27.6%) and Peru (20.9%) (Figure 1 and Table 1). Most of the patients had adenocarcinoma (82%) followed by squamous cell carcinoma (12.5%). Smoking behavior was reported for 3,515 patients. Never smokers included 35.3% of all cases, former and current smokers accounted for 56% and 8.7%, respectively. Quantitative tobacco-use information was available for 1,755 patients; on average they reported the consumption of 58 packs per year. Disease stage information (according to the 8th edition of the TNM classification for lung cancer [23]) was available for 3320 patients (69% stage IV, 18% stage III, 6% stage II, 7% stage I).

Standard of Care ALK (SofC) testing results in Chile, Brazil, and Peru. SofC ALK test was possible in 3,970 samples (94%), of which 3,739 (88.2%) had Ventana-ALK test and 231 (11.8%) had FISH (Table 1). A negative or positive result was obtained in 3730 (95%) of the samples. The reasons to exclude the SofC ALK test were insufficient tissue (3.1%), and ambiguous results (2.5%). In Chile, the prevalence of ALK rearrangements was estimated at 3.67% (CI95%: 2.94% - 4.58%); in Brazil, 4.05% (CI95%: 2.99% - 5.46%); and in Peru, 4.59% (CI95%: 3.23% - 6.47%). The prevalence of ALK-positive patients and the number of samples collected per city are shown in Figure 1A. An elevated prevalence was documented for the cities of Trujillo, Arequipa, and Rio de Janeiro (Figure 1A and Figure 2A), however, we can not exclude distortions as these cities contributed with a very small number of subjects (respectively 12, 15, and 15 patients). We found the age distribution (Figure 1B) to be similar between the countries, with a shifted distribution of ALK-positive cases towards younger subjects (14.8%, 18-33 years old), followed by the next age bin (34-50 years) with a 9.8% (Figure 1B). *ALK* rearrangements were more frequent in females (OR: 1.02) as well as in younger patients from 18 to 50 years and never smokers (OR: 1.02, Figure 2A). No significant differences were observed regarding biopsy procedures. Regarding histologies, adenocarcinoma tumors are more likely to be ALK-positive than squamous cell carcinoma (OR 1.02 versus 0.98). In adenocarcinoma only samples, SofC ALK+ prevalence in Chile is 4.3%, Brazil 4.6%, and

Peru 5.0% (Table 1). Only 14 ALK+ samples were detected in non-adenocarcinoma samples, 8 of them in squamous cell carcinoma (Supplementary Table 1).

Concordance Analysis. We analyzed a total of 1450 NSCLC tissue samples with valid results in SofC and OFA tests. As described in Table 2, 45 samples were true positives, 21 samples were false positives, and 38 samples were false negatives. The statistical measures of concordance shown in Table 2, support the hypothesis that the detection of rearrangements using OFA is equivalent to the use of SofC ALK tests (Kappa: 0.583, p-value: 0.037). Regarding performance metrics, OFA displays a PPV of 68.2% and an NPV of 97.3% (Table 3). Age at diagnosis, sample strata, Tobacco use, and Country Site, were significant covariates for the rate of concordance between OFA and SofC ALK, but with a very modest effect (OR: 0.98 - 1.03, Figura 2B).

Orthogonal analysis using EML4-ALK qPCR as a benchmark. To further explore the moderate agreement between OFA and SofC ALK tests, we performed an orthogonal analysis using as benchmark EML4-ALK qPCR in a subset of 50 samples. In this analysis, 44 samples passed the qPCR quality control and presented valid results for both SofC ALK and OFA; 20 cases were ALK-positive by SofC ALK, OFA, or both; and 24 were negatives by both tests (Supplementary Table 2). The results showed that when the qPCR is used as a benchmark, SofC ALK displayed 79% sensitivity, as compared to 63% observed in OFA. However, in terms of specificity and Positive Predictive Value (PPV), OFA showed higher values (100% for both) than SofC ALK tests (88% and 83%, respectively, Table 3). Post hoc statistical power analysis for this sample size revealed statistically significant differences with a power of 80% (Supplementary Table 3) for specificity and PPV. When all qPCR positive samples were analyzed, the SofC ALK tests failed to detect fusions in 2 samples that were correctly detected by OFA (Supplementary Table 4 and Figure 3A). These 2 rearrangements, v3a and v1[24], were detected in samples with moderated and high coverage in OFA (20-199 and >200 reads, respectively). For just one case a rearrangement was filtered out in OFA due to low coverage (<20 reads), however, this case does not affect the sensitivity as it was found in an

already OFA-Positive sample. When replicates or resequencing data passing the quality control were considered in the analysis, one sample had its status changed to OFA positive. These reanalyzes would result in an increment of OFA sensitivity to 68.4%, accuracy of 86.4%, and NPV of 80.6%.

ALK Variants detected by OFA. We found a total of 145 known ALK alterations in the cohort. 61 of them were detected in DNA-derived reads, and 85 were found from RNA (Supplementary Table 5). These DNA variants included several missense SNVs predictive of sensitivity to specific ALK inhibitors [25] including *ALK* G1123X, L1152V, F1174L, V1180I, L1198F, G1201X, and G1202X. At the RNA level, the most frequent fusions were EML4-ALK (Figure 3B), including EML4 Exon 13 fused to *ALK* exon 20, known as EML4-ALK (E13;A20, v1) followed by EML4-ALK (E6a; A20, v3) and *EML4-ALK* (E20; A20, v2) [24]. All these are actionable variants relevant to the prescription of ALK inhibitors [25].

DISCUSSION

The treatment of metastatic NSCLC patients has changed dramatically in the last decade as lung adenocarcinoma is now viewed as a disease with multiple molecular-defined subtypes such as EGFR-mutated and ALK-rearranged tumors, among others. Nevertheless, the efficacy of tailored, targeted therapies is entirely dependent on precise molecular diagnostic tests. Here we present a thorough analysis and comparison of the results of the current gold standard diagnostic test for *ALK* rearrangements against a sequencing-based assay. We also measure and elucidate reasons for discordances between different tests and assess the feasibility of NGS-based approaches, using South American samples sequenced in laboratories of this same continent. There are a few published reports that compare the performance of sequencing-based approaches versus IHC or FISH tests for *ALK* gene fusion diagnosis; moreover, these few publications were restricted to a limited sample size [26–28]. With a total of 4240 lung cancer patients enrolled, our work, along with the CLICaP study [29], is one of the largest NSCLC cohorts of Latin America, testing samples by the standard of care ALK test (IHC or FISH), and also by a novel approach such as the NGS-based Oncomine Focus Assay. Also, the number of samples

allowed us to determine, with exceptional confidence, demographic aspects like the ALK gene fusion prevalence in South American countries. Due to the large number of clinical sites, across the three participating countries in the study, we were able to answer epidemiological questions with statistical relevance. We concluded that there are no statistically relevant differences between Chile, Peru, and Brazil; regarding the prevalence of ALK gene fusion. Other studies in the region have found similar rates [30–32], based on 250 cases per country using solely FISH tests. The shifted distribution of the prevalence of the ALK towards younger non-smoker patients found with SofC tests correlates with the current literature for the United States population [30].

Just like similar studies [26,29,33,34] a significant number of samples were discarded for technical reasons, including sample quality and handling across the whole biopsy-pathology continuum. It has been widely reported that the success of technologies like IHC depends on pre-analytical aspects such as the quality standard of the collection procedure, management of sample storage, training, and experience of the specialists that evaluate the results. Even though we made great efforts to normalize and ensure the quality of the samples, the known challenges of the IHC test, like cutting and fixation times, type, and quality of fixatives, and scoring methods should not be taken for granted in the real-world conditions embraced in the study. Regarding qPCR and sequencing, these are primarily dependent on the type of sample (FFPE or Fresh Frozen), tumor cell content, and the conditions of tissue preservation [35,36]. Particularly, the intra-tumoral heterogeneity could play an important role over sequencing-based results, especially for small samples like fine-needle biopsies. Also, because the tumoral content across the paraffin block often fluctuates, this could result in significantly different mutant-allele frequencies among slices, potentially affecting the outcome of the assay.

In general, the agreement tests determined that the concordance between SofC ALK tests and OFA results was moderate, with almost perfect specificity and NPV estimates, which confirms OFA's robustness to discard clinical cases where the rearrangements are truly absent. This feature is

complemented with the multiplex capabilities of the assay, which quickly delivers a complete landscape of the disease progression and early insights for downstream therapies. Conversely, sensitivity and PPV indexes leave room to hypothesize about pre-analytical factors and key parameters of the process that could explain and improve the rate of truly positive case detection. Subjects and sample characteristics considered in the regression analyses (sex, smoking habits, and age), are customarily evaluated in lung cancer studies [31,32,37–39]. Although rather small OR magnitudes were identified, these features correlate independently, even when combined in multi-parametric analyses. In our results, aspects such as the procedures to obtain the biopsy, the histology or disease stage revealed no significant effects over the concordance.

The discordance between NGS and SofC ALK tests was addressed with an orthogonal analysis using the qPCR as a tiebreaker to elucidate which technology determines the true rearrangements. This test was selected as it is a certified methodology to clinically diagnose ALK alterations, unlike OFA. It also allows the detection of variants segregating between 3 pools of oligonucleotide primers, each one being capable of amplifying 4 to 9 ALK fusions, therefore providing a more detailed description of tumor mutation status, and insights into clinical actionability. While this is an advantage over SofC, it does not reach the level of resolution that NGS does. In this sense, we consider qPCR an intermediate grade between both technologies. According to the orthogonal analysis results, SofC for ALK displays a better sensitivity than OFA, while OFA provides a significantly better specificity. Interestingly, our sequencing data has shown that when 200+ reads flank the breakpoint of the fusion, SofC and OFA results were consistent, suggesting this parameter as an empiric threshold to validate the equivalency of the results. The scarce amount of tissues in the samples could induce a pathological misinterpretation of the SofC result [40]. It has been documented that specific biopsy procedures (such as small biopsies and FNA) would favor the performance of ALK-IHC compared to ALK-FISH [41]. Also, preanalytical conditions such as tissue fixation time or the quality of reagents could affect the performance of the different assays [42]. As in our

study, we used tumor samples procured as part of the standard of care processes, as opposed to samples obtained as part of a controlled clinical trial study, we hypothesized that standard of care samples are a much better representation of the lifelike conditions these assays face in daily regular clinical practice. Regarding the international experience in *ALK* gene fusion detection methods, the reported discordance rates, in 9 independent studies [28,43], vary between 0.2 to 19% for qPCR vs. IHC or FISH for ALK. Regarding NGS versus IHC or FISH, in two independent studies, discordances vary from 12.7% (IHC), 17.6% (FISH) to 22.6% (IHC)[28,44], and is 18% in the present study. It is worth noting that both NGS and IHC for ALK are useful to predict ALK inhibitors' benefit in those studies.

Targeted gene panels have proven useful for molecular diagnosis in clinical cancer settings in the United States and several European countries. Likewise, an OFA test is capable of detecting *ALK* gene fusions, and of precisely identifying the gene pairs involved in the fusion events. Also, through this technology, it is possible to obtain a wider landscape of the mutational state of 52 other clinically actionable genes in a single biopsy sample and assay. These features give valuable information to the physicians, enabling them to review additional aspects such as tumor mutational burden and combinatorial patterns of somatic mutations [45] that will ultimately allow precision diagnostics and treatment for cancer patients.

We expect that the NIRVANA study, based on real-world samples and patient data, will help to advance the molecular epidemiology of lung cancer in Chile, Peru, and Brazil, supporting healthcare professionals in the understanding of the clinical implications of ALK rearrangements and encouraging other initiatives for new diagnostic methods and precision cancer medicine for South American patients.

Acknowledgments

The authors thank the patients who consented to provide tumor material and clinical data that was used in this study. We thank the Institutional BioBank of the A.C.Camargo Cancer Center, São Paulo, Brazil. ED-N is a research fellow from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

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FIGURE LEGENDS

Figure 1. NIRVANA SofC *ALK* testing results. (A) SofC *ALK*-positive prevalence is shown at the city level. For each city, *ALK*-positive prevalence and number of subjects are shown. (B) Superior panel: Patient age distribution for specific age bins (18 to >84 years old). Inferior panel: *ALK* prevalence for each bin is shown with a green bar, including 95% CI in vertical gray shades.

Figure 2. Logistic regression analyses The Odd Ratios of significant regressors categories, and 95% CI are depicted. The Y-axis represents the categories for each covariate, while the X-axis is the Odd Ratio value. A vertical dashed line is plotted over OR= 1, where the positive ratio is equal to the negative. (A) Significant demographic and clinical regressors of SofC *ALK* testing results (positive or negative) were used as response variables. From top to bottom, the regressors are Sex, Age at diagnosis, Site Country, Site City, and Tobacco use. (B) Potential predictors of agreement between SofC ALK and OFA results. From top to bottom, the predictors are: Age at diagnosis, Site Country, Patient's Strata, and Tobacco use.

Figure 3. **Sankey plot showing the flow of test results obtained on 17 rearrangements in 19 positive samples according to the qPCR assay.** (A) From left to right, the first column shows the SofC *ALK results* with the number of samples in parenthesis, while the second column shows how these are paired to the OFA results. The left side of this column shows the number of samples, and the right corresponds to the number of rearrangements found in the samples; the third column named *Coverage range*, categorizes the previous OFA results on how many reads were obtained in each fusion call (>200, 199-20 and <20). Finally, the right-most column shows the distribution of variants categorized by IASLC [24]: v1: *EML4* (exon 13) – *ALK* (exon 20), v2: *EML4* (exon 20) – *ALK* (exon 20), v3a/b: *EML4* (exon 6) – *ALK* (exon 20); In addition to those classifications, two fusion variants involving exons 3 (Exon 3) and 18 (v5) of *EML4*; with Exon 20 of *ALK* were found. (B) *ALK* gene fusion variants type frequency Barplot, for OFA BPA (BreakPoint Assay).

TABLE 1. Nirvana cohort description		
Characteristics (number of subjects)	n	%
Age at Diagnosis (n=4240)		
Mean (years)	66.0	
Standard Deviation	11.5	
Range	23 - 94	
Sex (n=4240)		
Female	2011	47.4
Male	2229	52.6
NSCLC histology (n=4240)		
LUAD	3476	82
LUSC	530	12.5
OTHERS	124	2.9
NSCLC NOS	62	1.5
NEC NOS	24	0.6
LUPC	24	0.6
Country (n= 4240)		
Chile	2184	51.5
Brazil	1170	27.6
Peru	886	20.9
Tobacco Use (n=3515)		
Former	1968	56
Never	1242	35.3
Current	305	8.7
Tobacco Use (n=1755)		
Mean (pack per years)	58.43	
Sd	47.83	
Range	0 - 346.5	
Lung Cancer Stage (n=3320)		
Stage I	232	7
Stage II	200	6
Stage III	594	18
Stage IV	2294	69
Biopsy procedure (n= 3946)		
Fibrobronchoscopy	1466	37.1
Percutaneous Puncture	622	15.8
Tumor Resection	603	15.3
Surgical Biopsy	1121	28.4
Transthoracic Pleura Biopsy	134	3.4
ALK Standard of care testing type (n=4240)		
Ventana Alk (D5f3)	3739	88.2
Vysis Alk Break-Apart Fish	231	5.4

Not Tested / Not Informed	270	6.4
ALK Standard of care testing results (n=3730)		
Positive	147	3.9
Negative	3583	96.1
ALK Standard of care testing results on Adenocarcinomas (n=3064)		
Positive	133	4.3
Negative	2931	95.7
Chile (n=1636)		
Positive	65	4
Negative	1571	96
Brazil (n=824)		
Positive	38	4.6
Negative	786	95.4
Peru (n=604)		
Positive	30	5
Negative	574	95

Clinical, demographic, and pathological aspects of the subjects. The table shows the most relevant clinical aspects collected in the NIRVANA cohort. Histological classifications according to MSK Oncotree (http://oncotree.mskcc.org/ as of 12/21/2019): Lung Adenocarcinoma (LUAD), Lung Squamous Cell Carcinoma (LUSC), Pleomorphic Carcinoma of the Lung (LUPC), Neuroendocrine carcinoma (NEC), Not otherwise specified (NOS). For the ALK Standard of care result, 510 subjects, which represents 12% of the total recruitment, have an invalid outcome due to insufficient tissue, not tested/not informed, or others.

TABLE 2. Confusion matrix & statistical analyses

Contingency Table for SofC ALK versus OFA Results.

	SofC ALK Positive	SofC ALK-Negative
OFA ALK-Positive	45	21
OFA ALK-Negative	38	1346

OFA and SofC ALK Hypothesis Tests of Concordance

Statistical Test	Rank (value)	P-value
Chi-squared independence test	Real (4.339)	0.037
Cohen's kappa test	Moderate (0.583)	NA

Contingency table for SofC *ALK* versus OFA results using data from 1450 patients that have met all the quality criteria and OFA and SofC *ALK* Hypothesis Tests of concordance.

TABLE 3. Concordance analyses

Index of Concordance	Estimation (%)	95% CI
OFA and SofC *ALK* (n=1450)		
Sensitivity	54.2	43.5 - 64.5
Specificity	98.5	97.6 - 99.0
Accuracy	95.9	94.8 - 96.8
Positive Predictive Value	68.2	56.2 - 78.2
Negative Predictive Value	97.3	96.2 - 98.0
SofC *ALK*, and qPCR (n = 44)		
Sensitivity	78.9	56.1 - 92.0
Specificity	88.0	69.2 - 96.7
Accuracy	84.1	70.3 - 92.4
Positive Predictive Value	83.3	60.0 - 95.0
Negative Predictive Value	84.6	65.9 - 94.5
OFA and qPCR (n = 44)		
Sensitivity	63.2	40.9 - 81.0
Specificity	100.0	84.2 - 100.0
Accuracy	84.1	70.3 - 92.4
Positive Predictive Value	100.0	71.8 - 100.0
Negative Predictive Value	78.1	61.0 - 89.3

Concordance analysis is given for OFA and SofC ALK, including all patients used on the confusion matrix. The indexes of concordance of an orthogonal analysis (n=44) performed using qPCR as a reference standard are also provided.

Figure 1

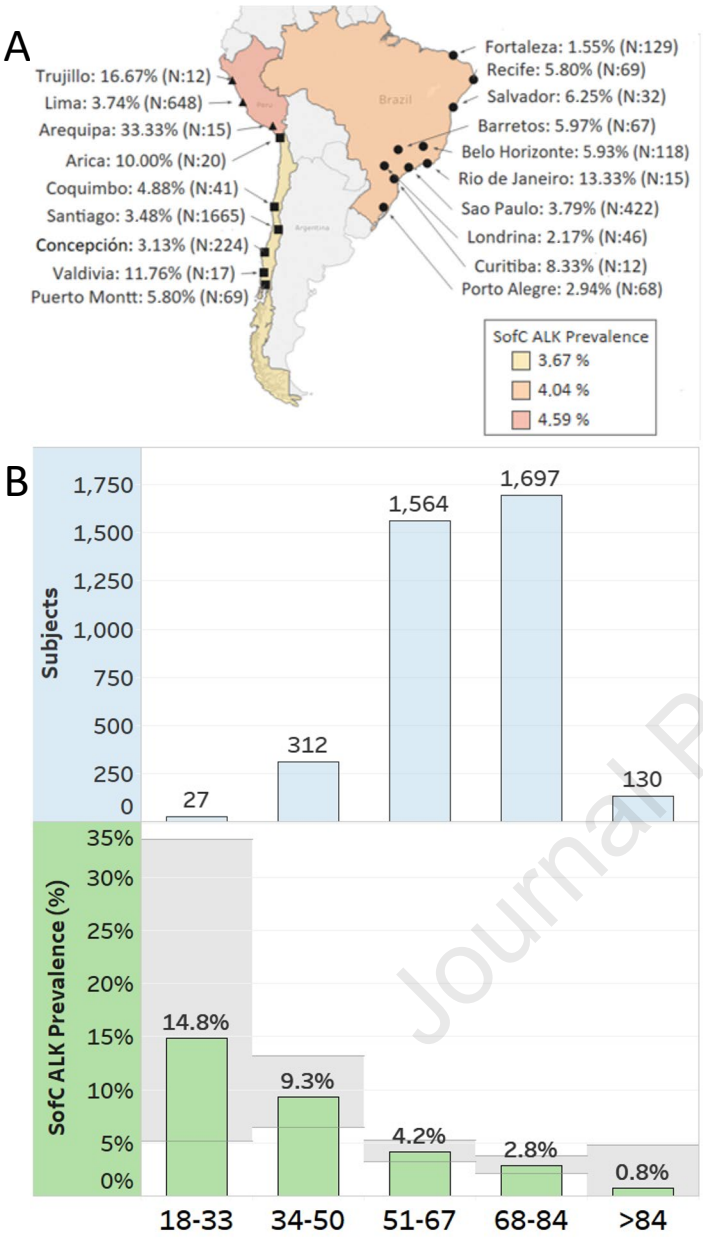

Figure 2

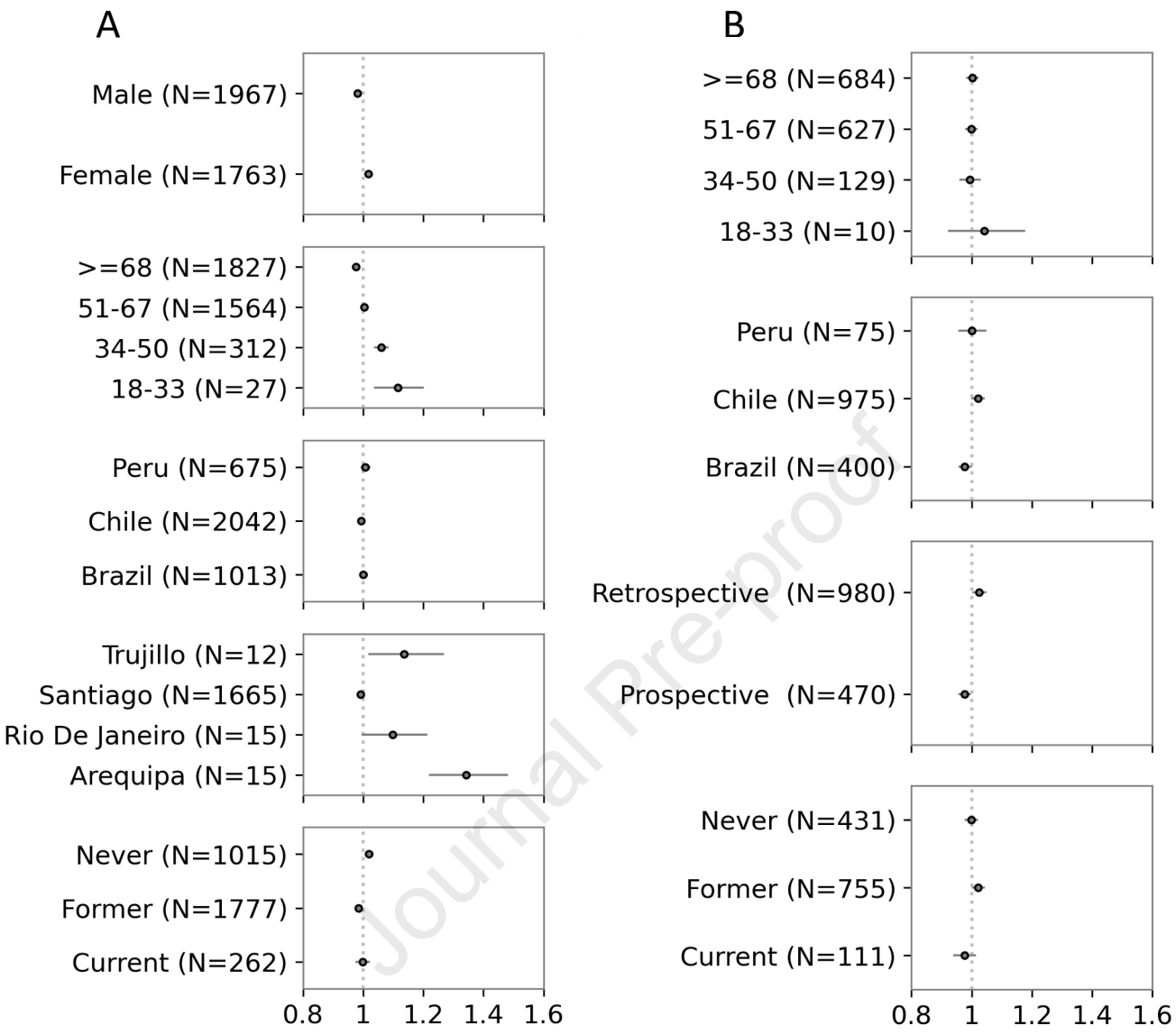

Figure 3