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ORIGINAL RESEARCH

# A Cross-Sectional Study of Variant Interpretation and Reporting of NGS Data Using Tertiary Analysis Software: Navify® Mutation Profiler

Francesco Pepe · Gianluca Russo · Nadia Barraco · Marco Bono · Angela Listì · Luisella Righi · Dario de Biase · Thais Maloberti · Claudia Scimone · Lucia Palumbo · Danilo Rocco · Giuseppina Roscigno · Enzo Gallo · Simonetta Buglioni · Michelina Coco · Lucia Anna Muscarella · Giancarlo Troncone · Umberto Malapelle

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## ABSTRACT

***Introduction***: Personalized medicine has revolutionized the clinical management of patients with solid tumors. However, the large volumes of molecular data derived from next-generation sequencing (NGS) and the lack of harmonized bioinformatics pipelines drastically impact the clinical management of patients with solid tumors. A possible solution to streamline the molecular interpretation and reporting of NGS data would be to adopt automated data analysis software. In this study, we tested the clinical efficiency of the Navify Mutation Profiler (nMP) software in improving the interpretation of NGS data analysis in diagnostic routine samples from patients with solid tumors.

***Methods***: This study included one coordinating institution (Federico II University of Naples) and five other Italian institutions. Variant call format (VCF) files from reference standard

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Francesco Pepe and Gianluca Russo contributed equally to the paper.

F. Pepe · G. Russo · C. Scimone · L. Palumbo · G. Troncone · U. Malapelle (✉)
Department of Public Health, Federico II University of Naples, Naples, Italy
e-mail: umberto.malapelle@unina.it

N. Barraco · M. Bono
Department of Precision Medicine in Medical, Surgical and Critical Care (Me.Pre.C.C.), University of Palermo, Palermo, Italy

A. Listì · L. Righi
Department of Oncology, University of Turin, San Luigi Hospital, Turin, Orbassano, Italy

D. de Biase
Department of Pharmacy and Biotechnology (FaBiT), University of Bologna, Bologna, Italy

D. de Biase · T. Maloberti
Solid Tumor Molecular Pathology Laboratory, IRCCS Azienda Ospedaliero—Universitaria di Bologna, Bologna, Italy

D. Rocco
Department of Pulmonary Oncology, AORN dei Colli Monaldi, 80131 Naples, Italy

G. Roscigno
Department of Biology, Federico II University of Naples, Naples, Italy

E. Gallo · S. Buglioni
Department of Pathology, IRCCS Regina Elena National Cancer Institute, Rome, Italy

M. Coco · L. A. Muscarella
Laboratory of Oncology, Fondazione IRCCS Ospedale Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

samples previously tested by the coordinating institution and from $n=8$ diagnostic routine samples ($n=2$ from colorectal carcinoma; $n=2$ from non-small cell lung cancer; $n=2$ from advanced melanoma; and $n=2$ from patients with gastrointestinal stromal tumors) and previously analyzed by each participating institution ($n=5$) with standardized internal analysis workflows were uploaded onto the Navify® Mutation Profiler (nMP) system (Roche Sequencing Solutions, Pleasanton, CA, USA) for automated analysis and interpretation of DNA and RNA molecular alterations analytical parameters, molecular profiling, and clinical interpretation were carried out by the nMP system and compared with the standard workflow data analyzed by the participating institutions.

***Results*:** Overall, all VCF files were successfully submitted and interpreted by the nMP system. A concordance agreement rate of 89.6% was observed between the automated and standard workflow systems. In particular, DNA and RNA molecular profiles obtained with the nMP system matched those obtained with standardized approaches in 44 out of 48 patients (91.7%) and in 11 out of 12 (91.7%) cases, respectively. In addition, the nMP system evidenced wild-type variants in 6 out of 7 (85.7%) cases.

***Conclusions*:** The nMP system represents a valid, easily manageable, and clinically useful system to interpret NGS data on diagnostic routine samples from patients with solid tumors.

**Keywords:** Navify® Mutation Profiler (nMP); Molecular pathology; Tumor biomarkers; Diagnostic techniques and procedures

**Key Summary Points**

In the era of precision medicine, the rapidly increasing number of predictive biomarkers drastically impacts the clinical interpretability of molecular alterations, thereby hindering the optimization of individualized cancer treatments.

Although automated bioinformatic pipelines can facilitate the interpretation of molecular records from next-generation sequencing (NGS) analysis, the current lack of standardized guidelines to assign clinical significance to molecular records remains an open challenge.

The Navify® Mutation Profiler (nMP) platform v.2.3.2.c090e09 (Roche Sequencing Solutions, Pleasanton, CA, USA) is cloud-based CE-IVD (In vitro diagnostic) software that can automatically provide a clinical interpretation of molecular records by matching these data with the Roche Cancer Genome Database, a knowledge-based database integrating several publicly available referral databases.

The nMP system showed a concordance rate of 89.6% with standard interpretation systems. In particular, nMP's interpretation of DNA- and RNA-based NGS analysis matched that of standardized diagnostic routine approaches in 44 out of 48 patients (91.7%) and 11 out of 12 (91.7%) of cases.

## INTRODUCTION

Precision medicine has radically modified the clinical paradigm for patients with solid tumors [1]. In the last decade, numerous predictive biomarkers have been approved in clinical practice for the clinical stratification of patients with solid tumors [2, 3]. For example, for patients with non-small cell lung cancer (NSCLC), a wide panel of "must test genes" has been developed for the molecular profiling of routine samples [4–10]. As of today, the panel includes the following mutations: epidermal growth factor receptor (*EGFR*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*) exon 2 p.G12C, v-Raf murine sarcoma viral oncogene homolog B *(BRAF*), anaplastic lymphoma receptor tyrosine kinase (*ALK*), ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*), rearranged during transfection (*RET*), neurotrophic receptor tyrosine kinase (NRTK), and MET proto-oncogene (MET) exon 14 skipping [4–10]. Moreover, detecting programmed

death-ligand 1 (PD-L1) expression is also key to selecting patients with NSCLC for immune-checkpoint inhibitors (ICIs) [11]. Remarkably, the rapidly evolving scenario of molecular targeted therapy is showing positive results in ongoing clinical trials, where human epidermal growth factor receptor 2 (*HER2*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), and neuregulin 1 (*NRG1*) are currently under investigation to guide treatment decision-making [12]. Moreover, complex biomarkers, including homologous recombinant deficiency status (HRD) and microsatellite instability (MSI), are also being routinely evaluated to identify genomic instability in clinical practice [2]. Hence, in the complex landscape of druggable and drug-resistant mutations, it should come as no surprise that many clinical laboratories are shifting from single- to multiple-gene testing approaches [13]. By far the most popular high-throughput sequencing technology is next-generation sequencing (NGS). Scores of studies in the last decade or so have evidenced the advantageous applicability of these platforms in cancer diagnostics and management. Indeed, in a single run, they can accurately carry out DNA/RNA massive parallel sequencing analysis of several biomarkers from tissue and liquid biopsy specimens in patients with multiple tumors, thereby reducing costs, turnaround times, and delays in targeted therapies [14]. Despite these advantages, the widespread use of NGS platforms in diagnostic routine practice has been hampered by several limitations [15]. One limitation is the vast number of molecular records churned out by NGS, requiring quality control checks to ensure reliability [16, 17]. In addition, variants from NGS analysis must also be inspected to determine their clinical significance [18]. In particular, a plethora of classification systems have been developed to clinically categorize inspected variants. However, controversial interpretations may often result from disharmonized classification systems set on different parameters (actionability, approved drugs, clinical impact), especially in the classification of variants of uncertain significance (VUS). Data analysis is currently automatically carried out by bioinformatics tools able to technically and clinically interpret molecular records. However, the reliability of these tools in diagnostic settings remains to be assessed in harmonized clinical trials. To overcome the challenges in the clinical interpretation of NGS data, several cloud-based decision support tools have recently been developed for clinical application. Among these platforms is the Navify® Mutation Profiler (nMP) platform v.2.3.2.c090e09 (Roche Sequencing Solutions, Pleasanton, CA, USA). This cloud-based CE-IVD (In vitro diagnostic) software is meant to facilitate the interpretation of clinically relevant molecular alterations detected by NGS platforms [19]. In particular, this system matches molecular records with clinical interpretations from the Roche knowledge-based database system. This system is instrumental for oncologists as it integrates several publicly available referral databases, including the Catalogue of Somatic Mutations in Cancer (COSMIC), The Cancer Genome Atlas (TCGA), Clinical Interpretation of Variants in Cancer (CIViC), biomedical literature, clinical trial results, and medical guidelines [20].

The aim of this paper was to evaluate the technical and clinical performance of nMP in interpreting NGS data in a retrospective series of patients with solid tumors previously diagnosed by a group of Italian referral institutions with internal clinical routine interpretation systems after NGS. In particular, concordance rates between the two systems were measured in terms of clinical interpretation and working time for the submission of the final report.

## STUDY DESIGN

This harmonization trial involved a series of $n=6$ Italian referral institutions: $n=1$ coordinating institution plus $n=5$ participating institutions. Our pathology laboratory at Federico II University of Naples served as the coordinating institution. To ensure proper handling of the nMP system, we provided a brief online training session to all institutions [21]. In general, the nMP software matches the NGS molecular profiling of tissue and liquid biopsy samples with the clinical interpretation of variants by integrating knowledge-based database records deriving from several public repositories. Essentially, this tool is based on the tiered variant classification system defined

by the Association of Molecular Pathology/American Society of Clinical Oncology/College of American Pathologists/The American College of Medical Genetics (AMP/ASCO/CAP/ACMG) guidelines; moreover, it provides updates on drug approvals and international recommendations by different pharmaceutical regulatory agencies, including the European Medicines Agency (EMA), SwissMedic, and the National Institute for Health and Care Excellence (NICE). In this study, before implementing the nMP software for the clinical interpretation of NGS test results for diagnostic routine samples, our coordinating institution shared a variant call file (VCF), generated from the sequencing analysis of $n=2$ reference standard samples (DNA and RNA), with each participating center. The concordance rate was calculated by comparing the nMP interpretation of DNA and RNA samples with that from the standardized internal data analysis workflows pertaining to each participating institution. The institutions' workflows were based on an NGS platform combined with manual and/or automated data-interpreting systems. Moreover, each investigator uploaded and analyzed VCFs of $n=8$ diagnostic routine samples ($n=2$ from colorectal carcinoma [CRC]; $n=2$ from NSCLC; $n=2$ from advanced melanoma [MM], and $n=2$ from patients with gastrointestinal stromal tumors [GIST]) previously analyzed with their standardized internal analysis workflows. For each case, each investigator reviewed the somatic variants inspected by the nMP software in accordance with the tier classification system used to evaluate the level of clinical significance. Molecular records, clinical interpretation, and analytical run parameters—(1) the time taken to create and submit the case; (2) the time between case uploading and automated results; and (3) the time taken to review the results and generate a final clinical report—were shared with the coordinating center within 1 month from the start of the study (Fig. 1). Written informed consent was acquired from all patients involved in the study and documented in accordance with the general authorization for processing personal data for scientific research purposes from the Italian Data Protection Authority (http://www.garanteprivacy.it/web/guest/home/docweb/-/docwebdisplay/export/2485392). All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/).

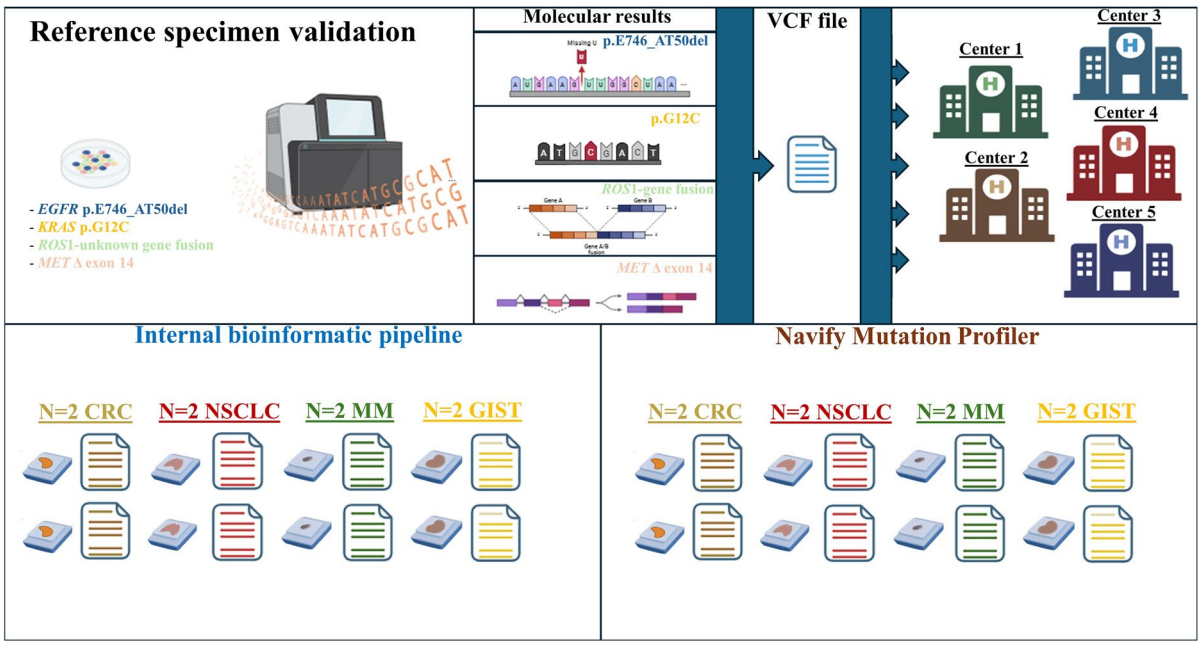


**Fig. 1** Schematic representation of the study design

# METHODS

### Standard Sample Analysis

A standard reference sample was previously validated by the coordinating institution [22]. Briefly, the artificial control was built by mixing $n=4$ engineered cell lines harboring actionable molecular alterations in patients with NSCLC (exon 19 *EGFR* p.E746_AT50del and exon 2 *KRAS* p.G12C hotspot mutations; *ROS*1-unknown gene fusion; and *MET* Δ exon 14 skipping molecular alterations). Nucleic acids were manually purified with the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) and All Prep DNA/RNA mini kit (Qiagen GmbH) by following the manufacturer's instructions. NGS analyses were independently carried out on the Ion S5™ Plus (Thermo Fisher Scientific) and Genexus (Thermo Fisher Scientific) platforms by adopting a customized and a commercially available NGS panel, respectively [22]. Each institution selected two distinct workflows on the nMP system to process VCF files for DNA- and RNA-based analysis. For the RNA-based workflow, an optimized analysis pipeline was required to detect RNA molecular alterations correctly.

### Diagnostic Specimen Analysis

In total, $n=8$ ($n=2$ CRC, $n=2$ NSCLC, $n=2$ MM, and $n=2$ GIST) diagnostic routine samples were retrieved from the internal archives of each institution; the molecular and clinical records are reported in Table 1. Each archival VCF file was uploaded to the nMP software to generate new ready-to-process samples. In this section, clinical information (age, sex, ethnicity) and pathological records (histological type, histological site, tumor purity of neoplastic cells) were filled out. The diagnostic workflows for DNA or RNA analysis (DNA or RNA, DNA/RNA) were selected before nMP analysis. Four processing run parameters were collected for each case: (1) the working time spent to prepare and submit the case; (2) the working time taken to upload files and generate results using the software; (3) the working time spent to manually inspect, review, and elaborate the clinical reports; and (4) any observations regarding the ease of use of nMP. The data interpretation was inspected if requested by oncologists; in the case of both DNA and RNA biomarker analysis, two distinct workflows were activated. Finally, all molecular records were shared with the coordinating center by adopting a dedicated Excel file containing molecular records and matched tiered interpretations.

# RESULTS

### Standard Sample Analysis

Overall, all the molecular data and matched tiered interpretations from the participating institutions were successfully shared with the coordinating center within the requested working time (15 days). The standard samples harbored druggable molecular alterations in referral genes for the clinical management of patients with NSCLC (*EGFR* exon 19 p.E746_AT50del, *KRAS* exon 2 p.G12C, *ROS1-SLC34A2* aberrant transcript, and *MET* Δ exon 14 skipping). In brief, standard reference specimens were successfully uploaded and interpreted by the nMP platform in each participating institution. In particular, whereas the nMP system successfully analyzed DNA-based molecular alterations in all cases, it failed to do so for RNA-based molecular alterations in a single institution (see Supplementary File 1). The clinical inspection of the molecular data integrated into the molecular reports overlapped with the data analysis previously obtained in the diagnostic routine of each participating institution (Table 2).

### Diagnostic Specimen Analysis

Overall, the molecular data and matched tiered interpretation were successfully shared with the coordinating center within the requested working time (15 days). The series of diagnostic routine cases was successfully uploaded to and interpreted by the nMP platform in all cases. Overall, a concordance rate of 89.6% (43 out of 48 submitted cases) was observed between the

**Table 1** Molecular results obtained at each center after reference DNA and RNA VFC file analysis provided by the coordinating center

| Center ID | Sample | Navify result |
|---|---|---|
| Coordinating center | Reference DNA | ***EGFR*** p.E746_A750del (77.0%); ***KRAS*** p.G12C (32.0%) |
| | Reference RNA | ***SLC34A2-ROS1*** (3327 reads); ***MET-MET*** (349 reads) |
| Center 1 | Reference DNA | ***EGFR*** p.E746_A750del (77.0%); ***KRAS*** p.G12C (32.0%) |
| | Reference RNA | ***SLC34A2-ROS1*** (3327 reads); ***MET-MET*** (349 reads) |
| Center 2 | Reference DNA | ***EGFR*** p.E746_A750del (77.0%); ***KRAS*** p.G12C (32.0%) |
| | Reference RNA | ***SLC34A2-ROS1*** (3327 reads); ***MET-MET*** (349 reads) |
| Center 3 | Reference DNA | ***EGFR*** p.E746_A750del (77.0%); ***KRAS*** p.G12C (32.0%) |
| | Reference RNA | ***SLC34A2-ROS1*** (3327 reads); ***MET-MET*** (349 reads) |
| Center 4 | Reference DNA | ***EGFR*** p.E746_A750del (77.0%); ***KRAS*** p.G12C (32.0%) |
| | Reference RNA | ***SLC34A2-ROS1*** (3327 reads); ***MET-MET*** (349 reads) |
| Center 5 | Reference DNA | ***EGFR*** p.E746_A750del (77.0%); ***KRAS*** p.G12C (32.0%) |
| | Reference RNA | *Data file error* |

Bold indciates Gene namesto clearly point on the type of genes inspected by software

*DNA* deossiribonuclei acid, *EGFR* epidermal growth factor receptor, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *MET* MET proto-oncogene, *RNA* ribonucleic acid, *ROS1* ROS proto-oncogene 1, *SLC34A2* solute carrier family 34 member 2

routine data interpretation and the nMP automated interpretation systems in all participating institutions. In addition, the DNA molecular profiles generated by the nMP system matched those generated by the standard interpretation workflows in 91.7% of cases (44 out of 48 patients); similarly, the RNA molecular profiles performed by the nMP software mirrored those of the standard interpretation workflows in 91.7% of cases (11 out of 12 submitted cases). Noticeably, the nMP system detected wild-type patients for actionable molecular alterations in 85.7% of cases (6 out of 7 patients). The molecular variants that were not detected by nMP or that had a Variant Allele Fraction (VAF) of < 1.0% were filtered out to calculate percentage agreement rates. A comparable VAF level was observed between internal data-reporting workflows and nMP analysis (± 2.3, ranging from 0.1 to 22.0). Clinical inspection of the molecular data integrated into the molecular report by the nMP system matched the data interpretation of the internal diagnostic routine workflows in 8/8 (100%), 8/8 (100%), 6/8 (75.0%), 8/8 (100%), 7/8 (87.5%), and 6/8 (75.0%) of cases for the coordinating center and centers 1–5, respectively. Regarding the discordant cases, the nMP system identified additional druggable alterations in a single case (*KRAS* p.G12V vs. *KRAS* p.G12V plus *NRAS* p.G13D [center 2]), whereas the routine standard interpretation workflows did not. In addition, $n = 2$ cases harboring *KIT* activating mutations detected by standard interpretation workflows were discordant with those reported by the nMP system (c-KIT p.A502_Y503dup [center 2] and p.T574_W577dup [center 5]).

**Table 2** Clinical data for the analyzed samples and related requests for molecular alteration assessment

| Centre ID | Sample | Sex | Age | Histological diagnosis | Neoplastic cells (%) | Clinical request |
|---|---|---|---|---|---|---|
| Coordinating center | Lung 1 | M | 67 | NSCLC | 20 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET; NTRK |
| | Lung 2 | M | 60 | NSCLC | 70 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET; NTRK |
| | Colon 1 | M | 80 | CRC | 60 | KRAS; NRAS; BRAF |
| | Colon 2 | M | 63 | CRC | 30 | KRAS; NRAS; BRAF |
| | GIST 1 | M | 74 | GIST | 80 | c-KIT; PDGFRA |
| | GIST 2 | F | 81 | GIST | 40 | c-KIT; PDGFRA |
| | Melanoma 1 | M | 63 | Melanoma | 30 | BRAF; NRAS; c-KIT |
| | Melanoma 2 | M | 88 | Melanoma | 90 | BRAF; NRAS; c-KIT |
| Center 1 | Lung 1 | F | 65 | NSCLC | 70 | BRAF; EGFR; ERBB2; KRAS; MET |
| | Lung 2 | F | 70 | NSCLC | 30 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET; NTRK; ERBB2 |
| | Colon 1 | M | 61 | CRC | 70 | KRAS; NRAS; BRAF |
| | Colon 2 | M | 72 | CRC | 15 | KRAS; NRAS; BRAF |
| | GIST 1 | M | 34 | GIST | 90 | c-KIT; PDGFRA |
| | GIST 2 | F | 66 | GIST | 90 | c-KIT; PDGFRA |
| | Melanoma 1 | M | 41 | Melanoma | 80 | BRAF; NRAS; c-KIT |
| | Melanoma 2 | F | 54 | Melanoma | 30 | BRAF; NRAS; c-KIT |
| Center 2 | Lung 1 | M | 71 | NSCLC | 60 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET |
| | Lung 2 | F | 82 | NSCLC | 20 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET |
| | Colon 1 | M | 73 | CRC | 60 | KRAS; NRAS; BRAF; PIK3CA; NTRK |
| | Colon 2 | M | 63 | CRC | 70 | KRAS; NRAS; BRAF; PIK3CA; NTRK |
| | GIST 1 | F | 58 | GIST | 100 | c-KIT; PDGFRA; BRAF |
| | GIST 2 | M | 61 | GIST | 100 | c-KIT; PDGFRA; BRAF |
| | Melanoma 1 | M | 47 | Melanoma | 80 | BRAF; NRAS; c-KIT |
| | Melanoma 2 | M | 77 | Melanoma | 50 | BRAF; NRAS; c-KIT; GNAQ |

**Table 2** continued

| Centre ID | Sample | Sex | Age | Histological diagnosis | Neoplastic cells (%) | Clinical request |
|---|---|---|---|---|---|---|
| Center 3 | Lung 1 | M | 52 | NSCLC | 40 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET; NTRK |
| | Lung 2 | F | 72 | NSCLC lymphonodal metastasis | 40 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET; NTRK |
| | Colon 1 | M | 85 | CRC | 60 | KRAS; NRAS; BRAF |
| | Colon 2 | M | 76 | CRC | 20 | KRAS; NRAS; BRAF |
| | GIST 1 | F | 66 | GIST | 90 | c-KIT; PDGFRA |
| | GIST 2 | F | 74 | GIST | 90 | c-KIT; PDGFRA |
| | Melanoma 1 | M | 68 | Melanoma | 60 | BRAF; NRAS; TERT |
| | Melanoma 2 | F | 75 | Melanoma | 70 | BRAF; NRAS; TERT |
| Center 4 | Lung 1 | M | 67 | NSCLC | 90 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET; NTRK |
| | Lung 2 | F | 47 | NSCLC | 85 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET; NTRK |
| | Colon 1 | M | 72 | CRC | 90 | KRAS; NRAS; BRAF |
| | Colon 2 | F | 77 | CRC | 85 | KRAS; NRAS; BRAF |
| | GIST 1 | F | 61 | GIST | 90 | c-KIT |
| | GIST 2 | M | 63 | GIST | 90 | c-KIT; PDGFRA; BRAF |
| | Melanoma 1 | M | 65 | Melanoma | 75 | BRAF |
| | Melanoma 2 | F | 72 | Melanoma | 85 | BRAF; NRAS; c-KIT |
| Center 5 | Lung 1 | M | 49 | NSCLC | 50 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET; NTRK |
| | Lung 2 | F | 30 | NSCLC | 80 | EGFR; BRAF; KRAS; ALK; ROS1; MET; RET; NTRK |
| | Colon 1 | M | 60 | CRC | 60 | KRAS; NRAS |
| | Colon 2 | M | 53 | CRC | 5 | KRAS; NRAS; BRAF |
| | GIST 1 | M | 61 | GIST | 80 | c-KIT; PDGFRA |
| | GIST 2 | M | 49 | GIST | 80 | c-KIT; PDGFRA |
| | Melanoma 1 | F | 54 | Melanoma | Not microdissected | BRAF; NRAS; c-KIT |
| | Melanoma 2 | M | 79 | Melanoma | 80 | BRAF; NRAS; c-KIT |

**Table 2** continued

*ALK* anaplastic lymphoma kinase, *BRAF* v-Raf murine sarcoma viral oncogene homolog B, *c-KIT* KIT proto-oncogene, *CRC* colorectal cancer, *EGFR* epidermal growth factor receptor, *EML4* EMAP like 4, *ERBB2* erb-B2 receptor tyrosine kinase 2, *GIST* gastrointestinal stromal tumors, *GNAQ* G protein subunit alpha Q, *ID* identifier, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *MET* MET proto-oncogene, *NRAS* v-ras neuroblastoma RAS viral oncogene homolog, *NSCLC* non-small cell lung cancer, *NTRK* neurotrophic receptor tyrosine kinase 1, *PDGFRA* platelet-derived growth factor receptor alpha, *PIK3CA* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, *RET* proto-oncogene tyrosine-protein kinase receptor Ret, *ROS1* ROS proto-oncogene 1, *WT* wild-type

Finally, discordant cases between standard interpretation workflows and nMP analysis were also observed in colon 1 from center 5 (wild type vs. *BRAF* p.V600E). Regarding RNA-derived molecular alterations, the nMP software did not identify EML4(6)–*ALK*(20) (lung 1, center 4). These alterations were classified as tier I–II according to the diagnostically, prognostically, and therapeutically significant levels established by the AMP/ASCO/CAP guidelines (Table 3). From a technical point of view, it took approximately 6.3 min (ranging from 1.5 to 15.0 min) to submit queries to the platform, 10.7 min (ranging from 4.5 to 13.5 min) between case upload and the generation of initial results by the software, and 9.7 min (ranging from 2.0 to 15 min) to review the platform's molecular and clinical data to elaborate valuable reports compared with. In the same way, medians of 2.7 min (ranging from 1.0 to 6.0 min) for preparing and submitting cases, 32.7 min (ranging from 1.0 to 152.0 min) between case upload and the generation of initial results by the software, and 17.5 min (1.5 to 30 min) for reviewing and clinically interpreting molecular records were observed (Tables 4 and 5).

## DISCUSSION

In the era of precision medicine, NGS systems have radically modified the diagnostic paradigm for the clinical management of patients with solid tumors, allowing for more thorough mutational profiles and accurate test results than conventional approaches. Amazingly, these panels can cover between 2 and > 500 clinically relevant genes. However, interpreting the vast range of test results generated by NGS panels is a dauting (but nonetheless necessary) task for oncologists [24]. Indeed, identifying treatable concomitant pathogenetic/activating mutations drastically impacts the clinical management of patients with tumors [25]. By the same token, identifying "unknown" molecular alterations is equally paramount as it allows us to expand the molecular and clinical landscape of informative variants in patients with solid tumors [26, 27]. In recent years, having acknowledged the practical pitfalls in the clinical reporting of molecular alterations from routine diagnostic specimens, international societies have underlined the pivotal role of data interpretation systems in overcoming the challenges of interpreting NGS data [25, 27]. Among these systems are bioinformatics pipelines. Generally, bioinformatics pipelines allow for the automated clinical interpretation of detected variants based on dedicated clinical databases. Today, a rapidly growing number of technical solutions include cloud-based systems that can be either integrated or not into cloud-based platforms. Among the currently available tools for the molecular and clinical interpretation of NGS data are open-source NGS tools, commercial NGS tools, and alignment tools. An advantage of these NGS bioinformatics tools is that they can automatically perform three fundamental analyses, thereby reducing the overall working time. The primary analysis consists of base calling and scoring base quality through FASTQ or uBAM files; the secondary analysis involves read alignment or de novo assembly; finally, the tertiary analysis involves data annotation by means of the SIFT, PolyPhen-2 CADD, ANNOVAR, and Condel prediction tools.

The clinical interpretation of molecular variants, which are ranked on the basis of their pathogenicities, is assessed through several approaches. Among these approaches is PHIVE.

**Table 3** Molecular results provided by the centers, mutations detected with the nMP system, and relative VAFs

| Center ID | Sample | Internal workflow result | Navify result |
|---|---|---|---|
| Coordinating center | Lung 1 | ***KRAS*** p.G12C (17.6%) | ***KRAS*** p.G12C (18.0%) |
| | Lung 2 | ***RET*** (ex12)—KIF5B (ex15) (4025 reads) | ***RET*** (ex12)—KIF5B (ex15) (4025 reads) |
| | Colon 1 | ***KRAS*** p.G12D (7.3%) | ***KRAS*** p.G12D (3.3%) |
| | Colon 2 | ***KRAS*** p.G12C (51.8%) | ***KRAS*** p.G12C (52.0%) |
| | GIST 1 | WT | WT |
| | GIST 2 | ***c-KIT*** p.V559D (86.7%) | ***c-KIT*** p.V559D (87.0%) |
| | Melanoma 1 | WT | WT |
| | Melanoma 2 | ***NRAS*** Q61K (61.9%) | ***NRAS*** Q61K (61.9%) |
| Center 1 | Lung 1 | ***EGFR*** p.L747_A750delinsP (54.0%); p.C797S (34.0%) | ***EGFR*** p.L747_A750delinsP (54.0%); p.C797S (34.0%) |
| | Lung 2 | ***ALK*** EML4(6)—ALK(20) (870 reads) | ***ALK*** EML4(6)—ALK(20) (870 reads) |
| | Colon 1 | ***KRAS*** p.G12D (26.0%) | ***KRAS*** p.G12D (26.0%) |
| | Colon 2 | ***KRAS*** p.Q61H (11.0%) | ***KRAS*** p.Q61H (11.0%) |
| | GIST 1 | ***c-KIT*** p.Q556_V560del (50.0%) | ***c-KIT*** p.Q556_V560del (50.0%) |
| | GIST 2 | ***c-KIT*** p.K558_V559delinsN (42.0%) | ***c-KIT*** p.K558_V559delinsN (42.0%) |
| | Melanoma 1 | ***BRAF*** p.V600E (44.0%) | ***BRAF*** p.V600E (44.0%) |
| | Melanoma 2 | ***BRAF*** p.V600E (43.0%) | ***BRAF*** p.V600E (43.0%) |
| Center 2 | Lung 1 | ***EGFR*** p.L858R (32.5%) | ***EGFR*** p.L858R (34.0%) |
| | Lung 2 | ***EGFR*** p.G719C (35.5%), p.L861Q (33.0%) | ***EGFR*** p.G719C (35.0%); p.L861Q (34.0%) |
| | Colon 1 | ***KRAS*** p.G12V (41.1%) | ***KRAS*** p.G12V (42.0%); ***NRAS*** p.G13D (1.2%) |
| | Colon 2 | ***BRAF*** p.V600E (26.0%) | ***BRAF*** p.V600E (22.0%) |
| | GIST 1 | WT | WT |
| | GIST 2 | ***c-KIT*** p.A502_Y503dup (39.7%) | WT |
| | Melanoma 1 | ***BRAF*** p.V600E (20.8%) | ***BRAF*** p.V600E (26.0%) |
| | Melanoma 2 | ***GNAQ*** p.Q209R (51.3%) | ***GNAQ*** p.Q209R (50.0%) |

**Table 3** continued

| Center ID | Sample | Internal workflow result | Navify result |
|---|---|---|---|
| Center 3 | Lung 1 | ***KRAS*** p.G12D (9.8%) | ***KRAS*** p.G12D (9.8%) |
| | Lung 2 | ***EGFR*** p.E746_A750del (30.9%) | ***EGFR*** p.E746_A750del (31.0%) |
| | Colon 1 | ***KRAS*** p.G13D (54.5%) | ***KRAS*** p.G13D (54.0%) |
| | Colon 2 | ***BRAF*** p.K601N (42.6%) | ***BRAF*** p.K601N (43.0%) |
| | GIST 1 | ***c-KIT*** p.V559D (21.0%) | ***c-KIT*** p.V559D (21.0%) |
| | GIST 2 | ***c-KIT*** p.V560D (95.6%) | ***c-KIT*** p.V560D (96.0%) |
| | Melanoma 1 | ***BRAF*** p.V600K (89.5%) | ***BRAF*** p.V600K (89.0%) |
| | Melanoma 2 | ***NRAS*** p.Q61K (34.6%) | ***NRAS*** p.Q61K (35.0%) |
| Center 4 | Lung 1 | ***ALK*** EML4(6)—ALK(20) (3003 reads) | WT |
| | Lung 2 | ***EGFR*** p.E746_A750del (58.1%); p.T790M (25.9%); p.C797S (13.1%) | ***EGFR*** p.E746_A750del (51.0%); p.T790M (26.0%); p.C797S (13.0%) |
| | Colon 1 | WT | WT |
| | Colon 2 | ***KRAS*** p.G13D (42.9%) | ***KRAS*** p.G13D (43.0%) |
| | GIST 1 | ***c-KIT*** p.W557R (38.4%) | ***c-KIT*** p.W557R (39.0%) |
| | GIST 2 | ***PDGFRA*** p.N659K (36.2%) | ***PDGFRA*** p.N659K (36.0%) |
| | Melanoma 1 | ***NRAS*** p.Q61R (30.8%) | ***NRAS*** p.Q61R (31.0%) |
| | Melanoma 2 | ***BRAF*** p.V600E (32.5%) | ***BRAF*** p.V600E (34.0%) |
| Center 5 | Lung 1 | ***EGFR*** p.L858R (11.0%) | ***EGFR*** p.L858R (12.0%) |
| | Lung 2 | ***EGFR*** p.L858R (25.0%); p.T790M (28.0%) | ***EGFR*** p.L858R (45.0%); p.T790M (50.0%) |
| | Colon 1 | WT | ***BRAF*** V600E (9.8%)* |
| | Colon 2 | ***KRAS*** p.G13D (27.0%) | ***KRAS*** p.G13D (26.0%) |
| | GIST 1 | ***c-KIT*** p.Q556_W577del (60.0%) | ***c-KIT*** p.Q556_W577del (61.0%) |
| | GIST 2 | ***c-KIT*** p.T574_W577dup (40.0%) | WT |
| | Melanoma 1 | ***NRAS*** p.Q61K (30.0%) | ***NRAS*** p.Q61K (46.0%) |
| | Melanoma 2 | WT | WT |

Bold indciates Gene namesto clearly point on the type of genes inspected by software

*ALK* anaplastic lymphoma kinase, *BRAF* v-Raf murine sarcoma viral oncogene homolog B, *c-KIT* KIT proto-oncogene, *EGFR* epidermal growth factor receptor, *EML4* EMAP like 4, *GIST* gastrointestinal stromal tumors, *GNAQ* G protein subunit alpha Q, *ID* identifier, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *MET* MET proto-oncogene, *NRAS* v-ras neuroblastoma RAS viral oncogene homolog, *PDGFRA* platelet-derived growth factor receptor alpha, *RET* proto-oncogene tyrosine-protein kinase receptor Ret, *WT* wild type

*Other molecular alteratons not included in clinical request by medical oncologist

**Table 4** Timetable for overviewing turnaround time (TAT) from query submission to diagnostic report using Navify® Mutation Profiler

| Center ID | Time taken to prepare and submit case | Time between case upload and provision of the initial results by the software | Time taken to review the results and prepare a final report for the case |
|---|---|---|---|
| Coordinating center | 6 min (5.0 to 7.0) | 12.5 min (10.0 to 15.0) | 12.5 min (10.0 to 15.0) |
| Center 1 | 4 min (3.0 to 5.0) | 13.5 min (12.0 to 15.0) | 13.5 min (12.0 to 15.0) |
| Center 2 | 3.5 min (2.0 to 5.0) | 12.5 min (10 to 15) | 10 min (5.0 to 15.0) |
| Center 3 | 1.5 min (1.0 to 2.0) | 4.5 min (4 to 5) | 2 min (1.0 to 3.0) |
| Center 4 | 15 min (1.0 to 30.0) | 11 min (7 to 15) | 5 min (1.0 to 10.0) |
| Center 5 | 7.5 min (5.0 to 10.0) | 10 min (5.0 to 15.0) | 15 min (10.0 to 20.0) |

**Table 5** Timetable for overviewing turnaround time (TAT) from query submission to diagnostic report using the centers' internal workflows

| Center ID | Time taken to prepare and submit case | Time between case upload and provision of the initial results by the software | Time taken to review the results and prepare a final report for the case |
|---|---|---|---|
| Coordinating center | 1 min (0.5 to 1.5) | 10 min (5.0 to 15.0) | 20 min (15.0 to 25.0) |
| Center 1 | 1 min (0.5 to 1.5) | 12 min (10.0 to 15.0) | 30 min (20.0 to 40.0) |
| Center 2 | 6 min (5.0 to 7.0) | 15 min (13.0 to 17.0) | 15 min (13.0 to 17.0) |
| Center 3 | 1.2 min (1.1 to 2.0) | 1 min (0.6 to 1.5) | 1.5 min (1.0 to 2.0) |
| Center 4 | 1.4 min (1.0 to 1.5) | 6.3 (3.0 to 10.0) | 6.5 min (4.0 to 10.0) |
| Center 5 | 6 min (5.0 to 7.0) | 152 min (140.0 to 164.0) | 30 min (25.0 to 35.0) |

PHIVE investigates the clinical actionability of molecular variants by comparing human disease phenotypes with data from knockout animal models. Other approaches include VarSeq/VSClinical (Golden Helix), ingenuity variant analysis (Qiagen), and the use of Alamut® software (interactive biosoftware) or VarElect software. These bioinformatics tools provide a clinical data repository of pathogenic alterations across solid tumors, hence representing a user-friendly, time-saving, and cost-effective diagnostic tool in clinical practice.

The downside of such heterogeneous landscape of databases is the lack of standardized interpretation workflows. Indeed, disharmonized parameters (in terms of record availability, clinical actionability, and interpretation systems) may often negatively affect the clinical interpretation of molecular records and, thus, the clinical outcomes of patients [28, 29]. A failure to detect or to accurately interpret tumor biomarkers is the reason why too many people are molecularly misdiagnosed, thereby missing the opportunity to benefit from targeted therapies [28]. In this paper, we evaluated the technical and clinical performance of Navify® Mutation Profiler—a tertiary genomic data analysis software that generates an automated tiered interpretation of molecular alterations through VCF files. Notably, built on publicly available evidence tiers, nMP automatically inspects molecular records by consulting multiple databases simultaneously [21]. Moreover, it integrates recommendations and updates on drugs approved by different agencies, including the European Medicines

Agency, SwissMedic, and the National Institute for Health and Care Excellence [21]. Of note, the nMP system also allows "missed variants" to be reinterpreted manually, thereby assigning a tiered clinical interpretation of these variants in the final report.

In this study, we investigated the technical performance of the nMP software in interpreting and reporting NGS data from solid tumor samples. For this purpose, we used standard reference specimens [22] covering four different types of NSCLC actionable alterations (point mutation, deletion, aberrant rearrangement, and exon skipping mutation).

All involved institutions successfully carried out the molecular analysis. Before the interpretation of real-world samples, a reference VCF file was shared with the participating institutions. In this training session, high concordance rates were observed between the nMP software and the institutions' bioinformatics pipelines for DNA-based alterations; however, in one institution (center 5), nMP failed to detect RNA-based alterations in a single case. A possible reason for the discrepancy between center 5 and the other institutions is that center 5 used the first version of the nMP software to analyze the reference VCF file; instead, the other institutions adopted a more recent bioinformatics pipeline (Oncomine_Fusions_only) optimized for the interpretation of RNA-based alterations (Supplementary File 1). In detail, the nMP software efficiently interpreted DNA and RNA molecular alterations, as evidenced by its overall concordance rates with the internal workflows, namely, 91.7% (44 out of 48) and 91.7% (11 out of 12), respectively. Moreover, a high percentage agreement of 85.7% (6 out of 7) was also seen between the nMP software and standard bioinformatics pipelines on the molecular interpretation of a wild-type series according to the referral interpretation approach. Regarding the discordant cases, in a single case, only the nMP system highlighted concomitant clinically relevant alterations in key genes for the clinical administration of patients with solid tumors (*KRAS* p.G12V plus *NRAS* p.G13D, center 2). We speculate that this discrepancy was due to the fact that the internal workflow systems filtered out the p.G13D hotspot mutation because the VAF threshold value was below the clinical value of 5.0% that is generally established for the interpretation of variants in tissue samples (Table 3). Conversely, the nMP system failed to detect two *c-KIT* pathogenetic duplications (c-KIT p.A502_Y503dup and p.T574_W577dup) in patients with GIST. The literature demonstrates that the detection of short modifications of repetitive DNA sequences requires optimized bioinformatics pipelines that are able to recover filtered-out variants from final reports [30]. Of note, the nMP software detected p.V600E *BRAF* mutations in a single instance (colon 1, center 5). By contrast, although the same *BRAF* mutation was identified by the internal workflow, it was not included in the final report out of clinical request (Table 3).

## CONCLUSIONS

Unfortunately, the interpretation of RNA-based alteration data is currently limited by the lack of optimized bioinformatics pipelines that are able to detect aberrant transcripts starting from scant diagnostic input material [31, 32]. Moreover, technical parameters like the variant calling stringency and the identification of unbalanced target regions require optimized data-interpreting pipelines [32]. In our study, although nMP successfully detected up to 91.7% (11 out of 12) of the RNA-based alterations, it did miss a clinically relevant *ALK* aberrant rearrangement. Such a shortcoming underlines the urgency of further optimizing analysis software to improve the detection rates of aberrant transcripts from routine diagnostic samples. Surprisingly, the nMP platform failed to successfully interpret pathogenic insertions that are able to clinically stratify patients with solid-tumors. A possible way to optimize the detection of such complex molecular alterations (such as indels, tumour mutational burden (TMB), MSI status, and HRD score) would be to create multidisciplinary working groups made up of different professionals, including molecular pathologists, clinicians, bioinformatics, and informatic engineers.

Lastly, the time efficiency of the nMP platform was also technically evaluated by calculating the median times needed to submit the query to the platform, to upload the cases, and to elaborate the clinical report. Interestingly, the nMP software was time-saving thanks to its rapid analytical procedures (Tables 4 and 5).

Finally, although this harmonized trial provides encouraging preliminary evidence regarding the analytical efficiency of nMP in identifying clinically relevant molecular alterations in diagnostic routine samples, some limitations should be pointed out. One limitation is inherent to the retrospective nature of the study. Indeed, having examined only a small number of selected cases, we were unable to assess any perspective clinical data from patients with tumors. Another limitation of the study is that the small series of patients yielded only a few preliminary results. Such a drawback drastically influenced the statistical analysis of the technical data, thereby requiring further confirmation in future investigations. Finally, the last limitation is that the wide series of molecular data interpreted by nMP should have undergone a visual inspection to ensure the reliability of the clinical reports. Despite these limitations, these preliminary data demonstrate that the nMP system is a technically valid, time-saving, and user-friendly tool able to consistently interpret NGS molecular data in clinical practice. Hence, we are adamant that future research involving larger cohorts of patients will further validate the adoption of the nMP system as a tertiary data analysis tool for the diagnostic and clinical management of patients with solid tumors.

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***Data Availability.*** All data generated or analyzed during this study are included as supplementary information files.

## *Declarations*

***Conflict of Interest.*** Francesco Pepe has a relevant relationship (advisory fees, honoraria, travel accommodation and expenses, grants, and non-financial support) with Menarini and Roche that is unrelated to the current work. Luisella Righi reports personal fees (for speaker's bureaus or acting as an advisor) from Roche, MSD, Boehringer Ingelheim, Eli Lilly, BMS, AstraZeneca, Amgen, and Bayer that are unrelated to the current work. Giancarlo Troncone reports personal fees (for speaker's bureaus or acting as an advisor) from Roche, MSD, Pfizer, Boehringer Ingelheim, Eli Lilly, BMS, GSK, Menarini, AstraZeneca, Amgen, and Bayer that are unrelated to the current work. Umberto Malapelle has received personal fees (for acting as a consultant and/or speaker's bureaus) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientific, Eli Lilly, Diaceutics, GSK, Merck, AstraZeneca, Janssen, Diatech, Novartis, and Hedera that are unrelated to the

current work. Umberto Malapelle is an editorial board member of *Oncology and Therapy*. Umberto Malapelle was not involved in the selection of peer reviewers for the manuscript nor in any of the subsequent editorial decisions. All other named authors confirm that they have no conflicts of interest to declare.

***Ethical Approval.*** Written informed consent was acquired from all patients involved in the study and documented in accordance with the general authorization for processing personal data for scientific research purposes from the Italian Data Protection Authority (http://www.garanteprivacy.it/web/guest/home/docweb/-/docwebdisplay/export/2485392). All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/).



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