INTRODUCTION

In today’s rapidly evolving field of molecular cytopathology, modern cytopathologists play a key role in bridging the gap between conventional microscopy and novel molecular technologies. The advent of targeted and personalised therapies has completely modified the way advanced cancer patients are managed. As opposed to standard therapeutic regimens (eg, radiochemotherapy), these novel drugs have proven highly effective in dramatically improving the overall clinical outcomes of advanced cancer patients while reducing unwanted severe adverse events and toxicities. Despite these remarkable advantages, the administration of these therapies is strictly dependent on the identification of specific molecular “targets.” Nowadays, several biomarkers have already been approved as predictors of response to targeted treatments while other promising ones are currently under investigation. Because surgical biopsies in advanced cancer patients often require impracticable and invasive procedures, cytological samples frequently represent the only available tissue material for morph-molecular purposes. Besides its predictive role, cytological sampling is also a useful diagnostic approach for superficial or deep-seated nodules. In this setting, nucleic acids extracted from cytological samples may be exploited to refine the malignancy risk of cases classified as “atypical” or as “of undetermined significance.” However, several pre-analytical factors may influence the reliability of NGS clinical analysis. Here, we briefly review the challenges of NGS in cytology practice, focusing on those pre-analytical factors that may negatively affect NGS success rates and routine diagnostic applications. Finally, we address the future directions of the field.

KEYWORDS
biomarkers, cytopathology, FNA, molecular cytopathology, next generation sequencing
technologies are based on three main sequencing approaches: by synthesis, by hybridisation, and by ligation. In spite of these differences, all three approaches employ the same exact four-step workflow: (a) library generation; (b) clonal amplification of the single generated fragments; (c) massive parallel sequencing, and (d) data analysis. Currently, because of its affordable cost and time-saving protocols, many molecular laboratories around the world are implementing NGS platforms in their routine practice. However, despite its popularity, a number of different pre-analytical factors seem to compromise NGS success rates on cytological specimens. For instance, although cytological samples are generally suitable for NGS analysis (Table 1), care must be taken to validate protocols developed for non-formalin-fixed and paraffin-embedded (FFPE) specimens, such as smears and liquid-based cytological (LBC) samples.

In this review, we briefly describe the current role of NGS in cytology particularly by focusing on critical pre-analytical factors and routine diagnostic applications, and address the future directions of the field.

2 | PRE-ANALYTICAL VARIABLES IN CYTOLOGY

One of the most common pre-analytical factors capable of negatively affecting NGS results is the fixative adopted. In particular, smears and LBC samples feature the remarkable advantage of providing better nucleic acid quality than FFPE samples, such as cell blocks (CBs). The problem with CBs though is that fixation in formalin, particularly if prolonged over time, may give rise to C > T sequence artifacts. Among non-formalin fixation modalities, whereas some studies have reported that air-dried and Diff Quik-stained smears yield higher quality nucleic acids than ethanol-fixed and Papanicolaou-stained smears, others have reported divergent evidence. In this setting, despite these differences, fixation and staining modalities are not likely to hamper accurate NGS analysis.

In the case of LBC preparation, as expected, a higher nucleic acid yield and quality has been reported when alcohol-based fixatives, such as CytoLyt (Hologic), have been adopted as opposed to formaldehyde-based ones, including CytoRich Red (Thermo Fisher Scientific).

In addition, cytopathologists try to avoid sacrificing the morphology of non-replicable diagnostic slides for molecular analysis. Thus, a preliminary evaluation of the most relevant factors that may affect NGS success rates of cytological specimens is crucial. In this regard, cytopathologists should review the pathological material on the slides to select the best high-quality smears or representative CB sections. In particular, careful attention should be paid to select slides displaying the highest tumour cell content while simultaneously avoiding, if possible, any contaminant that may interfere with the NGS analysis. This procedure is carried out for each selected slide, with the selection of the tumour-enriched areas. At this point, the cytopathologist should evaluate whether the tumour cell content of the sample can satisfy the analytical sensitivity of the molecular assay employed.

To this end, despite the lack of a universally accepted cut-off for NGS analysis, it has been proposed that the tumour fraction should be more than twice the limit of detection recommended by the assay.

On the other hand, when samples fail because of low cellularity, additional CB sections or smears can be used. However, when choosing among different cytopreparations, cytologists should bear in mind the distinct advantages and disadvantages that each one entails. For example, both direct smears and LBC specimens have an advantage over CB types because they contain whole cells and whole nuclei from which to extract high-quality nucleic acids. However, only direct smears are suitable for rapid on-site evaluation (ROSE), a technique that enables cytopathologists to better manage the aspirated material for morph-molecular purposes. The downside of direct smear preparations is that the number of available slides is very limited. This may lead to the sacrifice of valuable diagnostic morphological material and require a careful time-consuming

| TABLE 1 Different cytological preparations: Pros and cons |
| --- | --- | --- |
| Preparation | Pros | Cons |
| Direct smear | High-quality nucleic acids | Necessity of careful additional validation steps for any given molecular approach |
|  | Possibility to perform ROSE, useful to triage the aspirated material | Unique and unrepeatable |
| CB | Possibility to perform ancillary studies without the need of additional validation | Low quality nucleic acids due to formalin fixation |
|  | Ensure the preservation of diagnostic slides | Impossibility to perform ROSE |
| LBC | Avoid inadequate administration of the aspirated material by untrained physicians | Impossibility to perform ROSE |
|  | Aspirated material can be rapidly collected and preserved in alcohol-based media | Variable yield and quality of nucleic acids depending on the fixative adopted |
| Supernatant | High yield and quality of nucleic acids | Impossibility to perform ROSE |
|  | Ensure the preservation of diagnostic material | Impossibility to perform morphological evaluation |
|  | Enabling molecular analyses even when cytological slides are inadequate or insufficient | |

Abbreviations: CB, cell block; LBC, liquid-based cytology; ROSE, rapid on-site evaluation.
Comparing NGS results with those obtained on matching FFPE fixed smears for NGS analysis was demonstrated by Velizheva et al.15 Instead, recommended the use of CB samples over other types of cytological preparations for advanced-stage NSCLC molecular testing. The success of NGS testing in cytological samples from advanced cancer patients has been widely demonstrated.21 For instance, corroborating evidence has highlighted the possibility of assessing clinically relevant predictive biomarkers in non-small cell lung cancer (NSCLC) patients by applying NGS to different cytological preparations. Indeed, the guidelines of the first edition of the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology recommended the use of CB samples over other types of cytological preparations for advanced-stage NSCLC molecular testing. Instead, the guidelines reported in the subsequent updated version point out the usefulness of smears for molecular purposes.6

In a recent study, we described and validated a newly developed narrow NGS panel, which we called SiRe.8 This panel, which was designed to cover 568 clinically relevant mutations in six different genes, enabled us to analyse a total of 164 (91.1%) out of 180 routine NSCLC cytological samples, showing an 18.3% rate of mutated cases for Epidermal Growth Factor Receptor (EGFR), 28.0% for Kirsten Rat Sarcoma Viral Oncogene homolog (KRAS), 0.6% for Neuroblastoma RAS Viral Oncogene Homolog (NRAS), and for 4.9% V-Raf Murine Sarcoma Viral Oncogene homolog B (BRAF).25 A similar NGS success rate was reported by Zhang et al. Interestingly, in this limited experience, as it was performed on only 16 CBs derived from lung adenocarcinomas (pleural effusions or fine needle aspiration [FNA]), NGS succeeded in analysing almost all samples (93.8%, 15/16).25 In particular, it detected nine EGFR mutations, one Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) mutation, and one KRAS mutation.26 Likewise, the suitability of non-formalin-fixed smears for NGS analysis was demonstrated by Velizheva et al.15 Comparing NGS results with those obtained on matching FFPE histological and CB specimens, Velizheva and colleagues reported an overall success rate of 100.0% for DNA analysis and 92.0% for RNA analysis on stained smears. Interestingly, the smears showed a high sensitivity (100.0% and 100.0%) and specificity (96.0 and 100.0%) for both DNA and RNA analysis, respectively.15 In line with these results, Karnes et al.27 reported that despite the lower DNA input, cytological samples (both Diff-Quik- and Papanicolaou-stained smears) and matching FFPE histological specimens featured similar results in terms of sequencing run parameters and single nucleotide variants detection (overall concordance of 99.5%).

In addition to direct smears and CBs, LBC may also play a crucial role in biomarker assessment for predictive purposes in advanced-stage NSCLC patients. For example, Reynolds et al.28 evaluated the possibility of adopting archived residual cell pellets from LBC preparations as a reliable starting material for NGS analysis. Overall, 20 archived LBC cell pellet samples were retrieved and underwent NGS analysis. Focusing their attention on EGFR mutations, 12 mutated cases were reported. Of note, in all instances the EGFR mutations were further confirmed by real-time polymerase chain reaction.

As we mentioned above, NGS analysis in cytological samples may be a valid tool not only to predict treatment response to targeted treatments in advanced cancer patients but also to refine the risk of malignancy in cases classified as “atypical” or as “of undetermined significance.”29 A few years ago, we reported our experience with an NGS-targeted panel of 50 genes. Remarkably, the NGS panel was successfully applied to 34 (91.8%) out of 37 Diff-Quik-stained smears, including indeterminate cases retrospectively retrieved from our routine thyroid FNA practice. Overall, 22 (64.7%) out of 34 analysed samples harboured BRAF, NRAS, or Rearranged During Transfection (RET) alterations. Interestingly, the NGS approach demonstrated high sensitivity (89.4%), specificity (85.7%), and accuracy (88.4%) rates.29 Consistently, Le Mercier et al used the same 50-gene NGS panel on CBs and stained smears from thyroid FNAs to evaluate the risk of malignancy in patients with morphologically indeterminate diagnosis. As in the previous studies, they reported that NGS successfully identified gene mutations strictly associated with thyroid cancer development (eg, BRAF, NRAS, KRAS, and Phosphatase And Tensin Homolog [PTEN]), thus suggesting that NGS may increase the sensitivity of FNA diagnosis of indeterminate lesions.30 The analytical performance of NGS on FNA samples was further confirmed by Nikiforov et al. Using the ThyroSeq v2.1 panel to analyse a large series of 465 consecutive thyroid FNAs with indeterminate diagnosis, they detected 6.7% mutated cases. Interestingly, NGS was able to adequately assess a high risk of malignancy in almost all (20/22) nodules with indeterminate cytological diagnosis. In particular, NGS showed an overall accuracy of 91.8%, a sensitivity of 90.9%, a specificity of 92.1%, a positive predictive value of 76.9%, and a negative predictive value of 97.2%.31 In a subsequent experience the same group evaluated the analytical performance of a new version of the ThyroSeq panel, the ThyroSeq v3, an NGS DNA- and RNA-based panel able to cover 112 gene alterations and to distinguish malignant from benign lesions. For the evaluation, they applied ThyroSeq v3 to 413 specimens (including 175 thyroid FNA samples with indeterminate diagnosis) and to matching
histological surgical resections. Significantly, the panel showed an overall accuracy of 90.9%, a sensitivity of 98.0%, and a specificity of 81.8% compared with the histological samples.32

Besides lung cancer and thyroid neoplasms, NGS technology may also be applied to other types of cytological samples. For instance, Harris et al33 showed that NGS is a valid ancillary approach for atypical urine cytology samples and a potential screening tool for low-grade urothelial carcinomas, which are commonly missed by both cytology and cystoscopy approaches. Similarly, Carrara et al34 demonstrated that the application of NGS to pancreatic FNAs for the identification of \textit{KRAS} alterations may be a valid complementary diagnostic strategy to traditional morphological and ancillary approaches for the diagnosis of pancreatic ductal adenocarcinomas. Further, Yamamoto et al demonstrated that NGS can be useful in evaluating the malignant potential in salivary gland tumour cytological specimens. Indeed, NGS yielded a sensitivity and a specificity

| Platform | Panel | Sample type | Number of analysed samples | Adequate sample rate | Clinical application | Reference |
|----------|-------|-------------|----------------------------|----------------------|----------------------|-----------|
| Ion S5 System™ (Thermo Fisher Scientifics) | Custom Panel (7 genes) | Direct smear, Cell block | 180 | 91.1% | Therapy | 25 |
| Ion PGM™ (Thermo Fisher Scientifics) | NextDaySeq Lung panel (7 genes) | Cell block | 16 | 93.8% | Therapy | 26 |
| Ion PGM™ (Thermo Fisher Scientifics) | Oncomine DNA panel for Solid Tumors and Fusion Transcripts (26 genes) | Direct smear | 8 | 92.0% (RNA-based) 100.0% (DNA-based) | Therapy | 15 |
| HiSeq 2000 (Illumina) | WU-CaMP27 panel (27 genes) | Direct smear | 5 | 100.0% | Therapy | 27 |
| Ion PGM™ (Thermo Fisher Scientifics) | Ion AmpliSeq Cancer Hotspot Panel (50 genes) | Liquid-based cytology | 49 | 77.5% | Therapy | 28 |
| Ion PGM™ (Thermo Fisher Scientifics) | Ion AmpliSeq Cancer Hotspot Panel (50 genes) | Direct smear | 37 | 91.8% | Refine the risk of malignancy | 29 |
| Ion PGM™ (Thermo Fisher Scientifics) | Ion AmpliSeq Cancer Hotspot Panel (50 genes) | Direct smear, Cell block | 34 | 85.2% | Refine the risk of malignancy | 30 |
| Ion Torrent PGM or Ion Proton (Thermo Fisher Scientifics) | ThyroSeq v2.1 panel (56 genes) | Supernatant | 465 | 100.0% | Refine the risk of malignancy | 31 |
| Ion Proton (Thermo Fisher Scientifics) | ThyroSeq v3 panel (112 genes) | Supernatant | 175 | 100.0% | Refine the risk of malignancy | 32 |
| Next-Seq500 (Illumina) | AmpliSeq Comprehensive Panelv3 (161 genes) | Supernatant | 33 | 97.0% | Diagnosis | 34 |
| iSeq platform (Illumina) | AmpliSeq for Illumina Cancer Hotspot Panel v2 (50 genes) | Direct smear | 32 | 100.0% | Refine the risk of malignancy | 35 |
| Ion S5 System™ (Thermo Fisher Scientifics) | ‘Oncomine BRCA Research Assay (2 genes) | Direct smear | 11 | 100.0% | Therapy | 36 |
| Ion Proton (Thermo Fisher Scientifics) | Ion AmpliSeq Cancer Hotspot Panel v2 (50 genes) | Supernatant, Cell block | 35 | 100.0% | Diagnosis, Refine the risk of malignancy, Therapy | 38 |
| Ion PGM™ (Thermo Fisher Scientifics) | Solid Tumor Focus Assay (69 genes) | Supernatant | 30 | 100.0% | Therapy | 39 |
| Ion Proton (Thermo Fisher Scientifics) | Ion AmpliSeq Cancer Hotspot Panel v2 (50 genes) | Supernatant | 116 | 89.7% | Therapy | 40 |
| NextSeq (Illumina); digital droplet PCR | IAseq Targeted ActionableSolid Tumor Panel (20 genes) | Supernatant | 17 | 100.0% | Therapy | 41 |
| Ion Proton (Thermo Fisher Scientifics) | Ion AmpliSeq Cancer Hotspot Panel v2 (50 genes) | Supernatant | 156 | 83.0% | Refine the risk of malignancy | 42 |
of 71% and 94%, respectively. Notably, only 4/14 malignant cases showed no alterations, whereas only 1/18 benign lesions showed a mutation. Fumagalli et al highlighted the suitability of NGS for breast cancer (BRCA) 1/2 gene analysis on cytological samples derived from neoplastic ascites of ovarian cancer patients. In this study, NGS of cytological samples showed a success rate of 100.0%. Indeed, complete concordance was seen between cytological and histological analyses. In particular, two cases were wild types, and nine cases harboured BRCA 1/2 somatic or germline alterations. The results are summarised in Table 3.

4 | SALVAGING THE Supernatant

Preserving irreplaceable and irrepresentable diagnostic cytological specimens still remains a major issue for molecular analysis. One possible solution to this pitfall is to exploit the nucleic acids from supernatant fluids, primarily nucleic acid residues from FNA needle rinses or those obtained after cell pelleting and centrifugation during cytological specimen preparations. Moreover, the nucleic acids recovered from supernatant fluids could also be harnessed when diagnostic cytology slides are inadequate or insufficient for molecular purposes. In fact, in these cases, despite the evidence of diagnostic tumour cells for morphological purposes, the extremely low tumour cell content in terms of percentage of neoplastic cells may not be suitable for NGS analysis.

Several studies have been conducted to validate the feasibility of exploiting nucleic acids extracted from supernatant fluids for NGS analysis. A couple of years ago Roy-Chowdhury et al performed NGS analysis on DNA extracted from post-centrifuged supernatants from FNA needle rinses collected in an RPMI medium. They were able to analyse 13 (54.2%) out of 24 malignant cases featuring a limited volume (n = 6) or inadequate cytological tissue material (n = 7) for molecular purposes on tissue cytological samples. A similar line of research was followed a year later by Janaki et al. To further validate the idea of using discarded specimen fluids for molecular analysis, they compared the molecular results of NGS from tissue samples with those from supernatants in 30 endobronchial FNAs. Interestingly, they reported a complete concordance rate (100.0%) between the two groups. Hannigan et al confirmed the practicality of applying NGS analysis to supernatant-derived DNA for predictive purposes. Overall, they detected somatic mutations in the vast majority (81.7%) of the analysed samples. Interestingly, half of the patients harboured a druggable mutation. They also obtained concordance rates of 100.0% and 96.0% between the mutation identified in the supernatants and that found in the matching FNAs or core needle biopsies. Likewise, Guibert et al adopted the NGS approach to analyse DNA extracted from FNA supernatants of 12 lung adenocarcinomas. Of note, they obtained a perfect concordance rate between FNA-tissue derived DNA and supernatant-derived DNA obtained from newly diagnosed patients and those with adenocarcinomas who became resistant to tyrosine kinase inhibitors (TKIs). Finally, Ye et al recently adopted the same strategy by applying NGS analysis to DNA extracted from supernatants of FNA of thyroid nodules. Remarkably, they were able to improve the diagnostic accuracy in indeterminate nodules. Indeed, they found that two (6.5%) out of 31 cases with indeterminate cytological diagnosis harboured BRAF exon 15 p.V600E, a somatic mutation highly associated with papillary thyroid carcinoma. Among these two cases only one had a histological follow-up, featuring a papillary thyroid carcinoma diagnosis, further confirming the key role of molecular testing in cases with indeterminate cytological diagnosis. The results are summarised in Table 2.

5 | QUALITY CONTROL IN CYTOLOGY

Although cytological samples provide a higher quality of nucleic acids than histological samples, air-dried or ethanol-fixed cytological smears usually require a careful validation process before being considered suitable for NGS analysis in routine clinical practice. In addition, owing to their unique and unrepeatable nature, smears are generally not suitable for quality control studies that evaluate the consistency and reproducibility of NGS results among different laboratories. Thus, to circumvent loss of specimen, LBC preparations may play a relevant role in generating quality controls that may be distributed across different laboratories. For this reason, the international Molecular Cytopathology Meeting Group, a consortium composed of laboratories with very high expertise in molecular analysis of cytological samples, has designed, developed, and validated artificial genomic reference standards in cytocentrifuge/cytospin format. In brief, in the "first round" of this international ring trial, cell lines were engineered to harbour mutations in EGFR exon 19 p.E746_A750del, KRAS exon 2 p.G12D, NRAS exon 3 p.Q61L, BRAF exon 15 p.V600E, and PIK3CA exon 20 p.H1047R at different dilution points (10%, 5%, and 1% allele frequency, and a wild type control). Slides, containing 2x10⁶ cells, were distributed to each laboratory. Overall, all laboratories adopting NGS were able to detect all the engineered mutations at an allele frequency of 10% and 5% (the minimum adopted for clinical relevance on tissue specimens). Major issues were reported for low allelic frequency (1%). Interestingly, no false positive results were reported in the wild type specimens. In the "second round," slides were created to contain a lower number of cells (2x10⁶); the cell lines were engineered to harbour EGFR exon 19 p.E746_A750del, EGFR exon 20 p.T790M, KRAS exon 2 p.G12D, and BRAF exon 15 p.V600K. As in the first round, the major issue was represented by mutations at low allelic frequency (1%). However, in these difficult cases visual inspection of sequencing data was pivotal to avoid missing gene alterations of clinical interest.
retain cells on the slide surface, and non-frosted slides. These latter do not feature specialised surface or coating to enhance cellular adhesion and may be useful in high cellularity cases. However, dislodging tumour cells from fully frosted slides, either by scraping or by cell-lifting techniques, is particularly challenging. For this reason, fully frosted slides are less frequently used for nucleic acid extraction.24

Regarding the extraction techniques, scraping and cell lifting are the ones most commonly used. Evidence shows that direct scraping of archival slides achieves a higher nucleic acid yield than cell lifting with Pinpoint solution.24 Interestingly, the use of low-hazard, organic, polymer-based mounting medium Pertex (CellPath) provides a higher DNA yield than the xylene-based mounting medium (BioCare Medical LLC) for morphologic analyses. To circumvent this problem, scientists have resorted to using various cytopreparations, with results that are highly suitable for NGS analysis.14,15 Indeed, cytological samples applied to NGS have proven to be a valuable alternative to conventional histological samples both for predicting treatment response and for refining the risk of malignancy. In addition, studies have shown that both CBs and smears are suitable for more complex NGS analyses, such as tumor mutational burden profiling for immune-checkpoint inhibitor administration.45,46 Lastly, in an attempt to salvage the limited quantity of cytological specimens, several studies have recently demonstrated the feasibility of adopting supernatant fluids for NGS analysis. Beyond DNA, cytological samples may be also a valuable source of RNA that can be a suitable starting material for NGS analysis. As with DNA, RNA analysis is significantly influenced by several pre-analytical factors. It has been reported that the adoption of non-cross-linking alcoholic reagents may yield superior results in terms of quality and quantity of extracted RNA with respect to formalin.47 Interestingly, we have recently reported that our custom SiRe fusion NGS panel is a valid and robust tool for the detection of clinically relevant gene fusions and splicing events in advanced NSCLC patients, starting from RNA extracted from cytological (CB) preparations.48 However, in this field of investigation further studies are warranted.

In conclusion, in this review, we briefly highlighted the suitability of cytological samples for NGS analysis in a myriad of cancer types. In particular, NGS analysis may be a valid option for assessing molecular biomarker status for predictive and diagnostic purposes in patients with advanced-stage cancer. In addition, this review has highlighted that supernatant fluids, typically discarded after cytological sample preparation, represent a valid source of high-quality nucleic acids for NGS analysis.

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CONFLICT OF INTEREST
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AUTHOR CONTRIBUTIONS
Pasquale Pisapia, Francesco Pepe, Umberto Malapelle, and Giancarlo Troncone conceived the review. All Authors collected the literature data, wrote the original draft, and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
Data sharing is not applicable for this article as no datasets were generated or analysed during the current study.

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