

NTRK gene fusions in solid tumors: agnostic relevance, prevalence and diagnostic strategies

Antonio Marchetti, Benedetta Ferro, Maria Paola Pasciuto, Claudia Zampacorta, Fiamma Buttitta, Emanuela D'Angelo

Diagnostic Molecular Oncology Section, Center for Advanced Studies and Technology (CAST), University of Chieti-Pescara, Italy

Summary

A number of innovative drugs, developed for precision medicine, have shown impressive activity in neoplastic patients with rare molecular targets, independently from the site and type of tumor. This gave rise to the concept of agnostic treatments in oncology. The detection of such rare targets is a prerequisite for these treatments and is nowadays one of the main challenges in diagnostic molecular pathology. Various algorithms, new diagnostic strategies and pathological workflows have been suggested to help pathologists in the detection of these rare molecular alterations. An emblematic example of biological targets for agnostic treatments is represented by genetic rearrangements affecting members of the Neurotrophic Tyrosine Receptor Kinase (NTRK) gene family. These gene rearrangements have an unusual dual mode of distribution: the first, at high frequency in some very rare neoplasms, and the second with extremely lower frequencies in more common tumors. Even in the context of an agnostic approach, knowledge of site, histotype and prevalence of the tumors carrying these genetic lesions may be helpful to guide the pathologist in the daily effort in search of these molecular alterations. This review examines the prevalence of NTRK gene fusions in different forms of solid tumors, based on the largest studies to date, reports a comprehensive diagnostic algorithm and an innovative pathological workflow for rapid screening.

Key words: Neurotrophic Tyrosine, Receptor Kinase (NTRK), Next Generation Sequencing (NGS), targeted therapy, tumor agnostic treatments

Mutational oncology has deeply changed the management of patients affected by various forms of solid tumors, allowing the birth of a new diagnostic-therapeutic paradigm. In this context, the concept of anti-tumor therapy with agnostic drugs has emerged: it implies a therapeutic choice that involves the use of drugs based on the “driver” mutation that characterizes the neoplasm. The therapeutic indication is, therefore, independent of the site and type of tumor, and is strictly guided by the mutational profile.

The evolution of precision oncology with agnostic treatments has been made possible by the recent development of new technologies such as Next Generation Sequencing (NGS), as well as by advances in genomics that have allowed for gene profiling of the tumor to guide the therapeutic approach. As part of this new prototype in oncology, the morphological aspects of the tumor are integrated and enriched with the information obtained by genomic profiling. Mutational data, therefore, is integrated with histopathological and immunophenotypical features, for a comprehensive characterization of the neoplasm.

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Correspondence

Antonio Marchetti
Laboratorio di Diagnostica Molecolare
Oncologica, Center for Advanced Studies and
Technology (CAST), Università di Chieti-
Pescara, via dei Vestini, 66100 Chieti
E-mail: amarchetti@unich.it

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The process that led to this new model of management in oncology began with the so-called “histological model”, according to which the site of the tumor and its histopathological characterization guided the therapeutic strategy. Today, the new “mutational model”, thanks to the evolution of targeted therapies, is based on genomic profiling as the fulcrum of the therapeutic plan to complement information obtained from histopathological analysis ¹. In this way, genomics, and in particular the agnostic approach, has made it possible to develop a unified model based on the patient and genotypic features of the tumor to obtain increasingly personalized and effective therapeutic plans.

The concept of agnosticism in oncology is fully appreciated in cancer types with higher prevalence in which rare mutations, that function as target of agnostic drugs, are present. If such rare genomic targets are not routinely analyzed, these neoplasms remain inaccessible to effective therapeutic options. Therefore, a new diagnostic-therapeutic paradigm is needed, which opens up the possibility of targeted therapies for these tumors. Patients affected by these neoplasms should be tested with innovative approaches in order to rapidly screen for rare mutations in clinical

practice ². Methodological approaches and diagnostic algorithms will be discussed in next sections.

In the field of mutational oncology and agnostic therapy, an emblematic example is represented by the fusions affecting members of the Neurotrophic Tyrosine Receptor Kinase (NTRK) gene family, as recently reviewed ³⁻⁶. Innovative drugs, developed for precision oncology and now available in clinical practice, are showing impressive activity in patients carrying NTRK gene fusions in their tumors.

These gene rearrangements have an unusual dual mode of distribution: the first, at high frequency in some very rare neoplasms, and the second with extremely lower frequencies in more common tumors. Rare cancers which frequently have mutations in NTRK genes include infantile fibrosarcoma, congenital mesonephroma, secretory mammary carcinoma and the analogous neoplasm in salivary glands (Fig. 1).

The concept of agnosticism, however, is mainly applied for common cancer forms in which mutation of the NTRK genes is less frequent. Large-scale retrospective analyses were used in a genomic screening program at the Memorial Sloan Kettering Cancer Center (NY, USA)

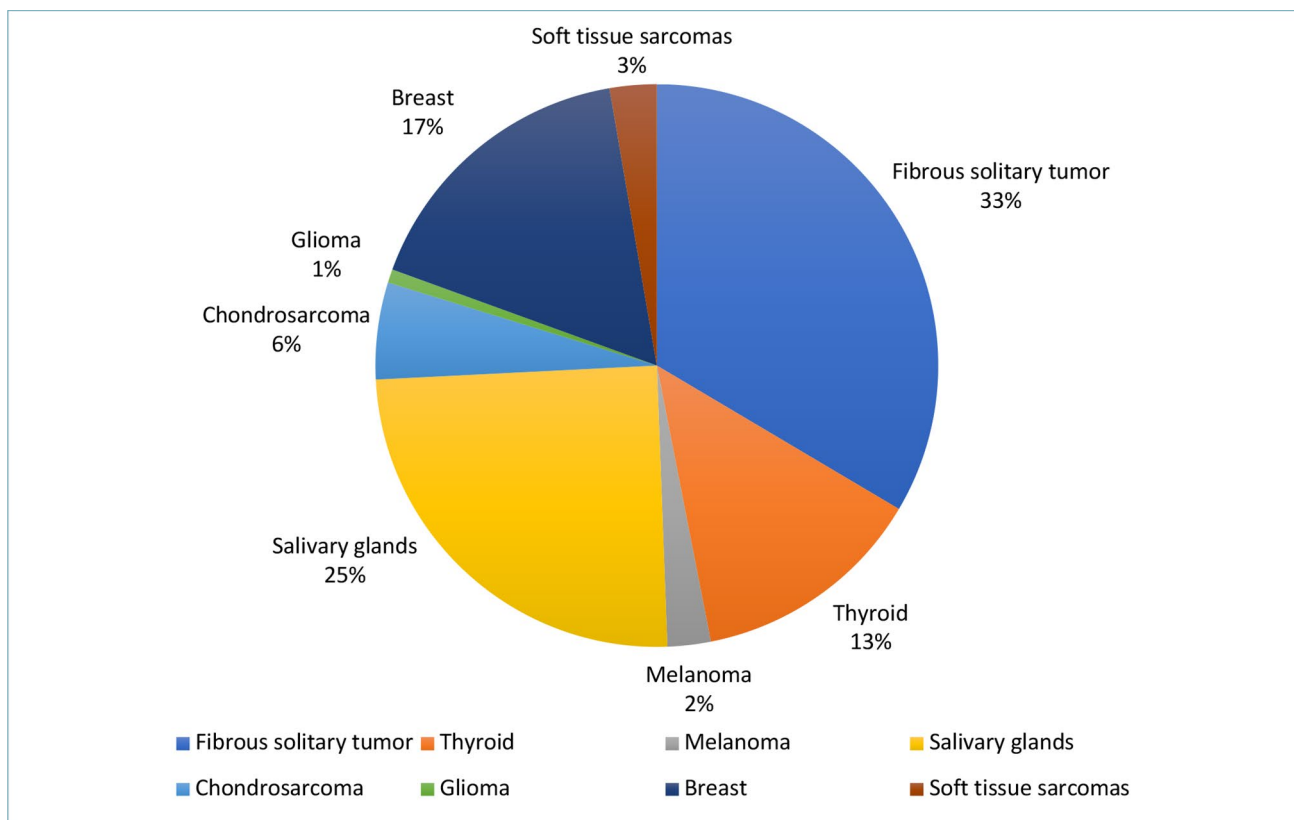


Figure 1. Frequency of NTRK gene fusions in rare tumors (data obtained from Westphalen et al.).

with the aim of understanding the prevalence, distribution, and genomic context of NTRK fusions in different tumor forms. Of note are the two studies conducted by Solomon et al. in 2019 and by Rosen et al. in 2020^{3,4}. The study by Solomon et al. involved 33,997 patients for a total of 38,095 samples and 87 cases (0.25%) with NTRK gene fusions were found. In common cancers, the prevalence of NTRK was 5.08% for salivary gland neoplasms and 0.13% for invasive breast cancer. Moreover, it has been observed that fusions in NTRK genes are mutually exclusive with respect to the most common driver mutations, such as KRAS, BRAF, NRAS, and EGFR³. Rosen et al, in a cohort of 26,000 cases from the same reference cohort as Solomon et al, found 76 NTRK fusions for an overall prevalence of NTRK gene rear-

rangements of 0.28%. Furthermore, NTRK-mutated tumors have been observed to show a tumor mutational burden (TMB) that is generally low, except for colorectal carcinomas with microsatellite instability (MSI-H)⁴. Both studies showed that tumor heterogeneity, a characteristic feature of agnostic therapy, represents a critical element in clinical trials since the cohorts under study may not be representative of the reference population (referral bias). Similar results were also obtained in a study by Gatalica et al. in 2019 on 11,502 tissue samples evaluated by NGS using a panel of 592 genes to detect 53 gene fusions: 31 cases were positive for NTRK fusion (0.27% of the entire cohort). According to the results of other studies, the most common fusions detected were ETV6/NTRK3 and TPM3/NTRK1⁵. In 2021, Westphalen et al. expanded the cohort under

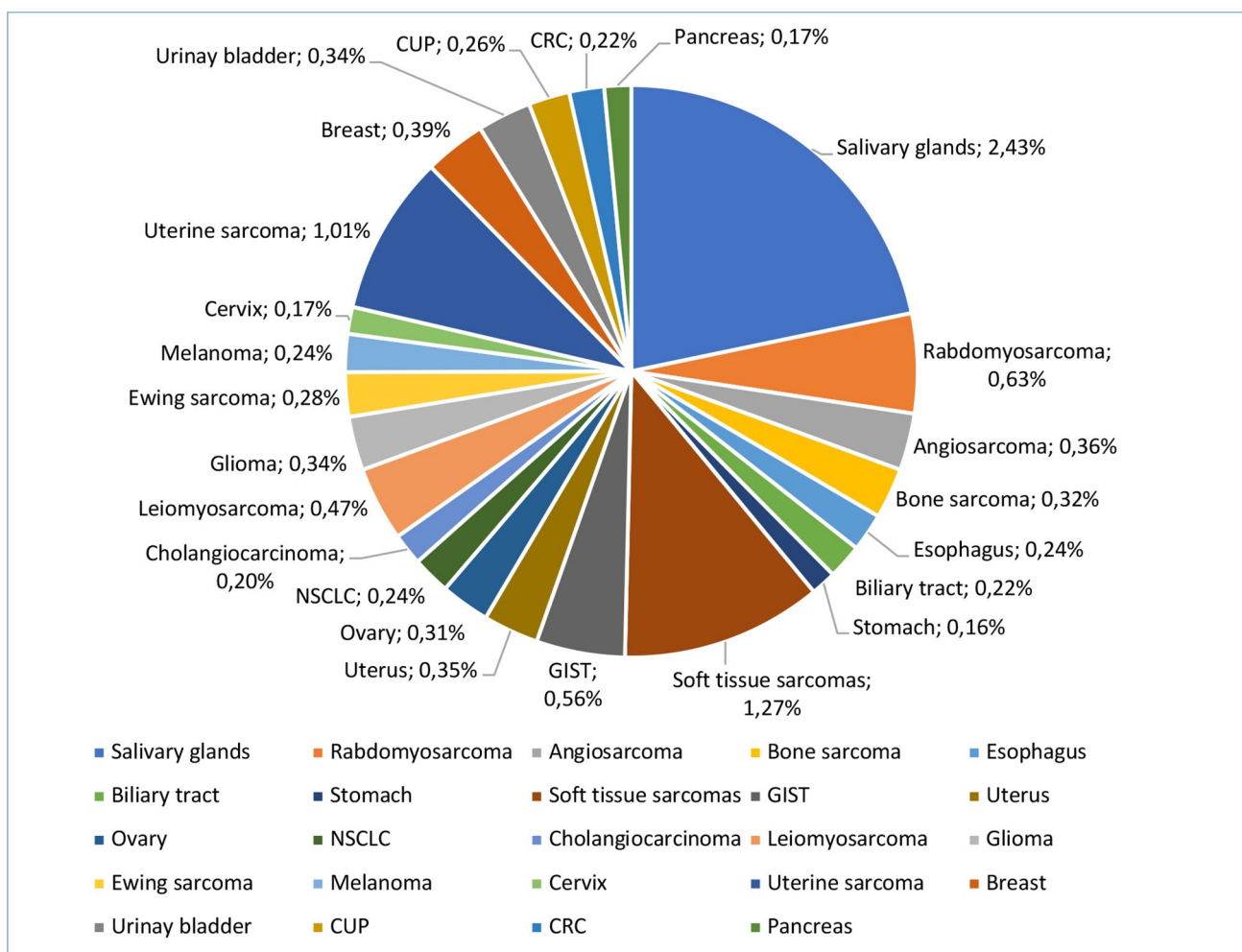


Figure 2. Prevalence of NTRK gene fusions in tumors in the adult population (data obtained from Westphalen et al, Rosen et al, Solomon et al.) CRC, colorectal cancer; CUP, cancer of unknown primary; GIST, gastrointestinal stromal tumor; NSCLC, non-small cell lung cancer.

study, in order to limit selection bias and therefore obtain results that are as meaningful as possible in clinical practice⁶. This was the largest study on NTRK1, NTRK2, and NTRK3 rearrangements in solid tumors. In particular, Westphalen et al. collected data on 295,676 adult and pediatric patients with different neoplasms using a database from the FoundationCORE[®] (Foundation Medicine Inc, Cambridge, MA, USA). The study evaluated the prevalence of NTRK gene rearrangements, the coexistence of alterations in other oncogenic drivers and the association with different fusion partners in various tumor types and histologies: 889 (0.3%) cases were positive for NTRK fusions in 45 different tumor forms for a total of 134 different histological subtypes. In the adult population (> 18 years old), the prevalence of fusion-positive tumors was 0.28%; in the pediatric cohort 1.34% had gene fusions with a peak incidence of 2.28% in children under 5 years of age. In adults, the cancers with the highest prevalence of NTRK fusions were salivary gland cancers (2.43%), soft tissue sarco-

mas (1.27%), and thyroid tumors (1.25%) (Fig. 2). Furthermore, all neoplasms that were positive for NTRK fusions were categorized by tumor type and frequency of NTRK fusions. NSCLC was the most common type of adult cancer with NTRK fusions (136 cases of which 95 adenocarcinomas), followed by breast cancer (117 cases of which 71 carcinomas not otherwise specified and 42 invasive ductal carcinomas), soft tissue sarcomas (79 cases including 37 sarcomas not otherwise specified and 13 liposarcomas), and colorectal carcinoma (77 cases including 73 colon adenocarcinomas) (Fig. 3)⁶.

In particular, 88 possible rearrangements with different fusion partners were identified, of which 58 (65.9%) had never been described previously. Among these, ETV6-NTRK3 was the most common gene fusion in both adults (78/295 cases, 26.4%) and children (17/52 cases 32.7%) for a total of 95 cases of 349 (n = 27, 2%), followed by TPM3-NTRK1 (21.5%) and LMNA-NTRK1 (9.5%) (Fig. 4)⁶.

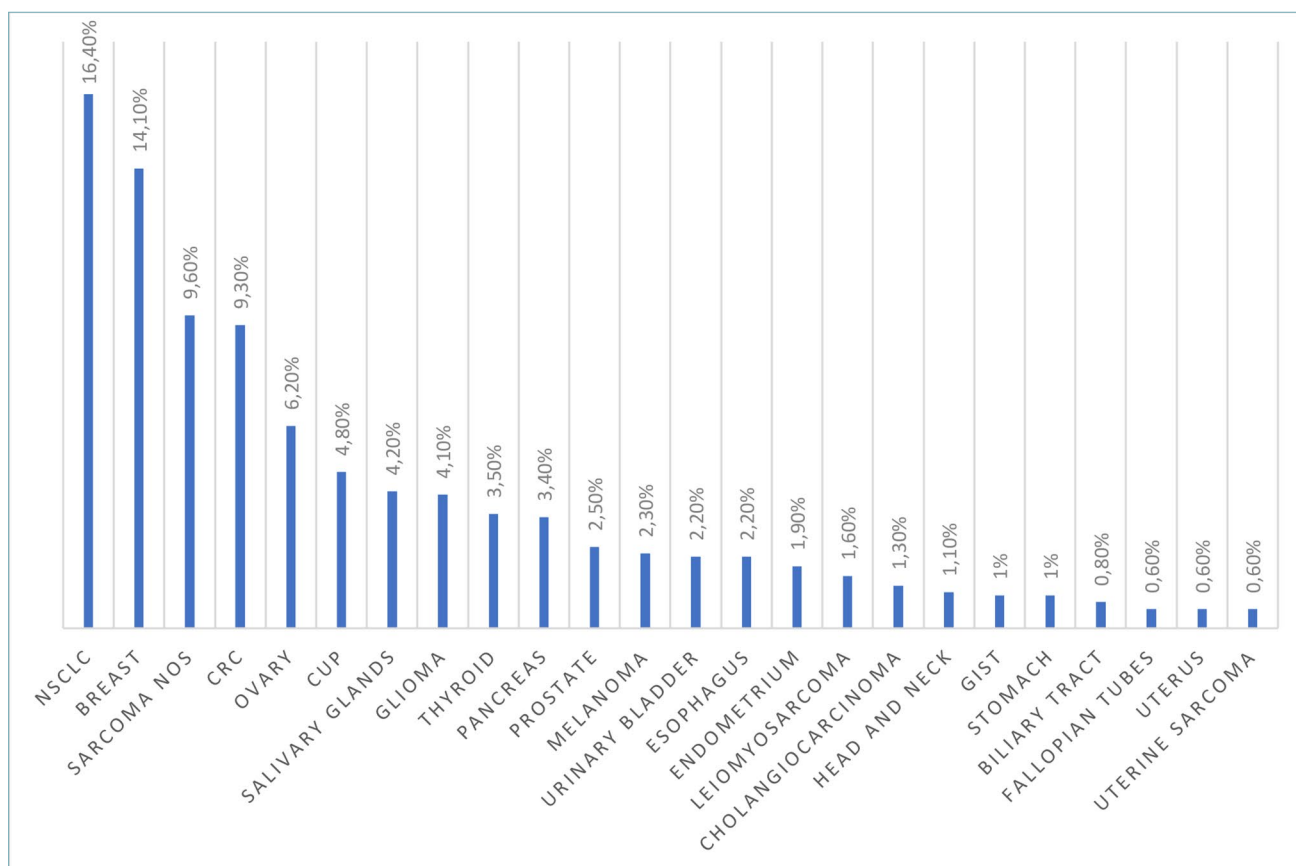


Figure 3. Prevalence of the different tumor types that were positive for NTRK gene fusions in the adult population (data obtained from Westphalen et al.). CRC, colorectal cancer; CUP, cancer of unknown primary; GIST, gastrointestinal stromal tumor; NSCLC, non-small cell lung cancer.

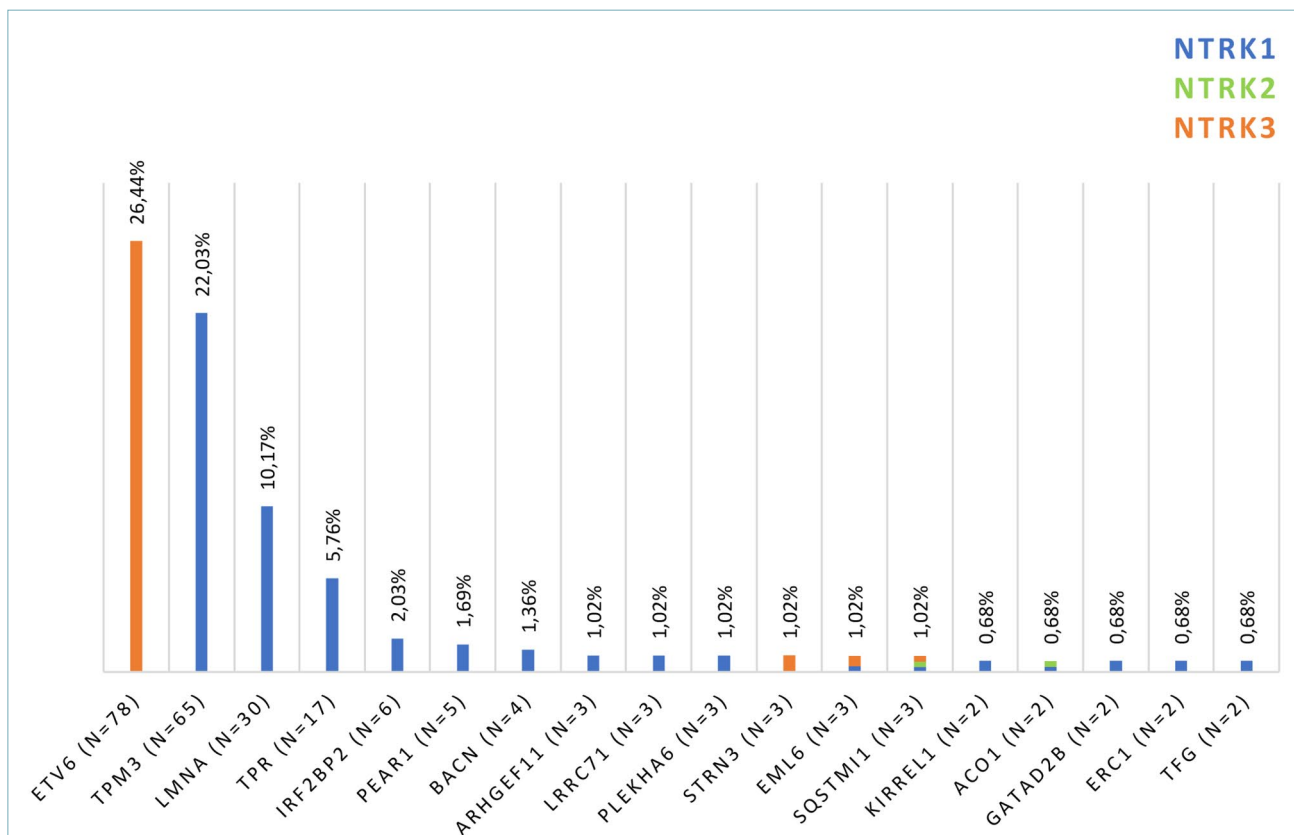


Figure 4. Major fusion partners of NTRK genes in the adult population by frequency (modified from Westphalen et al.).

NTRK mutations rarely coexisted with mutations such as KRAS, APC, TP53, and PIK3CA and appeared to be mutually exclusive with the most common tumor drivers (EGFR, ERBB2, RET, ALK, MET), especially in breast, colon and lung tumors. Another noteworthy aspect was the relationship with the tumor mutational burden which was similar in tumors with and without NTRK fusions, especially in NSCLC; on the contrary,

this feature was increased in colorectal carcinomas with mutated NTRK ⁶.

In addition, Westphalen et al. compared their results with those obtained in phase I and II clinical trials (ALKA-372 -001, STARTRK-1, STARTRK-2) to validate safety and efficacy of entrectinib in patients with advanced solid tumors or metastatic cancers positive for a NTRK fusion ⁷⁻¹⁰. The aim of this analysis was to con-

Table I. Prevalence of NTRK gene fusions in the most common solid tumors, as reported in different studies. NOS, not otherwise specified.

Histotype	Westphalen et al. (2021) (n = 290.431)		Rosen et al. (2020) (n = 26.312)		Solomon et al. (2019) (n = 33.997)		Gatalica et al. (2019) (n = 11.502)	
	n	%	n	%	n	%	n	%
Salivary gland carcinoma	35/1440	2.43%	12/227	5.29%	13/256	5.08%	/	/
Sarcoma NOS	79/6216	1.27%	9/770	1.17%	13/1915	0.68%	1/478	0.2%
Thyroid carcinoma	29/2314	1.25%	10/451	2.22%	13/571	2.28%	4/70	6%
Uterine sarcoma	5/5494	1.01%	2/174	1.15%	/	/	1/478	0.2%
Breast carcinoma	117/30075	0.39%	3/3775	0.08%	6/4458	0.13%	1/769	0.1%
Melanoma	19/8028	0.24%	5/932	0.54%	4/1125	0.36%	/	/
Lung adenocarcinoma	136/56440	0.24%	6/3658	0.16%	9/3993	0.23%	4/4073	0.1%
Biliary tract cancer	7/3150	0.22%	2/553	0.36%	2/787	0.25%	/	/
Colorectal carcinoma	77/34590	0.22%	8/2306	0.35%	9/2929	0.31%	2/1272	0.2%
Pancreatic adenocarcinoma	28/16769	0.17%	4/1315	0.30%	5/1492	0.34%	/	/

firm the validity of the results obtained in the general population. Adult patients from the FoundationCORE database were compared with the 11 NTRK fusion positive patient groups in the three clinical trials highlighting that the frequency of patients with sarcoma, NSCLC, pancreatic carcinoma, cholangiocarcinoma, and endometrial carcinoma was similar in the two cohorts. Conversely, the frequency of secretory breast cancer was higher in the cohort of patients participating in clinical trials, which is most likely due to screening bias. A different trend was demonstrated in breast, colorectal, and ovarian cancers. The distribution by sex and age was similar in the various studies¹⁰. Table I reports the frequency of NTRK gene fusions in the most common solid tumors, detected in different studies. A detailed description of the prevalence of NTRK gene fusion in some of the major forms of solid tumors is reported in Table I.

Thyroid carcinoma

Thyroid carcinoma is one of the highly prevalent cancers in which NTRK gene fusions are found. In particular, these rearrangements are present in different histotypes such as papillary carcinoma (PTC), Hürtle cell carcinoma (HCC), poorly differentiated carcinoma (PDTC), and anaplastic carcinoma (ATC). A peculiar feature is the different frequency found in adult patients (2.3-3.4%) compared to pediatric cases, where fusions are eightfold more common (18.3-25.9%). The genes most affected by mutations are NTRK1 and NTRK3, and fusions involving NTRK3 are fivefold more frequent than those involving NTRK1. In particular, in the Cancer Genome Atlas database, ETV6-NTRK3 rearrangements are the most frequent, especially in papillary carcinomas related to radiation exposure¹¹.

These mutations are a significant event in thyroid cancer and have received growing interest given the possible use of targeted drugs. Pekova et al. analyzed a cohort of 989 patients with thyroid cancer of which 59 (6%) were positive for NTRK fusion¹². In these cases, differences emerged between tumors with NTRK1 fusion or NTRK3 fusion: the first ones had a mixed growth pattern (both papillary and follicular) compared to the latter ones that had a predominantly follicular pattern of growth; moreover, NTRK1 mutations were associated with a high frequency of multifocality, extra-thyroid extension, vascular invasion, distant and lymph node metastases (80% vs 49% for NTRK3)¹². In summary, tumors with NTRK1 mutations appear to be different from those with NTRK3 mutations due to greater tumor aggressiveness. Thus, it is possible to consider NTRK1 gene fusions as a prognostic indica-

Table II. Types of NTRK gene fusions most frequently reported in thyroid tumors (modified from Pekova et al.)

NTRK fusion	Frequency (%)
ETV6-NTRK3	64.4 %
TPM3-NTRK1	8.4 %
SQSTM1-NTRK3	6.8 %
EML4-NTRK3	6.8 %
RBPMS-NTRK3	5.1 %
IRF2BP2-NTRK1	5.1 %
SQSTM1-NTRK1	1.7 %
TPR-NTRK1	1.7 %

tor which, together with the size of tumor, presence of metastases and late mutational events, is relevant for the assessment of outcomes. Therefore, the analysis of NTRK supports diagnosis and choice of surgical and pharmacological intervention. Table II lists the types of fusions most frequently reported in thyroid tumors.

Colorectal carcinoma

Another common tumor involving NTRK fusions is colorectal cancer (CRC): TPM3-NTRK1 was the first fusion found more than 35 years ago and a low prevalence of NTRK1 or NTRK3 rearrangements was subsequently reported¹³. Recent larger studies on metastatic CRC have identified several driver mutations, including those involving NTRK. In a randomized cohort study, Lasota et al. investigated NTRK fusions using IHC and molecular methods: of 7008 patients, 16 cases of CRC (0.23%) were positive for NTRK fusions¹⁴. Most of the tumors with a mutation in NTRK were in women (13 of 16, 81%) with a mean age of 63 years and at late stage (T3-T4). Tumors with NTRK mutations involved different portions of the large bowel. In addition, the mutation frequently involved tumors that had low or moderate differentiation, focal or extensive solid growth, presence of lymphovascular invasion and numerous tumor-infiltrating lymphocytes. Finally, a focal mucinous component was found in 8 cases¹⁴. From a molecular standpoint, 81% of NTRK fusion positive cases were also characterized by mutation of genes involved in mismatch repair (MMR), in particular MLH1 and PMS2. The most commonly encountered rearrangement was TPM3-NTRK1 (60%), followed by LMNA-NTRK1 (20%), and TPR-NTRK1 (13%). Other fusion partners, such as PLEKHAG and SCYL3 were not seen in this study but were observed in previous reports¹⁴. NTRK3 fusions are very rare in CRC, with only a few cases involving ETV6 (the most common), COX5A, EML4, and VPS18 genes. The types of fusion

Table III. Types of NTRK gene fusions most frequently reported in colorectal cancer (modified from Lasota et al.).

NTRK fusion	Number of cases (%)
LMNA-NTRK1	6 (14%)
PLEKHAG-NTRK1	1 (2.3%)
SCYL3-NTRK1	1 (2.3%)
TPM3-NTRK1	22 (51.2%)
TPR-NTRK1	3 (7%)
COX5A-NTRK3	1 (2.3%)
ELM4-NTRK3	2 (4.6%)
ETV6-NTRK3	6 (14%)
VPS18-NTRK3	1 (2.3%)
Total	43 (100%)

partners most frequently reported in CRC are listed in Table III.

From these analyses, it also emerged that most CRCs with NTRK gene fusion have features similar to tumors with microsatellite instability: female predominance, higher frequency in the right colon, presence of mucinous differentiation and high level of tumor-infiltrating lymphocytes. The co-presence of Wnt/ β -catenin and p53 mutations should also be underlined. Another important finding is the absence of mutations in BRAF, KRAS, NRAS, and PIK3CA, and for this reason it is recommended to carry out IHC and molecular investigations for NTRK in all patients with advanced CRC or wild-type metastatic for BRAF and RAS.

The association of NTRK fusions with microsatellite instability has emerged in several other studies on CRC. In a series of CRC patients investigated by Deihimi et al, of which 26 cases were with MSI-High and 558 non-MSI-High, NTRK rearrangement was detected in 40% in CRCs with MSH2/MLH1 mutations compared to 16% of cases with non-MSI-High CRC¹⁵.

Similarly, Yamashiro et al. found fusions of the NTRK1 gene in three CRC cases with microsatellite instability of 971 cases examined (0.31%): the study was per-

formed using an IHC screening followed by further analysis of positive cases with RNA sequencing¹⁶.

In conclusion, some cases of CRC, and especially those characterized by microsatellite instability, have fusions of the NTRK1 and NTRK3 genes with different partners, some of which are responsive to target therapy. For this reason, it is advisable to use IHC as screening investigation followed by NGS in order to identify cases that may benefit from target therapy¹⁷.

NSCLC

Fusions of NTRK genes in non-small cell lung cancer (NSCLC) were first detected in 2013 by Vaishnavi et al. by NGS analysis of 36 samples from patients with unknown genetic alterations, thus making possible to identify two mutations involving NTRK1 with two different fusion partners: MPRIP and CD74¹⁸. Later, other studies involving NSCLC were carried out revealing a frequency of mutation of NTRK genes less than 1% (Tab. IV)¹⁹.

One of the first large studies on NTRK mutations in NSCLC was carried out by Farago et al. in 2018 on 4872 NSCLC patients evaluated for NTRK fusion with an NGS panel²⁰. A mutation frequency of 0.23% was reported. NTRK rearrangements were thus less frequent than fusions affecting the ALK1 (4-6%), ROS1 (1-2%), and RET (1-2%) genes. Compared to the latter, however, NTRK fusion seems to be present in different tumor histologies and to be independent of tobacco smoking. Furthermore, NTRK fusions appear to have a greater role than driver mutations in ALK1, ROS1, and RET, also considering that inhibition of their signal with target therapies in preclinical models leads to cell death and tumor regression²⁰. Although involving a limited number of patients with NSCLC and NTRK fusion, this study provided the possibility to build an initial database on clinical-pathological aspects related to this

Table IV. Frequency and type of NTRK gene fusions in NSCLC in various studies (Russo et al.). ADC, adenocarcinoma, NSCLC, non-small cell lung cancer.

Study	Population (n)	Frequency (%)	NTRK	Fusion partner
Farago, 2018	NSCLC (4872)	0,23%	NTRK1 NTRK3	SQSTM1, TPR, IRF2BP2, TM3, MPRIP, ETV6
Vaishnavi, 2013	ADC (91)	3,3%	NTRK1	MPRIP, CD74
Stransky, 2014	ADC (513)	0,19%	NTRK2	TRIM24
Miyamoto, 2019	NSCLC (non-squamous) (4874)	0,05%	NTRK3	NR
Gatalica, 2018	ADC (4073)	0,1%	NTRK1 NTRK3	TPM3, SQSTM1, ETV6
Ou, 2019	NSCLC (42791)	0,1%	NTRK1 NTRK3	IRF2BP2, TPM3
Xia, 2019	NSCLC (21155)	0,056%	NTRK1	CD74, IRF2BP2, LMNA, PHF20, SQSTM1, TPM3, TRP

Table V. Types of NTRK gene fusions in patients with NSCLC according to the histotype and smoking history (modified from Da Farago et al.). ADC, adenocarcinoma; NE, neuroendocrine carcinoma.

Case	NTRK fusion	Histotype	Smoking history
1	NTRK1-SQSTM1	ADC	30 pack-years
2	NTRK1-TPR	ADC/NE	/
3	NTRK1-IRF2BP2	ADC	/
4	NTRK1-TPM3	ADC	2 pack-years
5	NTRK1-MPRIP	ADC	/
6	NTRK3-ETV6	ADC	/
7	NTRK1-IRF2BP2	ADC	30 pack-years
8	NTRK3-ETV6	SCC	58 pack-years
9	NTRK1-SQSTM1	ADC	/
10	NTRK3-ETV6	ADC	/
11	NTRK3-SQSTM1	NE	1 pack-years

Table VI. Types of NTRK gene fusions in patients with NSCLC according to the gender and the different histologic variants of adenocarcinoma (ADC) (modified from Ruiying et al.). M, male; F, female; ADC, adenocarcinoma.

Case	Sex	Age	NTRK fusion	Histotype
1	M	53	NTRK1-C14orf2	ADC
2	M	41	NTRK1-RRNAD1	ADC (papillary)
3	F	64	NTRK1-NBPF25P	ADC (acinar)
4	M	54	NTRK1-ARHGEF11	ADC
5	F	56	NTRK1-FMN2	ADC
6	F	31	NTRK1-TPM3	ADC in situ
7	M	51	NTRK1-TPM3	ADC (papillary)
8	F	39	/	ADC (acinar)
9	M	36	/	ADC minimally invasive

gene alteration. In fact, seven fusions of NTRK1 with five different fusion partners and four fusions of NTRK3 with two different fusion partners were found; 55% of patients were male with a median age at diagnosis of approximately 47 years and a variable history of smoking; 73% of patients had metastatic cancer at diagnosis. NTRK fusions were found to be mutually exclusive with mutations in KRAS, EGFR, ALK1, ROS1, or other known oncogenic drivers. Regarding histotypes, nine patients had an adenocarcinoma and among these two invasive mucinous adenocarcinomas and an adenocarcinoma with neuroendocrine features (the latter with fusion of TPR-NTRK1). In addition, several histological subtypes were found among patients with adenocarcinoma, including poorly differentiated solid pattern forms and signet ring cells. One patient with a mutated NTRK (ETV6-NTRK3 fusion) had squamous carcinoma confirmed by p40 expression and negativity for TTF1. In addition, a well-differentiated large cell neuroendocrine carcinoma with high mitotic index and brain metastasis harbored a NTRK fusion (SQSTM1-NTRK3) (Tab. V) ²⁰. A study in 2021 by Ruiying et al. collected data from 4619 lung adenocarcinoma samples from Chinese patients who underwent lung biopsy or resection at

Shanghai Cest Hospital from 2017 to 2019 (2651 surgical samples and 1968 small biopsies) ²¹. All samples were initially studied with NGS for NTRK1 rearrangements; cases positive for NTRK1 and those negative for the most common driver mutations were subsequently analyzed for alterations in NTRK1/2/3 by TNA-NGS (Total Nucleic Acid-NGS) and IHC ²¹. NTRK1 fusions were detected in seven patients (0.15%). Of these, two had TPM3 as fusion partners, while five had a mutation with uncommon fusion partners (Tab. VI). The two canonical mutations were mutually exclusive with respect to the other potential driver mutations; in contrast, three of the five uncommon partner fusions were detected together with EGFR or KRAS mutations. Of note is the appearance of a non-canonical NTRK mutation in a patient who underwent previous therapy with an EGFR-TKI, which was likely induced by therapy ²¹. Another relevant feature was the finding of two NTRK fusions (TPM3-NTRK1 and KIF5B-NTRK2) in a case of adenocarcinoma in situ and in an early stage adenocarcinoma, respectively). This is a novel finding compared to previous studies that identified NTRK mutations only in advanced tumor stages or in poorly differentiated forms. It is generally recognized that driver gene fusions occur

more frequently in young individuals. The comparison with ALK1 and ROS1 mutations showed that the mean age of patients with NTRK fusions in this study was significantly lower (39 years versus 54 for ALK and 56 for ROS1) ²¹.

The coexistence of aberrations in NTRK and other driver mutations remains controversial. The study by Ruiying et al. suggests mutual exclusivity. In contrast, Xia et. al suggested that the NTRK1 mutation may emerge as a mechanism of resistance in patients with an EGFR mutation treated with a TKI ²².

Sarcomas

Sarcomas are neoplasms of mesenchymal origin representing 1% of adult cancers and up to 20% of pediatric cancers. These neoplasms frequently originate from soft tissue (80%) or bone tissue (20%) and include about 70 subtypes, each with well-defined biological and clinical characteristics ²³: The subtypes of soft tissue sarcomas most frequently encountered are liposarcoma, leiomyosarcoma, undifferentiated soft tissue sarcoma, fibrosarcoma, and synovial sarcoma, while the most frequent bone sarcomas are Ewing's sarcoma and osteosarcoma ²⁴.

Sarcomas with NTRK gene rearrangements arise more frequently in superficial or deep soft tissues of extremities, with a greater incidence in pediatric and pubertal ages and a higher prevalence of alterations in the NTRK1 gene. The 2020 classification of the World Health Organization (WHO) of soft tissue tumors includes in the so-called "spindle cell neoplasms with NTRK gene rearrangements" two sarcomatous entities that can raise suspicion for the presence of a NTRK fusion, according to their morphology and immunophenotypic expression:

- neural tumor with lipofibromatosis-like aspects (LLNT), characterized by monomorphic, spindle cell elements, infiltrating subcutaneous adipose tissue with immunoreactivity for S100 and CD34;
- malignant tumor-like neoplasms of the peripheral nerve sheaths, consisting of spindle cells in a context of stromal and perivascular hyalinization ^{25,26}.

NTRK gene rearrangements are present in less than 1% of sarcomas in both pediatric and adult patients. ETV6-NTRK3 fusion, one of the earliest discovered and best characterized alterations, is present in 90% of childhood fibrosarcomas and appears to be recurrent in cellular mesoblastic nephroma. It is also found in a subgroup of GIST of the small intestine and rectum that occur in adulthood and which are negative for classic driver mutations ²⁷.

Another fusion frequently found in sarcomas, espe-

cially in spindle cell variants, is LMNA-NTRK1 alteration, which appears to arise with greater prevalence in pediatric age and in young adults with peripherally localized mesenchymal neoplasms. The same genomic alteration has been reported in low-grade sarcomas with myopericytoma-like features, uterine spindle cell sarcomas, generalized eruptive histiocytosis, and in neoplasms of epithelial origin such as colon adenocarcinoma ²⁷.

Considering the prevalence of NTRK gene fusions, especially in children, Zhao et al. analyzed 1347 tumors from 1217 pediatric patients and identified alterations of NTRK genes in 29 tumors from 27 patients. The frequency of fusions involving the NTRK3 gene was higher in soft tissue sarcomas (Tab. VII): some of these had the typical morphology of infantile fibrosarcoma, densely cellular with a fasciculated and infiltrating growth pattern, while others presented histological and immunophenotypic features of myofibroblastic sarcoma ²⁸.

Overall, the study by Zhao et al. is qualified as one of the major analyses in the field of pediatric neoplasms with NTRK gene rearrangements, highlighting that these fusions are present in 2.22% of all tumors and 3.08% of solid neoplasms. These data support the idea that genomic alterations in NTRK genes are more frequent in pediatric patients than in adults and stress the clinical utility of screening for NTRK gene rearrangements in all pediatric malignancies ²⁸.

These observations result in the importance of providing detailed histological and molecular characteriza-

Table VII. Prevalence and type of NTRK gene fusions in pediatric cancers (data from Zhao et al.).

Histological diagnosis	Prevalence of fusions in NTRK genes	Type of fusion
Papillary thyroid cancer (PTC)	10/76 cases (13%)	ETV6-NTRK3 IRF2BP2-NTRK1 SQSTM1-NTRK1 TPR-NTRK1
Tumors of the central nervous system (CNS)	7/364 cases (1.9%)	KANK1-NTRK2 C2orf44-NTRK2 QKI-NTRK2 KCTD16-NTRK2 TRIM24-NTRK2 SPECC1L-NTRK2 ETV6-NTRK3
Solid tumors, non-CNS, non-PTC, including sarcomas of soft tissues	8/435 cases (1.8%)	TFG-NTRK3 RBPMS-NTRK3 ETV6-NTRK3 SPECC1L-NTRK3 STRN3-NTRK3 PRDX1-NTRK1
Hematological tumors	2/472 cases (0.4%)	RBPMS-NTRK3 TPM3-NTRK1

tion of these neoplasms in order to assist the clinician in the choice of target therapy, especially in cases of severe post-surgery morbidity or impossibility to excise the neoplastic mass. In this respect, it is to note that the largest series of pediatric sarcomas treated so far belong to clinical studies on larotrectinib²⁸.

Moreover, rearrangements in NTRK genes have been detected in stromal tumors found to be negative for other driver mutations which are known to frequently affect these neoplasms: among these, of particular interest are “wild-type” GISTs or other tumors in which characteristic oncogenic mutations are not found. In particular, as part of the NCT02576431 trial, genomic analysis of about 190 “wild-type” GISTs was performed identifying two cases with an ETV6-NTRK3 rearrangement in adult male patients, with neoplasms localized in the small and large bowel²⁹.

Furthermore, a classification of GISTs with NTRK fusions has been proposed, dividing them into:

- tumors with NTRK3 gene fusions, including high-grade fibrosarcoma-like childhood cancers;
- tumors with NTRK1 gene fusions, divided into:
 - low-grade neoplasms positive for CD34 and S100;
 - high grade unclassified sarcomas²⁹.

Altogether, it was observed that compared to stromal neoplasms with NTRK1 gene fusions, sarcomas with NTRK3 gene rearrangements show more aggressive biological behavior with a metastatic attitude, even in the low/intermediate grade forms²⁹. The importance of NTRK gene rearrangements in stromal neoplasms is under investigation and substantial efforts are necessary to understand the role of these aberrations in the biological and clinical behavior of these tumors.

Several investigations were carried out on uterine sarcoma which accounts for about 3% of malignant tumors of the uterus³⁰. According to recent observations, the high-grade form, with uncertain origin from the endometrial stroma, is related to rearrangements of NTRK genes and in particular NTRK1 and NTRK3 genes³¹.

The pilot study that first highlighted this correlation was conducted by Chiang et al. in 2018, identifying different fusion partners of NTRK genes through RNA sequencing of four uterine sarcomas, including:

- RBPMS-NTRK3
- TPR-NTRK1
- LMNA-NTRK1
- TPM3-NTRK1³².

The most frequent NTRK gene fusions in high-grade uterine sarcomas are shown in Table VIII.

A relationship was later reported between rearrangements in NTRK genes and sarcoma of the uterine cervix: in particular, in five cases the rearrangement involved exon 6 of TPM3 gene and exon 10 of NTRK1 gene; in one case the fusion involved exon 4 of TPM3

Table VIII. NTRK gene rearrangements in high-grade uterine sarcomas-endometrial stroma sarcomas (modified from Akaev et al.).

Gene partner involved	Most frequent NTRK fusion	Traslocation
TPR	TPR-NTRK1	1q31.1-1q23.1
LMNA	LMNA-NTRK1	1q22-1q23.1
TPM3	TPM3-NTRK1	1q21.3-1q23.1
RBPMS	RBPMS-NTRK3	t(8;15) (p12;q25.3)
EML4	EML4-NTRK3	t(2;15) (p21;q25.3)
STRN	STRN-NTRK3	t(2;15) (p22.2;q25.3)

gene and exon 10 of NTRK1 gene. Rearrangement of NTRK3 gene was found in one case, with fusion of exon 2 of EML4 gene and exon 14 of NTRK3 gene³³. These studies highlighted the clear prevalence of NTRK genes rearrangements in uterine sarcoma of the uterine cervix, neoplasms with fibrosarcoma-like morphology characterized by spindle cells or, more rarely epithelioids, with ovoid nuclei, scant cytoplasm and occasional focal vacuolations. The degree of nuclear atypia is generally mild to moderate with mitotic activity that appears extremely variable as the presence of necrosis³⁴.

After further immunohistochemical investigations, Croce et al. proposed a diagnostic algorithm for patients with uterine sarcoma that are negative for hormonal and smooth muscle markers and positive for S100 and CD34. For these cases it is recommended the evaluation of the immunophenotypic profile and NTRK gene rearrangements³⁴. As patients affected by this tumor are generally young, premenopausal and with an advanced disease tendency, early detection of NTRK gene fusions would be desirable to start treatment with TRK inhibitors and to give the opportunity of undergoing neoadjuvant therapy in order to inhibit the TRK pathway and preserve fertility³².

Melanocytic lesions of spitz and melanoma

Melanoma and melanocytic lesions have been the subject of several studies, especially following the discovery of characteristic driver mutations and the possibility of administering target therapies to improve clinical outcomes: despite of the large interest on this topic, the available studies on NTRK gene fusions in melanocytic lesions are limited. Special interest has been placed on melanoma and spitzoid neoplasms,

especially atypical Spitz tumors and spitzoid melanoma, neoplastic forms that do not harbor typical oncogenic mutations of conventional melanoma³⁹.

One of the first studies was conducted by Wiesner et al. in 2014 who evaluated NTRK fusions frequency in 140 spitzoid neoplasms, finding a prevalence of NTRK1 gene rearrangements in 10.7% of Spitz nevi, in 25% of atypical Spitz tumors, and in 21 % of spitzoid melanomas³⁸. The latter data is highly similar to that reported by Wu et al. in malignant/biologically indeterminate spitzoid tumors in which the prevalence of NTRK1 gene fusions, in a small cohort of cases, was 28%⁴¹.

Moreover, these data are similar to those of the study by Yeh et al. in which, in a cohort of 1202 difficult to classify melanocytic tumors, NTRK3 gene rearrangements were found in 0.7% of cases. In addition to the well-known ETV6-NTRK3 fusion, other rearrangements have been identified, such as MYO5A-NTRK3 and MYH9-NTRK337 in Spitz tumors and TUBGCP3-NTRK3 in acral melanoma³⁶. These observations suggest that in primary BRAF and NRAS wild-type melanomas, NTRK3 gene fusions arise at an early stage of neoplastic progression and in a mutually exclusive manner with respect to the classic oncogenes involved in melanoma³⁷.

Recurrent fusions of NTRK3 gene have also been identified by Wang et al. in four childhood melanocytic tumors, three with spitzoid morphology, suggesting that, even if NTRK gene rearrangements are prevalent in spitzoid melanomas, they can also be identified in melanocytic neoplasms of infancy with morphology not classified as “Spitz-like”³⁹.

Lezcano et al. identified four metastatic amelanotic melanomas with NTRK gene rearrangements in a cohort of 751 cases: three had cutaneous origin and one perianal origin, with respective prevalences of 0.8% and 0.9%; both showed neoplastic elements with epithelioid features³⁵.

It has been suggested that NTRK gene fusions in melanoma, especially when coexisting with driver mutations, represent a secondary mutational event not strictly necessary for tumor growth, even if the identified fusion partners are primarily involved in mechanisms responsible for tumorigenesis and tumor survival. However, the finding of NTRK gene rearrangements in metastatic melanomas could be a crucial element for potential therapeutic implications³⁵.

Data on the frequency of NTRK gene fusions in different melanoma types and the rearrangements observed in the studies conducted to date are shown in Table IX. The available data reinforce the importance of detecting NTRK gene rearrangements as they have additional diagnostic value and allow the pathologist to guide the clinician in therapeutic choices for patients with metastatic melanoma. This is especially relevant in consideration of the high rate of response to therapy with TRK inhibitors in neoplasms harboring these gene fusions^{35,36,40}.

Tumors of the bilio-pancreatic tract

Malignant diseases of the bilio-pancreatic tract are mainly represented by carcinomas of the biliary tract, in particular cholangiocarcinoma, and pancreatic adenocarcinoma. Several studies found a low prevalence (0.67%) of fusions of NTRK genes in tumors of the bilio-pancreatic tract. Demols et al. analyzed 162 formalin-fixed paraffin-embedded samples from surgical resections, biopsies, or cytological samples of biliary tract tumors including intrahepatic, extrahepatic, perihilar cholangiocarcinoma, and gallbladder tumors. An immunohistochemical screening was carried out, followed by NGS with an RNA-based panel to determine the prevalence and characteristics of NTRK gene fusions⁴².

Table IX. Frequency and type of fusion of NTRK genes in melanomas (modified from Forscher et al.).

Type of melanoma	Frequency of NTRK gene fusions	Types of NTRK fusions
Spitzoid melanoma (Wiesner et al. 2014)	7/33 cases (21.2%)	LMNA-NTRK1 TP53-NTRK1
Spitzoid melanoma (Wu et al. 2016)	2/7 cases (28.5%)	TPM3-NTRK1
Acral melanoma (Yeh et al. 2019)	3/122 cases (2.5%)	MYO5A-NTRK3 TUBGCP3-NTRK3
Difficult to classify melanocytic lesions (Yeh et al. 2016)	22/1202 cases (1.8%)	ETV6-NTRK3 MYO5A-NTRK3 MYH9-NTRK3
Metastatic amelanotic mucosal/paramucosal melanoma (Lezcano et al. 2018)	1/751 cases (0.9%)	TRIM63-NTRK1 DDR2-NTRK1
Metastatic amelanotic cutaneous melanoma (Lezcano et al.2018)	3/751 cases (0.8%)	GON4L-NTRK1 TRAF-NTRK2

By IHC, 17 samples were positive: the intensity of staining was weak in 16 samples and moderate in one. Furthermore, staining was frequently cytoplasmic with a diffuse and not focal pattern. NGS of the positive samples revealed a single rearrangement of NTRK3 with the fusion partner ETV6. The tumor was a case of perihilar cholangiocarcinoma with weak and focal IHC staining, both cytoplasmic and nuclear ⁴².

In pancreatic adenocarcinoma, 319 samples were analyzed, and 19 were positive by IHC. Similarly, in these samples the intensity of staining was frequently weak, cytoplasmic, and diffuse, but no fusion was detected by NGS ⁴².

In the pancreatic cancers, one case of a 61-year-old patient with pancreatic ductal adenocarcinoma and liver metastases was reported ⁴³. After standard chemotherapy, liver biopsy revealed a CTSC-NTRK1 fusion which allowed therapy with larotrectinib: the drug was well tolerated and the patient had a partial response to treatment and excellent quality of life. However, after 6 months, the patient developed drug resistance associated with a new oncogenic mutation onset (BRAF-V600E). Despite of therapy with dabrafenib and trametinib, the tumor progressed and the patient died after 2 months ⁴³.

Therefore, targeted inhibition of TRK with larotrectinib in pancreatic ductal adenocarcinoma with CTSC-NTRK1 fusion is well tolerated and can improve the quality of life. However, resistance to treatment may emerge, with still unknown frequency. Despite of NTRK gene fusions are rare in bilio-pancreatic tumors, the possibility of treatment with specific TRK inhibitors is significant.

Clinical laboratory techniques for the identification of tumours with NTRK gene fusions

The analysis of NTRK gene fusions for the selection of patients for targeted treatment can be performed in clinical specimens by different techniques such as immunohistochemistry (IHC), Fluorescence in situ hybridization (FISH), Real Time-PCR (RT-PCR), and Next generation Sequencing (NGS) ⁴⁴. As an increasing number of biomarkers for therapeutic decisions are required in oncology and biopsy material is limited, a method that allows the simultaneous detection of multiple types of genomic alterations, such as NGS, would be preferable. However, for a number of reasons that are mainly dealing with costs and availability of the technology in pathology labs, this option is not always feasible at the present time. Alternatively, the pathologist can integrate different methods such as IHC, FISH and RT-PCR (Tab. X).

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TRK1, TRK2, and TRK3 proteins are codified by the corresponding members of the NTRK gene family. In the presence of NTRK gene fusions, a high expression of TRK proteins is detectable directly on tissue sections by IHC, a fast, easy and widely used method in pathological diagnostics with the restriction of being able to analyse only one or a few biomarkers at a time. In vitro diagnostics (IVD) tests for the analysis of all three TRK proteins (pan-TRK) are now commercially available for detection of all translated fusion products. Several studies have suggested that pan-TRK IHC is an effective method to identify tumours harbouring

Table X. Advantages and disadvantages of the described techniques.

	Advantages	Disadvantages
IHC	Evaluation of actual protein expression Low costs Short turnaround time (TAT) High sensitivity (95%) and specificity (100%)	Inability to identify the fusion partner Can only be used on formalin-fixed, paraffin-embedded samples Reduced specificity in nervous system cells (constitutive expression of NTRK) Evaluation of a single analyte only
FISH	Widespread and well recognized method Commercially-available kits Useful to evaluate NTRK3-ETV6 fusion	Complex interpretation of results Higher costs compared to IHC Evaluation of a single gene
RT-PCR	Commercially-available kits Specificity (specific primers) Low cost	Can detect only a limited number of already known fusions Impossible to detect translocations > 200 bp Variable sensitivity and specificity (based on quality of the nucleic acid)
NGS	Possibility to identify fusion partners High sensitivity and specificity Can analyse small quantities of samples Simultaneous analysis of other clinically relevant markers	High costs Longer TAT vs. other techniques Operators need a high level of training Limited territorial diffusion

NTRK gene fusions, with high sensitivity and specificity. However, assessment of sensitivity and specificity of IHC is currently limited by the small number of cases available for comparative analysis with different technologies. The available data suggest that larger studies are needed to determine sensitivity and, in particular, specificity of immunohistochemical tests for NTRK gene alterations. Therefore, IHC must be considered as an inconclusive screening analysis that requires confirmation, in case of positivity, with an orthogonal method (NGS, FISH, or RT-PCR). Particular attention should be paid to malignancies with constitutional expression of TRK proteins (tumours with muscular or neuronal/neuroendocrine differentiation) in which false positives are more frequently observed⁴⁵.

FLUORESCENCE IN SITU HYBRIDIZATION

FISH assays are useful diagnostic tools in pathology laboratories to detect chromosomal alterations on formalin-fixed, paraffin-embedded tumor specimens.

The use of dual-colour FISH probes, such as break-apart or dual-fusion probes, allows for detection of chromosomal rearrangements in tumor samples. In particular, dual-fusion probes can detect chromosomal translocations with specific known fusion partners with high sensitivity. In cases where fusion partner genes are variable or not known, break-apart probes are required. However, several factors can influence the sensitivity and specificity of these methods, such as the genomic distance between the probes on the target chromosome, type of rearrangement, whether the aberration is intra- or inter-chromosomal and the threshold chosen to determine positivity.

FISH allows evaluation of a single gene at a time. Therefore, in order to fully determine the fusion status of all three NTRK genes, three separate FISH assays must be performed.

Suspected cases of infantile fibrosarcoma, congenital mesoblastic nephroma and secretory carcinoma of the breast and salivary glands should be routinely evaluated for ETV6-NTRK3 fusions to help confirm histopathological diagnosis. Given the high prevalence of this fusion in the aforementioned tumours, in some cases diagnosis is based solely on positive FISH for ETV6-NTRK3 fusions. However, the identification of a high number of different fusion patterns in these tumour types suggests that such a diagnostic approach would lead to the inability to identify all lesions carrying NTRK gene fusions⁴⁶.

RT-PCR

RT-PCR analysis can be used to identify tumours with NTRK gene fusions. In particular, this test is effective in detecting ETV6-NTRK3 fusions in infantile

fibrosarcoma, secretory breast cancer and congenital mesoblastic nephroma. However, in order to obtain valid results, the pre-analytical variables (time of cold ischaemia, conditions of fixation and method for RNA extraction) and the presence of internal controls for evaluation of the quality of the RNA extracted from tumor samples must be optimised. The sensitivity of RT-PCR is reduced in samples with degraded RNA. Furthermore, the approach fails to identify all the different fusions present in various tumours given the large and growing number of possible fusion partners and breakpoints identified to date⁴⁶.

NEXT-GENERATION SEQUENCING

The capacity for massive parallel sequencing provided by NGS allows detection of multiple genetic alterations in routine clinical practice even on limited quantities of tissue thanks to its high processivity and sensitivity. NGS assays can be performed on DNA or RNA and can analyse the entire genome, exome, or transcriptome, or can be targeted to groups of genes of interest using specific panels, thus improving the accuracy and the sensitivity required in clinical diagnostics. With regards to the main sequencing strategies, these can be traced back to the analysis of genomic libraries produced by PCR amplification with multiple primers (amplicon sequencing) or by chemical or mechanical fragmentation and hybridisation with specific probes and capture (hybridisation and capture).

A distinct advantage of DNA-based NGS, using the hybridization and capture approach, is the ability to simultaneously evaluate mutations, amplifications, deletions, gene fusions, microsatellite instability and tumour mutational burden. For the detection of genetic rearrangements, including those involving NTRK genes, the high sensitivity and specificity of the method, in addition to the ability to detect new fusion partners, are further advantages of massive parallel sequencing.

However, at the level of specificity, NGS based on DNA analysis can identify genomic rearrangements that may or may not result in a functional fusion protein. Consequently, an additional NGS RNA test may be required to confirm a positive result. In fact, by carrying out sequencing starting from RNA, intronic regions removed by splicing are avoided, this allows for easier capture and amplification of functional fusions.

With regard to the sensitivity of NGS based on RNA analysis, in a classical approach with amplicons at known fusion sites (breakpoints), this is determined by the ability of the gene panel used to cover the fusion breakpoints. The analysis can give rise to false negatives in case of gene panels of limited size. In-

stead, with an RNA-based hybridisation and capture approach, it is possible to obtain an in-depth analysis of fusions, even if not known beforehand, with unexpected fusion partners and variable breaking points. This is particularly important for NTRK gene fusions which involve more than 100 different fusion partners. The major drawback of RNA-based approaches is the highly variable quality of RNA extracted from formalin-fixed, paraffin-embedded tissues. This disadvantage is significant and requires stringent controls to understand when a result can be considered non-informative rather than negative.

These complementary strengths and weaknesses between DNA- and RNA-based NGS techniques demonstrate the need to carefully consider a combined approach. This is made possible by platforms that are capable of evaluating both DNA and RNA extracted from the same formalin-fixed, paraffin-embedded sample.

Another consideration on NGS assays is that they require adequate purity and optimal technical processing of the neoplastic sample. Furthermore, the average time for reporting is generally longer (about two weeks) than that required for other methods.

Notwithstanding, a multiplex and comprehensive analytical tool such as NGS is the diagnostic technique of choice for genomic testing to obtain as much information as possible on biomarkers, especially from small samples. An accurate and clinically effective alternative, if it is not possible to obtain a tissue biopsy, is genotyping of the tumour starting from circulating tumour DNA (ctDNA). ctDNA also provides a non-invasive approach to monitor the onset of tumor resistance in patients with NTRK gene fusions receiving TRK inhibitor therapy. Although the study of ctDNA has high diagnostic specificity, its sensitivity can sometimes be problematic since detection of genetic alterations requires sufficient diffusion of tumour cells into circulation to allow their detection. Finally, circulating RNA is also a potential type of sample for diagnostic tests, although it is less stable than DNA⁴⁷.

Diagnostic algorithms

Inhibition of TRK proteins has been shown to be very effective in giving rise to lasting responses that are observed regardless of the patient's age, tumor site and fusion gene partner. Therefore, it is crucial to define the optimal strategies to identify NTRK fusions and accordingly choose the most suitable therapy. Various algorithms have been suggested to help pathologists in the detection of these rare molecular alterations which can be present in different forms of cancer⁴⁴⁻⁴⁷.

In consideration of the low prevalence and wide diffusion of such molecular alterations, it is first necessary to evaluate the efficiency and costs of the methods described above. In this regard, it has been shown that pan-TRK IHC is an efficient, reliable and rapid first level test to detect expression of TRK in clinical practice. In cases showing any degree of protein expression, a multigene panel testing by NGS is then recommended to confirm or deny the suspected genetic alteration. Furthermore, the type of TRK immunohistochemical staining may help to identify the type of NTRK gene involved, such as in tumours harbouring NTRK1 rearrangements that typically exhibit diffuse cytoplasmic staining. In contrast, tumours harbouring NTRK3 rearrangements may have weaker cytoplasmic expression and focal nuclear staining.

NGS analysis with genetic panels offers the possibility to evaluate a relatively large number of genes in a single test, thus analysing a spectrum of genomic alterations of the tumour under consideration and plan the best therapeutic strategy. Among the different options for gene panels, with regards to the detection of NTRK fusions, sequencing methods based on RNA analysis may represent the gold standard, provided that the quality of the RNA is adequate. Furthermore, it must also be considered that, whenever the availability of tissue is limited, a combined DNA/RNA approach may be preferable, extracting both nucleic acids at the same time.

Given these premises on testing technologies and based on their availability in clinical practice as well as costs and efficiency, we propose an algorithm in which tumours are subdivided into two groups, each with a different diagnostic approach: those with a high prevalence of NTRK gene fusions and those with a low prevalence of NTRK gene fusions⁴⁴ (Fig. 5).

- 1 Tumours with a high rate of NTRK gene fusions (> 50%) include infantile fibrosarcoma, secretory carcinomas of the breast and salivary glands, and congenital mesoblastic nephroma. Such neoplasms should be routinely analysed for NTRK gene fusions, typically with FISH using break-apart/dual-fusion probes and/or NGS, with particular attention to the ETV6-NTRK3 fusion. It should be emphasised that negativity by FISH does not exclude the presence of a fusion, which is why a second level NGS test should be considered in such cases.
- 2 Tumours with a low prevalence of NTRK gene fusions (< 5%) include the remaining malignancies. In some of these neoplasms, multigene analysis is routinely planned in clinical practice (e.g. in NS-CLC, melanoma, colorectal cancer, thyroid cancer etc.) with the aim of identifying specific mutations

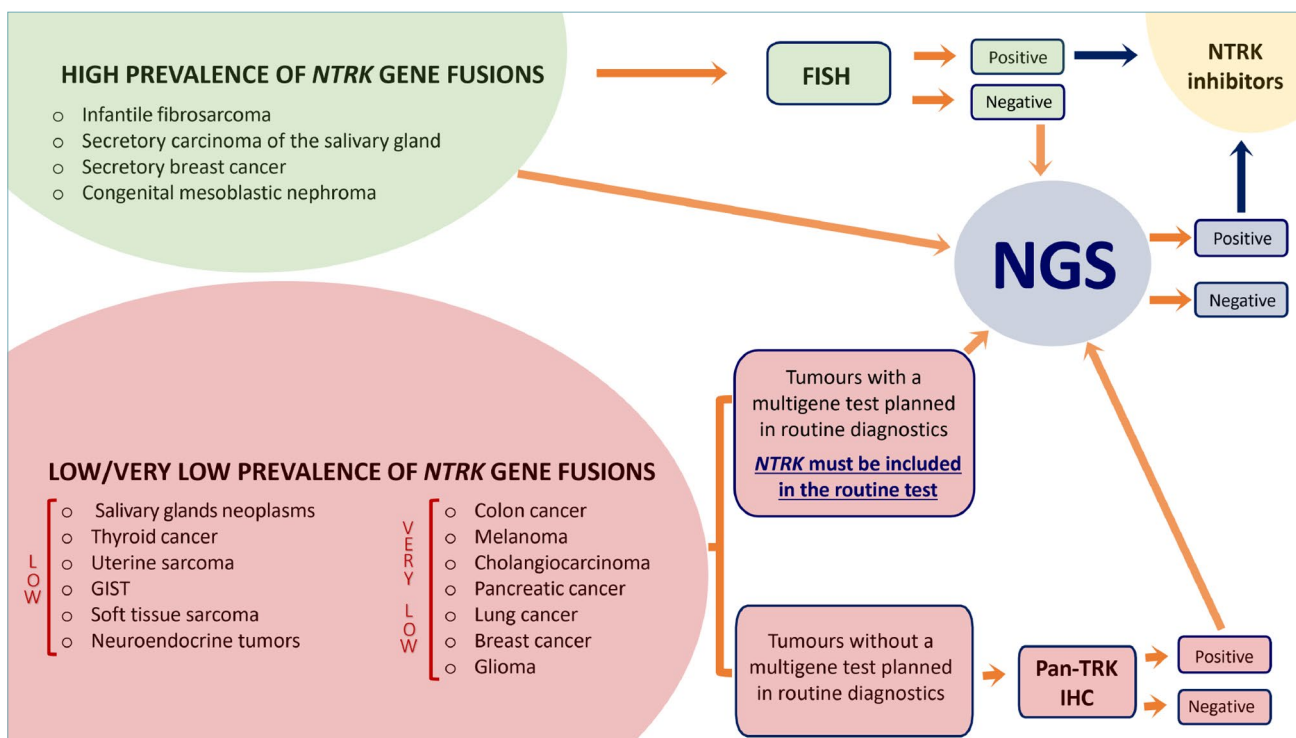


Figure 5. Diagnostic algorithm based on the prevalence (high, > 50%: low, 1-5%; very low, < 1%) of NTRK gene fusions and the molecular tests planned in routine diagnostics.

for targeted therapy. In such neoplasms, therefore, a broad NGS analysis that also includes NTRK genes is indicated. On the other hand, in tumours in which a multigene test is not yet routinely recommended (e.g. pancreatic cancer), the use of pan-TRK IHC can identify, albeit with limited specificity, patients for whom further diagnostic investigation with NGS is appropriate. It is important to underline that the immunohistochemical test in some of these tumours (such as GIST, sarcomas and neuroendocrine tumours) must be evaluated with particular attention in the light of the possible constitutive expression of TRK proteins.

A new diagnostic strategy for early detection of rare targets and tumor-agnostic treatments

Ideally, the test to identify rare targets for agnostic treatments, including NTRK gene fusions, should be extended to all solid tumours at an early stage. This can allow for early identification of patients who may benefit from targeted anti-NTRK treatment. However, extending the test to all invasive solid tumours would

require high costs and execution times that are currently unsustainable. Even a simple IHC screening test on all solid tumours at the time of diagnosis (reflex test) is not currently feasible in routine clinical practice. In order to use IHC as a screening method on a wide range of tumours and to carry out routine reflex testing, in our previous pilot study we presented a method that favours the application of an IHC panTRK test on a large scale, using tissue microarrays, which allows the simultaneous analysis of tumours from many patients². A diagnostic workflow has been implemented to favour a routine screening in clinical practice (Fig. 6). The application of this new diagnostic approach has made it possible to identify a series of rare molecular targets to select patients for tumour-agnostic treatments, including NTRK fusions. This technique can analyse dozens of samples at the same time with considerable reduction in costs and the time needed for screening; positive cases must be subsequently subjected to an orthogonal method (NGS or FISH) for confirmation.

On the basis of this pilot study, the Italian Society of Pathological Anatomy and Cytopathology (SIAPeC) is currently carrying out a multicentre project on about 10,000 patients (VITA SIAPeC-IAP Projects) to identify rare molecular alterations, including fusions of

A pathological workflow for screening of rare genomic alterations on TMA samples in routine diagnostics

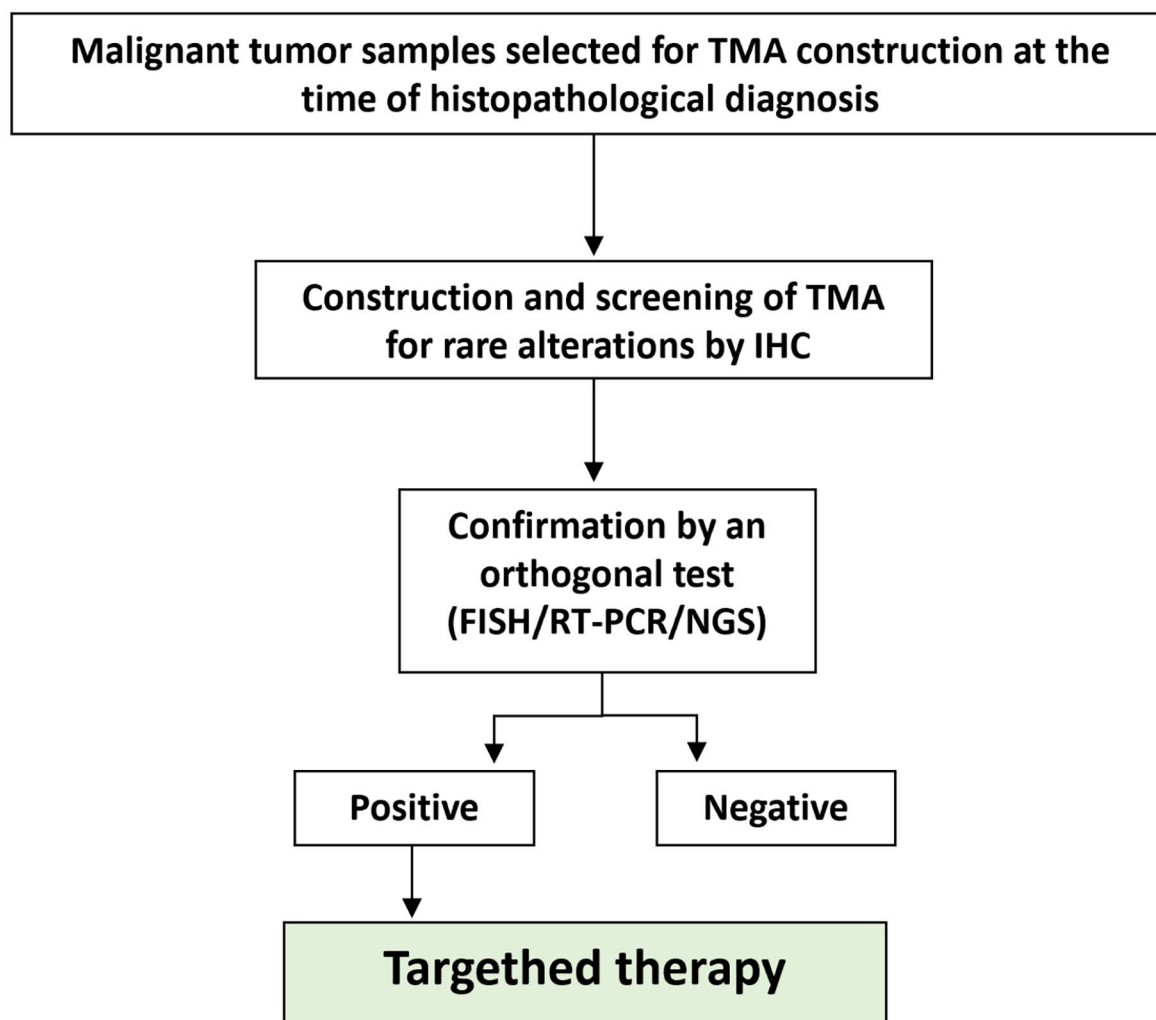


Figure 6. A simplified diagnostic workflow for testing rare biomarkers and select patients for tumor-agnostic treatments. A more detailed workflow for diagnostic screening in clinical practice is reported in Marchetti et al, 2019², from which the figure was derived.

NTRK genes, mainly focusing on tumours in younger patients (< 50 years of age).

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CONFLICT OF INTEREST

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ETHICAL CONSIDERATION

No ethical considerations are needed.

AUTHOR CONTRIBUTIONS

All the authors contributed to writing. AM, FB and ED revised and edited the manuscript.

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