

***ROS1* rearrangements are uncommon in biliary tract cancers**

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Abstract. Biliary tract cancers (BTCs) are a pool of diseases with poor prognosis and there is no orphan drug available. Currently, no molecular targets have been tested as druggable oncogenic drivers. C-ros oncogene 1 (*ROS1*) rearrangements have been previously described in various tumors, including BTCs; however, data regarding their incidence and biological significance are controversial. Therefore, a retrospective multicenter study was performed to assess the incidence of *ROS1* rearrangements in BTCs by means of immunohistochemistry and fluorescence *in situ* hybridization (FISH). The present study failed to demonstrate *ROS1* expression in a multicenter series of 150 cases with BTCs and revealed that D4D6 was the most specific clone compared with other *ROS1* primary antibodies, namely PA1-30318 and EPMGHR2. Notably, negative results obtained with D4D6 completely matched to data sorted out by FISH analysis, thus confirming a lack of *ROS1* gene rearrangements in BTCs and false positive results when PA1-30318 and EPMGHR2 clones were used. These results

suggest that *ROS1* rearrangements may not be targets for molecular therapy of BTCs with specific inhibitors.

Introduction

Biliary tract cancers (BTCs) are rare tumors arising from the biliary tree epithelium, from the small peripheral hepatic ducts to the distal common bile duct. The progress on the treatment strategies for patients with BTCs has been slow in the past decades. The disease prognosis remains poor, with a modest improvement from 11 to 17% in terms of 5-year overall survival (OS) rates (1).

Complete surgical resection or liver transplantation, when feasible, are the only potentially curative treatments in the early stages of BTCs (2). In advanced stages, standard chemotherapy (CT) in combination with palliative supportive care, such as biliary drainage or stenting, is the only available therapeutic option, providing a survival advantage with a modest impact and benefit in terms of quality of life (3,4). Gemcitabine plus cisplatin regimen is the current standard first-line treatment, with a median OS of less than 1 year (4). However, no standard second-line CT regimens have been established.

In recent years, whole-genome tumor profiling studies have identified a wide variety of genetic alterations, many of them considered targetable therapeutic options (*HER2*, *BRAF*, *FGFR1-3*, *IDH1/2*, *MET* and *MEK*) (5,6). In addition, preliminary trials in selected populations treated with targeted therapies have demonstrated promising improvement in survival outcome (7-9). Immunotherapy is considered a revolutionary treatment method for selected patients and preliminary preclinical data have revealed encouraging results, even in BTCs (10).

C-ros oncogene 1 (*ROS1*), a proto-oncogene, encodes a receptor tyrosine kinase (RTK) without a known ligand. *ROS1*

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shares a high structural homology with the insulin receptor family and the anaplastic lymphoma kinase (ALK) (11). When *ROS1* is constitutively activated by gene rearrangement, the RTK is overexpressed and is likely detected using immunohistochemistry (IHC). It has been reported that chromosomal rearrangements lead to fusion of *ROS1* with several partner genes, resulting in the formation of a constitutively active fusion kinase (12). This kinase induces mitogen-activated protein kinase, signal transducer and activator of transcription 3 and phosphoinositide 3-kinase pathways, among others, subsequently promoting cellular transformation (12). These rearrangements, also evidenced by the aberrant expression of the RTK ROS1, have been detected in several types of cancer, including 1-2% of lung adenocarcinoma cases, glioblastoma, cholangiocarcinoma (CAC) and others (13,14). In lung cancer, clinical and epidemiological published trials have already described the incidence and prevalence of *ROS1* as well as its predictive and prognostic role. However, there is currently a lack of consistent evidence regarding *ROS1* gene rearrangements and its protein expression in other neoplasms, including BTCs (14).

It has been shown that ROS1 and ALK share significant homology within their respective tyrosine kinase (TK) domains. This finding led to the hypothesis that ALK tyrosine kinase inhibitors (TKIs) may also inhibit ROS1 expression (15). Based on promising preclinical data with different ALK TKIs, several clinical studies have been performed in ROS1-positive NSCLC patients with interesting results. For example, Shaw *et al* (15) demonstrated a progression free survival (PFS) of 19.2 months and a response rate of 72% in 50 ROS1-positive lung cancer patients treated with crizotinib (16-19).

In a case series of various tumors, *ROS1* rearrangements were detected in 2 out of 23 patients (8.7%) with BTCs (20). However, in a cohort of 56 Chinese CAC patients no *ROS1* rearrangements were observed (21). Additionally, Graham *et al* (22) reported one case with *ROS1* rearrangements among 100 CAC cases. Of note, the *ROS1* positive case also harbored an *IDH1* mutation (22,23). Recently, two additional studies on Asiatic cohorts of BTCs patients reported *ROS1* rearrangements in 0 and 1.1%, respectively (24,25). The present study aimed to identify the incidence of *ROS1* rearrangements in a retrospective, Italian and multicentric cohort of patients with BTCs. All cases were tested using IHC and the results from three different commercially available ROS1 primary antibodies (Abs) (clones D4D6, PA1-30318 and EPMGHR2) were compared. Positive cases were further analyzed by fluorescence *in situ* hybridization (FISH) to confirm the presence of *ROS1* rearrangements.

Materials and methods

Study aim and design. The present multicenter, retrospective study was conducted by the Italian Clinical Oncology Research Group (GOIRC) and included eight Italian centers as follows: Azienda Ospedaliero-Universitaria Careggi, Florence; Regional Hospital Parini, Aosta; Santa Maria delle Croci Hospital, Ravenna; Santa Chiara Hospital, Pisa; Santa Maria Nuova Hospital, Reggio Emilia; IRSSST, Meldola; Maggiore Hospital, Parma; and San Luca Hospital, Lucca. In the present study, 150 cases of BTCs, diagnosed between January 2012

and December 2015 using surgical specimens (n=98) or liver biopsy (n=52), were enrolled. All cases were eligible for inclusion in the study and sufficient material was available for IHC and FISH analyses. At the time of diagnosis patients were ≥ 8 years old. All subjects provided written informed consent according to the Local Ethical Committees.

Histopathological samples were centrally reviewed and analyzed at the Pathology Units of the Regional Hospital Parini and Santa Maria delle Croci Hospital. The clinicopathological characteristics, including age, sex, disease stage, treatment and survival rate, were analyzed from the patients' medical records and referring physicians.

The association between ROS1 expression and survival parameters and clinicopathological characteristics, collected from the available medical records, was considered as the secondary endpoint of the study. OS was defined as the time between diagnosis and death, resulting from any cause. In particular, the secondary end point was to evaluate the potential prognostic role of ROS1 expression by comparing the ROS1-positive population with ROS1-negative tumors. The study was conducted in accordance with the precepts of the Good Clinical Practice guidelines and the Declaration of Helsinki. In addition, the present study was approved by the Ethics Committee of each institute and written informed consent was obtained from all participants.

IHC staining. IHC staining was performed on 4- μ m sections obtained from formalin-fixed and paraffin-embedded tissue blocks that were subsequently mounted on charged slides. Following deparaffinization and rehydration, antigen retrieval was carried out using a Cell Conditioning 1 (CC1) solution for 64 min at 95°C. ROS1 IHC assay was performed using three different primary Abs, monoclonal EPMGHR2 (1:200 dilution; Abcam), monoclonal D4D6 (1:50 dilution; Cell Signaling Technology, Inc.) and polyclonal PA1-30318 (1:200 dilution; Thermo Fisher Scientific, Inc.). All assays were carried out using an automated immunostainer (VENTANA, BenchMark ULTRA system; Ventana Medical Systems, Inc.). The intensity of immunostaining in samples was scored by an experienced pathologist using the H-score method. The scoring system was based on intensity (0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining) and the percentage of positive tumor cells. Therefore, a H-score range of 0-300 was recorded in each case. Based on a previous study in NSCLC (26), a case was considered positive when the H-score was >100 . In all batches, a negative (without a primary antibody) and a positive (lung adenocarcinoma previously evaluated as ROS1-positive using IHC and FISH analyses) control were employed to evaluate the appropriateness of the IHC analysis.

FISH analysis. In the present study FISH analysis was performed using a commercially available assay (The ZytoLight® SPEC ROS1 Dual Color Break Apart Probe; ZytoVision GmbH) according to the manufacturer's recommendations. At least 50 tumor cells from each sample were analyzed and scored according to the guidelines of the European recommendations (27).

The probes labeled the 5' (telomeric) and 3' (centromeric) ends of the fusion breakpoint with green and orange

fluorochromes, respectively. The criteria for ROS1 FISH interpretation in the tested tumors were the following: i) The break-apart pattern ('conventional' pattern) with one fusion signal and two separated 3' and 5' signals; and ii) an atypical pattern showing an isolated 3' signal (usually one fusion signal and one isolated 3' green signal without the corresponding 5' signal). The cut-off of rearranged signals to quote ROS1 positivity was based on detection of $\geq 15\%$ among 50 neoplastic nuclei.

Statistical analysis. No statistical analysis was reported since the expression value of ROS1 with FISH analysis was negative in all cases and the association between ROS1 expression and no associations with clinicopathological parameters of patients was determined.

Results

Study population. In this study, 150 CAC samples were collected, including 98 surgical specimens and 52 biopsies. The samples were collected from the medical archive corresponding to cases diagnosed between January 2012 and December 2015. The available clinical data were derived from 100 patients' medical records and referring physicians, including 69 males and 31 females with a median age at diagnosis of 70 years (range, 35-84 years). The Eastern Cooperative Oncology Group (ECOG) performance status at diagnosis was 0 in 59, 1 in 27, 2 in 11 patients and unknown in the remaining 3 cases. The primary tumor origin was: intrahepatic bile ducts (n=56); hilar (n=4); extrahepatic (n=32); gallbladder (n=6); while in 2 patients the tumor arised from an unknown primary site. In addition, the clinical stage distribution at diagnosis was as follows: 48 cases exhibited locally recurrent disease; 24 locally advanced disease; 26 metastatic disease; and 2 cases were undetermined. Furthermore, 67 patients underwent surgery and among them, 58 patients experienced radical R0 resection. At the time of data collection (April 2018), 22 patients were still alive, while 78 died, including 50 who exhibited disease relapse or progression. Patients' baseline characteristics are presented in Table I.

According to the 2015 revised classification of intrahepatic CAC, pathological diagnosis was consistent with conventional small duct type (15 cases), bile ductular type (1 case), intra-ductal papillary type (1 case), intraductal tubular type (1 case), squamous/adeno squamous cell type (1 case), mucinous/signet ring cell type (5 cases) and undifferentiated type (2 cases). Other rare types were reported in 9 cases, whereas in 13 cases the pathological subtype was undetermined. Regarding perihilar and distal CAC, pathological subtype was conventional in 10 cases, intraductal papillary type in 1 case, squamous/adeno squamous cell type in 1 case, mucinous/signet ring cell type in 1 case, while rare and undetermined types were reported in 3 and 12 cases, respectively. Finally, 6 cases of gallbladder adenocarcinoma were included in the study.

Considering treatment strategy, 45/48 patients (93.75%) with early stage BTC at diagnosis, received radical surgery as first-line treatment. In 41 of these cases (91%), no residual tumor (R0) following surgery was observed, while 4 cases exhibited positive surgical margins (R1). Following surgery, 18 patients were treated with gemcitabine-based adjuvant

Table I. Clinical characteristics of patients with biliary tract cancer.

Parameters	Value, n (%)
Sex	
Male	69/100 (69)
Female	31/100 (31)
Age, years (median, range)	70 (35-84)
Eastern Cooperative Oncology Group Performance Status	
0	59/100 (59)
1	27/100 (27)
2	11/100 (11)
Unknown	3/100 (3)
Disease stage	
Localized	48/100 (48)
Locally-advanced	24/100 (24)
Metastatic	26/100 (26)
Unknown	2/100 (2)
Surgery	67/100 (67)
R0	58/67 (87)
R1	5/67 (7)
R2	2/67 (3)
Unknown	2/67 (3)
Site	
Intrahepatic bile ducts	56/100 (56)
Hilar ducts	4/100 (4)
Extrahepatic ducts	32/100 (32)
Gallbladder	6/100 (6)
Unknown	2/100 (2)

CT. At follow-up, 31 patients experienced disease relapse and among them, 21 patients were subsequently treated with alternative platinum- or fluoropyrimidine-based CT treatment.

Furthermore, 24 patients were diagnosed with locally advanced disease, therefore 19 of them underwent surgery and R0 status was observed in 16 cases. Of the remaining 3 patients, 1 was treated with R1 surgery and 2 patients exhibited R2 positive margins. Additionally, 7/24 locally advanced patients were treated with gemcitabine-based adjuvant CT after surgery. Conversely, 2 patients received induction CT with platinum/gemcitabine regimen prior to surgery. At follow-up, 12 patients experienced disease relapse with 10 cases being subsequently treated with alternatively platinum- or fluoropyrimidine-based CT. In addition, 26 patients were diagnosed with metastatic disease. Nevertheless, 3 patients received surgical treatment and among them, 1 patient exhibited R0 status.

Overall, 55 patients received a first-line CT for advanced disease (alternatively platinum, gemcitabine- or fluoropyrimidine-based; single agent or combination regimen). Furthermore, following progression, 27 patients were treated with a second-line CT.

Table II. C-ros oncogene 1 antibodies tested in the cohort of patients with biliary tract cancer.

Primary antibody	Dilution	Commercial source	Epitope	Isotype
Monoclonal D4D6	1:50	Cell Signaling Technology, Inc.	Carboxy terminal domain	Rabbit IgG
Monoclonal EPMGHR2	1:200	Abcam	aa 2050-2150	Rabbit IgG
Polyclonal PA1-30318	1:200	Thermo Fisher Scientific, Inc.	aa 39-57	Rabbit IgG

aa, amino acid; IgG, immunoglobulin G.

Table III. Distribution of C-ros oncogene 1 expression according to different primary antibody clones used in immunohistochemistry experiments.

Sample type	Clone D4D6	Clone EPMGHR2	Clone SP384
Biopsy, n	0/52	7/52	5/52
Surgical sample, n	0/98	21/98	17/98
Total, n (%)	0/150	28/150 (18.6)	22/150 (14.6)

Table IV. Literature review: C-ros oncogene 1 expression (%) in cholangiocarcinoma using different antibodies and techniques.

Author, year	IHC			FISH				Refs.
	D4D6	SP384	EPMGHR2	Break-apart	Exon 30	RT-qPCR	Immunoaffinity	
Gu <i>et al</i> , 2011	-	-	-	-	-	-	8.7	(20)
Liu <i>et al</i> , 2013	-	-	-	-	-	0	-	(21)
Graham <i>et al</i> , 2014	-	-	-	1	-	-	-	(22)
Arai <i>et al</i> , 2014	-	-	-	-	-	0	-	(28)
Lee <i>et al</i> , 2015	37.1	-	-	-	0	-	-	(24)
Lim <i>et al</i> , 2017	19.1	-	-	1.1	-	-	-	(25)

IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; RT-qPCR, reverse transcription-quantitative PCR.

ROS1 IHC and FISH findings. Although several studies with selected Abs have shown significant correlation between molecular (extractive or *in situ*) and IHC assays for ROS1 detection, the present study is the first to directly compare various primary Abs against ROS1. Therefore, a group of 150 BTCs was evaluated for ROS1 positivity, using three different primary Ab clones in order to determine their relative sensitivity and specificity. ROS1 protein expression was evaluated with clone D4D6 and two clones tested for the first time, namely EPMGHR2 and PA1-30318. The main characteristics of the three primary Abs used in the study are summarized in Table II. The scoring distribution of IHC results for each primary Ab is presented in Table III. ROS1 was differentially expressed, based on primary Ab clones used, whereas no ROS1-positive tumors were observed when clone D4D6 was applied. By contrast, 22 (14.6%) and 28 (18.6%) cases showed positive staining when polyclonal PA1-30318 and monoclonal EPMGHR2 Abs were used, respectively. Furthermore, both Abs exhibited higher immunoreactivity in surgically-derived samples compared with that noted to biopsies (Table III and Fig. 1).

Subsequently, FISH analysis was performed in 75 BTC cases, including 25 randomly selected negative cases that were tested with all clones and, 22 and 28 ROS1 positive tumors evaluated with PA1-30318 and EPMGHR2 Abs, respectively. Overall, FISH was negative in all cases. The results obtained with clone D4D6 were consistent with FISH analysis. Therefore, clone D4D6 exhibited the highest specificity and association with FISH assay in detecting *ROS1* rearrangements, while false positive results were obtained using the other clones. The results of ROS1 immunostaining reported to date in the literature are summarized in Table IV.

Discussion

Due to controversial results having previously been reported, *ROS1* rearrangements in a large cohort of BTCs were screened. To date, *ROS1* rearrangements have been identified only in 6 patients with BTCs (20,22,25). In particular, Gu *et al* (20) showed the presence of *FIG-ROS1* rearrangement in 2/23 patients with CAC (8.7%), whereas Graham *et al* (22) reported a single

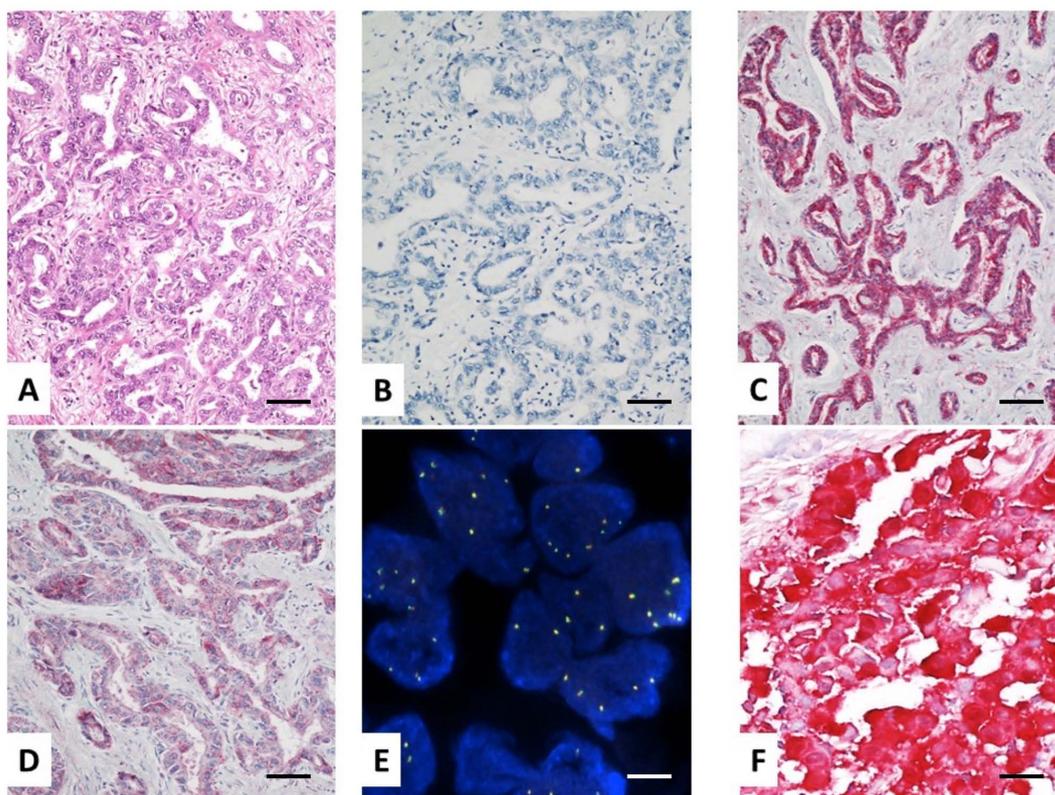


Figure 1. Representative case of intrahepatic CAC. (A) H&E staining of intrahepatic CAC tissue. (B) IHC assay characterized by negative ROS1 staining using the monoclonal D4D6 Ab. (C) IHC assay characterized by positive ROS1 expression using the polyclonal PA1-30318 and (D) the monoclonal EPMGHR2 Abs. (E) Break-apart fluorescence *in situ* hybridization probe did not reveal gene rearrangements. Normal fused signals are indicated. (F) A case of *ROS1* rearrangement detected with the D4D6 Ab in lung adenocarcinoma served as a positive external control in each bath. (A-D and F) Scale bar, 50 mm; (E) Scale bar, 5 mm. CAC, cholangiocarcinoma; H&E, hematoxylin-eosin; IHC, immunohistochemistry; Ab, antibody; *ROS1*, c-ros oncogene 1.

case (1/100, 1%) with *ROS1* translocation and concurrent *IDH1* mutation. In addition, Lim *et al* (25) demonstrated a frequency of *ROS1* rearrangements of 1.1% (3/261), in the largest cohort to date. However, the frequency of *ROS1* rearrangements has not been fully elucidated. Arai *et al* (28) and Lim *et al* (25) revealed that no *FIG-ROS1* fusion was detected in CAC when screened by RT-qPCR and FISH, respectively. Screening of *ROS1* rearrangements is generally performed using IHC or FISH assays. In lung cancer, the application of both assays is recommended in order to confirm positive results (29).

In particular, we aimed to investigate the incidence of *ROS1* rearrangements in BTCs by exploiting IHC and FISH techniques. The efficiency of IHC method was examined by comparing three different commercially available primary Abs. To date, only a study conducted by Conde *et al* (27) compared *ROS1* expression using two different *ROS1* primary Abs, namely D4D6 and SP384, in a selected cohort of NSCLC patients. This study suggested that SP384 clone was less specific compared with D4D6, as a higher number of false positive results were obtained (25 and 9% for each Ab, respectively) (27). The results of the present study confirmed that the D4D6 clone was more accurate compared with polyclonal PA1-30318 and monoclonal EPMGHR2 Abs. Furthermore, negative IHC results obtained with the D4D6 Ab, were consistent with the results emerged by FISH analysis. By contrast, the positive results obtained using PA1-30318 (14.6%) and EPMGHR2 (18.6%) Abs were not confirmed by FISH analysis. Unlike previous studies, the present study did not detect *ROS1* expression in 150 Italian patients

with BTCs (20,22,25,30). Previous works by Lee *et al* (24) and Lim *et al* (25) have detected *ROS1* by IHC with clone D4D6 in 37.1 and 19.1%, respectively. None out of 102 cases (24) and 3 out of 261 cases (1.1%) (25) were finally resulted as positive at FISH analysis, respectively. Nevertheless, the authors used a very low immunohistochemical cut-off of expression, namely any staining in the study of Lim *et al* (25) and at least 5% of stained tumor cells with at least 2+ of staining intensity in the report by Lee *et al* (24). In the present study we quoted a positive expression using a more robust cut-off, namely H-score >100, then explaining the discrepancy of positive cases between the present and previous observations (24,25).

In a recent study by Lowery *et al* (31), 195 patients with intrahepatic CAC were prospectively examined by targeted next generation sequencing (NGS) assay, including exons and selected introns from 410 tumor genes. The results of this study demonstrated that the most commonly altered genes in CAC were *IDH1* (30%), *ARID1A* (23%), *BAP1* (20%), *TP53* (20%) and *FGFR2* gene fusions (14%) (31). In addition, the most common genetic alterations in extrahepatic CAC were detected in *KRAS*, *SMAD4* and *STK11* genes. Of note, the authors did not reveal *ROS1* gene alterations in their large cohort of patients, although an advanced sequencing technique was performed (31).

In summary, the present study suggests that *ROS1* rearrangements in BTCs are not considered reliable molecular targets for the development of novel and selective therapeutic approaches. *ROS1* gene alterations in BTCs, that have been

reported in previous studies, may be considered sporadic cases or false-positive results. Therefore, no further studies are required to detect *ROS1* rearrangements in BTCs. However, further research on alternative pathways for the detection of consistent genetic alterations driving to BTC carcinogenesis is needed. These pathways may serve as promising prognostic biomarkers and therapeutic targets for BTCs.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

FM and LA conceived the study design. FM and SP performed manuscript drafting. PP and SP were responsible for data collection and analysis. EV, MP, ACG, FN, AL, CV, EG, GLF, LM, GJ and AB collected data, enrolled patients and performed data interpretation. GR performed data interpretation and wrote the manuscript. LA revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with Good Clinical Practice guidelines, the Declaration of Helsinki, and with approval from each institutional Ethical Committee of the Centers involved (Careggi Hospital-Florence, Regional Hospital Parini-Aosta, Santa Maria delle Croci Hospital-Ravenna, Santa Chiara Hospital-Pisa, Santa Maria Nuova Hospital-Reggio Emilia, IRSSST-Meldola, Maggiore Hospital-Parma and San Luca Hospital-Lucca). Written informed consent was obtained from all patients involved.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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