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ORIGINAL ARTICLE

Confocal endomicroscopy accuracy in identifying central nervous system tumors tissue at the infiltration margins: results from a prospective clinical trial

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ABSTRACT

BACKGROUND: We have previously shown the usefulness of a new confocal endomicroscopy system (CONVIVO®) in providing a quick and reliable method for intraoperative diagnosis *ex vivo* in glioblastoma (GBM). In this study, we aimed to assess the intraoperative usefulness of CONVIVO® in an *in-vivo* setting, focusing on its capability to explore the presence of residual tumor at the resection margins of Central Nervous System (CNS) tumors.

METHODS: We consecutively enrolled patients submitted to fluorescein-guided CNS-tumor removal (May 2020 to December 2022). CONVIVO® was used in vivo to obtain images from virtual biopsies at the central tumor core and at its margin of resection, evaluating its ability to offer a histological diagnosis at the center and a tumor tissue identification at the periphery, with respect to corresponding standard histological sections. CONVIVO® images were analyzed before interpretation of permanent or frozen sections, with the pathologist being totally blinded to histological results.

RESULTS: Seventy-five patients were studied. The most frequent diagnoses were GBM (50.6%) and metastasis (13.3%). At the tumor margins, on a total of 169 biopsies, we obtained an overall accuracy in tumor tissue identification of 82.2% (95% CI 75.0-89.5) in GBM/Grade 4 IDH-mutated astrocytomas, and 85.8% (95% CI 80.5-91.1) considering all tumors together. At the tumor center, a correct intraoperative diagnosis was obtained in 67.6% (95% CI 56.9-78.2) of all the cases, and in 80.9% (95% CI 69.1-92.8) of the GBM/Grade 4 IDH-mutated astrocytoma subgroup.

CONCLUSIONS: CONVIVO® allowed to accurately assess the presence of pathological marginal tissue remnants during resection of aggressive CNS tumors. More studies are needed to evaluate if this could possibly improve the extent of resection.

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KEY WORDS: Multiphoton, fluorescence, microscopy; Fluorescein; Glioblastoma.

Extent of resection (EOR) is one of the most important factors associated with overall survival in central nervous system (CNS) tumors.[1-3] Therefore, in order to improve EOR, various tools have been suggested, such as neuronavigation, intraoperative imaging and fluorophores usage.[4, 5] Such technological advancements are nowadays the mainstay in neuro-oncological surgery, helping in the tumor removal, even beyond the limits of contrast-enhancement.[6, 7] Nevertheless, although the direct intraoperative identification of tumor tissue at the margin of resection would represent the ideal way to improve EOR, an on-the-fly visualization of tumor cells during CNS tumor removal is still not available.[8] Frozen section, although providing a reliable intraoperative diagnosis,[4, 9, 10] has still several drawbacks, including the time needed for tissue transportation to the pathology department and for its histological evaluation.[11, 12]

Confocal laser endomicroscopy (CLE), a technique able to visualize tissues at a microscopic level without fixation or staining, has been recently introduced as a valuable tool for obtaining histological data during neuro-oncological surgical procedures.[13-15] Already studied in other specialities,[16] the actual clinical usefulness of CLE remains unknown in neurosurgery. We recently evaluated the accuracy of a newly available CLE system (CONVIVO®, Carl Zeiss Meditec, Oberkochen, Germany) in offering *ex vivo* an intraoperative diagnosis during Glioblastoma (GBM) removal, based on a real-time, blinded interpretation of the pathologist, directly in the operating room, obtaining very promising results.[17] Hence, in this study, we assessed the *in vivo* capability of CONVIVO® in identifying CNS tumor tissue at the margins of resection.

Materials and methods

Study design, clinical and surgical protocol, primary and secondary endpoints

This prospective, observational clinical study was approved by the local Institutional Review Board. The study protocol has been already described elsewhere.[18] Briefly, the study included patients older than 18 years old, harboring a possible aggressive CNS tumor, according to Italian Drug Agency (AIFA) determination, located in a not-primary-eloquent region (Figure 1). All patients gave their authorization to FNa-administration and to participate to this study. Primary endpoints were: 1) the accuracy of CONVIVO® in the identification of tumor tissue at the tumor margins, compared to standard histology, only in aggressive glioma subtypes (GBM and Grade 4 IDH-mutated Astrocytoma); 2) the concordance of diagnosis between CONVIVO ® images and histological analysis, in all tumor subtypes, at the central core of the tumor. Secondary endpoints were: 1) the concordance of diagnosis between CONVIVO® images and frozen section analysis and between frozen section and histological analysis, in all tumor subtypes, at the central core of the tumor; 2) the accuracy of CONVIVO® in the identification of tumor tissue at the tumor margins, compared to standard histology, in all tumor subtypes; 3) procedural aspects, such as time spent for tissue analysis by CONVIVO® in the operating room, along with time needed for picture interpretation by the pathologist.

The removal of the tumor was performed with the aid of intraoperative images and FNa-guided technique.[9] Surgical removal was aimed at the maximal safe resection, including all the fluorescent area, as visualized by microscope-integrated specific filter (YELLOW-560, Pentero900 or Kinevo Microscope, Carl Zeiss Meditec, Oberkochen, Germany). Specifically, most of the CNS tumors were removed in an inside-outside fashion, while metastases were removed *en bloc*, then maximizing the resection by also including the fluorescent infiltrating tissue outside the main tumor area.[9, 19, 20]

During surgery, "virtual" CONVIVO® biopsies were performed by applying the probe, covered by a specifically designed sterile sheath (Figure 1), directly on the tissue (*in vivo*). A first virtual biopsy with CONVIVO® was performed during the initial phases of resection, at the tumor central core (*i.e.* when the more centrally located area of the tumor was reached, or just few mm below the surface for the superficially located lesions). Then, at the final stage of resection, up to four virtual biopsies (two in fluorescent areas and two in non-fluorescent areas as shown by YELLOW-560 filter evaluation) were again performed with CONVIVO® at the tumor margin.

The results coming from CLE evaluation was only used for comparison with histological techniques and not to guide surgical resection strategy. All biopsies were also recorded on the neuronavigation system (StealthS8, Medtronic, Minneapolis, MN, USA), to check them on pre-operative radiological images. All recorded data and pictures were collected in an anonymized database.

Postoperative MRI was performed within 48 hours from surgery; patients were scheduled for clinical follow-up and adjuvant therapies based on histological diagnosis, following the standard clinical practice, without a specific follow-up for this study.

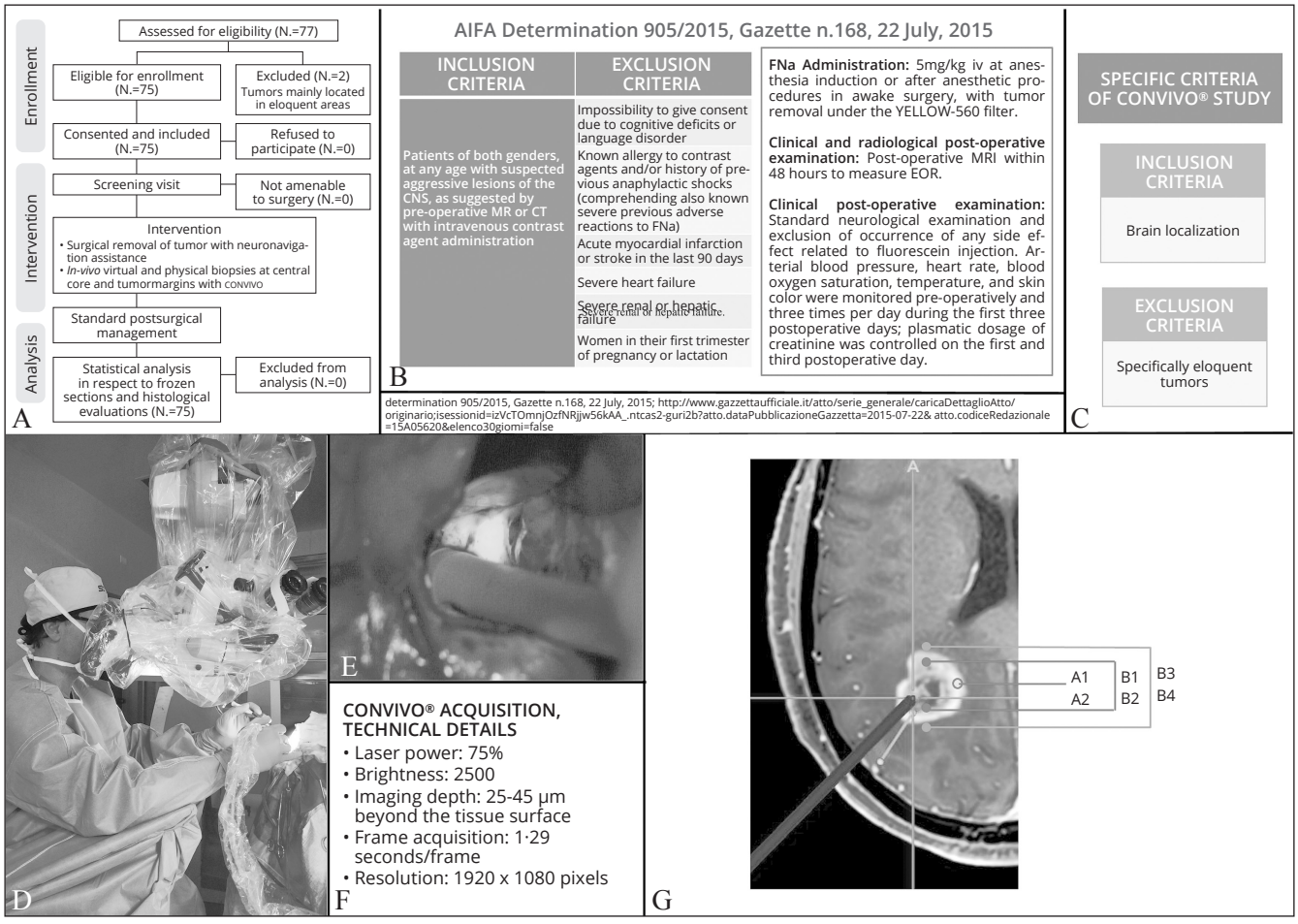

Figure 1.—A) Flow chart of the study; B) AIFA Determination July 15th, 2015, with specifications regarding FNa use for CNS tumor removal in Italy; C) specific inclusion and exclusion criteria of the *in-vivo* CONVIVO® study; D) intraoperative CONVIVO® use. The surgeon may use CONVIVO® stylet similarly to an endoscopic probe. The specific sterile sheat allows the probe to be applied directly to the brain parenchyma for the *in vivo* tissue analysis; E) intraoperative view under YELLOW-560 filter visualization of an intensely fluorescent intra-axial glial lesion. CONVIVO® probe was placed at the center of the tumor for obtaining virtual biopsies. The same position was checked with intraoperative neuronavigation; F) CONVIVO® acquisition technical details of the *in vivo* study; G) scheme of the *in vivo* biopsy protocol. During tumor resection, one virtual biopsy was performed with CONVIVO® at the central core of the tumor, then cut in two parts and processed by frozen section technique and common histological analysis by one pathologist (A1 and A2 samplings). Then, during the final part of the tumor resection, at its margins, up to 4 virtual biopsies were performed by CONVIVO® system, 2 in fluorescent areas (B1 and B2 samplings) and 2 in non-fluorescent areas (B3 and B4 samplings), as shown by YELLOW-560 filter evaluation. In the same locations, tissue samplings were collected and sent to the pathology department for histological analysis only, following standard protocols available at our Institution. During CONVIVO® analysis, the pathologist was blinded regarding the characteristics of fluorescence of the tissue and regarding the histological analysis of the corresponding CONVIVO® images.

CONVIVO® characteristics and imaging acquisition

Technical details of CONVIVO® have been already discussed in previous works.[17, 21-25] Briefly, the machine consists of a miniatured distal confocal microscope. A laser source delivers blue laser light (488 nm) to a lens system which excites FNa located in the tissue, providing a fluorescence signal that is detected and elaborated as a digital image. As a result, cells are visualized as "negative" images above a contrast-enhancing background, given by the FNa extravasation in the interstitial space.[17, 23, 26-28] For every virtual biopsy, images were recorded continuously as a time series, and sequentially, as a "z-stack" series, to scan along the z-axis to a depth of up to 30 µm above and below the plane of interest.

Blinded intraoperative interpretation of CONVIVO® images and other procedural variables analyzed

A dedicated pathologist (BP) judged in near real-time the images obtained from the *in-vivo* tissue analyzed

with CONVIVO®. Specifically, at the tumor center the pathologist was asked to provide intraoperatively tumor diagnosis. At the periphery, the pathologist was asked to judge the margins of resection, assessing for the presence of residual pathological tissue. Both at the center and at the margins, the pathologist was asked also to categorize various morphological patterns according to the following categories: tumor tissue, necrosis, reactive changes, marginal infiltrated tissue, vascular proliferation, healthy tissue, and cellularity. Thus, CONVIVO® images were analyzed before interpretation of permanent or frozen sections, with the pathologist being totally blinded to histological results.

Other variables studied included the presence of artifacts from movements, surgery time with CLE, time from FNa injection and time from biopsy sampling to CONVIVO® scanning, and median time needed for CONVIVO® images interpretation.

Tissue sampling, frozen section and histopathological processing and interpretation

Physical tissue samplings were performed immediately after CONVIVO® virtual biopsies interpretation. The tissue sampled at the tumor center was cut in two parts and processed by frozen section technique (specimen "A1") and standard histological analysis (specimen "A2"). The tissue samplings performed at the marginal area of the tumor were sent for histological analysis only (specimens "B1" to "B4"). Histological diagnosis and analyses were completed following standard protocols, and according to the 2021 WHO Classification.[29] In each biopsy, the elements of the microscopic image were categorized as done intraoperatively with the CONVIVO® system. To note, the pathologist performing histological analysis was strictly blinded regarding both the fluorescence characteristics of the sample and the results of CONVIVO® evaluation.

Comparison between CONVIVO® and histological evaluation

Concordance at the tumor core between CONVIVO® and standard histology or frozen section was defined if both techniques allowed the same diagnosis or only minimal grading diagnostic mismatch was found among the two techniques (*i.e*. Astrocytoma IDH-mutated grade 3 *vs*. GBM). Concordance at the tumor margin between CONVIVO® and standard histology was defined if both techniques were coherent in identifying tumoral or non-tumoral tissue.

Regarding the evaluation of morphological characteristics at the tumor core and at its margins, a concordance between CONVIVO® and standard histology or frozen section was defined if the same morphological patterns or cellularity grade were identified by the two compared techniques (complete concordance) or if only a difference in no more than one morphological characteristic or slightly different cellularity were identified by the two compared techniques (partial concordance).

Statistical analysis

The sample size of 75 patients was based on an expected concordance of diagnoses in terms of tumor categorization between CONVIVO® and standard histology of at least 80%, based on the Binomial Wald method. This was considered to guarantee a margin of error of no more than ±10% of the relative 97.5% confidence interval. Following the same method, based on an expected accuracy of CONVIVO® of at least 80% in the identification of pathological tissue at the margin of resection in aggressive gliomas (*i.e*. GBM or Grade 4 IDH-mutated Astrocytoma), an expected number of 53 aggressive gliomas patients (>70% of the entire population), for a total of 210 biopsies (4 for each case), were considered adequate for providing a margin of error of no more than ±6% of the relative 97.5% confidence interval. Descriptive statistics were provided in terms of absolute values and percentages for categorical data and means with standard deviations and medians with value ranges for continuous data.

Concordance was expressed in percentage and calculated by comparing the diagnostic results of a CONVIVO® virtual biopsy and the histological analysis (frozen section and definite histology) of a physical biopsy obtained at the same location in the tumor core. Regarding results at the tumor margin, accuracy (ACC) was defined as the sum of the number of positive and negative concordant CONVIVO® biopsies over the total number of biopsies; sensitivity (SN) was defined as the number of positive concordant CONVIVO® biopsies over the total number of histologically pathological biopsies, while specificity (SP) as the number of negative concordant COVIVO® biopsies over the total number of histologically healthy biopsies. Positive predictive value (PPV) and negative predictive value (NPV) were defined as the total number of positive concordant CONVIVO® biopsies and negative concordant CONVIVO® biopsies over the total number of CONVIVO biopsies considered tumoral or healthy, respectively. For proportions comparison, appropriate Chi-square Test was applied (STATA, StataCorp. LLC 2019).

Results

Descriptive epidemiological analysis

Seventy-five patients (46 males, mean age 52.08±17.41 years, range 19-82) affected by suspected aggressive lesion of the CNS were prospectively enrolled between May 2020 and December 2022 (Table I).[29]

A total of 74 biopsies were performed at the tumor center (in one patient, this was not feasible due to extensive brain swelling after dural opening, requiring fast tumor removal). At the tumor margins, 169 biopsies were performed (107 in the GBM and Grade 4 IDH-mutated astrocytomas).

TABLE I.—*Histological results of the prospective cohort (N.=75).*[29]

Histological results	N.
Glioblastoma	38
Metastasis	10
Grade 4 IDH-mutated astrocytoma	4
Grade 3 oligodendroglioma	4
Grade 2 IDH-mutated astrocytoma	3
Grade 3 IDH-mutated astrocytoma	2
Grade 2 oligodendroglioma	2
Ganglioglioma	2
Germ cells tumor	1
Medulloblastoma (desmoplastic/nodular)	1
Grade 2 pleomorphic xanthoastrocytoma	1
Grade 3 pleomorphic xanthoastrocytoma	1
Pilocytic astrocytoma	1
Radionecrosis	1
Grade 2 oligoastrocytoma	1
Grade 2B posterior fossa ependymoma	1
Immature teratoma	1
Angioleiomyoma	1

Accuracy of CONVIVO® in the identification of tumor at the margins

Considering only GBM and Grade 4 IDH-mutated Astrocytoma, we found an accuracy in identifying tumor tissue at the margins of resection of 82.2% (SN 86.5%, SP 72.7%, PPV 87.7% and NPV 70.6%, Table II). In addition, a complete/partial concordance in identifying gross morphological characteristics and pattern of cellularity was found respectively in 80.4% and 96.1% of the biopsies.

Considering all tumors together, the accuracy of CONVIVO® in identifying tumor tissue at its margins was 85.8% (SN 89.6%, SP 79.4%, PPV 88.0% and NPV 82.0%, Table II), with a complete/partial concordance in gross morphological characteristics and the pattern of cellularity resulted of 83.4% and 97.5%, respectively.

TABLE II.—*Analytical results of the study. Results subdivided according to primary/secondary endpoints and tumor margins/tumor center.*

Endpoints	Results	Morphological and cellularity patterns
Primary endpoints		
Tumor margins: tumor identification at the margin of resection in aggressive gliomas ° (CONVIVO® *vs.* HISTOLOGY*)	Accuracy: 82.2% [95% CI 75.0-89.5%] Sensitivity: 86.5% [95% CI 76.5-93.3%] Specificity: 72.7% [95% CI 54.5-86.7%] Positive predictive value: 87.7% [95% CI 77.9-94.2%] Negative predictive value: 70.6% [95% CI 52.5-84.9%]	Complete/partial concordance for morphological discrimination: 80.4% Complete/partial concordance for cellularity discrimination: 96.1%
Tumor center: diagnosis at the tumor center in all tumor subtypes (CONVIVO® *vs.* HISTOLOGY*)	Concordance: 67.6% [95% CI 56.9-78.2] (80.9% [95% CI 69.1-92.8] for aggressive gliomas° only)	Complete/partial concordance for morphological discrimination: 87.8% (100.0% for aggressive gliomas° only) Complete/partial concordance for cellularity discrimination: 91.4% (92.5% for aggressive gliomas° only)
Secondary endpoints		
Tumor margins: tumor identification at the margin of resection in all tumor subtypes (CONVIVO® *vs.* HISTOLOGY*)	Accuracy: 85.8% [95% CI 80.5-91.1] Sensitivity: 89.6% [95% CI 82.2-94.7] Specificity: 79.4% [95% CI 67.3-88.5] Positive predictive value: 88.0% [80.3-93.4] Negative Predictive Value: 82.0% [70.0-90.6]	Complete/partial concordance for morphological discrimination: 83.4% Complete/partial concordance for cellularity discrimination: 97.5%
Tumor center: diagnosis at the tumor center in all tumor subtypes (CONVIVO® *vs.* FROZEN)	Concordance: 61.1% (73.2% for aggressive gliomas° only)	Complete/partial concordance for morphological discrimination: 84.7% (95.1% for aggressive gliomas° only) Complete/partial concordance for cellularity discrimination: 86.8% (89.7% for aggressive gliomas° only)
Diagnosis at the tumor center in all tumor subtypes (FROZEN *vs.* HISTOLOGY*)	Concordance: 88.9% (87.8% for aggressive gliomas° only)	

° GBM and Grade IV IDH-mutated astrocytoma; *for each biopsy and for each comparison histological analysis was always used as the reference exam.

Concordance of diagnosis between CONVIVO® images and histological analysis at the central core

A 67.6% of diagnostic concordance was found comparing the intraoperative results with CONVIVO® with postoperative histological analysis. In 5.4% (four cases) a completely different diagnosis between CONVIVO® and histology was made; in 4.0% (three cases) CONVIVO® images did not allow any diagnosis due to movement artifacts during CONVIVO® sampling (one case, 1.3%) or poor fluorescent contrast (two cases, 2.7%). Furthermore, in 22.9% (17 cases) a comparison was not possible since the pathologist could not provide a definitive intraoperative diagnosis based on CONVIVO® images. However, correct histo-morphological and cellularity pattern identification at the tumors center was 87.8% and 91.4%, respectively, with histology as the reference (Table II).

Diagnostic concordance of CONVIVO® with respect to histology at the tumor center of only GBM and Grade 4 IDH-mutated Astrocytoma (42 biopsies, 56.8% of total biopsies) resulted to be higher (80.9%) with 100.0% and 92.5% of correct CONVIVO® assessment of morphological characteristics and grade of cellularity, with respect to histology.

Concordance between CONVIVO® and frozen sections at the tumor center was found in 61.1% of the total biopsies (73.2% considering GBM/Grade 4 IDH-mutated Astrocytoma only).

As a control, we compared diagnostic accuracy between frozen and histological sections, finding a concordance of 88.9% for the entire group of tumors, and 87.8% for GBM/Grade 4 IDH-mutated astrocytoma.

Procedural aspects

FNa was administered iv at anesthesia induction in all cases (5 mg/kg). We observed just one case of yellow-colored skin in the peri-intravenous access soon after FNa administration, that resolved withing few minutes spontaneously. No patient experienced adverse events from FNa administration or intraoperative CLE use (high ergonomics and versatility of the probe, permitting to hold it in one hand, placing it upon cerebral surface without traumatizing it, looking at the movable monitor mounted on top of the CONVIVO® at the same time).

Looking at operative data, considering the entire cohort, time from FNa injection to in vivo CONVIVO® scanning was 94.4±2.4 (range 40.0-366.0) minutes for biopsies taken at tumor core, 125.5±2.4 (range 50.3-378.2) minutes for biopsies taken at tumor margin with a mean value of 107.2±2.5 (40.0-378.2) minutes, taken together. Mean time needed for each surgical procedure for performing the CONVIVO® analysis was 10.5 minutes, with a mean of 2.1 minutes for each biopsy site. Mean time needed for CONVIVO® images interpretation by the pathologist at the tumor center was 3.6±0.7 minutes, while identifying the presence or absence of tumor tissue and its morphological characteristics at the periphery never required more than a minute per virtual biopsy (40.9±8.3 seconds).

Discussion

Accuracy of CONVIVO® in evaluation of CNS tumor margins

For the first time, we demonstrated that the *in vivo* application of a newly available CLE system (CONVIVO®) allows for an accurate assessment of the margins of resection during removal of different types of CNS tumors, permitting to correctly identify the tissue characteristics at the margin of resection, discriminating between tumor remnants and peritumoral brain parenchyma, in more than 80% of the cases. In addition, SN and SP were also particularly high, respectively 89.6% and 79.4%, in the whole tumor population. Therefore, CONVIVO®, at the margin of resection, could act as a "*screening test*", permitting to identify the presence of even minimal number of infiltrative tumor cells, increasing the rate of microscopic resection, particularly in aggressive gliomas (Figure 2, Table II). This aspect owes a high translational relevance, since the direct correlation existing among EOR and survival.[3]

These results appear very interesting considering two aspects, strictly connected to the design of our study. First, this was the first time in neurosurgery where a prospective and blinded-to-histological section protocol was carried out to obtain *in vivo* data from a new generation CLE system, specifically regarding the margins of resection. Recently, Xu *et al*. published a first experience about the assessment by confocal endomicroscopy of the marginal tumoral area in 28 gliomas, reporting interesting preliminary results about sensitivity.[30] However, all the analyses in these cases were performed retrospectively, with multiple confocal systems, and with a different FNa injection protocol (*i.e*. 5 mg/kg within minutes before confocal evaluation of the tissue). As a matter of fact, our study represents the very first where CONVIVO® images were evaluated intraoperatively by a pathologist who was blinded both to the intraoperative tissue fluorescence and to the corresponding histological images derived from the same area. Such aspect strengthens the reliability of CONVIVO® as

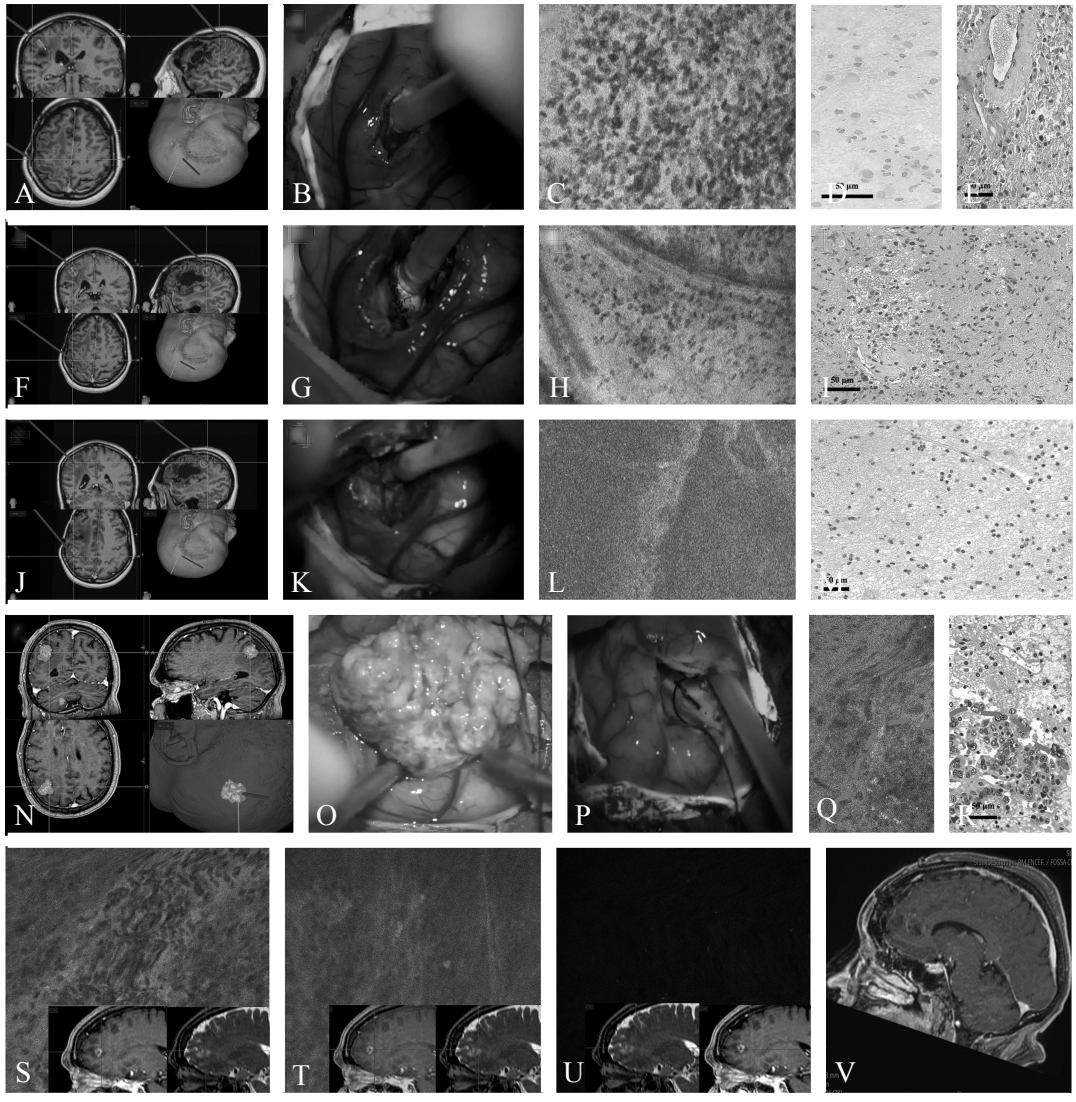

Figure 2.—Case samples from the study cohort. A-M) GBM analyzed with CONVIVO® at the tumor center and margins. In this patient a left frontoparietal GBM was removed under FNa guidance. A-E) Center of the tumor. The position of the biopsy was recorded with neuronavigation (A); in the same position the optical biopsy with CONVIVO® was obtained (B, C) and exactly in the same place tissue biopsies for frozen section (D) and histological sections (E) were performed, confirming the presence of neoplastic cells, characterized by a very high cellularity. F-I) Fluorescent tumor margin. The position of the biopsy was recorded with neuronavigation (F); in the same position the optical biopsy with CONVIVO® (G, H) and histological sections were performed (I), still confirming the presence of neoplastic cells with clear necrosis foci. J-M) Not fluorescent tumor margin. The position of the biopsy was recorded with neuronavigation (J); in the same position an optical biopsy with CONVIVO® (K, L) and histological analysis (M) were performed. Both CONVIVO® and histological analysis disclosed the absence of tumor cells, with presence of reactive astrocytes, visible on histological sections. Negative CONVIVO® images could witness the presence of a large amount of fluorescence spots, probably related to cells with phagocytic activity.[8, 15] N-R) Brain metastasis analyzed with CONVIVO® at the margins. This patient was affected by a left parieto-occipital metastasis from breast carcinoma (multiplanar T1 after contrast administration on neuronavigation system, N), that was macroscopically completely removed under FNa guidance, following our Institutional protocol (intraoperative view under YELLOW-560 filter). O. En-bloc metastasis removal under white light illumination. At the end of nodule resection, macroscopically the tumor was completely removed. Nevertheless, still fluorescent tissue at a certain border was found. In that position CONVIVO® virtual biopsy was performed (P, Q), suggesting the presence of sparse neoplastic cells with cuboidal features that were then confirmed by the corresponding histological section analysis (R, red dotted line demonstrating the nodule of tumor cells as outlined by same-section virtual and histological biopsy). Further radicalization lead to complete removal of the tumor tissue. S-V) Right frontal Ganglioglioma analyzed with CONVIVO® at the margins. Similarly, in this case after tumor removal CONVIVO® demonstrated the presence of frank tumor tissue at the FNa negative margin, characterized by nests of cells on a fluorescent background (S). Interestingly, in this position still some tumor cells on a darker background were found with CONVIVO® (T). A further radicalization of the removal permitted to reach a CONVIVO®- and histologically-negative margin (U), confirmed also by postoperative T1 with contrast MRI images (V), demonstrating how CONVIVO® owes the ability to go beyond FNa, enlightening the presence of tumor cells also in areas of frank FNa negativity.

an effective intra-operative tool to correctly guide surgical strategy regarding resection. Secondly, our results may be confidently considered even underestimated, as this study was designed accordingly to the only possible neurosurgical application of FNa in Italy (Figure 1). Keeping such FNa dosage and timing of injection may be beneficial considering the oncological aspect of tumor resection, as already previously demonstrated.[9, 31] However, this strict protocol could reduce the quality of images during confocal assessment, as suggested by various authors.[24, 28] For instance, Belykh et al demonstrated that the administration of 40 mg/kg of FNa at anesthesia induction in a low-grade glioma patient improved CLE visualization of identification of tumor cellularity.[22, 24] In addition, the same group showed that FNa redosing during surgery, closer to confocal assessment, allowed for brighter and more contrasted images, with a possible impact on tissue examination.[28] In light of this aspect, we could hypothesize that a supplementary administration of FNa at the end of tumor removal would have probably increased the overall quality of images obtained at tumor margins, possibly empowering the overall results.

Another aspect linked to FNa injection protocol was that the CONVIVO® images interpreted as not pathological at the margin of resection (*i.e.* considered as peritumoral brain parenchyma) were those in which no cells could be recognized. Specifically, these images were characterized by the absence of interstitial fluorescence in the analyzed tissue: a completely black background, with only few scattered fluorescence spots, possibly related to phagocytic cells, was disclosed (Figure 2).[8, 15] In fact, as FNa IV injected at low dosage and at the induction of anesthesia could only concentrate in tumoral areas characterized by blood-brain barrier disruption (BBB), it was not possible to identify normal parenchymal cells in areas beyond the tumoral infiltration. One could consider such kind of "indirect" evaluation of the negative margin a limit of our protocol, as we could not directly visualize normal cellular structure like in standard histological techniques. Nevertheless, the NPV obtained in CNS tumors marginal biopsies resulted to be notably high (82.0%), meaning that when CONVIVO® images were considered as negative, histology confirmed the absence of tumor cells in the great majority of cases. In addition, also this potential limitation could be overcome by re-administering FNa at the end of macroscopic tumor resection, allowing for a larger amount of unbound interstitial FNa passage through the BBB, possibly permitting a better morphological characterization of the healthy margin of resection.[26]

Accuracy of CONVIVO® intra-operative diagnosis at CNS tumor center

Regarding the possibility to obtain a direct intraoperative tumor diagnosis by CLE analysis, with the present *in-vivo* protocol we sought to overcome the intrinsic limitation of our previous *ex-vivo* experience,[17] such as the reduced readability of CLE images when time from FNa injection to image interpretation was too high, and the discrepancy that exists between the small field of view of CLE and the one of histological sections.[13] As a matter of fact, the mean time from FNa injection to CONVIVO® scanning was significantly reduced in this study (94.4 minutes) compared to our previous *ex vivo* experience (134.0 minutes); in addition, the *in vivo* application directly at the tumor bed could enlarge the area of the scanned tissue by CLE, making it more comparable to the larger section assured by standard histological analysis. Nonetheless, although a high overall rate of concordance comparing CONVIVO® and histology regarding morphological characteristics of the sample was found at the center of the tumor (87.8% and 100.0% for all CNS tumors and GBM/Grade 4 IDH-mutated Astrocytoma subgroups, respectively), a concordant histological diagnosis was found in 67.6% in the entire cohort of patients. To note, as a "baseline" control, we also calculated the concordance between frozen and histological sections, which resulted around 89%, in line with current literature.[12] These results were somehow unexpected, considering also data coming from other studies.[32] Nonetheless, this may be explained by the inclusion of rare tumors lacking clear CLE characterization (Figure 3).[15] In fact, the diagnostic concordance when analyzing only GBM/Grade 4 IDH-mutated Astrocytoma was significantly higher (80.9%). Indeed, among the 17 cases with CLE-undefined diagnosis at the center, the great majority (76%) was represented by histologies different from aggressive gliomas (seven metastasis, one medulloblastoma, two oligodendrogliomas, one pleomorphic xantoastrocytoma Grade 2, one angioleiomyoma, and one posterior fossa ependymoma grade 2). In addition, also the 5.4% of cases where the analysis of CONVIVO® images produced completely different intraoperative diagnosis with respect to histology were represented by pathologies, that, due to their peculiar histological characteristics, could pose difficulties in CONVIVO® analysis: two ganglioglioma misinterpreted as GBM and two GBM (one of whom being an epithelioid GBM) misinterpreted as metastasis. Therefore, we could hypothesize that, with more experience using CONVIVO® also in rarer histological subtypes, this tool could give more significant results also in diagnostic accuracy at the center

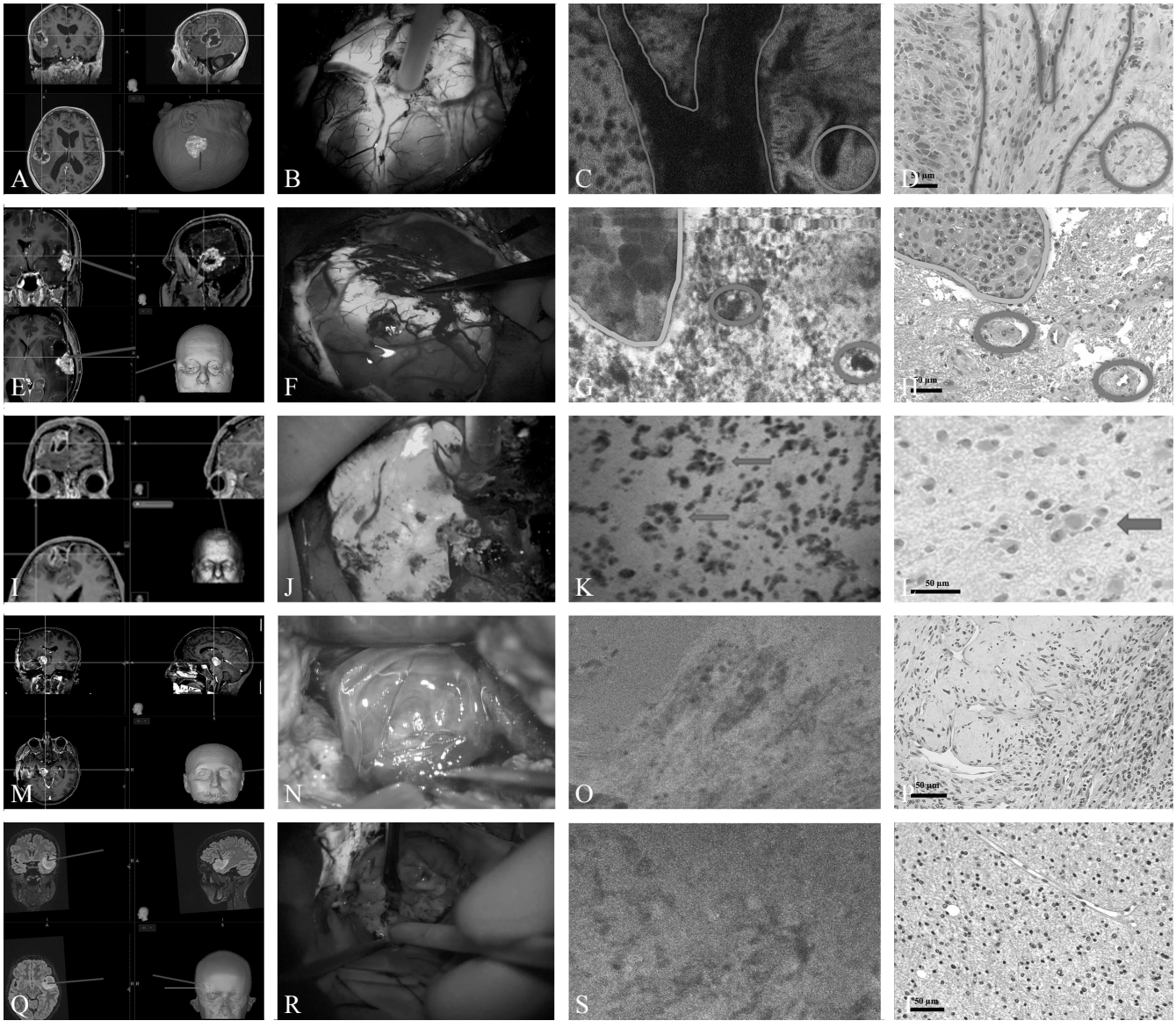

Figure 3.—Hallmarks of different CNS tumors as observed under CLE CONVIVO® imaging at the tumor center. A-D) GBM, neuronavigation (A) and intraoperative YELLOW-560 visualization (B) of a left frontotemporal GBM case, which was analyzed with CONVIVO® at the tumor center (C). The common histological findings (D) in GBM could be clearly seen under CONVIVO® imaging, such as the presence of neoplastic cells all around a neoplastic vessel (blue lines) and an area of focal necrosis (red circle). E-H) Breast carcinoma metastasis. Neuronavigation (E) and intraoperative YELLOW-560 visualization (F) of a right temporal carcinoma metastasis, which was analyzed with CONVIVO® at the tumor center (G). Also in this case, the common histological pattern (H) of breast carcinoma metastasis, such as lobular cells organized in a glandular structure (green line), could be easily seen with both the visualization methods. Higher density areas were also visible (red circles). I-L) Grade 3 oligodendroglioma. Neuronavigation (I) and intraoperative YELLOW-560 visualization (J) of a left frontal parasagittal oligodendroglioma, which was analyzed with CONVIVO® at the tumor center (K). In this patient, CLE imaging demonstrated the presence of perineuronal satellitosis, confirmed on corresponding histological sections (blue arrows in K and L, respectively). M-P) Intracranial angioleiomyoma. Neuronavigation (M) and intraoperative YELLOW-560 visualization (N) of a left temporal-mesencephalic region angioleiomyoma, which was analyzed with CONVIVO® at the tumor center (O). In this specific case, intraoperative images suggested the presence of a vascular lesion, such as an intracranial aneurysm or a vascular malformation (N). Nevertheless, CONVIVO® permitted to identify the presence of blood vessels, smooth muscle cells and collagen tissue at a microscopic level (O), which were confirmed in the corresponding histological sections (P), permitting a confident tumor removal. Q-T) Grade 2 astrocytoma. Neuronavigation (Q) and intraoperative YELLOW-560 visualization (R) of a left temporal intra-axial lesion characterized only by slight contrast enhancement on preoperative MRI, which was analyzed with CONVIVO® at the tumor center (S). Intraoperatively, such lesion demonstrated only a slight uptake of FNa (R). However, subsequent CONVIVO® assessment demonstrated the presence of neoplastic cells, in low cellularity, also in a low-grade glioma case. This result was then confirmed by the histological study of the corresponding biopsy (T).

of the tumor. Moreover, as it has been previously suggested,[33] also our study confirmed that the workflow assured by using CLE can be much more efficient, compared to the use of frozen section, with a reduction of workload for the neuropathologist (<4 minutes in average for CONVIVO® images interpretation) and a significant shorter timing for the whole process to be completed.[12, 33]

Limitations of the study

This study suffers from some limitations. First, the great majority of the surgical cohort was represented by GBM/Grade 4 IDH-mutated astrocytoma. To address this, our aim is to create an international shared database for CLE-using centers to improve our knowledge also on rarer histological subtypes. In addition, further developments in Artificial Intelligence for sure will be the basis on which future research also on CLE will be built.[8, 34] Moreover, although the number of biopsies at the tumor margin were notably high, providing significant data about ACC, SN and SP, they were slightly less than expected (around 19%), due to the difficulties in performing all the four biopsies in each case, for anatomical and surgical reasons. Another aspect that should be underlined is that intraoperative CLE use is an operator-dependent technique. Erroneous dosages or timings in FNa administration, erroneous timings in reaching the lesion and, above all, incorrect or unsteady positioning of the probe could affect readability of the images. For instance, in our cohort around 4% of images produced by CONVIVO® at the tumor center were considered as uninterpretable due to movement artifacts or darkness related to longer timing between FNa administration and confocal analysis. Such events should be considered as an inevitable consequence of the progressive learning that an "operator-dependent" technique requires, as suggested by the fact that they occurred among the first 37 cases, although no significant "learning effect" was observed in this study. It is important to stress that, for in vivo use, probe positioning should be always kept tangential in respect to the analyzed surface, following manufacturer instructions and to avoid blood interposition, trying to indulge normal brain pulsation that, if hampered, produces movement artifacts, as depicted in our previous work.[17] Moreover, as already underlined, a long timeframe between FNa administration and CLE imaging is one of the factors which could mostly influence image quality. Regarding FNa utilization with CLE in neurosurgery, as stated above, clearer CLE images could be obtained when shorter times between FNa administration and CLE imaging and higher doses of FNa are considered, although the exact timing appears to be dependent on the specific tumor type and must be linked to local regulations.[24, 28] We recognize that this aspect may be considered a clear limitation for the spreading of this technology in neurosurgery, at least until clear shared administration protocols permitting a standardized "quality" of the images will not be available. In addition, in this work we could not analyze any possible correlation among CLE use and EOR, as our study was only powered to measure CLE accuracy in identification of tumor tissue in a prospectively enrolled cohort of patients, without a correspondent control group. However, we believe that the results of this study may be considered a preliminary step toward a future assessment of the possible role of CLE on EOR, at least in aggressive gliomas.

Conclusions

In this work, for the first time, we showed that the in vivo application of a newly available CLE system (CONVIVO®) allows for an accurate assessment of the margin of resection during removal of different types of CNS tumors, raising the possibility of exploring CLE meaning in increasing EOR in CNS tumors.

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Conflicts of interest

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Authors' contributions

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Congresses

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History

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