microRNAs combined to radiomic features as a predictor of complete clinical response after neoadjuvant radio-chemotherapy for locally advanced rectal cancer: a preliminary study

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Abstract

Objective To define a predictive Artificial Intelligence (AI) algorithm based on the integration of a set of biopsy-based microRNAs expression data and radiomic features to understand their potential impact in predicting clinical response (CR) to neoadjuvant radio-chemotherapy (nRCT).

Summary background data The identification of patients who would truly benefit from nRCT for Locally Advanced Rectal Cancer (LARC) could be crucial for an improvement in a tailored therapy.

Methods Forty patients with LARC were retrospectively analyzed. An MRI of the pelvis before and after nRCT was performed. In the diagnostic biopsy, the expression levels of 7 miRNAs were measured and correlated with the tumor response rate (TRG), assessed on the surgical sample. The accuracy of complete CR (cCR) prediction was compared for i) clinical predictors; ii) radiomic features; iii) miRNAs levels; and iv) combination of radiomics and miRNAs.

Results Clinical predictors showed the lowest accuracy. The best performing model was based on the integration of radiomic features with miR-145 expression level (AUC-ROC = 0.90). AI algorithm, based on radiomics features and the overexpression of miR-145, showed an association with the TRG class and demonstrated a significant impact on the outcome.

Conclusion The pre-treatment identification of responders/NON-responders to nRCT could address patients to a personalized strategy, such as total neoadjuvant therapy (TNT) for responders and upfront surgery for non-responders. The combination of radiomic features and miRNAs expression data from images and biopsy obtained through standard of care has the potential to accelerate the discovery of a noninvasive multimodal approach to predict the cCR after nRCT for LARC.

Keywords Locally advanced rectal cancer (LARC) · Artificial intelligence · microRNA · Complete clinical response · Neoadjuvant radio-chemotherapy

Locally advanced rectal cancer (LARC) is defined as a transmural (cT3-4) and/or node-positive (cN +) cancer. The optimal treatment for LARC is based on neoadjuvant chemoradiotherapy (nRCT) followed by surgery after at least 6–8 weeks [1, 2].

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MRI provides the most accurate imaging of the rectum, allowing the definition of the pretherapeutic staging.

nRCT allows disease downstaging as well as reduction of the local recurrence rate and preservation of sphincter integrity [1]. After nRCT, 50–60% of patients are downstaged, with approximately 15–20% of pathological complete response (pCR) [3–6]. On the other hand, at the end of nRCT approximately 7% of patients do not show any response and more than 20% develop a grade 3–4 toxicity [7]. Non-responder patients can also develop tumor progression or distant metastases during nRCT [7].

Complete clinical response (cCR) assessment could be achieved by means of clinical examination, endoscopy, and, in a more objective way, by MRI and endoscopic rectal ultrasound (ERUS), although the estimated accuracy in predicting good responders at restaging is 52–72% and 65–73%, respectively [8, 9].

The identification of patients who will really benefit from nRCT could be crucial for the improvement of a tailored therapy. Currently, no specific biomarker alone is able to accurately predict the response to nRCT.

For that reason, there is an increasing trend toward the evaluation of tumor response using a variety of techniques.

Artificial intelligence and molecular markers could play an interesting role as a potential predictive tool, and microRNAs (miRNAs) and radiomics have been proposed for this purpose [10–12].

miRNAs are short non-coding RNAs [10] and they cause target degradation, translational repression, or gene silencing and thus affect subsequent protein expression [13].

Radiomics refers to the high-throughput extraction and analysis of quantitative features from standard-of-care medical images [14]. Radiomic features (tumor signal intensity, shape characterization, and texture) represent quantitative and objective measures and could reflect tumor heterogeneity and sub-regional habitats.

Prior studies have evaluated the use of radiomics analysis in MR imaging to distinguish cancer from benign tissue or add information about cancer aggressiveness [15–18], as well as to predict response after nRCT [19, 20].

The primary aim of this study was to understand if (i) radiomics analysis could improve the qualitative assessment in order to differentiate patients following the regression rate and (ii) specific miRNAs, extracted in pre-treatment tissue biopsies of LARC, could be considered a potential response biomarker.

The secondary aim was to test the combined miRNAs and radiomics data for a more accurate prediction of cCR to nRCT.

Material and methods

Study sample

160 consecutive rectal cancer patients, treated between October 2007 and June 2017 in the Surgical Clinic Unit of Trieste's University Hospital, were retrospectively analyzed.

We include in the present study 40 patients who fully reach the inclusion criteria (imaging data, RNA isolation, protocols, and staff are strictly standardized):

1. Locally advanced rectal cancer patients were included (cT3-4 and/or node-positive (cN+) cancer);
2. Staging process (included CEA, Ca 19.9, colonoscopy, thoraco-abdominal CT scan, and pelvic-MRI);
3. A high-field Magnetic Resonance (MR) of 1.5 Tesla performed before and after nRCT analyzed by the same two radiologist; several patients performed a pelvic-MRI in private diagnostic center or in other hospitals;
4. Patients treated with regular course RT were included;
5. Only patients with diagnostic biopsy specimen available were included

The other patients were excluded due to the missing of one or more cited inclusion criteria (i.e., patients who underwent to colonoscopy in other center and/or performed MRI in other center or MRI less than 1.5 Tesla and/or small or missing biopsy).

Following a multidisciplinary discussion, all patients received nRCT with capecitabine and pelvic locoregional radiotherapy (5 weeks, 45–50 Gy); subsequently, they underwent to a restaging pelvic-MRI after 6 weeks and submitted to surgery 8–10 weeks after nRCT completion. The surgical specimens were evaluated and classified according to TNM-seventh edition and pathological response was graded according to the Dworak classification of tumor regression grade (TRG) [21, 22].

Based on TRG, subjects were divided into two groups: complete and almost complete responders (TRG 3–4, *RESP*) and incomplete and non-responders (TRG 0–2, *NO-RESP*).

microRNAs selection methods

In the diagnostic biopsy [10, 13], the expression levels of 7 miRNAs (let-7a, let-7f, miR-21, miR-29a, miR-145, miR-320a, and miR-520d) were measured. As for the miRNA selection, a literature research was performed using the electronic databases of PubMed, Scopus, WoS and EMBASE, and the Cochrane Register of Controlled Trials on December 1st, 2019.

From 2008 to 2019, 188 papers were published regarding the miRNA expression in colorectal cancer. Of them, less than 20 try to measure miRNAs levels directly into the first biopsy sample. A total of 31 miRNAs were found to be upregulated or downregulated in patients with rectal cancer compared to controls. After a comprehensive and thorough review of the selected papers, the seven reported miRNAs associated with rectal cancer were selected.

MR image acquisition and texture analysis for radiomics features

A high-field Magnetic Resonance (MR) of 1.5 Tesla was performed before and after nRCT. T2-weighted sequences in the three planes of space and Diffusion weighted images (DWI) were at least requested.

After the anonymization of the images, the DICOM files were uploaded in the Health-Myne software, a provider of integrated solution for radiomics' studies.

Two abdominal radiologists, who were aware that all the patients had a histologically confirmed diagnosis of rectal cancer, first revised the images through the software and then identified the lesions in the pre-treatment images; finally, the segmentation of the lesion in the pre-treatment images (in the T2-weighted images, excluding the lumen of the rectum) was performed. In addition, a sphere of 1 cm [3] volume was traced in the area which was considered as the most representative of the tissue composition of the tumor.

The same software extracted the radiomic features from the volumes obtained after the segmentation of the entire lesion (EL) and from the single spheres. 3 groups of features were extracted: morphological features, gray-level co-occurrence-based features, and intensity-based statistical features.

RNA extraction and Real-Time PCR analysis

Total RNA was isolated starting from three FFPE (Formalin-Fixed Paraffin-Embedded) tissue Sects. (10 μm) using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific). RNA quantity and quality were assessed by NanoDrop ND-1000 (Thermo Fisher Scientific).

Expression of the selected miRNAs was measured using TaqMan quantitative real-time PCR (Applied Biosystems (AB)). Single-stranded cDNA was synthesized from 10-ng total RNA using specific miRNA primers (TaqMan MicroRNA Assay, AB). The following TaqMan microRNA Assays were used in this study and obtained from AB: hsa-let-7a-5p (000377), hsa-let-7f-5p (000382), hsa-miR-21-5p (000397), hsa-miR-29a-3p (002112), hsa-miR-145-5p (002278), and hsa-miR-320a-3p (002277). Furthermore, hsa-miR-520d-5p (002393) and hsa-miR-16-5p (000391) were used as an endogenous control for data normalization by measuring expression in all the samples.

Statistical analysis

Clinical characteristics were compared between the two groups using t test on continuous variables (or the non-parametric Mann–Whitney test when necessary) and Chi-square test (or Fisher's exact test when necessary) for categorical ones.

Given the high number of features, radiomic data underwent a three-step processing. First, pairwise correlation analysis was performed considering all pairs of variables in order to identify those highly correlated (Pearson correlation > 0.9) and to remove redundant features. Second, univariate association test between features and TRG class was carried out and only variables showing association (Mann–Whitney test, alpha $= 0.05$) were retained in the analysis. Third, Principal Component Analysis (PCA) was performed on the remaining features. PCA is a useful method for the multivariate setting in which a transformation is applied to data with two objectives: to represent correlated variables with principal components (PC) that are uncorrelated and to reduce the dimension of the variable space by selecting a restricted number of PCs. In this study the first component (PC1) was extracted [17]. The association between PC1 and TRG class was investigated with a logistic regression (LR) model. This strategy was applied for both EL and sphere segmentation. In the case of spherical segmentation, features were additionally filtered based on coefficient of variation (cv $<$ 1e.4) to remove those which were meaningless for spherical shape.

As for miRNAs data, the expression level was normalized subtracting the level of the control miRNA. Correlation analysis was conducted using Pearson correlation method. Shapiro–Wilk test was performed for normality of data and Levene's test for homogeneity of variance. Comparison of means between RESP and NO-RESP groups was performed using the t test.

Variables of the different domains (clinical, radiomics, genomics) were used as predictors to estimate LR models, and the predicted probabilities were used to calculate the Area Under the ROC curve (AUC-ROC). In order to obtain an internal validation of the models' performance, we applied a bootstrapping resampling strategy, which is a data-based simulation method that involves repeated random sampling with replacement from the original data to obtain multiple random samples, each of which provides an estimate of the parameter of interest. In this study, sampling was repeated for 150 times and the resulting ROC curves were used to obtain an estimate of AUC-ROC. Once the bootstrapped estimates were obtained, we subtracted the mean of the estimates to the AUC-ROC of the original dataset to

correct for the "optimism" in performance evaluation that should be always considered when the same dataset is used to both develop and validate a model [23].

All computations were carried out with the statistical software R version 4.0.3, including libraries *rms* and *pROC* [24].

Results

Baseline clinical and pathologic characteristics of the cohort are resumed in Table 1. Forty consecutive patients were analyzed, 24 males and 16 females, with a median age of 68 years. The study population was homogeneous, and all patients were treated with 50.4 Gy in 28 fractions.

At diagnostic workup, clinical stage higher than cT2 was present in 36 patients (90%), and 33 patients (82.9%) had lymph-node metastasis. Downstaging of the disease after nRCT was observed in 13 (32.5%) patients; non-responders were 27 (67.5%). As for responder patients, all TRG4 patients had ypT0No and all TRG3 ones had ypT1-2N0.

Analysis of microRNAs expression

For hsa-miR-145-5p (miR-145), no deviance from normality was detected (p-value = 0.16), neither for homogeneity of variance (p = 0.15). Groups RESP and NO-RESP showed a significant difference in the expression level (t = 2.71, df = 33, p-value = 0.010 – Fig. 1). The difference between NO-RESP and RESP means was 0.743, which corresponds to a fold change of 1.675 (RESP group had miR-145 expression level increased by 67.5% compared to No-RESP group).

No significant differences were identified in the expression levels of the other six miRNAs (Fig. 1).

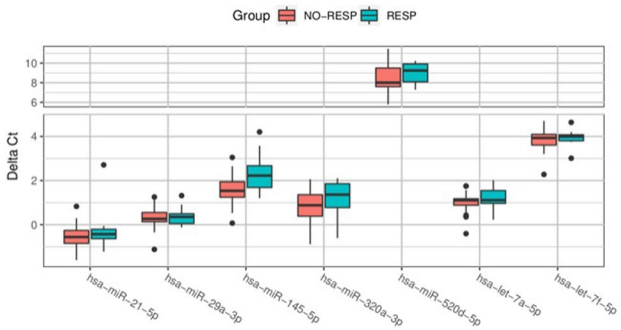

Fig. 1 Distribution of the 7 miRNAs in RESP and NO-RESP groups. Data for has-miR-520d-5p are referred to a different scale for a better visualization

Analysis of radiomic features

For T2 images, 206 features were extracted. After pairwise correlation analysis that identified redundant variables, 60 of them were tested and 16 showed association with the TRG class. As reported in Table 2, PC1 has a significant effect on the TRG class (OR = 0.38, 95% CI [0.17, 0.67]). For sphere images, pairwise correlation analysis reduced the set to 59 features, of which 7 demonstrated an association with TRG class. In this case as well, PC1 has a significant impact on the outcome (OR = 6.25, 95% CI [2.22, 28.9]).

Internal validation

The results of 6 different models are reported in Table 3 and represented in Fig. 2. The first includes age and stage; the second and the third, radiomic PC1 for EL and sphere segmentation, respectively; the fourth, level expression of miR-145; ultimately, the last two include both miR-145 expression level and radiomic PC1 (EL and sphere, respectively). Considering single-domain models, the top performing ones are those based on spherical segmentation radiomics

Table 1 Main clinical characteristics of the patients included in the study

	Total population (n = 40)	RESP (n = 13)	NO-RESP (n = 27)	p-value
Age	68 (45–85)	66 ± 9	69 ± 11	0.39
Male	24 (60%)	8 (61.5%)	16 (59.2%)	1
ASA > = 3	9 (22.5%)	4 (30.7%)	5 (59.2)	0.43
Stage (III–IV)	13 (32.5%)	2 (15.4%)	11 (40.7%)	0.12
TNM				
T > 2	36 (90%)	11 (84.6%)	25 (92.6%)	0.58
N +	33 (82.5%)	10 (*63.5%)	23 (85.2%)	1
ypTNM				
ypT0-1	17 (42.5%)	13 (100%)	4 (14.8%)	0.005
ypT2-4	23 (57.5%)	0 (0%)	23 (85.2%)	0.001
ypN0	31 (77.1%)	13 (100%)	17 (63%)	0.63
ypM +	2 (5.7%)	0 (0%)	2 (7.4%)	0.03

Values are mean ± SD, %, or median [interquartile range]

*NX in one individual

Table 2 Analysis of radiomic features

Model	OR	95% CI	p-value
Radiomics (EL)	0.38	[0.17–0.67]	0.005
Radiomics (sphere)	6.25	[2.22–28.9]	0.004

The table include the result of the logistic model based on the segmentation of the entire lesion (Radiomics (EL)) and the one based on the spherical segmentation (Radiomics (Sphere)). CI: confidence interval; OR: odds ratio

Table 3 Comparison of predictive performance

Model	AUC-ROC	95% CI
Clinical	0.76	[0.59–0.93]
Radiomics (EL)	0.83	[0.69–0.97]
Radiomics (Sphere)	0.85	[0.71–0.98]
miR145	0.74	[0.55–0.93]
Radiomics (EL) + *miR145*	0.90	[0.80–1]
Radiomics (Sphere) + *miR145*	0.91	[0.81–1]

Predictive performance in terms of AUC-ROC is reported for model based in clinical features (Clinical), radiomics on entire lesion (Radiomics (EL)), radiomics on spherical segmentation (Radiomics (Sphere)), miR-145 (miR145), radiomics on entire lesion and miR-145 (Radiomics (EL) + miR145), and radiomics on entire lesion and miR-145 (Radiomics (Sphere) + miR145). AUC: Area Under the Curve; other abbreviations as in Table 2

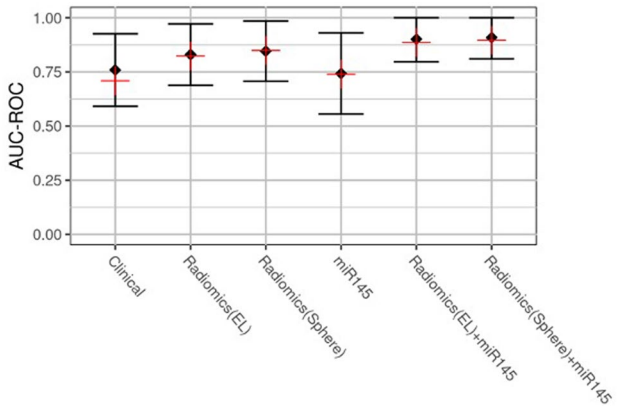

Fig. 2 Models' performance. The performance measured in terms of AUC-ROC and relative confidence interval are reported in black for each model. The red cross indicates the AUC-ROC corrected for optimism

(AUC-ROC = 0.85, 95%CI [0.71–0.98]) and EL segmentation radiomics (AUC-ROC = 0.83, 95%CI [0.69–0.97]). Instead, we observed lower accuracy using clinical predictors (AUC-ROC = 0.76, 95%CI [0.59–0.93]) and mirR-145 (AUC-ROC = 0.74, 95%CI [0.55–0.93]). Of note, the best performing model was the one which integrated two domains: EL segmentation radiomic PC1 and mirR-145 expression level (AUC-ROC = 0.90, 95%CI [0.80–1.0]) and sphere segmentation radiomic PC1 and mirR-145 expression level (AUC-ROC = 0.91, 95%CI [0.81–1.0]). Corrected estimates differ very slightly from the raw AUC-ROCs (Fig. 2).

Discussion

Main findings

In a world where medicine is becoming more and more personalized and resources are not unlimited, predicting the correct therapeutic pathway is becoming a crucial point of scientific interest. The discrimination of patients between non-responders and responders to nRCT could, in fact, spare ineffective and possibly harmful harming treatments and design *ad hoc* therapeutic strategies. The integration of miR-145 expression and radiomics features seems to be a positive predictive factor in recognizing good responders to nRCT.

The main strength of the study was to define, for the first time, to our knowledge, a predictive model based on the integration of a set of biopsy-based miRNAs expression data and radiomic features: two completely different approaches combined together into a statistical model which could potentially provide a robust and noninvasive predictor of clinical and pathological response after nRCT for LARC, in a framework of a new concept of personalized surgery.

Radiomic and miRNAs in LARC patients

As known, the ability of imaging techniques to exclude complete response is superior to their ability to confirm it [8, 9], which makes it extremely difficult to detect patients who could really benefit from a 'Watch and Wait' approach. To address this problem, a promising concept of AI analysis, such as radiomics analysis, has been recently developed [25]. With the analysis of the metadata obtained, diagnostic pathways could be improved, and information about prognosis and response to treatment could be easily assessed [14]. To our knowledge, few studies are available and report that radiomics analysis for LARC showed a good performance in identifying cCR after nRCT, which increased when combined with standard clinical evaluation [26, 27].

To this account, Horvat et al. demonstrated that the radiomics' performance in detecting cCR after nRCT was higher than MRI ($p < 0.0001$), with a sensitivity of 100% and specificity of 91% [27]. The results are encouraging but further studies with larger and independent datasets are needed in order to validate the potential of radiomics in this topic.

On the other hand, many molecular markers have been studied in order to identify patients who could really benefit from nRCT:genetic mutations [28], protein biomarkers, tumor immune microenvironment [28], or non-coding RNAs, such as microRNAs [29]. miRNAs are involved in many different cellular processes [10], including carcinogenesis, radiosensitization, and radioresistance [30, 31]. Moreover, they remain stable even in extreme environmental conditions (e.g., boiling, extreme pH levels), and they can be secreted intact in body fluids [32]. To advocate their possible use as biomarker, it has also been demonstrated that the expression of miRNAs' levels in tissues and plasma, the so-called circulating miRNAs, are concordant [33].

MiRNAs are negative regulators of gene expression and are dysregulated in many cancers, including colorectal cancer. In general, in our study, we do not observe

significant differences regarding six over seven screened miRNAs. Although, for miR-145, we observed that expression level is significantly higher in RESP compared to NO-RESP group (the fold change is 1.675 that correspond to an increasing in 67.5% expression level in RESP group).

From our findings, several studies try to understand the role of the over- or under-expression of microRNAs in CCR in response to nCRT, but there are difficult to compare for several reasons: in literature the sample sizes are mostly small (< 50 patients), the miRNA extraction methods used are disparate and the tumor regression grading system is different in each study and, in some of them, TRG are different in the same cohort.

Drebber et al. conducted one of the first studies on miRNAs expression related to nRCT in rectal cancer [34]. They evaluated the levels of miR-21, miR-143, and miR-145 in tumor tissue of 40 patients before and after therapy and demonstrated a significant correlation between a high miR-145 expression in the post-therapeutic tumor tissue and a major response to nRCT. However, the expression of miR-145, which gave significant results, was evaluated in the post-therapeutic tissue sample, not focusing on predicting the response to therapy in advance. For this reason, their results are not comparable neither with ours nor with the others in the available literature.

Eriksen et al. analyzed the role of both miR-145 and miR-21 in a test cohort of 55 patients and a subsequent validation cohort of 130 patients in surgical specimen of LARC after nRCT [35]. In the test cohort, under-expression of miR-145 was significantly associated with major response to therapy ($p < 0.001$) but, in the validation cohort, this result was confirmed without reaching statistical significance ($p = 0.085$). This could seem in contrast with our data, but the absence of statistical significance regarding miR-145 expression levels makes this report less consistent.

Concerning the role of miR-145, it has been demonstrated that it directly inhibits insulin receptor substrate-1 (IRS-1) and type 1 insulin-like growth factor (IGF-IR) [36–38]. In rectal cancer cells, miR-145 is frequently downregulated, promoting the pro-mitotic role IGF-IR and IRS-1 [39]. Hence, the upregulation of miR-145 is presumed to contribute to the suppression of tumor proliferation. As mentioned before, miRNAs are secreted intact in body fluids, including blood and plasma, making them the perfect noninvasive biomarkers to differentiate patients into responders and non-responders before nRCT. However, only a few studies evaluated the role of circulating miRNAs in LARC [38, 40, 41].

The combination of radiomic features and miRNAs expression data from images and biopsy obtained through standard of care has the potential to accelerate the discovery of a noninvasive multimodal approach to predict the cCR after nRCT for LARC.

Our work presents several limitations. The study is based on a retrospective design and a validation cohort for the proposed model is absent. The number of patients included in the analysis is small and we cannot exclude that other associations between TRG class and miRNAs were not identified because of the low statistical power. Moreover, the application of PCA on the radiomics data made possible to include in the model information from multiple features and to increase the predictive performance, but the same time made the relationship between radiomics and outcome difficult to be interpreted. Future developments of the study include an external validation of the model in an independent cohort.

The monocentric design of our study provides a homogeneous approach in terms of a therapeutic pathway to LARC, pathologic analysis of the surgical specimens, along with a uniform TRG classification, and a well-structured miRNAs extraction technique and radiomics analysis.

Conclusion

If confirmed by future evidence, patients with a high probability of a cCR could be proposed for a Total neoadjuvant therapy (TNT) regimen. At the same time, non-responders could directly undergo upfront surgery, reducing the risk related to nRCT, including the hypothesis of tumor progression. Three therapeutic-targeted pathways could be proposed for our daily practice: TNT, nRCT and surgery, and surgery alone. Supported by these promising results, in a multicentric prospective study, we will extend the analysis to the whole-human miRNAome, integrate radiomics, and apply advanced classification algorithms to identify LARC patients to be addressed to TNT or nRCT or upfront surgery.

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Data availability The manuscript has not been published nor is considered for publication or elsewhere in any language. There are no other works in preparation, submitted, in press, or published that are potentially overlapping the actual presented report.

Declarations

Disclosures Drs. Pasquale Losurdo, Ilaria Gandin, Manuel Belgrano, Ilaria Fiorese, Roberto Verardo, Fabrizio Zanconati, Maria Assunta Cova, and Nicolò de Manzini have no conflicts of interest or financial ties to disclose.

Ethical approval The present study was conducted in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments

or comparable ethical standards. Data were stored anonymously in our colorectal cancer database, not open access. A proper broad informed consent for unspecified use of data was obtained for each patient. Patients' data were stored only once anonymized, therefore confidentiality of the information linked to the data is guaranteed. Nobody among the researchers had access to the identification of patients' information. According to local legislation (GDPR 679/2016, Par.26), anonymized data do not require data protection and ethical committee approval.

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