Claudin 18.2: An attractive marker in pancreatic ductal adenocarcinoma

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Abstract. Pancreatic ductal adenocarcinoma (PDA) is a highly aggressive tumor with limited treatment options. Zolbetuximab, a monoclonal antibody against the tight junction protein Claudin 18.2 has recently been developed. At present, few and conflicting data have been reported regarding the clinical-pathological features of Claudin 18.2 expression in PDA. The present study investigated the expression of Claudin 18.2 in histological samples from PDA patients with the aim of verifying its utility as a therapeutic biomarker. Claudin 18.2 immunoreactivity was assessed by immunohistochemical staining on 70 formalin-fixed, paraffin-embedded PDA specimens (28 surgical specimens and 42 fine needle aspiration biopsies). The results obtained were associated with the clinicopathological characteristics and the survival rate of patients. Claudin 18.2 was detected only in neoplastic cells, not in normal pancreatic tissue. Claudin 18.2 was positive in 50% of samples and a higher expression was associated with well- and moderately-differentiated tumors and lymph node-negative status. The high expression of Claudin 18.2 in neoplastic tissue and absence in normal cells suggested that this protein had an attractive role in PDA as both a diagnostic and a prognostic-therapeutic marker. High expression of Claudin 18.2 in neoplastic tissue was associated with more favorable prognostic parameters and the high percentage of positive samples obtained suggests that Zolbetuximab may be suitable for a large number of patients.

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Introduction

Pancreatic ductal adenocarcinoma (PDA) is the most frequent malignant tumor of the exocrine pancreas (1,2) and the incidence continues to increase yearly (3). Due to the difficulty of obtaining an early diagnosis, the rapid progression of the disease and the poor prognosis, the majority of patients are already in an advanced stage of cancer at the time of diagnosis (4). When curative surgical resection is possible, the 5-year survival rate is less than 5% (5,6). Chemotherapy is a cornerstone treatment for patients with unresectable or metastatic PDA. The development of combined regimens, including Gemcitabine with albumin-bound paclitaxel and FOLFIRINOX, has improved the survival of patients with metastatic PDA (7). Other therapeutic strategies are adopted for KRAS-mutated and wild-type PDA patients. KRAS is mutated in >90% of PDA cases (8), therefore ongoing clinical trials have been Only focused on inhibiting the more common allele variants G12D, G12V. Only a small percentage of patients KRAS wild-type are eligible for therapies targeting molecular alterations such as BRCA1, BRCA2, NTRK, ROS1, ALK, RET, NRG1, BRAF, MSI-H status (8).

The Claudin protein family is composed of no less than 27 transmembrane proteins, which are assigned by category into classical and non-classical types based on their sequence characteristics (9-11). Claudins are important components of tight junctions and form a paracellular barrier to control the flow of molecules between cells (10). Claudin 18 belongs to the non-classical type and is normally expressed in gastric and lung cells (11). Claudin 18 has two isoforms: Claudin 18.1 (expressed in lung tissues) and Claudin 18.2 (CLDN18.2) (expressed in gastric tissues) (12-14).

CLDN18.2 is expressed in normal gastric mucosa cells and is retained in most gastric and gastroesophageal junction adenocarcinomas (15,16). Moreover, it is aberrantly expressed in 60-90% of PDA (17,18).

After malignant transformation, CLDN18.2 can undergo phosphorylation and exposure to the membrane cell surface, becoming enabled to bind monoclonal antibodies (19,20).

Based on these characteristics, CLDN18.2 was considered optimal for therapeutic target (21), and Zolbetuximab (ZBT) was developed as a first chimeric immunoglobulin G1 monoclonal antibody highly specific for CLDN18.2 (22). ZBT binds to CLDN18.2 on the tumor cell surface and stimulates cellular and soluble immune effectors that activate both antibody-dependent cytotoxicity and complement-dependent cytotoxicity (23). ZBT is currently being undergoing clinical testing in gastric and pancreatic tumors.

Our study aimed to evaluate CLDN18.2 expression on both pancreatic surgical specimens and FNABs, to investigate its possible prognostic role, as well as therapeutic together with upcoming targeted drugs.

Materials and methods

The current study enrolled 70 patients diagnosed with PDA between February 2015 and November 2023 at the National Institute of Gastroenterology of Castellana Grotte, Italy.

PDA specimens included either fine needle aspiration biopsies (42 FNABs from metastatic cancers) or surgical samples (28 resections from non metastatic cancers). For each surgical specimen, the chosen block included normal as well as neoplastic tissue. Follow-up data and the dates of patients' deaths were collected from the Institute records.

Serum CEA and CA19-9 levels (Elecsys Cobas 8000, Roche, Basel Switzerland) were known for all patients.

Tissue specimens were sectioned into 4 μm thickness slices, mounted on Apex Bond Slides (Leica Biosystems), and used for immunohistochemical analysis. Immunohistochemical staining procedures were carried out on a BOND III automated immunostainer (Leica Biosystems, Wetzlar Germany), from deparaffinization to counterstaining with hematoxylin, using the Bond Polymer Refine Detection Kit (Leica Biosystems). For Claudin 18.2 detection, a rabbit monoclonal antibody (clone EPR19202, Abcam, Cambridge, UK) at 1:200 dilution was used. Antigen retrieval was performed using BOND Epitope Retrieval Solution 2 (Leica Biosystems).

CLDN 18.2 immunostaining was evaluated using the Histoscore (H-score) (24), defined as a method combining both percentages of positive-expression cells in the tissue and immunostaining intensities (1+, 2+, 3+). Only membranous staining was retained for scoring. The H-score was calculated according to the formula: (0 x percentage of no reactive cells) + (1 x percentage of weakly stained cells) + (2 x percentage of intermediately stained cells) + (3 x percentage of strongly stained cells). Thus, the H-score ranged from 0 to a maximum of 300. A sample was considered positive with an H-score ≥5.

Statistical analysis. Patients' characteristics are reported as mean and standard deviation (M±SD) for continuous variables, and as frequency and percentage (%) for categorical variables. To test the association between the independent groups (Claudine Positive vs. Claudine Negative), Chi-Squared test or Fisher's test where necessary were used for categorical variables, while the Wilcoxon ranksum test (Mann-Whitney) was used to compare continuous variables.

Survival probability was explored using the non parametric Kaplan-Meier method, and the equality of survival curves was analyzed with the log-rank test.

To test the null hypothesis of non-association, the two-tailed probability level was set at 0.05. The analyses were conducted with Stata Statistical Software: Release 18, StataCorp, 2023, StataCorp LLC.: College Station, TX, USA.

Results

The clinicopathological features of the 70 patients (32 women and 38 men) are listed in Table I. Median patient age was 69 years (range 44-84 ys). Most patients (67.14%) had pancreatic head cancer, that was well- or moderately-differentiated (Table I). Twelve (17.14%) cancers were located at the isthmus, eleven (15.71%) at the body/tail and were all poorly differentiated. The pathological tumor stage and node stage were assessed only in surgical specimens. Seven (25%) of 28 surgical specimens were classified as pT3/4 vs. 21 (75%) as pT1/T2. Twenty-one (75%) cases had lymph node invasion (pN1/2) vs. 7 (25%) classified as N0. Serum carcinoma embryonic antigen (CEA) levels were positive (cut-off >3 ng/ml) in 44 patients (69%) at the time of diagnosis and carbohydrate antigens 19-9 (CA 19-9) (cut-off >27 U/ml) in 53 patients (82%).

CLDN18.2 staining was not detectable in any of the normal pancreatic tissue cells (Fig. 1B). The expression of CLDN18.2 was evaluated solely in PDA cells, excluding its expression in precancerous lesions like pancreatic intraepithelial neoplasia. CLDN18.2 was positive in 35 (50%) PDA patients. Twenty-one (60%) of these had an H-score >50. Twelve (34%) samples were scored up to 3+, fifteen (43%) were scored up to 2+, eight (23%) were scored up to 1+ (representative images are shown in Fig. 1). A higher number of positive cases was observed in the FNAB group (23 samples) compared to the surgical specimens (12 samples) (67.71% vs. 34.29%) (Table I) (Figs. 2-3). The analytic results showed that histologic grading and node stage were significantly associated with CLDN18.2 expression (P=0.04; P=0.02) (Table I). Mean serum CEA values were lower in patients who were CLDN18.2 positive (Table I). The other clinicopathological characteristics showed no significant association (Table I). Positive CLDN18.2 immunostaining was not associated with survival outcomes (Fig. 4). Median OS was 5 months in the positive group vs. six months in the negative samples (Table I).

Discussion

To the best of our knowledge, ours is the first study that evaluated the expression of CLDN18.2 also on pancreatic FNAB. It is known that curative resection is not possible for the majority of these patients, so very often the diagnosis is made on FNAB, being the only material available for further evaluation. Unlike in other studies (17,22,24-27), we also evaluated the presence of the protein on biopsy slices, in order to verify its expression on samples with poor cellularity. We demonstrated a higher percentage of CLDN18.2 positive specimens in the FNAB group, likely due to hypofixation problems that are more frequent in surgical samples. Therefore, the increased expression of this marker on FNAB could be useful both to resolve a doubtful histological diagnosis and to decide the possible eligibility of patients for the target drug. Chemosensitivity of PDA is moderate and so targeted therapies are of high interest. This study was conducted to evaluate whether CLDN18.2

Table I. Clinicopathological patient characteristics by CLDN18.2 expression.

Parameters[a]	Total cohort (n=70)	Claudine-negative (n=35)	Claudine-positive (n=35)	P-value
Sex, male, n (%)	38 (54.29)	19 (54.29)	19 (54.29)	0.99
Age, years	68.90±8.42	70.46±8.55	67.34±8.10	0.15
Samples, n (%)				0.46
FNAB	42 (60.00)	19 (54.29)	23 (65.71)	
Surgical specimens	28 (40.00)	16 (45.71)	12 (34.29)	
Localization, n (%)				0.99
Head	47 (67.14)	24 (68.57)	23 (65.71)	
Isthmus	12 (17.14)	6 (17.14)	6 (17.14)	
Body + tail	11 (15.71)	5 (14.29)	6 (17.14)	
Histological grading, n (%)				0.04
G1 + G2	47 (67.14)	19 (54.29)	28 (80.00)	
G3	23 (32.86)	16 (45.71)	7 (20.00)	
Tumor stage, n (%)				0.66
T1 + T2	21 (75.00)	11 (68.75)	10 (83.33)	
T3 + T4	7 (25.00)	5 (31.25)	2 (16.67)	
Node stage, n (%)				0.02
N0	7 (25.00)	1 (6.00)	6 (50.00)	
N1 + N2	21 (75.00)	15 (94.00)	6 (50.00)	
CEA	17.82±32.61	26.57±44.74	10.20±13.10	0.06
CA 19-9	2402.25±4435.15	2015.95±3562.89	2774.24±5180.95	0.55
Status (died), n (%)	54 (78.26)	30 (85.71)	24 (70.59)	0.13
Median survival	5 (0.00-14.00)	6.00 (0.00-17.00)	5.00 (0.00-14.00)	0.90

[a]Presented as mean and standard deviation (M±SD) or median and interquartile range for continuous variables, and as frequency and n (%) for categorical. CEA, Carcinoembryonic Antigen; CA 19-9, Carbohydrate Antigen 19-9.

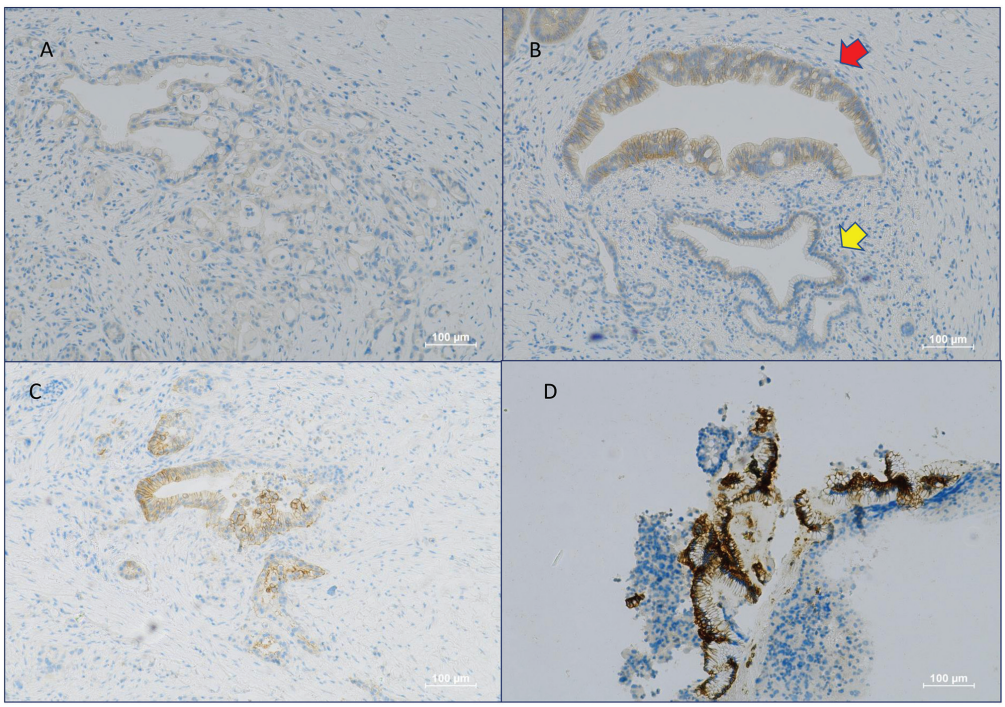

Figure 1. Immunohistochemical expression of CLDN18.2 in PDA samples. Examples of CLDN18.2 positive PDA with (A) 0/none, (B) 1+/weak (red arrow indicates a Claudin positive neoplastic gland with 1+ of staining intensity), (C) 2+/intermediate and (D) 3+/strong staining intensity. The yellow arrow indicates a normal negative pancreatic duct. Scale bar, 100 μm. PDA, pancreatic ductal adenocarcinoma; CLDN18.2, Claudin 18.2.

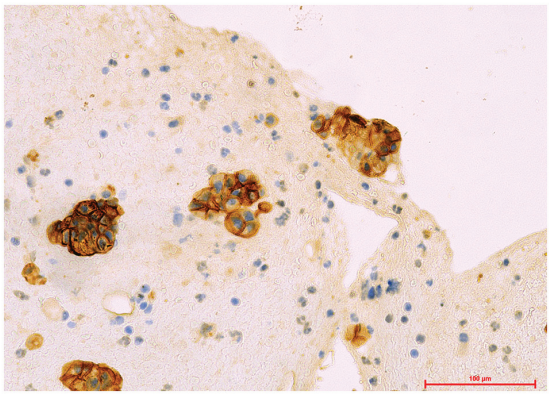

Figure 2. Membranous Claudin 18.2-positive immunostaining in pancreatic ductal adenocarcinoma with 3+/strong staining intensity from a fine needle aspiration biopsy (magnification, x10).

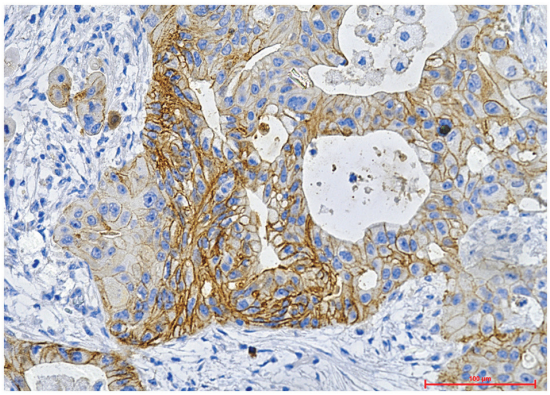

Figure 3. Membranous Claudin 18.2-positive immunostaining in pancreatic ductal adenocarcinoma with 3+/strong staining intensity from a surgical specimen (magnification, x20).

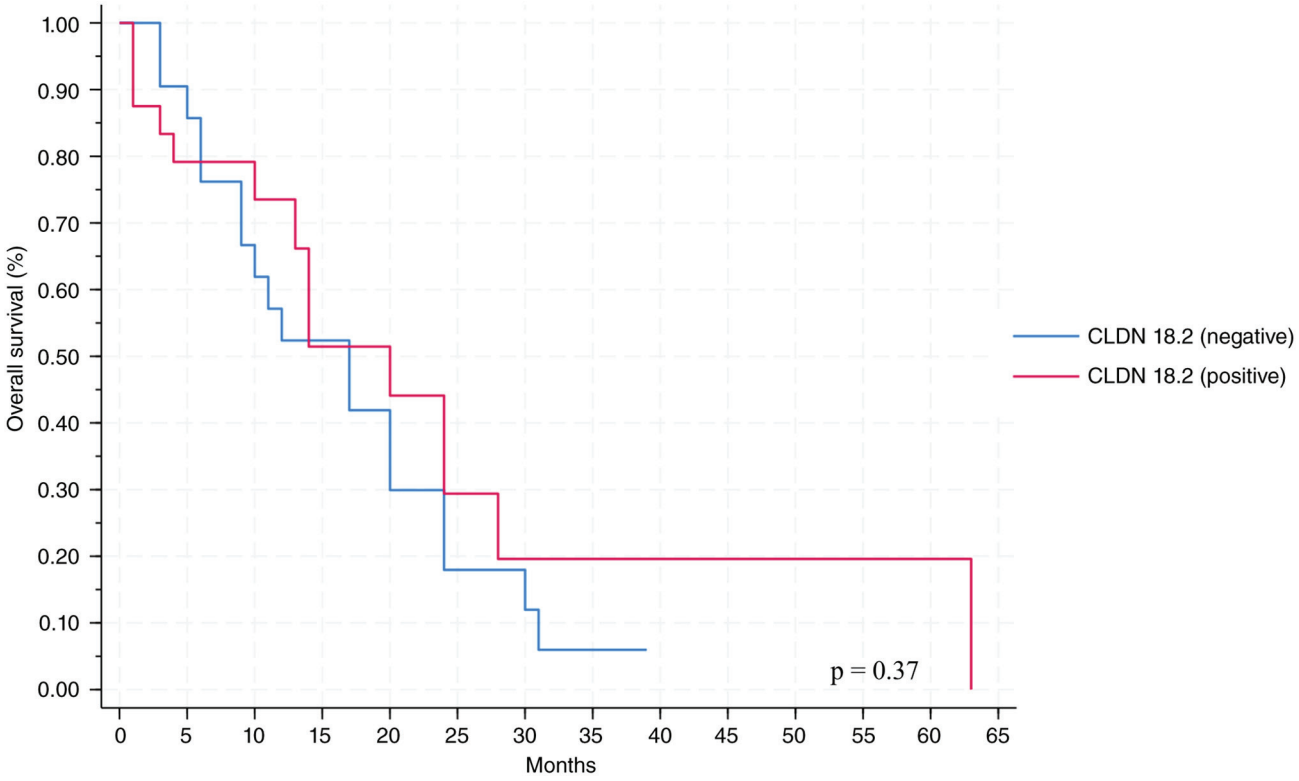

Figure 4. Overall survival in patients with pancreatic ductal adenocarcinoma according to CLDN18.2 expression. CLDN18.2, Claudin 18.2.

immunoreactivity can be considered an adequate indication for ZBT target therapy. Claudins are appropriate targets for anticancer treatment due to their dysregulated location following carcinogenesis (6). In fact, while in normal cells they are present at the level of tight junctions and therefore not reachable by the targeting antibodies, carcinogenesis alters their localization and makes them good targets (6).

The phase II clinical trial (28,29) demonstrated that ZBT in combination with first-line chemotherapy significantly improved the overall survival, progression-free survival and the objective response rate, with acceptable safety and tolerability in patients with CLDN 18.2-positive advanced recurrent gastric cancers and gastroesophageal junction cancers compared with those who received chemotherapy alone. Recently, ZBT combined with chemotherapy demonstrated a survival benefit in patients with CLDN18.2-positive and HER-2-negative gastric or gastroesophageal junction cancers in the global phase III SPOTLIGHT and GLOW trials (30,31).

Türeci *et al* (32) demonstrated that, using human peripheral blood mononuclear cells and serum as effectors, ZBT induced ADCC (Antibody-Dependent Cell-Mediated Cytotoxicity) and CDC (Complement Dependent Cytotoxicity) against human pancreatic cancer cells in *ex vivo* models. They also revealed that ZBT suppressed tumor development and lung metastasis formation in human pancreatic cancer cell lines transduced with lentiviral claudin-18.2 in mouse xenograft models (32). Furthermore, they demonstrated that CLDN18.2

expression on the cell surface was increased by gemcitabine or 5-fluorouracil *in vitro* administration (32). If this finding were supported by other studies it would mean that even if pancreatic cancer cells are not killed by chemotherapy, the patients could become newly eligible for ZBT therapy, owing to the increased expression of CLDN18.2.

More recently, a randomized open-label phase 2 study (NCT03816163) assessed the safety and efficacy of Gemcitabine and nab-paclitaxel alone or with ZBT in patients with PDA and high CLDN18.2 expression (>75% of positive tumor cells) (25).

Considering the importance that CLDN18.2 might have as a potential therapeutic marker for PDA, we correlated its expression with clinicopathological features and clinical outcomes. Pancreatic tumor samples showed heterogeneous CLDN18.2 expression as regards the level of surface expression measured as staining intensity of positive cells and also the fraction of stained cells within a single tumor sample. We defined tumors as CLDN18.2 positive if a proportion of ≥5% of all evaluable tumor cells showed membrane-specific staining. Noteworthily, PDA often has a prominent desmoplastic and stromal-dominant component with few tumor cells. Therefore, evaluating the sample for the expression of a target molecule and determining the positive fraction of the target molecule is surely a relevant analysis. Each sample was evaluated by two independent pathologists.

Our results showed that a considerable number of patients were immunoreactive to CLDN18.2 (Table I) and the majority of them (21/35, 60%) had a Histoscore >50. This indicates that even if clinical benefit will require a high expression of CLDN18.2, a considerable number of PDA patients will still be eligible.

Previous articles (22,24,25,33) reported controversial results for the expression of CLDN18.2 in normal pancreatic tissue. Some investigators have reported a weak expression of CLDN18.2 in normal pancreatic tissue (25), whereas others have found no expression (22,24,33). In agreement with the latter, our data confirm that it was not expressed in any type of normal pancreatic cell (Fig. 1B yellow arrow). Therefore, it resulted an ideal therapeutic target because it showed a high and specific expression in the tumor and no expression in normal pancreatic tissues.

In agreement with Zhang and Lyu (17,27), we found a significant correlation between CLDN18.2 expression and tumor histologic grading, with well- or moderately-differentiated tumors yielding a higher prevalence of positive samples (Table I). In our study, the node stage was assessed only in 28 samples. Correlation analysis highlighted that the proportion of CLDN18.2 positive tumors was significantly higher in lymph-node negative tumors, in contrast to previously reported findings (22,26) but in agreement with Park S *et al* (25). As already demonstrated in other studies (24,25), the expression of CLDN18.2 is associated with the prognostic factors mentioned above but was not correlated with patient survival. This result is possible because grading and nodal status do not always correlate with survival.

This study suffers from some limitations that need to be highlighted. First, this study has a small number of patients. Second, the detection method and the cut-off used are arbitrary. Therefore, more large-scale studies using detection methods based on the results of ongoing clinical trials of ZBT are needed in order to better identify patients eligible for targeted therapy.

In conclusion, the results of this study seem to suggest an attractive role for CLDN18.2 in PDA, for both diagnostic and prognostic-therapeutic purposes. In fact, its absence in normal tissue and high expression in neoplastic cells suggest that it may be a very useful marker for diagnostic and prognostic-therapeutic purposes. Its expression is correlated with grading and node stage and the high percentage of positive samples could indicate that a large number of patients may be eligible for ZBT.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

AMV conceptualized the study; IG and NL performed the IHC method; AMV analyzed and interpreted the results; RD performed the statistical analysis; GA and MTS analyzed and interpreted the data and investigated for data in the literature; PAI provided laboratory data; SV and CO enrolled patients and contributed to conception and design of study; AMV performed writing-review and editing; RA and CL contributed to the acquisition of data and performed the supervision of the manuscript. RA and CL confirm the authenticity of all raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the local scientific committee and by the institutional ethics committee of the Oncologic Institute Research Hospital of Bari, Italy, and was performed in accordance with the Declaration of Helsinki. The study individuals gave written consent for the laboratory investigations and recording of their clinical data.

Patient consent for publication

Patients provided written informed consent for publication of their data.

Competing interests

The authors declare that they have no competing interests.

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