Nerve Fibers in the Tumor Microenvironment as a Novel Biomarker for Oncological Outcome in Patients Undergoing Surgery for Perihilar Cholangiocarcinoma

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Keywords
Cholangiocarcinoma · Nerve fiber density · Oncological outcome · Biomarker

Abstract

Introduction: Perihilar cholangiocarcinoma (pCCA) is a biliary tract cancer with a dismal prognosis, with surgery being the only chance of cure. A characteristic aggressive biological feature of pCCA is perineural growth which is defined by the invasion of cancer cells to nerves and nerve fibers. Recently, nerve fiber density (NFD) was linked to oncological outcomes in various malignancies; however, its prognostic role in pCCA remains to be elucidated.

Materials and Methods: Data of 101 pCCA patients who underwent curative-intent surgery between 2010 and 2019 were included in this study. Extensive group comparisons between patients with high and low NFD were carried out, and the association of cancer-specific survival (CSS) and recurrence-free survival with NFD and other clinicopathological characteristics was assessed using univariate and multivariable cox regression models. Results: Patients with high NFD showed a median CSS of 90 months (95\% CI: 48–132, 3-year CSS = 77\%, 5-year CSS = 72\%) compared to 33 months (95\% CI: 19–47, 3-year CSS = 46\%, 5-year CSS = 32\%) in patients with low NFD (p = 0.006 log rank). Further, N1 category (HR = 2.84, p = 0.001) and high NFD (HR = 0.41, p = 0.024) were identified as independent predictors of CSS in multivariable analysis. Patients with high NFD and negative lymph nodes showed a median CSS of 90 months (3-year CSS = 88\%, 5-year CSS = 80\%), while patients with either positive lymph nodes or low NFD displayed a median CSS of 51 months (3-year CSS = 59\%, 5-year CSS = 45\%) and patients with both positive lymph nodes and low NFD a median CSS of 24 months (3-year CSS = 26\%, 5-year CSS = 16\%, p = 0.001 log rank). Conclusion: NFD has been identified as an important novel prognostic biomarker in pCCA patients. NFD alone and in combination with nodal status in particular allows to stratify pCCA patients based on their risk for inferior oncological outcomes after curative-intent surgery.

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Introduction

Perihilar cholangiocarcinoma (pCCA) is the commonest subtype among biliary tract tumors and is associated with a poor prognosis [1–3]. Liver resection with vascular reconstructions and radical lymphadenectomy emerged as the gold standard of therapy in resectable disease, and this yielded improved survival rates in selected cohorts [3–9]. Despite these encouraging outcomes, surgical therapy remains challenging and often displays significant perioperative mortality rates exceeding 10% due to the distinct anatomic location of the tumor and the close proximity to major vascular structures [8–11].

As the prognosis spans from poor prognosis to good prognosis, there is significant value in being able to identify prognostic features [3]. Tumor differentiation, R0 status, and lymph node status have been previously reported as important prognostic factors by Nagino et al. [12]. Our group and others have confirmed perioperative blood transfusion, serum albumin, and lymphovascular invasion (LVI) as independent variables predicting adverse outcomes following surgery for pCCA [3, 13, 14].

CCA is characterized by a large desmoplastic stroma component which might explain systemic therapy resistance [15]. Nerve fibers are a component of the tumor microenvironment (TME), and a significant proportion of cholangiocarcinoma patients display a tumor infiltration of the epineural, perineural, and endoneural space of the neural sheath [16, 17]. These features are termed perineural infiltration (PNI). PNI can be recognized on Hematoxylin and Eosin (H&E) staining and appears to be an independent predictor of prognosis in cholangiocarcinoma (CCA). In contrast, the prognostic role of nerve fiber density (NFD) referring to small nerve fibers in the TME, which are usually not visible on H&E staining and do not show invasion of cancer cells remains to be determined in pCCA patients [18, 19]. Therefore, we aimed to investigate NFD as a prognostic marker in a large European cohort of pCCA patients undergoing surgical resection.

Materials and Methods

Patients

All consecutive patients with pCCA who underwent surgical resection at the University Hospital RWTH Aachen (UH-RWTH) between 2010 and 2019 were eligible for this study. Of these patients (n = 127), 20 individuals were excluded (n = 14 perioperative mortality; n = 6 with missing NFD data). Subsequently, a cohort of 101 patients was included in this analysis (Fig. 1b). The study was conducted in accordance with the requirements of the Institutional Review Board of the RWTH-Aachen University (EK 106/18), the Declaration of Helsinki, and good clinical practice guidelines (ICH-GCP).

Assessment of NFD

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as previously described [19]. In brief, sections (2.5 μm thick) were cut, deparaffinized in xylene, and rehydrated in graded alcohols. Slides were boiled in citrate buffer (pH 6.0) at 95–100°C for 5 min and were cooled for 20 min and with endogenous peroxidase in methanol for 10 min. Sections were incubated with rabbit anti-human PGP 9.5 (DAKO 1:100) overnight at 4°C. A single digital image was uploaded in QuPath 0.1.6. As previously described, all slides were assessed by a trained pathologist who was blinded to the clinical outcomes of the individual patients, and NFD was evaluated by manually counting the number of nerve fascicles with diameters of <100 μm in 20 continuous visual fields at ×200 magnification [19]. An overview of
the workflow is presented in Figure 1a. Based on NFD results, patients were categorized into a low NFD group (<10 nerve fibers) and a high NFD group (≥10 nerve fibers).

Assessment of Origin of the Small Nerve Fibers
Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as previously described. Sections were incubated with neuropeptide Y (Abcam 1:200) and vasoactive intestinal peptide (Abcam 1:50) overnight at 4°C.

Statistical Analysis
The primary endpoint of this study was cancer-specific survival (CSS), which was defined from the date of resection to the date of tumor-specific death. Deaths not associated with the tumor, for example, cardiovascular events, were censored at the time of death. The secondary endpoint was recurrence-free survival (RFS), which was defined as the period from surgery to the date of first recurrence. Patients without tumor recurrence were censored at the time of death or at the last follow-up. Perioperative mortality was defined as in-hospital mortality. The cutoff level for NFD categorization was determined by the receiver operating characteristic analysis of CCS with respect to NFD as previously described [19]. Group comparisons were conducted by the Mann-Whitney U test in case of continuous variables, while the χ² test, Fisher’s exact test, or linear-by-linear association in accordance with scale and number count were used in case of categorical variables. The associations of CSS and RFS with clinicopathological characteristics were assessed using univariate and multivariable Cox regression analyses in a backward selection model. Survival curves were generated by the Kaplan-Meier method and compared with the log-rank test. The level of significance was set to p < 0.05, and p values were given for 2-sided testing. Analyses were performed using SPSS Statistics 24 (IBM Corp., Armonk, NY, USA).

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**Fig. 1.** Workflow and study cohort. a Overview of all steps from tissue to biomarker. FFPE, formalin-fixed paraffin-embedded. b Study cohort. CSS, cancer-specific survival; NFD, nerve fiber density; pCCA, perihilar cholangiocarcinoma.
| Demographics                              | Overall cohort (n = 101) | High NFD (n = 31) | Low NFD (n = 70) | p value |
|-------------------------------------------|--------------------------|-------------------|------------------|---------|
| Gender, male/female (%)                   | 68 (67)/33 (33)          | 22 (71)/9 (29)    | 46 (66)/24 (34)  | 0.604   |
| Age, years                                | 68 (57–74)               | 68 (56–72)        | 68 (57–74)       | 0.968   |
| BMI, kg/m²                                 | 25 (22–29)               | 25 (23–30)        | 25 (22–28)       | 0.439   |
| Neoadjuvant therapy, n (%)                | 4 (4)                    | 3 (10)            | 1 (1)            | 0.050   |
| PVE, n (%)                                | 43 (43)                  | 12 (39)           | 31 (44)          | 0.601   |
| ASA, n (%)                                |                          |                   |                  |         |
| I                                         | 4 (4)                    | 1 (3)             | 3 (4)            |         |
| II                                        | 44 (44)                  | 16 (52)           | 28 (40)          |         |
| III                                       | 50 (50)                  | 13 (42)           | 37 (53)          | 0.740   |
| IV                                        | 3 (3)                    | 1 (3)             | 2 (3)            |         |
| V                                         | 0                        |                   |                  |         |
| Bismuth classification, n (%)             |                          |                   |                  |         |
| I                                         | 4 (4)                    | 4 (13)            | 4 (6)            |         |
| II                                        | 10 (10)                  | 0                 | 6 (9)            |         |
| IIIa                                      | 28 (28)                  | 8 (26)            | 20 (29)          | 0.675   |
| IIIb                                      | 27 (27)                  | 9 (29)            | 18 (26)          |         |
| IV                                        | 32 (32)                  | 10 (32)           | 22 (31)          |         |
| Preoperative cholangitis, n (%)           | 27 (27)                  | 5 (16)            | 22 (31)          | 0.109   |
| Preoperative PBD, n (%)                   | 23 (23)                  | 5 (16)            | 18 (26)          | 0.289   |
| Preoperative EBD, n (%)                   | 78 (77)                  | 23 (74)           | 55 (79)          | 0.628   |
| Clinical chemistry                        |                          |                   |                  |         |
| Albumin, g/dL                             | 37 (33–42)               | 39 (34–42)        | 37 (32–41)       | 0.268   |
| AST, U/L                                  | 47 (36–81)               | 49 (32–135)       | 47 (36–77)       | 0.932   |
| ALT, U/L                                  | 68 (37–133)              | 88 (43–161)       | 63 (37–120)      | 0.318   |
| GGT, U/L                                  | 451 (221–774)            | 554 (240–982)     | 441 (209–756)    | 0.304   |
| Total bilirubin, mg/dL                    | 1.2 (0.6–2.8)            | 1.2 (0.8–2.6)     | 1.2 (0.5–2.8)    | 0.805   |
| Platelet count, /μL                       | 88 (228–393)             | 292 (212–392)     | 297 (230–399)    | 0.548   |
| Alkaline phosphatase, U/L                 | 254 (169–396)            | 251 (160–445)     | 254 (183–372)    | 0.747   |
| Prothrombin time, %                       | 96 (81–105)              | 99 (79–107)       | 96 (82–104)      | 0.868   |
| INR                                        | 1.02 (0.96–1.12)         | 1.01 (0.97–1.13)  | 1.03 (0.96–1.12) | 0.839   |
| Hemoglobin, g/dL                          | 12.5 (11.3–13.4)         | 13 (11.5–13.6)    | 12.3 (11.1–13.2) | 0.165   |
| CRP, mg/L                                 | 12 (6–35)                | 12 (6–35)         | 12 (6–36)        | 0.759   |
| Operative data                            |                          |                   |                  |         |
| Operative time, min                       | 404 (356–474)            | 415 (355–465)     | 400 (355–483)    | 0.947   |
| Operative procedure, n (%)                |                          |                   |                  |         |
| Limited bile duct resection               | 1 (1)                    | 0                 | 1 (1)            |         |
| Right hepatectomy                         | 11 (11)                  | 2 (7)             | 9 (13)           |         |
| Left hepatectomy                          | 11 (11)                  | 5 (16)            | 6 (9)            |         |
| Extended right hepatectomy                | 18 (18)                  | 6 (19)            | 12 (17)          |         |
| Extended left hepatectomy                 | 28 (28)                  | 9 (29)            | 19 (27)          | 0.475   |
| Right trisectionectomy                    | 20 (20)                  | 6 (19)            | 14 (20)          |         |
| Left trisectionectomy                     | 5 (5)                    | 3 (10)            | 2 (3)            |         |
| Hepatodudenoectomy                        | 6 (6)                    | 0                 | 6 (9)            |         |
| ALPPS                                      | 1 (1)                    | 0                 | 1 (1)            |         |
| Portal vein reconstruction                | 101 (100)                | 31 (100)          | 70 (100)         | 0.999   |
| Arterial reconstruction                   | 7 (7)                    | 2 (7)             | 5 (7)            | 0.900   |
| Intraoperative blood transfusion          | 0 (0–2)                  | 0 (0–2)           | 1 (0–2)          | 0.368   |
| Intraoperative FFP                        | 3 (0–5)                  | 3 (0–4)           | 3 (0–6)          | 0.463   |
| Pathological examination                  |                          |                   |                  |         |
| R1 resection, n (%)                       | 12 (12)                  | 3 (10)            | 9 (13)           | 0.606   |
| pT category, n (%)                        |                          |                   |                  |         |
| 1                                         | 7 (7)                    | 2 (7)             | 5 (7)            |         |
| 2                                         | 62 (61)                  | 18 (58)           | 44 (63)          | 0.678   |
| 3                                         | 26 (26)                  | 10 (32)           | 16 (23)          |         |
| 4                                         | 6 (6)                    | 1 (3)             | 5 (7)            |         |
Table 1 (continued)

| Demographics          | Overall cohort | High NFD (n = 31) | Low NFD (n = 70) | p value |
|-----------------------|---------------|------------------|-----------------|---------|
| **pN category**       |               |                  |                 |         |
| N0                    | 62 (61)       | 21 (68)          | 41 (59)         | 0.383   |
| N1                    | 39 (39)       | 10 (32)          | 29 (41)         |         |
| **Tumor grading, n (%)** |             |                  |                 |         |
| G1                    | 6 (6)         | 2 (7)            | 4 (6)           |         |
| G2                    | 73 (75)       | 27 (90)          | 46 (68)         | 0.099   |
| G3                    | 18 (18)       | 1 (3)            | 17 (25)         |         |
| G4                    | 1 (1)         | 0                | 1 (2)           |         |
| **MVI, n (%)**        |               |                  |                 |         |
| 71 (73)               | 4 (13)        | 21 (32)          |                 | 0.100   |
| LVI, n (%)            | 21 (22)       | 7 (23)           | 14 (22)         | 0.938   |
| PNI, n (%)            | 66 (81)       | 18 (82)          | 48 (80)         | 0.854   |
| **Postoperative data**|               |                  |                 |         |
| **Intensive care, days** | 1 (1–3)    | 1 (1–2)          | 2 (1–3)         | 0.233   |
| **Hospitalization, days** | 20 (13–36) | 14 (12–31)       | 24 (13–42)      | **0.017**|
| **Postoperative complications, n (%)** |          |                  |                 |         |
| No complications      | 14 (14)       | 6 (19)           | 8 (11)          |         |
| Clavien-Dindo I       | 8 (8)         | 3 (10)           | 5 (7)           |         |
| Clavien-Dindo II      | 27 (27)       | 10 (32)          | 17 (24)         |         |
| Clavien-Dindo IIIa    | 19 (19)       | 2 (7)            | 17 (24)         | 0.272   |
| Clavien-Dindo IIIb    | 22 (22)       | 7 (23)           | 15 (21)         |         |
| Clavien-Dindo IVa     | 7 (7)         | 3 (10)           | 4 (6)           |         |
| Clavien-Dindo IVb     | 4 (4)         | 0                | 4 (6)           |         |
| Clavien-Dindo V       | 0             | 0                | 0               |         |
| CCI                   | 35 (21–50)    | 23 (8–54)        | 39 (20–49)      | 0.124   |
| **Oncologic data**    |               |                  |                 |         |
| **Adjuvant therapy**  | 23 (23)       | 6 (19)           | 17 (25)         | 0.562   |
| **Median RFS, months (95% CI)** | 37 (18–56) | 83 (34–132)      | 24 (13–35)      | **0.004**|
| **Median CSS, months (95% CI)** | 49 (29–69) | 90 (48–132)      | 33 (19–47)      | **0.006**|

Data are presented as median and interquartile range if not noted otherwise. Bold numbers indicate statistical significance (p < 0.05). NFD, nerve fiber density; ALT, alanine aminotransferase; ASA, American Society of Anesthesiologists classification; AST, aspartate aminotransferase; CCI, comprehensive complication index; CSS, cancer-specific survival; EBD, endoscopic biliary drainage; FFP, fresh frozen plasma; GGT, gamma glutamyltransferase; INR, international normalized ratio; LVI, lymphovascular invasion; MVI, microvascular invasion; PBD, percutaneous biliary drainage; PNI, perineural invasion; RFS, recurrence-free survival; PVE, portal vein embolization.

Fig. 2. Oncological survival in pCCA. a CSS and OS in pCCA. The median CSS was 49 months (95% CI: 29–69) and the median OS 33 months (95% CI: 19–47), respectively. b RFS in pCCA. The median RFS was 37 months (95% CI: 18–56). c PGP staining of pCCA with high NFD. Zoomed in image of the tumor with a lot of small nerves in the stroma between the tumor glands (blue arrows). On the zoomed in image of the routine HE staining, these small nerve fibers are not visible. These results occur in patients corresponding to the blue line in the Kaplan-Meier curve in d and f (high NFD). d CSS in pCCA stratified by NFD. The median CSS was 90 months (95% CI: 57–123) in patients with high NFD and negative lymph nodes, 51 months (95% CI: 38–64) in patients with either positive lymph nodes or low NFD but not both, and 24 months (95% CI: 14–32) in patients with both positive lymph nodes and low NFD (p = 0.001 log rank). e PGP staining of pCCA with low NFD. Zoomed in image of the tumor without any small nerves in the stroma between the tumor glands. The zoomed in routine HE staining shows a positive big nerve trunk with perineural invasion. These big nerve fibers are also easily recognized on the zoomed in routine HE staining. f RFS in pCCA stratified by NFD. The median RFS was 83 months (95% CI: 43–132) in patients with high NFD compared to 24 months (95% CI: 13–35) in patients with low NFD (p = 0.004 log rank). g CSS in pCCA stratified by NFD and pN category. The median CSS was 90 months (95% CI: 57–123) in patients with high NFD and negative lymph nodes, 51 months (95% CI: 38–64) in patients with either positive lymph nodes or low NFD but not both, and 24 months (95% CI: 14–32) in patients with both positive lymph nodes and low NFD (p = 0.001 log rank). h RFS in pCCA stratified by NFD and pN category. The median RFS was 83 months (95% CI: 42–124) in patients with high NFD and negative lymph nodes, 45 months (95% CI: 8–82) in patients with either positive lymph nodes or low NFD but not both, and 10 months (95% CI: 0–21) in patients with both positive lymph nodes and low NFD (p = 0.001 log rank). CI, confidence interval; CSS, cancer-specific survival; RFS, recurrence-free survival; OS, overall survival; pCCA, perihilar cholangiocarcinoma; PGP, protein gene product 9.5; NFD, nerve fiber density.

(For figure see next page.)
Nerve Fiber Density in Perihilar Cholangiocarcinoma

Liver Cancer 2021;10:260–274
DOI: 10.1159/000515303

Survival, %

100 80 60 40 20 0

0 12 24 36 48 60 72

Time, months

Survival, %

100 80 60 40 20 0

0 12 24 36 48 60 72

Time, months

Number at risk

CCS 101 76 56 41 32 16 11

OS 101 76 56 41 32 16 11

Number at risk

RFS 101 61 46 36 25 14 9

Recurrence-free survival, %

100 80 60 40 20 0

0 12 24 36 48 60 72

Time, months

Recurrence-free survival, %

100 80 60 40 20 0

0 12 24 36 48 60 72

Time, months

Number at risk

High NFD 31 24 22 17 15 8 5

Low NFD 70 52 35 26 18 9 7

Number at risk

High NFD 31 22 18 15 7 4

Low NFD 70 39 29 22 13 8 6

Number at risk

High NFD 21 19 17 15 12 6 4

Low NFD or N+ 51 38 31 23 17 8 6

Low NFD/N+ 29 20 11 6 5 3 2

Number at risk

High NFD/N– 21 17 14 12 10 5 3

Low NFD or N+ 51 34 25 22 14 8 6

Low NFD/N+ 29 13 9 5 3 2 2

Nerve Fiber Density in Perihilar Cholangiocarcinoma

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DOI: 10.1159/000515303

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Results

Patient Cohort

The study cohort consisted of 68 men (67%) and 33 women (33%) with a median age of 68 years. The majority of patients presented with Bismuth type III (55%, 55/101) or IV (32%, 32/101) tumors and were assessed as ASA (American Society of Anesthesiologists classification) III or higher (53%, 53/101). Neoadjuvant therapy was applied in a small number of patients (4%, 4/101), while preoperative portal vein embolization was carried out in a significant proportion of patients (43%, 43/101). Mandatory portal vein resection and reconstruction was carried out in every patient (101/101), while additional arterial reconstruction was necessary in 7% (7/101). Also, the concomitant resection of the pancreatic head was needed in 6% (6/101) of the patients to achieve clear tumor margins. Accordingly, R1 resection was confirmed in 12% (12/101) of the overall cohort. Major complications after surgery were frequently observed with 33% (33/101) of the patients presenting with complications ≥Clavien-Dindo IIIb. Cases with perioperative mortality were excluded from the analysis as stated above. Further demographic and clinicopathological details of the cohort are outlined in Table 1.

Group Categorization and Comparative Analysis with Respect to NFD

A receiver operating characteristic analysis evaluating the total number of nerve fibers for patients who survived at least 4 years versus patients who died during follow-up was conducted. The corresponding area under the curve was 0.618 (95% confidence interval [CI]: 0.480–0.756). A cutoff value for NFD was determined with respect to optimized accuracy and equal weight for sensitivity and specificity errors (<10 nerve fibers and ≥10 nerve fibers). Using the established cutoff value, the median CSS was 90 months in patients with high NFD (≥10 nerve fibers) and 33 months in patients with low NFD (<10 nerve fibers, p = 0.006 log rank).

A comparative group analysis regarding NFD was further carried out between patients with high NFD (n = 31) and low NFD (n = 70). Extensive group comparisons revealed no significant differences in clinical characteristics expect a longer median hospitalization time in the low NFD group (14 vs. 21 days, p = 0.017). Of note, no statistical differences in pT category (p = 0.678), pN category (p = 0.383), tumor grading (p = 0.099), LVI (p = 0.938), microvascular invasion (MVI, p = 0.100), and PNI (p = 0.854) were observed between the groups. However, the median CSS (90 months [95% CI: 48–132] vs. 33 months [95% CI: 19–47], p = 0.006 log rank) and the median RFS (83 months [95% CI: 34–132] vs. 24 months [95% CI: 13–35], p = 0.004 log rank) were significantly longer in patients with high NFD compared to patients with low NFD. More details regarding the group comparisons are presented in Table 1.

Survival Analysis

After a median follow-up of 53 months, the median CSS of the whole cohort was 49 months (95% CI: 29–69), the median OS 33 months (95% CI: 19–47), and the median RFS 37 months (95% CI: 18–56, Fig. 2a, b). A Kaplan-Meier analysis with respect to NFD showed a median CSS of 90 months (95% CI: 48–132, 3-year CSS = 77%, 5-year CSS = 72%) in patients with high NFD compared to 33 months (95% CI: 19–47, 3-year CSS = 46%, 5-year CSS = 32%) in patients with low NFD (p = 0.006 log rank, Fig. 2c). Further, RFS was significantly lower in patients with low NFD (24 months [95% CI: 13–35]) compared to patients with high NFD (83 months [95% CI: 34–132], p = 0.004 log rank, Fig. 2d). Interestingly, in a subsequent Kaplan-Meier analysis, the combination of NFD with nodal status resulted in a median CSS of 90 months (95% CI: 57–123, 3-year CSS = 88%, 5-year CSS = 80%) in patients with high NFD and negative lymph nodes, 51 months (95% CI: 38–64, 3-year CSS = 59%, 5-year CSS = 45%) in patients with either positive lymph nodes or low NFD but not both, and 24 months (95% CI: 14–32, 3-year CSS = 26%, 5-year CSS = 16%) in patients with both positive lymph nodes and low NFD (p = 0.001 log rank, Fig. 2e). Accordingly, the median RFS was 83 months (95% CI: 42–124) in patients with high NFD and negative lymph nodes, 45 months (95% CI: 8–82) in patients with either positive lymph nodes or low NFD, and 10 months (95% CI: 0–21) in patients with both positive lymph nodes and low NFD (p = 0.001 log rank, Fig. 2f).

Cox Regression Analysis

In univariate analysis, intraoperative blood (p = 0.011) and FFP transfusion (p = 0.010), R1 resection (p = 0.019), nodal status (p = 0.002), tumor grading (p = 0.004), MVI (p = 0.022), LVI (p = 0.026), and NFD (p = 0.009) were significantly associated with CSS. All variables showing p value <0.05 were included in a multivariable Cox regression model. Here, intraoperative FFP transfusion (HR = 2.90, p = 0.004), nodal status (HR = 2.84, p = 0.001), and NFD (HR = 0.41, p = 0.024)
were identified as independent predictors of CSS (Table 2).

In univariate analysis, intraoperative blood (p = 0.034) and FFP transfusion (p = 0.009), R1 resection (p = 0.006), nodal status (p = 0.003), tumor grading (p = 0.001), MVI (p = 0.008), LVI (p = 0.022), NFD (p = 0.007), and adjuvant therapy (p = 0.028) showed significant associations with RFS. In the corresponding multivariable Cox re-

### Table 2. Univariate and multivariable analysis of CSS in pCCA

| Demographics          | Univariate analysis | Multivariable analysis |
|-----------------------|---------------------|------------------------|
|                       | HR (95% CI)         | p value                | HR (95% CI)         | p value |
| Sex (male = 1)        | 1.28 (0.71–2.29)    | 0.411                  |                      |         |
| Age (≤65 years = 1)   | 1.13 (0.64–2.00)    | 0.683                  |                      |         |
| BMI (≤25 kg/m² = 1)   | 1.17 (0.66–2.06)    | 0.591                  |                      |         |
| PVE (no = 1)          | 1.01 (0.57–1.80)    | 0.972                  |                      |         |
| ASA (I/II = 1)        | 1.27 (0.72–2.27)    | 0.411                  |                      |         |
| Preoperative cholangitis (no = 1) | 0.82 (0.44–1.53)   | 0.526                  |                      |         |
| EBD (no = 1)          | 1.29 (0.60–2.76)    | 0.515                  |                      |         |
| PBD (no = 1)          | 0.95 (0.50–1.84)    | 0.889                  |                      |         |
| Clinical chemistry    |                     |                        |                      |         |
| Albumin (≤40 g/L = 1) | 0.68 (0.36–1.28)    | 0.238                  |                      |         |
| AST (≤40 U/L = 1)     | 1.34 (0.72–2.48)    | 0.739                  |                      |         |
| ALT (≤40 U/L = 1)     | 0.87 (0.40–1.93)    | 0.354                  |                      |         |
| GGT (≤400 U/L = 1)    | 1.20 (0.66–2.20)    | 0.555                  |                      |         |
| Bilirubin (≤1 mg/dL = 1) | 1.26 (0.72–2.23)  | 0.423                  |                      |         |
| Alkaline phosphatase (≤250 U/L = 1) | 1.01 (0.56–1.86) | 0.964                  |                      |         |
| Platelet count (≤300/nL = 1) | 1.08 (0.59–1.96) | 0.813                  |                      |         |
| INR (≤1 = 1)          | 1.57 (0.84–2.95)    | 0.162                  |                      |         |
| Hemoglobin (≤13 g/dL = 1) | 0.63 (0.34–0.1.17) | 0.144                  |                      |         |
| CRP, mg/L (≤10 mg/L = 1) | 1.08 (0.59–1.95) | 0.813                  |                      |         |
| Operative data        |                     |                        |                      |         |
| Operative time (≤360 min = 1) | 1.68 (0.87–3.27)   | 0.125                  |                      |         |
| Right-sided hepatectomy | 1                    | 0.965                  |                      |         |
| Left-sided hepatectomy | 0.97 (0.53–1.74)   | 0.907                  |                      |         |
| Blood transfusion (no = 1) | 2.11 (1.19–3.75)  | Excluded               |                      |         |
| FFP transfusion (no = 1) | 2.38 (1.23–4.61)  | Excluded               |                      |         |
| Arterial resection (no = 1) | 1.85 (0.66–5.19) | 0.242                  |                      |         |
| Pathological data     |                     |                        |                      |         |
| R1 resection (no = 1) | 2.42 (1.16–5.06)    | 0.019                  | Excluded             |         |
| pT category (T1/T2 = 1) | 1.53 (0.85–2.78)   | 0.159                  |                      |         |
| pN category (N0 = 1)  | 2.52 (1.32–4.47)    | 0.004                  | Excluded             | 2.84 (1.52–5.31) | 0.001 |
| Tumor grading (G1/G2 = 1) | 2.10 (1.16–3.96)  | Excluded               |                      |         |
| LVI (no = 1)          | 2.14 (1.09–4.18)    | 0.026                  | Excluded             |         |
| PNI (no = 1)          | 1.24 (0.67–4.42)    | 0.260                  | Excluded             |         |
| NFD (low = 1)         | 0.57 (0.18–0.78)    | 0.009                  | Excluded             | 0.41 (0.19–0.89) | 0.024 |
| Oncological data      |                     |                        |                      |         |
| Neoadjuvant therapy (no = 1) | 0.58 (0.08–4.21)  | 0.589                  |                      |         |
| Adjuvant therapy (yes = 1) | 1.52 (0.78–2.97)  | 0.219                  |                      |         |

Various parameters are associated with overall survival. Bold numbers indicate statistical significance (p < 0.05). CSS, cancer-specific survival; pCCA, perihilar cholangiocarcinoma; ALT, alanine aminotransferase; ASA, American Society of Anesthesiologists classification; AST, aspartate aminotransferase; CCI, comprehensive complication index; CRP, C-reactive protein; EBD, endoscopic biliary drainage; FFP, fresh frozen plasma; GGT, gamma glutamyltransferase; INR, international normalized ratio; LVI, lymphovascular invasion; MVI, microvascular invasion; NFD, nerve fiber density; PBD, percutaneous biliary drainage; PNI, perineural invasion; PVE, portal vein embolization.
gression model, intraoperative FFP transfusion (HR = 3.10, p = 0.002), nodal status (HR = 2.92, p = 0.001), MVI (HR = 1.98, p = 0.048) and NFD (HR = 0.42, p = 0.031) were identified as independent predictors of RFS (Table 3).

**Table 3.** Univariate and multivariable analysis of RFS in pCCA

|                       | Univariate analysis | Multivariable analysis |
|-----------------------|---------------------|------------------------|
|                       | HR (95% CI)         | p value                | HR (95% CI)         | p value                |
| **Demographics**      |                     |                        |                      |                        |
| Sex (male = 1)        | 1.12 (0.62–2.02)    | 0.704                  |                       |                        |
| Age (≤65 years = 1)   | 0.89 (0.51–1.56)    | 0.691                  |                       |                        |
| BMI (≤25 kg/m² = 1)   | 1.46 (0.83–2.58)    | 0.189                  |                       |                        |
| PVE (no = 1)          | 1.23 (0.70–2.16)    | 0.478                  |                       |                        |
| ASA (I/II = 1)        | 1.00 (0.57–1.75)    | 0.990                  |                       |                        |
| Preoperative cholangitis (no = 1) | 1.71 (0.94–3.09)    | 0.077                  |                       |                        |
| EBD (no = 1)          | 1.23 (0.60–2.54)    | 0.576                  |                       |                        |
| PBD (no = 1)          | 1.12 (0.58–2.14)    | 0.741                  |                       |                        |
| **Clinical chemistry**|                     |                        |                      |                        |
| Albumin (≤40 g/L = 1) | 0.61 (0.33–1.13)    | 0.114                  |                       |                        |
| AST (≤40 U/L = 1)     | 1.49 (0.81–2.74)    | 0.200                  |                       |                        |
| ALT (≤40 U/L = 1)     | 1.00 (0.46–2.17)    | 0.997                  |                       |                        |
| GGT (≤400 U/L = 1)    | 1.25 (0.69–2.28)    | 0.463                  |                       |                        |
| Bilirubin (≤1 mg/dL = 1) | 1.26 (0.72–2.23)    | 0.423                  |                       |                        |
| Alkaline phosphatase (≤250 U/L = 1) | 0.96 (0.53–1.75)    | 0.901                  |                       |                        |
| Platelet count (≤300/nL = 1) | 1.26 (0.68–2.31)    | 0.464                  |                       |                        |
| INR (≤1 = 1)          | 1.75 (0.94–3.27)    | 0.077                  |                       |                        |
| Hemoglobin (≤13 g/dL = 1) | 0.56 (0.30–1.05)    | 0.069                  |                       |                        |
| CRP, mg/L (≤10 mg/L = 1) | 1.34 (0.74–2.43)    | 0.343                  |                       |                        |
| **Operative data**    |                     |                        |                      |                        |
| Operative time (≤360 min = 1) | 1.67 (0.85–3.27)    | 0.136                  |                       |                        |
| Type of hepatectomy   | 1                   | 0.466                  |                       |                        |
| Right-sided hepatectomy |                   |                        |                      |                        |
| Left-sided hepatectomy | 0.81 (0.45–1.44)    | 0.434                  |                      |                        |
| Blood transfusion (no = 1) | 1.84 (1.05–3.24)    | 0.034                  | 3.10 (1.42–6.30)     | 0.002                  |
| FFP transfusion (no = 1) | 2.33 (1.23–4.40)    | 0.009                  |                      |                        |
| Arterial resection (no = 1) | 0.88 (0.21–3.67)    | 0.862                  |                      |                        |
| **Pathological data** |                     |                        |                      |                        |
| R1 resection (no = 1) | 2.69 (1.33–5.44)    | 0.006                  | Excluded              |                        |
| pT category (T1/T2 = 1) | 1.64 (0.92–2.94)    | 0.095                  |                       |                        |
| pN category (N0 = 1)  | 2.33 (1.32–4.10)    | 0.003                  | 2.92 (1.55–5.48)     | 0.001                  |
| Tumor grading (G1/G2 = 1) | 3.51 (1.82–6.76)    | 0.001                  | Excluded              |                        |
| MVI (no = 1)          | 2.27 (1.23–4.19)    | 0.008                  | 1.98 (1.01–3.89)     | 0.048                  |
| LVI (no = 1)          | 2.13 (1.11–4.08)    | 0.022                  | Excluded              |                        |
| PNI (no = 1)          | 2.41 (0.86–6.80)    | 0.096                  |                       |                        |
| NFD (low = 1)         | 0.35 (0.16–0.75)    | 0.007                  | 0.42 (0.19–0.93)     | 0.031                  |
| **Oncological data**  |                     |                        |                      |                        |
| Neoadjuvant therapy (no = 1) | 0.49 (0.07–3.55)    | 0.479                  |                       |                        |
| Adjuvant therapy (yes = 1) | 0.50 (0.27–0.93)    | 0.028                  | Excluded              |                        |

Various parameters are associated with disease-free survival. Bold numbers indicate statistical significance (p < 0.05). RFS, recurrence-free survival; pCCA, perihilar cholangiocarcinoma; ALT, alanine aminotransferase; ASA, American Society of Anesthesiologists classification; AST, aspartate aminotransferase; CCI, comprehensive complication index; CRP, C-reactive protein; EBD, endoscopic biliary drainage; FFP, fresh frozen plasma; GGT, gamma glutamyltransferase; INR, international normalized ratio; LVI, lymphovascular invasion; MVI, microvascular invasion; NFD, nerve fiber density; PBD, percutaneous biliary drainage; PNI, perineural invasion; PVE, portal vein embolization.

Analysis of Nerve Fiber Origin

To further investigate the origin of the counted NFD, immunohistochemistry was carried out in a representative subset of patients (n = 20). Here, small nerve fibers counted to assess NFD were stained positive for vasoac-
Fig. 3. Origin of the nerve fibers. Schematic overview of tissue with cancers cells invading the nerve. 

**a** Routine HE staining showing perineural invasion of cancer cells invading a large nerve trunk (red arrow). 

**b** PGP immunohistochemistry staining being expressed in this cancer-invaded nerve (yellow arrow). NPY immunohistochemistry (sympathetic origin) is expressed (**c**), and VIP immunohistochemistry (parasympathetic) is not expressed in this large nerve trunk (**d**). Schematic overview of tissue with cancer cells not invading the nerve. 

**e** Routine HE staining showing the yellow arrow to the localization of the small nerve fibers that are not visible on the HE staining. 

**f** Those small nerve fibers stain positive in the PGP immunohistochemistry. 

**g** Negative staining of these small nerve fibers in the VPN immunohistochemistry. 

**h** Yellow arrow points to the positive staining in the VIP immunohistochemistry of these small nerve fibers. NPY, neuropeptide Y; VIP, vasoactive intestinal peptide.
tive intestinal peptide (to indicate parasympathetic origin) but negative for NPY (to indicate sympathetic origin). The larger pre-existing nerve fibers which also stained positive in the PGP marker showed expression of the NPY marker, and those nerve fibers were observed to be invaded by tumor cells (Fig. 3).

**Discussion and Conclusion**

Radical surgery with lymphadenectomy represents the current gold standard therapy of resectable pCCA [15]. However, oncological outcome remains heterogeneous after curative-intent surgery with some patients displaying long-term survival while other individuals suffer from early tumor recurrence [3]. Therefore, the identification of prognostic factors is of clinical and academic significance as it provides implications for clinical management and may give insights in the underlying tumor biology of the disease [24]. In this large European cohort of pCCA patients, we identified NFD as a novel and important prognostic biomarker for oncological outcome in these patients. The combination of NFD and nodal status demonstrated an excellent ability to stratify pCCA regarding their oncological prognosis after curative-intent surgery for pCCA.

CCA is considered to be a “neurotopic” cancer with a high frequency of PNI which is associated with impaired survival [17, 25]. The biliary tree is surrounded by a high frequency of PNI which is associated with impaired survival. Interestingly, Iwasaki et al. [19] have recently reported on the role of NFD in PDAC and found an inverse relationship with low NFD being independently associated with reduced survival after surgical resection. This observation is in line with our present findings, identifying low NFD as an important predictor of inferior CSS in pCCA. In our study, we were able to demonstrate a 5-year CSS of 72% in patients with high NFD compared to a 5-year CSS of 32% in patients with low NFD (Fig. 2c).

The underlying mechanisms of this clinical observation are yet to be explored. Iwasaki et al. [19] demonstrated a correlation of lower NFD to a higher tumor grade in PDAC patients suggesting a biologically more aggressive disease. In our group comparison, we observed the tendency for a higher tumor grading in the low NFD group which did not reach statistical significance (Table 1). Nonetheless, whether low NFD tumors represent a more aggressive tumor type per se or are just more prone to be resistant to standard treatment cannot be explored in our setting. However, these data suggest a major oncological role of NFD in pCCA which warrants further basic research and potentially novel clinical approaches to patients with low NFD tumors.

The prognostic role of lymph node metastases in pCCA is abundantly discussed elsewhere [3, 35–38]. In line with these previous observations, nodal status was also of major predictive value in our multivariable analysis of oncological outcome. As nodal status was not associated with NFD in our cohort and the combination of both major oncological predictors might therefore be of particular value, we created 3 distinct subgroups by combining NFD and pN category as a next step in our analysis. Here, patients with high NFD and no nodal metastases showed a median CSS of 90 months compared to 51 months in patients with either positive lymph nodes or low NFD and 24 months in patients displaying both high risk features (Fig. 2e). This statistically significant observation translates into an 80% 5-year CSS in the low-risk, 45% 5-year CSS in the median-risk, and 16% 5-year CSS in the high-risk subcohorts of our analysis. An interesting observation was the particularly compelling outcome of the abovementioned low-risk cohort (high NFD and N−), while our median-risk group (low NFD or N+) is in line with previous reports and the high-risk group (low NFD and N+) below the commonly reported outcome figures. This underlines the oncological role of NFD in pCCA [1–9, 15]. As such, the combination of NFD (which is assessable by inexpensive measures) and nodal status (which is routinely provided by the pathology report) does provide an easily applicable risk stratification for pCCA patients undergoing curative-intent surgery.
To further investigate the counted NFD, we conducted immunohistochemistry and identified the small nerve fibers to be of parasympathetic origin. This finding is novel and interesting as the role of the parasympathetic nervous system in cancer initiation and progression is not fully understood. A potential inhibitory effect of the parasympathetic nervous system on cancer initiation was historically observed in patients who underwent vagotomy as a treatment for gastric ulcers and subsequently displayed a higher incidence for gastric cancer [39]. A recent study investigated the impact of subdiaphragmatic vagotomy in a murine PDAC model. Here, vagotomy increased tumor growth and impaired survival in this mouse model, which was partly explained by increased expression of tumor necrosis factor alpha in tumor tissue [40]. Recently, Kamiya et al. [41] introduced an adeno-associated virus vector enabling the stimulation or inhibition of parasympathetic and sympathetic nerve fibers localized in the tumor tissue. In a xenograft model, they observed a decreased progression of the primary tumors and attenuation of the development of distant metastases after increasing parasympathetic neurotransmission. Additionally, the vector also showed antitumor activity in a chemically induced model of breast cancer [41, 42]. While the investigation of the mechanism of our observation regarding the protective effect of parasympathetic nerve fibers is beyond the scope of our clinically oriented analysis, these observations provide further evidence for the significant interaction of the parasympathetic nervous system and cancer cells and warrants further research. In pancreatic cancer, our team and another group have reported that a low NFD indicates a poor survival [19, 43]. Observational studies have shown differences in the role of an increased sympathetic innervation of tumors. For prostate cancer [44], breast cancer [41], and hepatocellular carcinoma [45], the presence of sympathetic nerve fibers was indicative for cancer progression. For gastric cancer [46] and colorectal cancer [47], the opposite has been described, and low sympathetic fiber density was associated with progression. This dual role of nerve fibers and their exact origin in pCCA are important research questions of ongoing studies of our group.

Tumor recurrence remains the major problem in pCCA patients who underwent curative-intent surgery [48, 49]. Local recurrence is commonly diagnosed concomitantly with a stenosis at the surgically performed hepaticojejunostomy resulting in recurrent cholangitis and life-threatening biliary sepsis [20]. In contrast, survival after metastatic recurrence is usually determined by the limited response and resistance to chemotherapy resulting in early disease progression and associated fatal outcome [15]. These limitations in the treatment of tumor relapse were also observed in our cohort with most of the individuals experiencing tumor recurrence (median RFS 37 months) deceasing shortly after the diagnosis (median CSS 49 months). This close relationship between RFS and CSS also explains our finding that NFD is also associated with RFS in multivariable analysis.

Interestingly, adjuvant therapy was a risk factor for RFS in univariate analysis. This might be explained by our historic approach to apply adjuvant therapy in patients with a high risk for tumor recurrence (e.g., positive nodal status or R1 resection) in the early study period [50, 51]. From 2018, patients with adequate performance status were subjected to adjuvant capcitabine-based therapy or referred for inclusion to the currently recruiting ACCTI-CA trial (Adjuvant Chemotherapy With Gemcitabine and Cisplatin Compared to Standard of Care After Curative Intent Resection of Biliary Tract Cancer, NCT02170090) in all cases if the patient was willing to participate in a clinical trial [23, 52].

Unfortunately, NFD cannot be assessed prior to surgery and is therefore not available for preoperative patient selection. However, NFD displayed a good prognostic ability for recurrence and reduced survival after surgery and might therefore be used for postoperative risk stratification. Based on our data, one could speculate that low NFD is associated with a biologically more aggressive tumor which might benefit from adjuvant therapy after surgery. Adjuvant therapy in biliary tract cancer is considered the standard of care in Europe after the results of the BILCAP trial but is not taken by every patient as the beneficial effects regarding survival appear marginal [23]. In other countries, patients are selected for adjuvant therapies if they display high-risk features as positive lymph nodes or residual tumor [53]. In this context, NFD could be seen as a high-risk feature similar to nodal status. Further, patients with low NFD should be closely monitored for signs of disease relapse irrespective of adjuvant therapy in order to facilitate an early treatment in case of recurrence.

For our statistical approach, we decided to exclude patients with perioperative mortality from our analysis and reported oncological outcome in terms of CSS. Liver resection for pCCA is a high-risk procedure with a perioperative mortality up to 15% [54, 55]. Also, as previously reported by our group, our local policies comprise portal vein reconstruction in every patient and arterial reconstruction and/or concomitant resection of the pancreatic head on demand within a cohort characterized by high ASA patients.
and complex tumors in terms of Bismuth staging [3, 9]. As the aim of this study was to unravel the prognostic role of the pathological parameter NFD, the exclusion of perioperative mortality appears reasonable. Also, our group comparison with respect to NFD showed no statistical difference between patients with low and high NFD regarding perioperative characteristics (Table 1). Further, CSS instead of OS was used to evaluate the oncological importance of NFD. Our monocentric cohort undergoes a detailed follow-up allowing to report CSS. Due to our aggressive surgical approach to the disease in general, our cohort comprises a median age of 68 years (range 49–80 years) and significant comorbidities (>50% patients assessed ASA III or higher). Therefore, a significant proportion of patients deceased due to cancer-unassociated circumstances, for example, cardiovascular events, secondary malignancies, or late surgical complications such as obstructive ileus. As our sample size is limited due to the monocentric nature of our study and might therefore not compensate these effects from a statistical point of view, CSS appeared more appropriate to investigate the true oncological effect of NFD on long-term outcome.

As with all oncological outcome studies, our analysis has some potential limitations. All patients included in this study were treated at a single institution reflecting the authors’ individual surgical approach, and all data were collected and analyzed retrospectively. This retrospective nature of our study resulted in some missing data which would have been interesting in the context of the oncological analysis, for example, CA19-9. The monocentric approach also results in a limited sample size which did not allow to split the cohort into a training and validation set. Therefore, our findings warrant further validation in an independent dataset.

Notwithstanding the abovementioned limitations, we have identified NFD as a novel and important prognostic biomarker in pCCA patients. NFD alone and in combination with nodal status in particular allows to stratify pCCA patients in terms of oncological outcome after curative-intent surgery. Larger, multicenter studies are needed to confirm and validate our findings.

Statement of Ethics

The study was conducted in accordance with the requirements of the Institutional Review Board of the RWTH-Aachen University (EK 106/18), the current version of the Declaration of Helsinki, and the good clinical practice guidelines (ICH-GCP). A written informed consent was obtained from all patients.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

X.T. was funded by the China Scholarship Council (CSC Grant No. 201806210074).

Author Contributions

L.R.H. and J.B. designed the study. L.R.H. and J.B. were responsible for data analysis and interpretation. X.T. and L.R.H. annotated all tumor samples manually. J.B and L.R.H. performed statistical analysis and interpretation. L.R.H., G.W., U.P.N., J.K., C.C., Z.C., and S.S. contributed to the first drafted initial manuscript. C.C. and L.R.H. provided tumor blocks and histology expertise. U.P.N. and F.U. provided infrastructure and supervised the study. All authors contributed to the data analysis and to writing the manuscript.

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