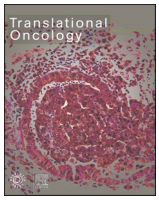
Translational Oncology 43 (2024) 101919

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon



Original Research

CT-guided high dose rate brachytherapy can induce multiple systemic proteins of proliferation and angiogenesis predicting outcome in HCC

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ARTICLE INFO

Keywords:
Growth factors
Angiogenesis
HCC
Interventional oncology
Biomarker
HDR-BT
Tumor ablation

ABSTRACT

Background and purpose: To determine the potential prognostic value of proliferation and angiogenesis plasma proteins following CT-guided high dose rate brachytherapy (HDR-BT) of hepatocellular carcinoma (HCC).
Materials and methods: For this prospective study, HDR-BT (1 × 15 Gy) was administered to 24 HCC patients. Plasma was obtained and analyzed using an Olink proteomics Target-96 immuno-oncology-panel that included multiple markers of angiogenesis and proliferation. Fold-change (FC) ratios were calculated by comparing baseline and 48 h post HDR-BT paired samples. Patients were classified as responders ($n = 12$) if they had no local progression within 6 months or systemic progression within 2 years. Non-responders ($n = 12$) had recurrence within 6 months and/or tumor progression or extrahepatic disease within 2 years.
Results: Proliferation marker EGF was significantly elevated in non-responders compared to responders ($p = 0.0410$) while FGF-2, HGF, and PlGF showed no significant differences. Angiogenesis markers Angiopoietin-1 and PDGF-B were likewise significantly elevated in non-responders compared to responders ($p = 0.0171$, $p = 0.0462$, respectively) while Angiopoietin-2, VEGF-A, and VEGFR-2 did not differ significantly. Kaplan-Meier analyses demonstrated significantly shorter time to systemic progression in patients with increased EGF and Angiopoietin-1 ($p = 0.0185$, both), but not in patients with one of the remaining proteins elevated (all $p > 0.1$). Pooled analysis for these 9 proteins showed significantly shorter time to systemic progression for FC $\geq$1.3 and $\geq$1.5 for at least 3 proteins elevated ($p = 0.0415$, $p = 0.0193$, respectively).
Conclusion: Increased plasma levels of EGF and Angiopoietin-1 after HDR-BT for HCC are associated with poor response and may therefore function as predictive biomarkers of outcome.

Abbreviations: ANG-1, angiopoietin-1; ANG-2, angiopoietin-2; BCLC, barcelona clinic liver cancer; CT, computed tomography; d, days; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; FC, fold-change; Gd-EOB-DTPA-enhanced MRI, gadolinium-ethoxybenzyl-diethylentriamine pentaacetic magnetic resonance imaging; HCC, hepatocellular carcinoma; HDR-BT, high dose rate brachytherapy; HGF, hepatocyte growth factor; hrs, hours; LOD, limit of detection; min, minutes; NPX, normalized protein expression; NR, non-responder; OS, overall survival; PEA, proximity extension assay; PDGF subunit B, platelet-derived growth factor subunit B; PlGF, placental growth factor; RFA, radiofrequency ablation; R, responder; rpm, rounds per minute; TTSP, time to systemic progression; VEGFA, vascular endothelial growth factor A; VEGFR-2, vascular endothelial growth factor receptor 2.

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https://doi.org/10.1016/j.tranon.2024.101919
Received 19 December 2023; Received in revised form 28 January 2024; Accepted 17 February 2024
Available online 23 February 2024

Introduction

In the past several decades, novel treatment approaches for hepatocellular carcinoma (HCC) have emerged [1–3]. Beside locoregional therapies, local treatment strategies including high dose rate brachytherapy (HDR-BT) have been successfully incorporated into multidisciplinary treatment algorithms and current guidelines for HCC [4–6]. Despite their multiple advantages including a minimally invasive nature and low complication rate, tumor recurrence remains a challenge to treatment efficacy [7]. Indeed, for local ablation, studies showed up to 25 % higher 5-year cumulative distant intrahepatic new tumor rate and worse disease-free survival rate compared to surgical resection [8]. Initial study of this phenomenon has rendered an increasing body of evidence indicating a complex interplay between proimmunogenic and protumorigenic effects following local tumor ablation mediated by cellular components, cytokines, growth factors, and epigenetic regulations such as micro-RNAs. For example, it has previously been elucidated that activation of an interlinked pathway of IL-6/HGF-c-Met/STAT3/VEGF is a key driver of post-ablation distant tumorigenesis [9,10]. Nevertheless, a wide diversity of growth factors of proliferative and angiogenic capability is associated with this post-ablation tumorigenesis phenomena. Consequently, single biomarkers do not fully cover the abundance of processes put into motion by local tumor therapies. Accordingly, a combination of multiple biomarkers is likely to be needed in order to select patients who need additional follow-up and potentially more aggressive treatment [11]. While the effect of ablation-induced tumorigenesis has been shown for thermal ablation [7,9] it has so far not been reported for brachytherapy. This study analyzed a panel of circulating tumorigenic proteins, particularly those associated with proliferative and angiogenic potential, and assessed their association with outcome of HDR-BT in HCC.

Materials & methods

Study design

24 patients were included in this prospective study cohort. Recruited patients presented between August 2017 and November 2019 at the Department of Radiology at LMU University Hospital, Munich, Germany. Written informed consent for local ablative treatment and study inclusion was provided by all patients. Patients were eligible for study inclusion if they presented with previously untreated HCC (Barcelona Clinic Liver Cancer (BCLC) A and B). Patient characteristics are demonstrated in Table 1.

Study procedures

Prior to procedure, all patients underwent baseline imaging through Gd-EOB-DTPA-enhanced liver MRI (Primovist®, Bayer Vital GmbH Gb Pharma, Leverkusen, Germany) and contrast-enhanced CT of chest, abdomen, and pelvis. HDR-BT was performed as previously described [12]. 5 mL of peripheral blood was collected at baseline (day before procedure) as well as 48 hrs after procedure in Monovette EDTA tubes (Sarstedt AG, Nümbrecht, Germany). Centrifugation was performed within one hour at 3000 rpm for 5 min at 4 °C. Plasma aliquots were stored at −80 °C until use. Follow-up imaging was performed in three months intervals through MRI and CT as described above.

Proximity extension assay of plasma proteins

Proteins were measured in 1 μL of EDTA plasma using the Olink proteomics Target 96 Immuno-Oncology panel (Olink Proteomics, Uppsala, Sweden) based on the Proximity Extension Assay (PEA) technology [13,14]. The panel includes Epidermal growth factor (EGF), Fibroblast growth factor-2 (FGF-2), Hepatocyte growth factor (HGF), and Placental growth factor (PlGF) representing the proliferation subpanel and Angiopoietin-1 (ANG-1), Angiopoietin-2 (ANG-2), Vascular endothelial growth factor A (VEGFA), Vascular endothelial growth factor 2 (VEGFR-2), and Platelet-derived growth factor subunit B (PDFG subunit B) representing the angiogenesis panel (see **supplementary Table 1** for the full list of proteins). Briefly, proteins are bound by two independent antibodies, each carrying an oligonucleotide complementary to each other. From the hybridization of the DNA sequence, the DNA polymerase can generate unique and specific DNA products, quantified by RT-qPCR. Samples were distributed randomly on a 96-well plate to avoid batch effects. Each plate includes interplate controls to adjust differences between different plates, and negative controls to calculate the limit of detection (LOD). Protein levels are expressed as a normalized protein expression (NPX) unit on a log2 scale. High and low NPX values correspond to high and low protein concentrations,

Table 1
Patient characteristics. NASH: Non-alcoholic steatohepatitis. AFP: Alpha-Fetoprotein. TTSP: Time to systemic progression in days.

	Overall number/median (%/range [IQR])	Responders number/median (%/range [IQR])	Non-responders number/median (%/range [IQR])	p-value
Basic characteristics				
Female	4 (16.7)	2 (16.7)	2 (16.7)	1
Male	20 (83.3)	10 (83.3)	10 (83.3)	
Age [years]	69 (50–84[21])	68.5 (58–87 [21.5])	68.5 (44–86 [32.5])	0,80
Tumor etiology				
Hepatitis B	4 (16.7)	1 (8.3)	3 (25.0)	0,59
Hepatitis C	6 (25.0)	4 (33.3)	2 (16.7)	0,64
Alcoholic	9 (37.5)	5 (41.7)	4 (33.3)	1
NASH	3 (12.5)	1 (8.3)	2 (16.7)	1
Unknown	6 (25.0)	3 (25.0)	3 (25.0)	1
Multiple	4 (16.7)	2 (16.7)	2 (16.7)	
Tumor characteristics				
Child-Pugh A	19 (79.2)	10 (83.3)	9 (75.0)	1
Child-Pugh B	5 (20.8)	2 (16.7)	3 (25.0)	
AFP [ng/ml]	5.25 (1.6–35.8 [7.0])	5.25 (1.6–21.5 [4.7])	5.45 (2.0–35.8[32.2])	0,77
AFP ≥ 20 ng/ml	4 (16.7)	1 (8.3)	3 (25.0)	0,59
TTSP	475.0 [377.3–897.3]	911.5 [837.5–1047.5]	396.5 [252.0–461.5]	0,01
Technical parameters				
Lesion diameter sum [cm]	3 (1.1–10.9[3.2])	2.9 (1.7–10.3[1.1])	3.5 (1.1–10.9[4.8])	0,49
Lesion diameter max. [cm]	2.5 (1.1–10.3[1.6])	2.7 (1.7–10.3[0.8])	2.3 (1.1–10.0[5.7])	0,31
Lesion number treated: 1	16 (66.7)	10 (83.3)	6 (50.0)	0,19
Lesion number treated: 2	8 (33.3)	2 (16.7)	6 (50.0)	

respectively.

Definitions of oncological outcome

Patients were stratified into responders and non-responders as previously described [12]. In brief, responders did not show limited or diffuse systemic tumor progression within a period of 6 months after procedure and had no diffuse systemic progression of more than 3 nodules with a diameter of > 3 cm within a period from 6 months to 24 months. By contrast, non-responders showed tumor recurrence within a period of 6 months and/or tumor progression with more than 3 nodules or at least one individual nodule exceeding a diameter of 3 cm or extrahepatic tumor disease within 24 months. The time point at which any of the above-mentioned criteria occurred defined the time to systemic progression (TTSP). Two patients underwent liver transplantation 9 months after procedure without prior tumor recurrence assessed in MR and CT imaging and no viable tumor detection in the liver explant. Accordingly, these two patients were stratified as responders.

Statistical analysis

NPX values from the protein plasma analysis were obtained for each sample. ΔNPX values were calculated as subtraction of the pre-therapy (baseline) NPX value from post-therapy NPX value. Additionally, fold change (FC) ratios were calculated based upon manufacturer instructions as the power of two from each absolute ΔNPX value in order to express differences on a linear scale. Data of baseline NPX value, post-therapy NPX value, and FC ratio is reported as median [1st quartile–3rd quartile]. Proteins were grouped into two different biological panels subsuming proteins involved in proliferation and angiogenesis pathways. Both panels were tested separately in their respective entirety to display differences according to therapy response. For this analysis, the sum of all respective FCs within each protein panel was compared between responders and non-responders using the Mann-Whitney-*U* test. Significance and trends to significance provided a basis for further hierarchical approach by testing single proteins separately. For the comparison of pre-therapy with post-therapy protein levels in both responders and non-responders, Wilcoxon signed-rank test was applied. Mann-Whitney-*U* test was used for the comparison of baseline protein levels between responders and non-responders and for comparison of FCs between responders and non-responders for each respective individual protein. Additionally, dichotomous analysis was performed by using Fisher's Exact test. For each protein, FCs with a decrease of <0.9 or an increase of >1.1 were compared between responders and non-responders. Accordingly, FCs were only considered if they showed a substantial alteration beyond a margin of error reaching from 0.9 to 1.1. For Kaplan-Meier analysis, patients were divided into two groups according to the median FC of each protein with TTSP being compared by applying the log-rank test. For pooled protein analysis, TTSP of patients with at least one out of nine protein FCs being increased ≥1.3 or ≥1.5 were compared with TTSP of all patients not fulfilling this criterion using the log-rank test. Moreover, TTSP of all patients with at least three out of nine protein FCs increased above median, ≥1.3, or ≥1.5 were compared with TTSP of all patients not fulfilling these criteria using the log-rank test. For the pooled analysis, weighted impact models with weighting factors of two-fold or three-fold were applied for the three proteins found to have individual significance (EGF, ANG-1, and PDGF subunit B). An individual total score for each patient was calculated by summing up all single proteins rendering a maximum score of 12 or 15. Based on these score cut-offs, patients were stratified into two groups and TTSP was compared by log-rank test using FC ≥1.3, or FC ≥1.5 thresholds. For patients without systemic progression, data was censored at the time point of the last available follow-up. A significance level of α = 5 % was applied. As all analyses were performed in an explorative manner, no further adjustment of α was done. Statistical analysis was performed with SAS version 9.4 (SAS Institute, Cary, NC, USA).

Results

Clinical outcomes

The cohort consisted of $n = 12$ responders and $n = 12$ non-responders. Median follow-up period was 475 days (d) ranging from a minimum of 183 d to a maximum of 1224 d None of the patients that responded to treatment experienced systemic progression during the follow-up period. The median time to systemic progression or loss to follow-up was 912 days for patients who responded to treatment and 397 days for non-responders.

Comparison of baseline plasma levels rendered no significant difference for all investigated proteins (all $p > 0.05$, **supplementary Table 2a, b**). Changes in protein plasma levels following HDR-BT were seen in a proliferation panel consisting of EGF, FGF-2, HGF, and PlGF, as well as in an angiogenesis panel consisting of ANG-1, ANG-2, VEGFA, VEGFR-2, and PDFG subunit B. Specifically within this proliferation panel, the entirety of protein FCs was significantly elevated in non-responders compared to responders (R: 6.68 [3.06–4.40], NR: 4.24 [3.63–4.66], $p = 0.0251$). Likewise, within the angiogenesis panel the entirety of protein FCs was elevated in non-responders compared to responders, even though only a trend was observed as the required level of significance was not met (R: 4.96 [4.07–5.51], NR: 5.69 [4.80–6.92], $p = 0.1020$).

Proliferation panel

Within the proliferation panel, EGF FC demonstrated a significant increase in non-responders after HDR-BT compared to responders (R: 0.67 [0.53–1.14], NR: 1.51 [1.06–2.16], $p = 0.0410$, Fig. 1a, Table 2a). For EGF FC, observation of patients with substantial differences of <0.9 or >1.1, revealed 11 responders of which 8 (73 %) showed decreasing FCs, and 10 non-responders of which 8 (80 %) had increasing FCs ($p = 0.0300$, Fig. 2a). No significant difference was observed in FCs of FGF-2, HGF and PlGF between responders and non-responders (all $p > 0.05$, **supplementary Fig. 1a, b, c, Table 2a**). Fisher's Exact test did not show differences in the proportion of responders and non-responders for decreasing or increasing FCs in FGF-2, HGF, and PlGF (all $p > 0.05$). Baseline protein levels did not differ significantly between responders and non-responders in any of the proteins investigated (all $p > 0.05$). PlGF in non-responders demonstrated slightly elevated levels after HDR-BT compared to baseline (pre: 9.88 [9.71–10.10], post: 9.99 [9.75–10.47], $p = 0.0425$, data not shown, Table 2a). Yet, no significant difference was observed in non-responders for EGF, FGF-2, and HGF comparing before and after therapy (all $p > 0.05$). Likewise, responders had, no significant difference found for EGF, FGF-2, HGF, and PlGF after HDR-BT compared to baseline (all $p > 0.05$, Table 2a).

Angiogenesis panel

Among the angiogenesis panel proteins, ANG-1 FC was significantly elevated in non-responders after HDR-BT compared to responders (R: 0.86 [0.74–1.10], NR: 1.29 [1.15–1.56], $p = 0.0171$, Fig. 1b, Table 2b). Patients with substantial differences in ANG-1 FC included 9 responders of which 6 (67 %) demonstrated decreasing FCs and 12 non-responders of which 10 (83 %) had increasing FCs ($p = 0.0318$, Fig. 2b). For PDGF subunit B, non-responders demonstrated a significantly increased FC compared to responders (R: 0.89 [0.62–1.17], NR: 1.25 [1.02–1.93], $p = 0.0462$, **supplementary Fig. 2d, Table 2b**). Patients with substantial PDGF subunit B FC differences included 11 responders of which 7 (64 %) showed decreasing FCs and 9 non-responders of which 8 (89 %) showed increasing FCs ($p = 0.0281$, Fig. 2c). FCs of ANG-2, VEGFA, and VEGFR-2 did not show significant differences between responders and non-responders (all $p > 0.05$, **supplementary Fig. 2a, b, c**) and Fisher's

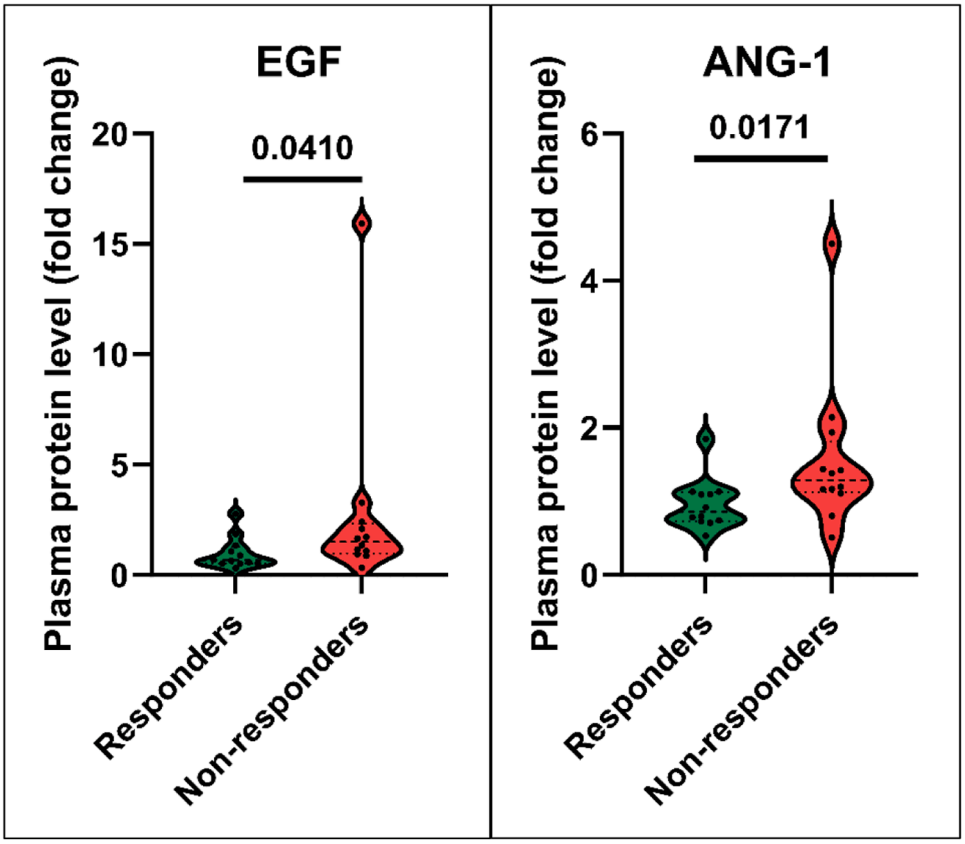

Fig. 1. Violin plots displaying plasma Fold change ratios (FC) of Epidermal growth factor (EGF) and Angiopoietin-1 (ANG-1) 48 h post-therapy compared to baseline in responders (red) and non-responders (green). EGF FC (a) showed significantly lower levels in responders ($n = 12$) compared to non-responders ($n = 12$) ($p = 0.0410$). Likewise, ANG-1 FC (b) showed significantly lower levels in responders compared to non-responders ($p = 0.0171$).

Exact test did not reveal differences in the proportion of responders and non-responders for decreasing or increasing FCs in ANG-2, VEGFA, and VEGFR-2 (all $p > 0.05$). ANG-1, ANG-2, VEGFA, VEGFR-2, PDGF subunit B had no significant differences of baseline levels between responders and non-responders, as well as between pre- and post-therapy levels for both responders and non-responders separately (all $p > 0.05$, Table 2b).

Changes in protein levels are associated with oncological outcome

Patients with an EGF and ANG-1 FC above their respective protein median FC presented with a median TTSP of 87d [563–1048d]. Patients with an EGF and ANG-1 FC below the respective protein median FC showed a median TTSP of 414d [252–461d] ($p = 0.0185$ both, Fig. 3). For the remaining proteins, median TTSP did not change significantly between patients with a protein FCs above or below their respective median (all $p > 0.05$, data not shown).

When a protein FC $\geq$1.3 was applied as a threshold to stratify patients significantly shorter TTSP was noted in patients with PDGF subunit B above this threshold compared to patients below ($p = 0.0320$, data not shown). ANG-1 showed a trend towards significantly shorter TTSP in patients with a FC $\geq$1.3 ($p = 0.0501$). The remaining proteins had no significantly altered TTSP when this FC threshold was applied for patient stratification (all $p > 0.05$).

Using a threshold of protein FC $\geq$1.5 rendered significantly shorter TTSP in patients with elevated EGF ($p = 0.0188$, data not shown), PlGF ($p = 0.0014$, data not shown), ANG-1 (0.0193, data not shown), and PDGF subunit B (0.0234, data not shown). For FGF-2, HGF, and VEGFA, no significant difference in TTSP was observed when a threshold of FC $\geq$1.5 was applied (all $p > 0.05$). No individual patient showed a protein FC $\geq$1.5 for ANG-2 and VEGFR-2, accordingly, these proteins were excluded from the analysis.

The pooled protein analyses showed that patients with at least one out of nine investigated protein FCs elevated $\geq$1.3 ($p = 0.0365$, Fig. 4a) or $\geq$1.5 ($p = 0.0142$, Fig. 4b) had a significantly shorter TTSP when compared to all patients not fulfilling this criterion.

All patients with at least 3 protein FCs elevated above median showed no difference in their TTSP compared to all other patients ($p = 0.5707$). However, patients with at least three protein FCs elevated $\geq$1.3 ($p = 0.0415$, Fig. 4c) or $\geq$1.5 ($p = 0.0193$, Fig. 4d) demonstrated a significantly shorter TTSP than patients not fulfilling this criterion.

For impact models using the FC $\geq$1.3 threshold to indicate protein elevation, one model showed a significantly shorter TTSP in patients with a high individual total score (weighting factor: 3, score cut-off: $\geq$3, $p = 0.0234$; **supplementary Table 3**). With an applied FC $\geq$1.5, four weighted impact models rendered significantly shorter TTSP in patients with high individual total scores (weighting factor: 2, score cut-off: $\geq$2, $p = 0.0352$; weighting factor: 2, score cut-off: $\geq$4, $p = 0.0083$; weighting factor: 3, score cut-off: $\geq$2, $p = 0.0352$; weighting factor: 3, score cut-off: $\geq$3, $p = 0.0352$; **supplementary Table 3**).

Discussion

Our proof-of-concept study provides initial evidence of altered circulating growth factors and angiogenesis markers with potentially protumorigenic effects following HDR-BT-treated HCC. Indeed, we demonstrated that increasing levels of EGF, ANG-1, and PDGF subunit B 48 h after local ablative therapy were associated with poor therapy response and shorter time to systemic progression. Our data further indicates that a panel of several biomarkers may be needed to detect various tumorigenic effects which may help to identify patients at risk.

Local tumor ablation with HDR-BT might induce multiple pathways of proliferation and angiogenesis with EGF being markedly increased in

Table 2a

Individual protein levels for proliferation markers Epidermal growth factor (EGF), Fibroblast growth factor 2 (FGF-2), Hepatocyte growth factor (HGF), and Placental growth factor (PlGF). Responders and non-responders displayed with their respective NPX protein level at baseline (Pre) and 48 h after therapy (Post). The Fold change ratio (FC) reflects the difference of protein levels after therapy compared to baseline. Median and interquartile range (IQR) are shown for responders and non-responders.

		EGF			FGF-2			HGF			PlGF		
Patient-ID	Response	Pre	Post	FC	Pre	Post	FC	Pre	Post	FC	Pre	Post	FC
1	Responder	6.73	6.83	1.08	2.34	2.31	0.98	11.09	11.12	1.02	10.33	10.62	1.23
2	Responder	7.26	6.32	0.52	2.18	1.38	0.57	9.46	9.55	1.06	9.17	9.53	1.29
3	Responder	6.20	5.23	0.51	2.26	1.72	0.69	10.47	10.37	0.93	9.38	9.52	1.10
4	Responder	8.06	6.34	0.30	3.32	1.62	0.31	10.77	10.83	1.04	10.10	10.41	1.24
5	Responder	7.95	8.36	1.34	3.40	3.57	1.12	10.51	10.56	1.03	11.15	11.15	1.00
6	Responder	7.71	7.55	0.89	2.35	1.80	0.69	9.94	9.84	0.94	9.24	9.34	1.08
7	Responder	5.84	7.31	2.78	1.08	1.56	1.39	9.57	10.04	1.39	9.07	9.59	1.43
8	Responder	7.33	6.82	0.70	2.64	2.26	0.77	10.38	10.61	1.17	9.82	10.01	1.14
9	Responder	8.54	7.63	0.53	2.57	0.75	0.28	10.49	10.02	0.73	10.32	9.98	0.79
10	Responder	9.13	8.32	0.57	3.63	3.11	0.70	11.20	10.32	0.55	11.03	10.66	0.78
11	Responder	7.39	6.75	0.64	0.81	0.43	0.77	9.43	9.94	1.42	9.65	10.03	1.30
12	Responder	9.65	10.55	1.86	2.55	3.18	1.55	10.44	10.43	0.99	9.65	9.71	1.05
	Median	**7.55**	**7.07**	**0.67**	**2.45**	**1.76**	**0.73**	**10.46**	**10.34**	**1.03**	**9.74**	**9.99**	**1.12**
	Mean	**7.65**	**7.33**	**0.98**	**2.43**	**1.98**	**0.82**	**10.31**	**10.30**	**1.02**	**9.91**	**10.05**	**1.12**
	SD	**1.12**	**1.34**	**0.71**	**0.84**	**0.96**	**0.39**	**0.60**	**0.45**	**0.24**	**0.69**	**0.56**	**0.20**
13	Non-responder	6.98	7.72	1.67	1.60	1.76	1.11	9.55	9.24	0.80	9.73	9.63	0.94
14	Non-responder	6.06	6.51	1.36	0.69	0.75	1.04	10.71	10.75	1.03	10.87	11.07	1.15
15	Non-responder	6.90	7.96	2.08	1.71	1.79	1.06	10.73	10.58	0.90	9.75	9.99	1.18
16	Non-responder	5.96	7.67	3.28	3.35	2.09	0.42	9.19	9.46	1.20	9.64	9.79	1.11
17	Non-responder	7.39	5.80	0.33	1.95	2.74	1.73	11.67	11.62	0.96	10.24	10.43	1.14
18	Non-responder	7.50	7.63	1.10	1.99	1.91	0.95	10.50	10.64	1.10	10.35	10.57	1.16
19	Non-responder	9.63	10.41	1.72	4.69	5.29	1.51	10.66	10.28	0.77	9.88	9.95	1.05
20	Non-responder	6.82	7.02	1.15	1.73	1.33	0.76	10.36	10.29	0.95	9.87	9.49	0.77
21	Non-responder	6.28	6.08	0.87	1.46	0.98	0.72	9.98	10.79	1.74	9.49	9.99	1.41
22	Non-responder	7.85	11.84	15.93	1.13	3.77	6.24	12.20	13.03	1.78	9.95	10.83	1.84
23	Non-responder	4.89	6.16	2.40	1.70	2.05	1.28	10.05	10.05	1.00	10.05	10.10	1.04
24	Non-responder	7.51	7.42	0.94	2.07	2.15	1.06	9.65	9.48	0.89	9.33	9.46	1.10
	Median	**6.94**	**7.52**	**1.51**	**1.72**	**1.98**	**1.06**	**10.43**	**10.43**	**0.98**	**9.88**	**9.99**	**1.13**
	Mean	**6.98**	**7.68**	**2.74**	**2.01**	**2.22**	**1.49**	**10.44**	**10.52**	**1.09**	**9.93**	**10.11**	**1.16**
	SD	**1.18**	**1.79**	**4.23**	**1.05**	**1.25**	**1.54**	**0.86**	**1.04**	**0.33**	**0.41**	**0.52**	**0.26**

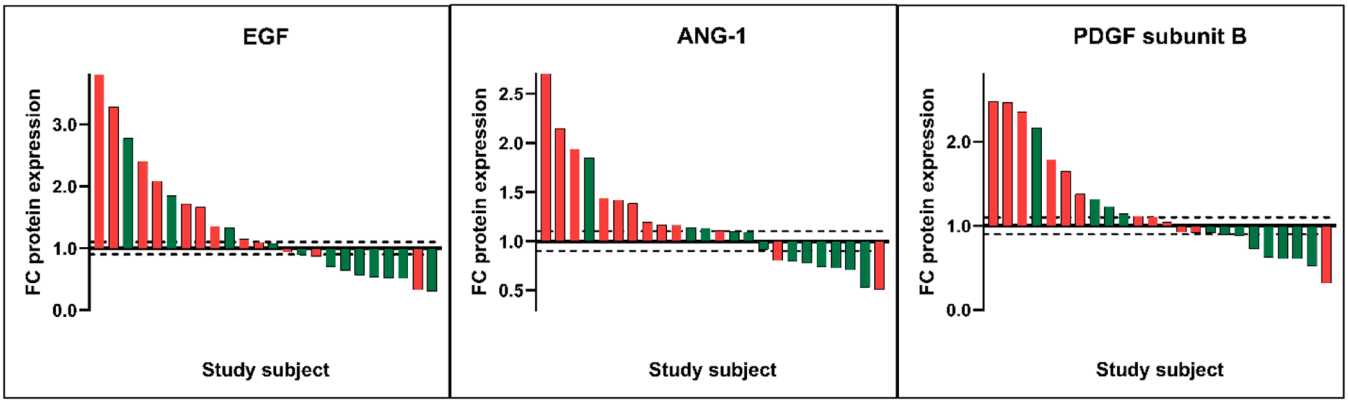

Fig. 2. Waterfall plots illustrating Fold change ratios (FC) per individual study subject between 48 h post-therapy compared to baseline for Epidermal growth factor (EGF), Angiopoietin-1 (ANG-1), and Platelet-derived growth factor subunit B (PDGF subunit B). EGF FC in patients with substantial FC differences (<0.9 or >1.1) was increased in 8 of 10 non-responders and decreased in 8 of 11 responders ($p = 0.0300$, a). ANG-1 FC in patients with substantial FC differences was increased in 10 of 12 non-responders and decreased in 6 of 9 responders ($p = 0.0318$, b). PDGF subunit B FC in patients with substantial FC differences was increased in 8 of 9 non-responders and decreased in 7 of 11 responders ($p = 0.0281$, c). FCs >1.0 reflect increasing, <1.0 reflect decreasing protein levels after HDR-BT compared to baseline. Dashed lines indicate the margin of error between 0.9 and 1.1. Green bars: responders ($n = 12$), red bars: non-responders ($n = 12$). Bars are displayed within the range of the respective y-axis.

non-responders. Activation of the EGF/EGFR pathway is associated with HCC promotion and metastasis formation [15]. EGFR is overexpressed in the majority of patients suffering from HCC [16]. The cellular origin of EGF after HDR-BT remains to be elucidated. In mice, EGF originating from salivary glands contributes to liver regeneration following partial hepatectomy [17] and EGFR seems to be a critical regulator of hepatocyte proliferation in early phases of liver regeneration [18]. Whereas EGFR is suggested to play an anti-tumorigenic role in hepatocytes, EGFR in Kupffer cells/liver macrophages may have a protumorigenic effect [19]. Therefore, early EGF increases following HDR-BT might fulfill a hepato-protective role in the acute phase of liver damage by irradiation, eventually contributing to hepatocarcinogenesis via a host of other pathways.

Regarding other growth factors of our panel, such as FGF-2, our findings differ with respect to other local ablation techniques. Following radiofrequency ablation, FGF-2 was found to be increased in a subset of

Table 2b

Individual protein levels for proliferation markers Angiopoietin-1 (ANG-1), Angiopoietin-2 (ANG-2), Vascular endothelial growth factor A (VEGFA), Vascular endothelial growth factor receptor 2 (VEGFR-2), and Platelet-derived growth factor subunit B (PDGF-B). Responders and non-responders displayed with their respective NPX protein level at baseline (Pre) and 48 h after therapy (Post). The Fold change ratio (FC) reflects the difference of protein levels after therapy compared to baseline. Median and interquartile range (IQR) are shown for responders and non-responders.

		ANG-1			ANG-2			VEGFA			VEGFR-2			PDGF subunit B	
Patient-ID	Response	Pre	Post	FC	Pre	Post	FC	Pre	Post	FC	Pre	Post	FC	Pre	Post	FC
1	Responder	7.03	7.16	1.10	7.53	7.61	1.06	10.61	10.86	1.19	8.71	8.93	1.17	8.88	9.08	1.14
2	Responder	7.99	8.12	1.09	6.12	6.39	1.20	10.22	10.20	0.98	8.38	8.54	1.12	9.49	9.32	0.89
3	Responder	7.44	7.12	0.80	7.38	7.11	0.83	9.86	9.76	0.93	9.20	8.92	0.83	8.63	7.95	0.63
4	Responder	8.43	7.93	0.71	7.31	7.26	0.97	10.47	10.53	1.04	9.34	9.15	0.88	10.31	9.37	0.52
5	Responder	8.38	8.56	1.13	7.18	6.90	0.82	11.50	11.51	1.01	8.98	9.11	1.09	10.43	10.83	1.32
6	Responder	8.33	8.20	0.92	6.39	6.28	0.92	10.13	10.18	1.03	8.94	8.89	0.97	9.93	9.77	0.89
7	Responder	7.78	8.67	1.85	6.17	6.44	1.21	9.55	10.17	1.54	8.53	9.02	1.40	9.11	10.22	2.16
8	Responder	8.11	7.65	0.73	7.43	7.39	0.97	10.34	10.48	1.11	8.64	8.60	0.98	9.83	9.37	0.73
9	Responder	9.52	8.61	0.53	6.94	6.49	0.73	10.76	10.41	0.78	9.19	8.81	0.77	10.92	10.20	0.61
10	Responder	8.93	8.50	0.74	8.61	8.26	0.78	11.52	10.75	0.58	9.62	9.09	0.69	11.08	10.37	0.61
11	Responder	8.15	7.80	0.78	7.04	7.51	1.38	10.35	10.47	1.09	8.93	8.97	1.03	9.37	9.24	0.91
12	Responder	10.41	10.59	1.13	6.84	7.15	1.24	11.25	11.42	1.13	9.39	9.39	1.00	11.12	11.42	1.23
	Median	**8.24**	**8.16**	**0.86**	**7.11**	**7.13**	**0.97**	**10.41**	**10.48**	**1.04**	**8.96**	**8.95**	**0.99**	**9.88**	**9.57**	**0.89**
	Mean	**8.38**	**8.24**	**0.96**	**7.08**	**7.06**	**1.01**	**10.55**	**10.56**	**1.03**	**8.99**	**8.95**	**0.99**	**9.92**	**9.76**	**0.97**
	SD	**0.91**	**0.91**	**0.34**	**0.68**	**0.60**	**0.21**	**0.62**	**0.51**	**0.23**	**0.38**	**0.23**	**0.19**	**0.86**	**0.91**	**0.46**
13	Non-responder	7.79	8.01	1.16	6.90	6.73	0.89	10.09	9.86	0.85	8.87	8.58	0.82	10.10	10.26	1.12
14	Non-responder	7.22	7.73	1.42	7.48	7.35	0.91	11.23	11.17	0.96	8.29	8.52	1.17	8.72	9.45	1.65
15	Non-responder	7.48	8.01	1.44	7.03	6.89	0.90	10.73	10.98	1.19	8.73	8.70	0.98	9.48	10.31	1.79
16	Non-responder	7.53	8.48	1.94	5.86	5.98	1.09	10.36	10.62	1.20	9.08	9.14	1.04	8.59	9.90	2.48
17	Non-responder	7.87	6.90	0.51	7.70	7.56	0.91	10.50	10.17	0.79	9.59	9.41	0.88	9.59	7.94	0.32
18	Non-responder	8.00	8.26	1.20	7.63	7.59	0.97	10.58	10.77	1.14	8.95	8.97	1.01	9.52	9.99	1.38
19	Non-responder	9.97	10.44	1.38	7.18	7.17	1.00	11.54	11.42	0.92	9.55	9.61	1.04	11.31	11.46	1.10
20	Non-responder	7.61	7.84	1.17	7.62	7.61	0.99	10.33	9.98	0.78	9.15	9.00	0.91	9.16	9.03	0.92
21	Non-responder	8.18	8.32	1.11	7.36	7.43	1.04	9.84	10.36	1.44	8.97	9.03	1.04	9.88	9.95	1.05
22	Non-responder	8.62	10.79	4.50	7.33	7.53	1.15	10.82	12.14	2.48	9.38	9.71	1.26	10.35	11.58	2.35
23	Non-responder	6.30	7.40	2.14	7.25	7.20	0.97	10.18	10.30	1.08	9.23	9.06	0.89	7.17	8.47	2.46
24	Non-responder	8.18	7.87	0.80	7.29	7.20	0.94	10.43	10.38	0.97	9.27	9.15	0.92	9.61	9.50	0.92
	Median	**7.83**	**8.01**	**1.29**	**7.31**	**7.28**	**0.97**	**10.46**	**10.50**	**1.03**	**9.11**	**9.04**	**0.99**	**9.56**	**9.93**	**1.25**
	Mean	**7.90**	**8.34**	**1.56**	**7.22**	**7.19**	**0.98**	**10.55**	**10.68**	**1.15**	**9.09**	**9.07**	**1.00**	**9.46**	**9.82**	**1.46**
	SD	**0.87**	**1.15**	**1.02**	**0.49**	**0.47**	**0.08**	**0.48**	**0.66**	**0.46**	**0.36**	**0.37**	**0.13**	**1.02**	**1.07**	**0.69**

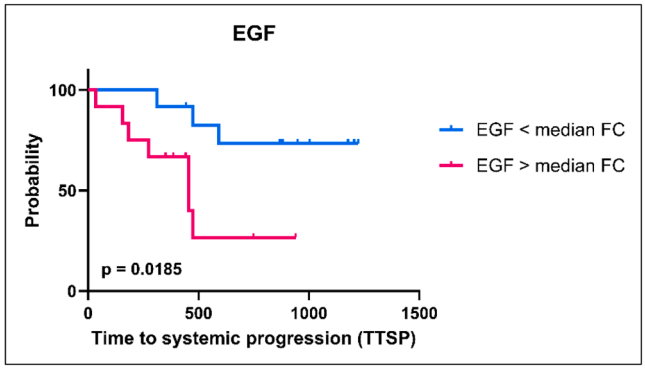

Fig. 3. Kaplan-Meier curves displaying time to systemic progression (TTSP) for patients according to the respective median protein Fold change (FC) 48 h post-therapy compared to baseline for Epidermal growth factor (EGF). Patients with EGF fold change ratio (FC) levels > median EGF FC demonstrated significantly shorter TTSP than patients with EGF FC < median EGF FC ($p = 0.0185$).

patients. In-vitro findings confirmed its contribution to proliferation, induced by thermally stressed hepatocytes [20]. This may be attributed to differences in the mechanism of activation by different energy sources and cell death.

It is well known that radiotherapy may induce rebound effects consisting of growth factor-induced angiogenesis as a consequence of vessel regression and vascular collapse caused by endothelial cell apoptosis and senescence [21]. In our study, we found that specifically ANG-1 was increased in non-responders, whereas no differences in protein level were found for the other main ligand of the receptor tyrosine kinase TIE2, ANG-2. This partially seems to be discordant to the literature. In a large HCC cohort, Pestana et al. [22] found elevated plasma ANG-1 to be associated with better overall survival (OS), whereas increased ANG-2 was associated with shorter OS. One potential explanation for our findings of increased ANG-1 after HDR-BT might be due to the modulating effect of ANG-1 in the stabilization and maturation of newly formed vessels. ANG-1 is increased at a relatively early time following damage to endothelial cells in the tumor and surrounding liver tissue [23] to execute its vasculoprotective effects [24]. Also, it has been shown that ANG-1 promotes endothelial cell survival by inhibiting apoptosis following irradiation [25]. Taken together, we hypothesize that ANG-1 is secreted for protective reasons in the early post-treatment phase but subsequently contributes to hepatocarcinogenesis [26,27].

ANG-2 was not found to be altered in our cohort. VEGFA and VEGFR-2 were also not significantly increased in either responders or non-responders. Even though pathways induced by VEGFR-2 and MET, the respective receptors for VEGFA and HGF, are associated with tumor growth and metastasis in HCC [28,29], we did not find a correlation of any of these three proteins with inferior outcome in our small cohort. Possibly the type of irradiation also determines the change in proteins, as increased angiogenic factors such as VEGFR-2 have been shown in low dose, but not high dose radiation schemes [30,31].

Closely related to VEGF, the PDGF family with its subform PDGF subunit B is known to promote liver fibrosis and HCC [32]. In HCC patients, tissue expression of PDGF subunit B in concert with VEGFR-3 is associated with short OS in HCC patients [33]. A main pathway triggered by PDGF subunit B is the PI3K/Akt/Stat-3 pathway [34,35]. Interestingly, Stat-3 was shown to be associated with tumorigenesis following local ablation of liver malignancies and to serve as a possible target for drug inhibition [36,37]. Moreover, PDGFR has been proven to be a key target of receptor kinase inhibitors such as Sorafenib or Sunitinib [38,39].

Despite successful integration in current guidelines, tumor

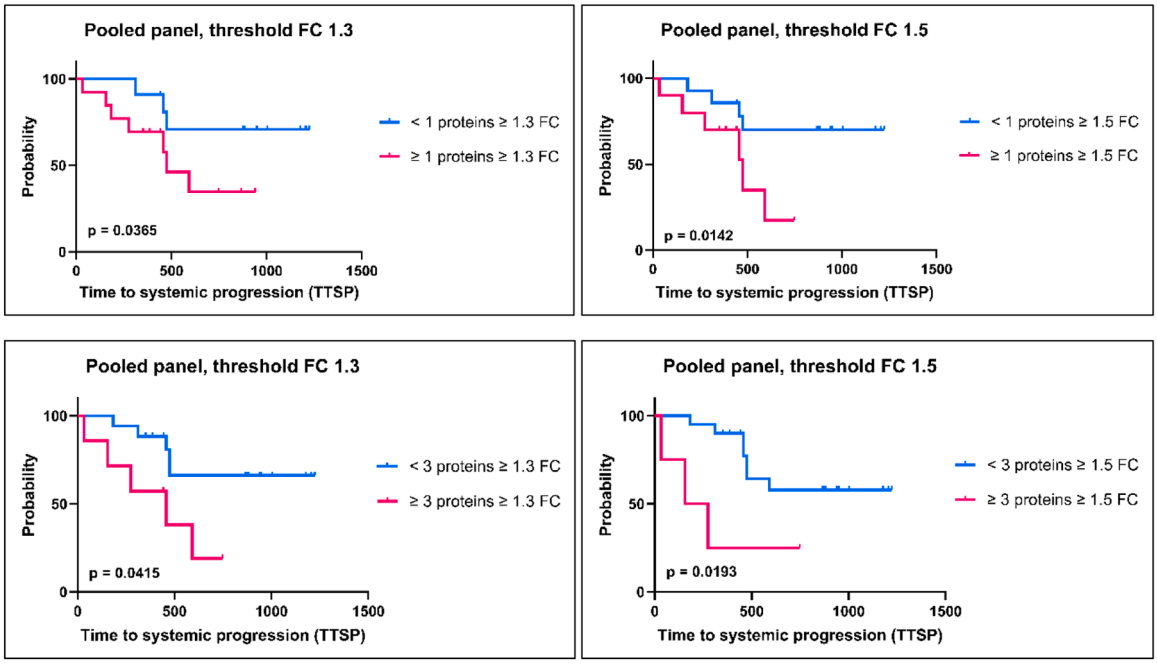

Fig. 4. Kaplan-Meier curves displaying time to systemic progression (TTSP) for patients according to the respective median protein Fold change (FC) 48 h post-therapy compared to baseline for pooled analysis. Patients with a fold change ratio (FC) of ≥1.3 or ≥1.5 in ≥1 out of 9 proliferation/angiogenesis markers demonstrated significantly shorter time to systemic progression (TTSP) than all patients not fulfilling this criterion ($p = 0.0365$, a; $p = 0.0142$, b, respectively). Furthermore, all patients with a FC of ≥1.3 or ≥1.5 in ≥3 out of 9 proliferation/angiogenesis markers demonstrated significantly shorter TTSP than all patients not fulfilling this criterion ($p = 0.0415$, c; $p = 0.0193$, d, respectively).

recurrence after local tumor ablation in HCC represents a major drawback in clinical practice. Our group has already shown secondary unwanted effects of HDR-BT by means of increased protumorigenic microRNA 21 which could serve as an additional tool to identify patients who are prone to progression [12]. Nevertheless, increases in growth factors have been found to be associated with outcome and contributing to tumorigenesis following radiofrequency ablation, whereas a single biomarker alone failed to sufficiently predict outcome in a small cohort [11]. However, we established various models of pooled panel analyses of proliferation and angiogenesis markers rendering promising association with TTSP that may qualify to predict clinical outcome.

The limited cohort size of 24 patients requires further confirmatory investigation with a larger study cohort. It must be acknowledged that the proposed biomarker set did not prove valuable for pre-therapeutic prediction parameter given its lack of capacity to differentiate between responders and non-responders. Even though it would have been desirable to have a powerful tool for predicting outcome even before therapy, we observed prognostic value of protein alterations only considering post-HDR-BT protein measurements to indicate patients at risk.

To date, it is unknown as to whether the described protein changes are dependent on the primary tumor or mainly are produced by heat-stressed or recruited cells within the reactive periablational margins. Information on protein changes in other tumor entities such as metastasized colorectal carcinoma after HDR-BT would indicate whether these biomarkers could be more generally applied. Additional time points of analysis after local ablation may be required to obtain a more comprehensive picture. Molecules after local ablation have characteristic dynamics in terms of induction and expression. Also, the characteristic profile of plasma protein dynamics varies among the proteins themselves. Presumably, each protein requires individualized study to identify peak plasma levels over time. Therefore, subsequent investigation of additional time points after HDR-BT is needed. Furthermore, our findings are based on plasma analysis only and may not reflect changes in the tumor microenvironment. Characterization of the tumor microenvironment is important for the understanding of locally induced effects and pathways, especially in terms of cellular origins. Finally, although the proteins investigated in this study have been linked to tumor growth distant from the ablation site, additional investigation of downstream analysis combined with the understanding of the cellular source of the described proteins will help to better understand the potential of the proposed biomarkers.

Conclusion

Our findings demonstrate unwanted systemic effects of proliferation and angiogenesis after HDR-BT of HCC and their association with patient outcome by the means of increasing EGF and ANG-1.

Ethics approval and consent to participate

In this monocentric clinical study, we analyzed prospectively acquired data of 24 patients from the "ESTIMATE" patient cohort (Studiennummer: DRKS00010587, Deutsches Register Klinischer Studien). Ethical approval was provided by the ethics committee "Ethikkommission bei der LMU München" (reference number "17–346″) on June 20, 2017 and August 26, 2020.

Declaration

The identical cohort of patients was analyzed for microRNA 21 and microRNA 210 expression with results published elsewhere in Radiation Oncology (Radiat Oncol. 2023 Jul 28;18(1):125).

Consent for publication

Patients provided written informed consent for both the local ablative treatment and study inclusion.

Data availability

Research data are stored in an institutional repository and will be shared upon request to the corresponding author.

CRediT authorship contribution statement

Lukas Salvermoser: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Shraga Nahum Goldberg:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Marianna Alunni-Fabbroni:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Philipp Maximilian Kazmierczak:** . **Moritz Nikolaus Gröper:** Writing – original draft, Investigation. **Jan Niklas Schäfer:** Investigation. **Elif Öcal:** Investigation. **Tanja Burkard:** Data curation. **Stefanie Corradini:** Supervision. **Najib Ben Khaled:** Investigation. **Agnese Petrera:** Methodology. **Moritz Wildgruber:** Supervision, Investigation, Data curation. **Jens Ricke:** Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Matthias Stechele:** Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The work of Shraga Nahum Goldberg was supported by the Israeli Science Foundation [grant number 904/2020]. All the other authors do not have to declare conflict of interests.

Acknowledgements

Not applicable

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2024.101919.

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