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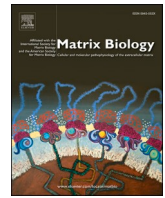
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## Tenascin-C targeting strategies in cancer

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### ABSTRACT

Tenascin-C (TNC) is a matricellular and multimodular glycoprotein highly expressed under pathological conditions, especially in cancer and chronic inflammatory diseases. Since a long time TNC is considered as a promising target for diagnostic and therapeutic approaches in anti-cancer treatments and was already extensively targeted in clinical trials on cancer patients. This review provides an overview of the current most advanced strategies used for TNC detection and anti-TNC theranostic approaches including some advanced clinical strategies. We also discuss novel treatment protocols, where targeting immune modulating functions of TNC could be center stage.

**Abbreviations:** ADA, anti-drug antibodies; ADCC, antibody-dependent cell mediated cytotoxicity; ADC, antibody-drug-conjugate; AgNPs, metallic silver nanoparticles; ApoE, apolipoprotein E; 211At, astatine-211; AML, acute myeloid leukemia; BALB, bagg albino; CAF, carcinoma associated fibroblasts; CAIA, collagen antibody induced arthritis; CAR-T, chimeric antigen receptor-T cell; CCR, C-chemokine receptor; CCL21, chemokine ligand 21; CD, cluster differentiation; CDR, complementarity-determining regions; ch, chimeric; CPTD, camptothecin prodrug; CR, complete response; Cri, incomplete count recovery; 64Cu, copper-64; CXCL, C-X-C motif chemokine ligand DAMP, danger associated molecular pattern; DNA, deoxyribonucleic acid; DOTA, dodecane tetraacetic acid; DOX, doxorubicin; ECM, extracellular matrix; EDA, extra domain A; EGFL, epidermal growth factor-like repeats; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial mesenchymal transition; 18F, fluorodeoxyglucose; Fab, fragment antigen-binding; FAM, fluorescein; FBG, fibrinogen like globular domain; Fc, fragment crystallin; FITC, fluorescein isothiocyanate; FN, fibronectin; FNIII, fibronectin type III repeat; g, gram; 68Ga, gallium-68; GBM, glioblastoma; HAMA, human-anti-mouse antibody; HCab, heavy-chain-only antibody; HER2, human epidermal growth factor receptor 2; HL, Hodgkin's lymphoma; HLA, human leucocyte antigen; HLE-B3, human lens epithelial cells; HNSCC, head and neck squamous cell carcinoma; HSCT, hematopoietic stem cell transplantation; hTNC, human TNC; HUVEC, human umbilical vein endothelial cells; IC50, inhibitory concentration 50; ID, injected dose; IF, immunofluorescence; IgG, immunoglobulin G; IHC, immunohistochemistry; 131I, iodine-131; IL, interleukin; IU, international unit; Kd, equilibrium dissociation constant; kDa, kilodalton; KPS, Karnofsky performance status; KRAS, Kirsten rat sarcoma viral oncogene; LNA, locked nucleic acid; 177Lu, lutetium-177; mAb, monoclonal antibody; mg, milligram; ml, milliliter; ng, nanogram; MAREMO, matrix regulating motif; MBS, MAREMO binding site; MFR, magnetic fluorescent radioisotope; mRNA, messenger RNA; miRNA, microRNA; MMTV-NeuNT, mouse mammary tumor virus-NeuNT; MR, magnetic resonance; MTD, maximum tolerated dose; Nb, nanobody; NB, nanobubbles; NHL, non-Hodgkin lymphoma; NK, natural killer cell; nM, nanomolar; 4NQO, 4-Nitroquinoline 1-oxide; NRP-1, neuropilin-1; NP, nanoparticle; NSLC, non-small lung carcinomas; NW, nanoworm; OS, overall survival; OSCC, oral squamous cell carcinoma; PAGRIT, pretargeted antibody-guided radioimmunotherapy; PC-3, prostate carcinoma cells; PDAC, pancreatic ductal adenocarcinoma; PD-1, programmed cell death protein -1; PD-L1, programmed death-ligand 1; PET, positron emission tomography; Poly-ICLC, polyinosinic-polycytidylic acid; PR, partial response; PTX, paclitaxel; RA, rheumatoid arthritis; RGD, Arginyl glycol aspartic acid; Rip1-Tag2, rat insulin gene promoter1-Tantigen 2; RNA, ribonucleic acid; scFv, single-chain fragment; SCRC, surgically created resection cavity; SD, stable disease; SELEX, systemic evolution of ligands by exponential enrichment; SIP, small immunoprotein; siRNA, short interfering RNA; SLE, systemic lupus erythematosus; SSL, nanoliposome; STAT, signal transducer and activator of transcription; TA, tenascin assembly; TGF, tumor growth factor; TIL, tumor infiltrating leukocyte; TLR4, toll-like receptor 4; t-Lyp-1, truncated Lyp-1; TME, tumor microenvironment; TNC, tenascin-C; TNCKO, TNC knock-out; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; VEGF, vascular endothelial growth factor; VHH, variable heavy domain of heavy chain; 90Y, yttrium -90.

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## Introduction

To date we still lack effective means for an early detection of some cancers with an extraordinary short survival and no cure. Also, for less malignant cancers, if detected sooner, anti-cancer treatment could start earlier thus enhancing the survival chances of the patient. The extracellular matrix (ECM) comprised of several molecules represents a major part of the tumor microenvironment (TME). The ECM is providing not only mechanical support but also is regulating cell behaviour and, often is a major obstacle in cancer therapy generating physical or biochemical barriers [1–3].

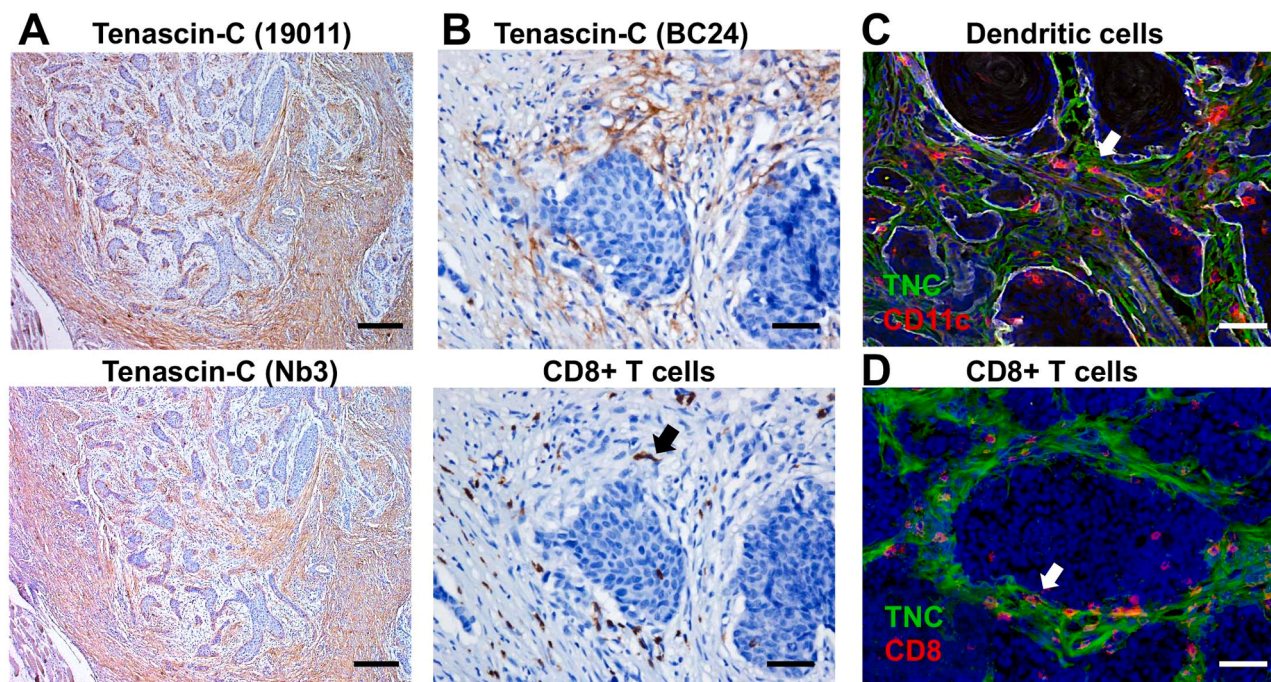
Based on the high abundance, some ECM molecules are good candidates for cancer diagnosis and therapy which applies to tenascin-C (TNC) that is highly expressed in most solid and hematological cancers forming stromal niches ([4–6], Fig. 1A–D). Human TNC (hTNC) is composed of an oligomerization TNC assembly (TA) domain, 14 ½ EGF-like repeats (EGFL), up to 17 fibronectin type III repeats (FNIII) and a C-terminal fibrinogen like globular domain (FBG) (Fig. 2A). Nine alternatively spliced FNIII domains (A1–A4, B, AD2, AD1, C, and D) can be inserted between the 5th and 6th FNIII domains in the human protein [7,8] (Fig. 2A). The insertion of these additional FNIII domains increases the size of the TNC protein giving rise to several TNC-long isoforms in comparison to a TNC-short isoform expressing only the constant FNIII domains 1–8. In contrast to normal tissue where TNC seems mostly composed of the constant FNIII domains, in tumors several large isoforms, including one or more of the alternatively spliced FNIII domains, are expressed (reviewed in [9–12]).

Whereas TNC is abundantly expressed during embryonic development, in adult tissues TNC expression is largely restricted to a few sites (reviewed in [29]). Here, TNC might have specific so far poorly understood functions likely providing physical and immunological barriers. TNC gets induced upon tissue damage and is promoting repair by acting as a danger associated molecular pattern (DAMP) molecule. However,

when TNC expression is not turned off timely or is induced out of control it promotes inflammatory pathologies such as rheumatoid arthritis (RA), myocarditis, chronic kidney disease and Systemic Lupus Erythematosus (SLE) in addition to cancer [29–34]. TNC can promote cancer by numerous mechanisms that have been reviewed elsewhere suggesting that TNC triggers survival, proliferation, migration, invasion, angiogenesis, metastasis and immune suppression promoting the immune exclusion phenotype (reviewed in [11,15,29]), (Fig. 1B–D). TNC can also form different proteoforms through proteolytic cleavage and post-translational modifications (e.g. glycosylation, citrullination), however little is known how these modifications impact the TNC functions. The average molar abundance of TNC monomers in the tumor proteome is more than two orders of magnitude higher than the level of human epidermal growth factor receptor 2 (HER2) which has been recognized as a valid target for Herceptin-based therapies [35,36]. Thus, high TNC levels and in particular expression of these additional FNIII domains have been recognized as excellent target and/or marker for tumor diagnosis and zip code for therapy [37] over 30 years ago (reviewed in [38]). Indeed, extensive research has delivered over hundreds of TNC-specific tools, some already examined in early-stage clinical trials (reviewed in [39,40]). Here, we will provide a historical and critical overview about the most important tools that target TNC as biomarker, for *in situ* tumor imaging and as zip code for the delivery of cytotoxic or immune stimulating agents (Fig. 2B). We will also describe recent discoveries and, finally will provide an outlook on how we could apply our constantly increasing knowledge and tools for a future tailored anti-cancer therapy boosting anti-tumor immunity.

### Full length monoclonal antibodies against TNC

Some polyclonal antibodies raised against full length TNC have the advantage to gain insight into the many isoforms of TNC expressed in tissues [29]. However, the just available technology of monoclonal antibody production largely promoted the discovery of the structure and



**Fig. 1.** (A) TNC expression in paraffin embedded HNSCC tissue detected in consecutive sections with the rabbit polyclonal antibody 19,011 and nanobody Nb3 as indicated. Scale bar 100  $\mu$ m. Reproduced with permission [13], (B) Expression of TNC (brown, upper image) in a human breast cancer. TNC is expressed in the stroma surrounding tumor cell nests with abundant CD8+ T cells (lower image). Scale bar, 50  $\mu$ m. Reproduced with permission [14], (C) Expression of TNC (green, MTn12) and laminin (white) organized inside the stroma encapsulating the tumor nests in a murine 4NQO induced tongue oral squamous cell carcinoma and entrapping dendritic cells (CD11c, red). Scale bar, 50  $\mu$ m. (D) Expression of TNC (green, MTn12) organized inside the stroma encapsulating the tumor nests in a murine MMTV-NeuNT mammary gland tumor entrapping CD8+ T cells (red). (C, D) Reproduced with permission [15].

function relationship of TNC (reviewed by [41]). Meanwhile, a vast number of monoclonal antibodies (mAbs) has been generated to determine TNC expression in tumor tissues by several technologies (e.g. ELISA, tissue staining by immunofluorescence (IF) or immunohistochemistry (IHC), immunoblotting) and by *in situ* tumor imaging.

Antibodies were raised in mice against purified or recombinant full length TNC or specific recombinant TNC domain molecules. Many of these mAbs can also be used as carriers to deliver tumoricidal agents such as toxins, cytotoxic or immune modulating molecules and radioisotopes in a so-called Antibody-Drug-Conjugate (ADC) format

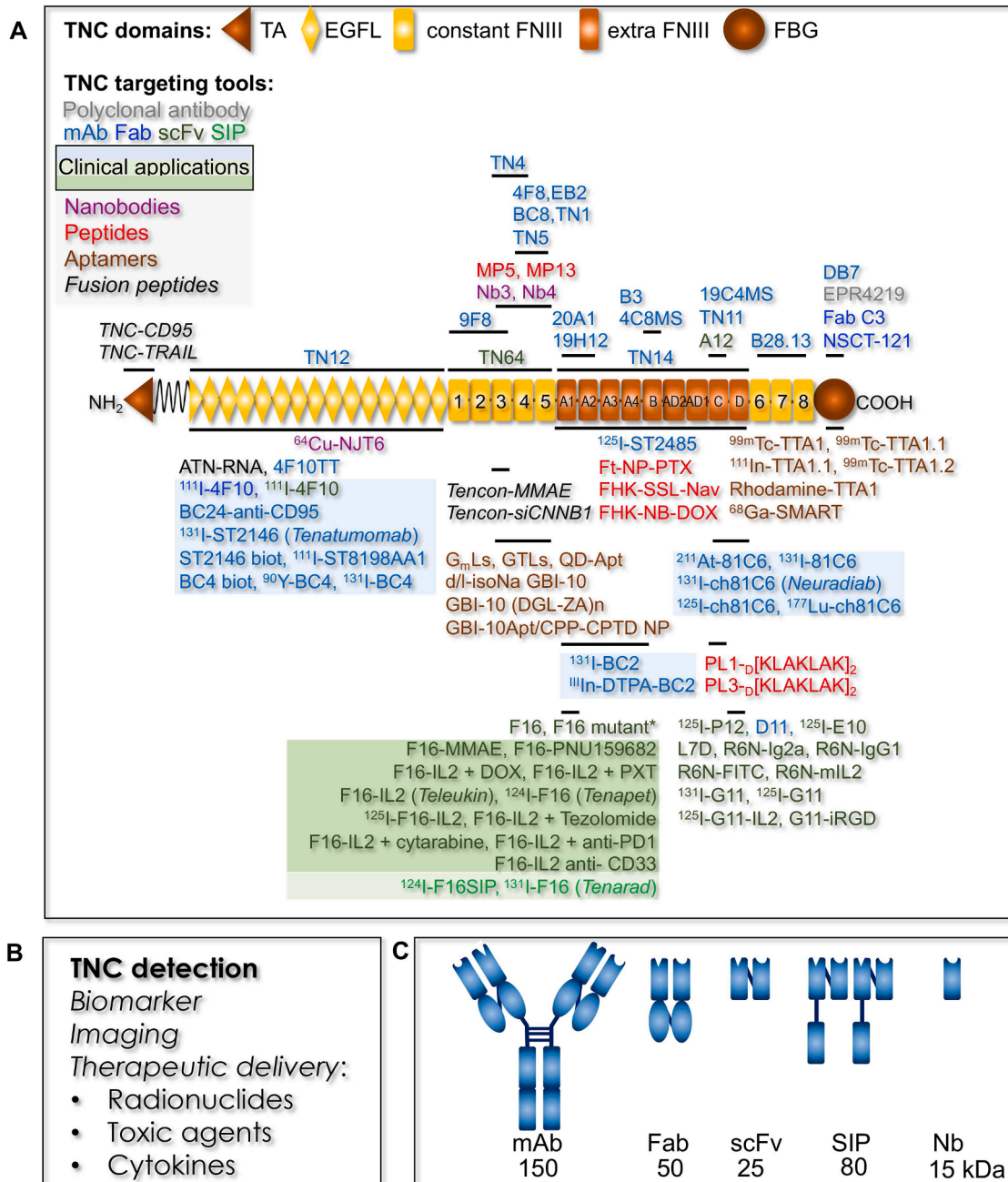


Fig. 2. Overview of TNC targeting and applications in cancer diagnosis and therapy

(A) The domain structure of TNC is schematically depicted as TA, TNC assembly domain, EGFL, EGF like domains, FNIII domains (constant and extra) and FBG, fibrinogen like globular domain. In this non-exhaustive summary of TNC binders the respective binding domain in TNC is marked with a black line. Binders with not further identified sequence in TNC are not shown. Different classes of TNC binding molecules are assigned by a color code for immune molecules: polyclonal antibody (light grey), monoclonal antibodies (light blue), Fab (dark blue), scFv (dark green), SIP (light green) and nanobodies (purple) and, for small molecules: peptides (red), aptamers (brown) and TNC fusion molecules (black). Also, the ATN-RNA reducing TNC expression is indicated. Green and blue background shading is marking TNC binders that have been used in clinical applications (case reports, clinical trials). References can be found in Table 1 and in the text. References for antibodies not present in Table 1 and 2, MAB1911, 9F8 [16], DB7 [17], EPR4219 (ABCAM), 4F8 (Merck Millipore), TN1 [18], TN4 [18], TN5 [18], TN11 [19], TN14 [20], D11 [21], EB2 [22], BC3 [23] and BC28.13 [24], F16 mutant\* [21], F16-MMAE (monomethyl auristatin E) [25], F16-PNU159682 [26], <sup>125</sup>I-ch81C6 [27], <sup>177</sup>Lu-ch81C6 [28]. (B) Detection of high TNC expression in malignant tumors can be exploited as biomarker (ELISA, tissue staining), for non-invasive tumor imaging (PET/CT, MRI) and as zip code for therapeutic delivery of death inducing radionuclides, toxic agents or immune stimulatory cytokines into the tumor. (C) Schematic representation of different immune molecules targeting TNC and indication of the molecular weight (kDa).

(reviewed in [39,40,42,43]). The two Tables and Fig. 2A summarize the most promising molecular tools specific for TNC where some prominent examples are described below.

In the early days of TNC research the TNC-specific antibodies **BC2**, **BC4** and **81C6** were generated, and then extensively investigated as radionuclides for a “pretargeted antibody-guided radioimmunotherapy” (PAGRIT) approach. These studies showed promising results in terms of good delivery and tumor uptake in patients with a variety of cancers detailed in Table 1 [44–49].

Another example is  $^{90}\text{Y}$ -“**BC4**”, recognizing the TNC *EGFL* repeats, that was well taken up and enriched in the tumor without any diffusion into the adjacent normal brain tissue. Whereas  $^{90}\text{Y}$ -BC4 did not show any clinical response on its own [48,99], when administered together with mitoxantrone (a cytostatic topoisomerase inhibitor blocking tumor cell division) to 26 patients with recurrent GBM, an increased median survival from 20 to 22 months was observed [60,61]. BC4 was also used for a three-step *in situ* imaging employing the avidin/biotin system that due to strong immunogenicity towards avidin and streptavidin led to discontinuation [62], (Table 1).

Another mAb “**81C6**”, recognizing the TNC *FNIII-C* and TNC *FNIII-D* domains, was extensively used for radiotherapy in human cancer patients (recently reviewed in [39,40]). One approach comprised the injection of the  $^{131}\text{I}$ -radiolabeled 81C6 ( $^{131}\text{I}$ -81C6) into the GBM resection area, however, the labeled antibody did not reach the required optimal effective dose because of toxic effects on the normal brain tissue (such as focal radiation necrosis and brain edema, hematological and neurological toxicity) and therefore other approaches with different radionuclides and humanized chimeric 81C6 antibody conjugates were established [72,73,100]. Also, different radioisotopes were tested as the radioactive dose emitted by each radioisotope as well as its decay are different. 81C6 was originally coupled to iodine-131 (routinely used as immunotracer), however, to reduce the patient confinement period, low- $\beta$  energy radionuclides have also been applied [28], (Table 1). In particular, the  $\alpha$ -particle emitting radionuclide Astatine-211 ( $^{211}\text{At}$ ) was used as  $^{211}\text{At}$  has an excellent stability [75] and a limited cellular retention (due to its relatively short half-life of approximately 7.2 h) [101].  $^{211}\text{At}$ -81C6 was injected into the surgically created resection cavity (SCRC) of 18 patients with recurrent GBM, causing no neurological toxicity but an increase of the median OS to 54.1 weeks compared to 23 weeks (placebo) and 31 weeks (carmustine) which is quite remarkable [75], (Table 1).

To minimize immunogenicity, the murine Fc-sequences were replaced with human IgG2 Fc-sequences [102] giving rise to humanized chimeric 81C6 (ch81C6) that was coupled to  $^{131}\text{I}$  ( $^{131}\text{I}$ -ch81C6, *Neuradiab*) and studied in NHL patients in phase I clinical trials. Only one of the nine patients achieved a complete response (CR), one a partial response (PR) and the others stable disease (SD) lasting from 2 to longer than 8 months which is remarkable and supporting TNC as a valid zip code for therapy [74]. Altogether, 81C6 has been labeled with  $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{177}\text{Lu}$  (lutetium), and  $^{211}\text{At}$  where each radionuclide has its own properties in terms of half-life, enrichment in the tumor and tumoricidal effects. Although the clinical trials with different parameters are difficult to compare, it appears that  $^{211}\text{At}$ -81C6 has the strongest therapeutic effect. Unfortunately, none of these 81C6 associated strategies is in the clinical practice today (reviewed in [39,40,43]).

Moreover, two mAbs derived by immunization of BALB/c mice “**ST2485**”, (directed against recombinant TNC *FNIII-A-D*), and “**ST2146**”, (directed against the TNC *EGFL* domains), were further engineered as radionuclide conjugates (Fig. 2A). In tumor mice,  $^{125}\text{I}$ -ST2146 showed the most prominent tumor accumulation (approximately 16 % of the injected dose (ID)/g of tumor) and very low levels in non-target tissues (blood, spleen, kidney, liver) [71], (Table 1).

Encouraged by these promising results, Homogenize: “**ST2146**” was investigated in a phase I clinical trial to deliver  $^{131}\text{I}$  in patients suffering from several treatment refractory TNC positive tumors (NCT02602067) (Fig. 2A, Table 1). However, as the uptake of  $^{131}\text{I}$ -ST2146

(*Tenatumomab*) into the tumor lesion was negligible, the clinical trial was discontinued. Results pointed at high inter-individual variable responses that may include the production of anti-drug antibodies (ADA) [103,104]. An improved version of ST2146 was subsequently generated by conjugation with three chelator moieties where ST2146-DOTA (ST8198AA1) was the best with over 50% immunoreactivity that was subsequently radiolabeled with  $^{111}\text{I}$  and applied in pre-treatment imaging and dosimetry giving rise to a radiolabeling efficiency of 77.0%, however no information about anti-tumor effects is provided [68].

These examples showed that full-length conventional antibodies may trigger immunogenicity and antibody-dependent cell mediated cytotoxicity (ADCC). Moreover, as most of these antibodies are of murine origin, they may not be suitable for repeated administration in human patients due to possible induction of human-anti-mouse antibodies (HAMA). The slow rate of tumor penetration of full-length antibodies [105] as well as their expensive manufacturing, are additional drawbacks that may explain why some of these highly TNC specific mAbs did not proceed beyond phase I/II clinical trials. Nevertheless, these studies have paved the way for the development of shorter and humanized anti-TNC mAbs or smaller mAb fragment molecules (scFv, Fab, SIP) using TNC as molecular zip code for tumor delivery of radionuclides or cytokines (so called “*immunocytokines*”) (Fig. 2A-C).

### Small anti-TNC antibody molecules

In addition to TNC specific full-length mAbs (150–160 kDa), composed of two heavy chains and two light chains, smaller TNC specific Fab molecules (around 50 kDa, representing one light chain and half of the heavy chain) and single chain variable molecules, scFv (around 25 kDa, representing only the two variable domains from the light and heavy chain) have been engineered altogether leading to many TNC specific smaller sized antibody molecules (reviewed in [39,43], Fig. 2C). As an example, the two small Fab’, Fab and scFv “**4F10**” antibody fragment molecules recognizing the TNC *EGFL* sequences were, upon radiolabeling ( $^{111}\text{I}$ -Fab’,  $^{111}\text{I}$ -Fab and  $^{111}\text{I}$ -scFv), applied to rats with myocarditis which allowed to image the damaged myocardium 24 h after injection, suggesting that scFv-4F10 may be useful for *in vivo* imaging of myocardial injuries and cancer [64,65].

For detection of constant domains in all TNC isoforms, the scFv “**TN64**” antibody was raised against bacterially expressed human TNC *FNIII-1–5*. This antibody reduced TGF $\beta$ 2-induced signaling in cultured lens HLE-B3 epithelial cells [69] and impaired TNC functions in a murine collagen antibody-induced RA (CAIA) model, where disease symptoms at tissue and cytokine/chemokine levels were reduced by TN64 [70]. Whether this mAb may have anti-tumor activity has still to be determined (see below).

To raise an scFv specific for *FNIII-C* highly expressed in many cancers (reviewed in [9,12]), antibody phage display technology was applied to retrieve “**G11**” (with the so far lowest dissociation constant of 4.2 nM) [91]. The  $^{125}\text{I}$ -labeled G11 selectively accumulated in U87MG GBM tumor xenografts highly expressing the TNC *FNIII-C* domain.  $^{125}\text{I}$ -G11SIP also detected atherosclerotic plaques in ApoE knockout mice and thus might also be useful for imaging of advanced atherosclerotic plaques [92], (Table 1).

The scFv antibody “**L7D**”, recognizing TNC *FNIII-D*, (also highly expressed in many malignant tumors (reviewed in [9,12]), was again isolated by antibody phage display technology, and further engineered giving rise to “**R6N**” (Kd value of 41 nM to 24 nM). Both antibodies are cross-reactive with murine TNC which could be valuable for applications in preclinical murine tumor models [21,94]. TNC specificity in the IgG2a-FITC format was confirmed by IF staining of xenograft tumors (Fig. 2A, Table 1). R6N was also found to localize inside the tumors with a high tumor-to-blood ratio. Finally, the *R6N/IL2 immunocytokine* fusion was generated that caused tumor regression [94]. However, despite these promising results in preclinical models, to our knowledge no R6N-conjugates have been investigated in clinical trials yet.

**Table 1**

Overview about TNC targeting antibodies and associated conjugates

Antibodies, antibody fragments and associated conjugates are sorted according to recognition of TNC sequences (N to C terminus). Information about the applied assay, results, clinical trials and reference is provided.

Targeting molecule	Compound name	Sequence in TNC	Applied assay	Disease context	Observation	Refs.
<b>Polyclonal antibody</b>						
	AB19011 rabbit	NN	ELISA	Exosomes from plasma of COVID-19 patients	Correlation of TNC enrichment in exosomes with COVID19 severity	[50]
	AB19011 rabbit	NN	Immunohistochemistry	Lungs from MRSA-infected mice	High TNC expression in airways of infected mice	[51] [52]
<b>Monoclonal antibody, monoclonal antibody derived antibody fragments and associated conjugates</b>						
	143DB7	NN	Immunohistochemistry	Human aortic valve stenosis	High TNC expression in stenotic aortic valves	[53]
	AB213831	EGFL (G23-P625)	ELISA	COVID19	Correlation of high TNC expression in bronchoalveolar lavage fluid with disease severity	[54]
	BC24	EGFL	Immunohistochemistry Immunostaining	Invasive breast carcinomas Renal cell carcinomas Hepatocellular carcinomas Pancreatic neuroendocrine tumors	Detection of high TNC expression in tumors and metastasis	[20] [55]
	BC24	EGFL	Collagen gel invasion assay	Human colorectal carcinoma HCT-8/11 cells	Inhibition of tumor cell invasion	[56]
	BC4	EGFL	Immunostaining	Human fibroblasts Human tumors (breast, brain, ovary, colon rectum, thyroid, prostate)	Detection of TNC expression in benign and malignant tissues	[57] [6]
	<sup>90</sup> Y-BC4	EGFL	Radio immunotherapy	GBM	Deep penetration into brain adjacent tissue No clinical response	[58,59]
	<sup>90</sup> Y-BC4 plus mitoxantrome	EGFL	Radio immunotherapy	Patients with recurrent GBM	Increased median survival (22 versus 20 months)	[60]
	Biotinylated BC4	EGFL	PAGRIT	Anaplastic astrocytoma, GBM	Disease stabilization Strong immunogenicity towards avidin and streptavidin	[61] [62]
	4F10TT	EGFL	Immunohistochemistry Immunoblotting	Human breast cancer tissue	High TNC expression	[63]
	<sup>111</sup> I-4FT10 (Fab)	EGFL	Biodistribution	Acute rat myocarditis	Specific binding to the damaged myocardium	[64]
	<sup>111</sup> I-4FT10 (scFv)	EGFL	Biodistribution	Acute rat myocarditis	Specific binding to the damaged myocardium	[65]
	ST2146	EGFL	ELISA Immunostaining	20 solid tumors (e.g. breast, liver, lung, rectum), over 60 normal tissues (e.g. skin, cornea, lachrymal glands, esophagus, uterine cervix)	High TNC levels in cancer tissues Low/no TNC expression in normal tissues Detection of recombinant and native TNC	[66]
	<sup>131</sup> I-ST2146 <i>Tenatumomab</i>	EGFL	Radio immunotherapy	Patients with cancer of the breast, HNSCC, skin, respiratory tract, urogenital tract, digestive system, pancreas, connective and soft tissue, lymphoma, NHL	Heterogenous inter-individual responses Anti-Drug Antibodies No significant antibody tumor uptake	Sigma Tau company, 2015 NCT02602067 Phase I trial terminated
	<sup>131</sup> I-ST2146 <i>Tenatumomab</i>	EGFL	Immunohistochemistry	Biopsies from NHL patients	Tumor detection	[67]
	<sup>111</sup> I-ST2146 -DOTAMA	EGFL	ELISA Different chelators (NCS-DTPA, NCS-DOTA, NHS-DOTA)	Breast cancer, colorectal carcinoma, lung cancer, ovary cancer, NHL tissue	Stable complex Residual immunoreactivity of ST2146 -DOTAMA	[68]
	TN64 (scFv)	FNIII1–5	Cell culture assays	Human lens epithelial cells	Decreased migration Decreased proliferation Reduction of cellular plasticity	[69]
	TN64 (scFv)	FNIII1–5	Cell culture assays	Patient-derived synovial fibroblasts	Inhibition of migration	[70]
	TN64 (scFv)	FNIII1–5	In vivo RA assay	CAIA mouse model	Reduced TNC expression Reduced inflammatory cytokine expression Reduced cartilage destruction	[70]
	ST2485	FNIIIA-D	Immunostaining	Breast cancer, colon carcinoma tissue	High TNC expression in the tumors	[71]
	ST2485	FNIIIA-D	ELISA	Recombinant TNC FNIIIA-D	Binding to TNC	[71]
	<sup>125</sup> I-ST2485	FNIIIA-D	Biodistribution study	HT29 colon carcinoma xenografts	Tumor detection High tumor to non-tumor ratio	[71]

(continued on next page)

Table 1 (continued)

Targeting molecule	Compound name	Sequence in TNC	Applied assay	Disease context	Observation	Refs.
	<sup>131</sup> I-81C6 <i>Neuradiab</i>	FNIII-A-D	Radio immunotherapy	Patients with recurrent brain and central nervous system tumors	Focal necrosis, brain edema, hematological and neurological toxicity Increased median patient survival (90.6 weeks <i>versus</i> 73 weeks)	[72] [73]
	<sup>131</sup> I-ch81C6	FNIII-A-D	Radio immunotherapy	NHL	One CR, one PR, 7 SD (2 to over 8 months)	[74]
	<sup>131</sup> I-81C6 plus Temozolomide	FNIII-A-D	Radio immunotherapy	Glioma grade IV	Unknown	NCT00615186 Phase III trial terminated, delay in site initiation and funding
	<sup>131</sup> I-81C6	FNIII-A-D	Radio immunotherapy	Primary or metastatic anaplastic glioma	Unknown	NCT00002752 Phase I/II trial completed
	<sup>131</sup> I-81C6	FNIII-A-D	Radio immunotherapy	Recurrent cystic anaplastic glioma	Unknown	NCT00002753 Phase I trial completed
	<sup>131</sup> I-81C6	FNIII-A-D	Radio immunotherapy	Comparison bolus injection <i>versus</i> microinfusion in patients with glioma grade III or IV	Unknown	NCT00003478 Phase I/II trial completed
	<sup>131</sup> I-81C6 plus Carmustine or Irinotecan	FNIII-A-D	Radio immunotherapy	Primary malignant brain tumor	Unknown	NCT00003484 Phase I trial completed
	<sup>211</sup> At-81C6	FNIII-A-D	Radio immunotherapy	Malignant recurrent brain tumors	No-dose limiting toxicity Increased median OS, 54.1 weeks <i>versus</i> 23 weeks (placebo) and 31 weeks (carmustine)	[75]
	BC2	FNIII-A1-A4	Immunostaining	Human fibroblasts Cancer of the breast, brain, ovary, colon rectum, thyroid, prostate	Detection of TNC expression in fibroblasts and cancer cells High TNC levels in the tumors	[57] [6]
	<sup>131</sup> I-BC2	FNIII-A1-A4	Radio immunotherapy	Patients with recurrent GBM	PR or CR in 30% of patients	[76]
	F16 (scFv)	FNIII-A1	Immunohistochemistry Immunostaining	Biopsies from bone-marrow of AML patients Tissue from non-small lung carcinoma, mesothelioma	High TNC expression	[77]
	F16SIP	FNIII-A1	Immunostaining	Lymphoma, renal cell carcinoma, lung cancer, HNSCC, GBM, melanoma tissues	High TNC expression	[78] [79] [80] [81] [82] [80]
	Biotinylated F16SIP	FNIII-A1	Immunohistochemistry	U87 MG GBM xenografts Human GBM tissue	High TNC expression	[80]
	<sup>125</sup> I-F16SIP	FNIII-A1	Biodistribution	U87MG bearing mice	Enrichment in the tumor	[21]
	<sup>131</sup> I-F16SIP <i>Tenarad</i>	FNIII-A1	Radio immunotherapy	Patients with recurrent refractory HL	Acceptable toxicity PR Partial SD	[83] [84] NCT01240720 Phase I/II trial completed
	F16-IL2 <i>Teleukin</i>	FNIII-A1	ELISA SPR	Serum from breast cancer patients with metastasis	No human anti-fusion protein antibodies No/low immunogenic potential Favorable dose-dependent pharmacokinetics	[85]
	<sup>125</sup> I-F16-IL2	FNIII-A1	Biodistribution study	MDA-MB231 xenografts in nude Balb-c mice	Tumor detection, tumor to non-tumor ratio 15:1	[86]
	F16-IL2 plus DOX or PXT	FNIII-A1	Toxicology and therapy	MDA-MB231 xenografts in nude Balb-c mice	Tumor growth retardation Involvement of NK cells	[86]
	F16-IL2 plus DOX or PXT	FNIII-A1	Safety study	Cynomolgus monkeys	No safety issues Moderate lymphocyte depletion	[86]
	F16-IL2 plus DOX	FNIII-A1	ELISA SPR	Serum from metastatic breast cancer patients	No human anti-fusion protein antibodies No/low immunogenic potential Favorable dose-dependent pharmacokinetics	[85]

(continued on next page)

Table 1 (continued)

Targeting molecule	Compound name	Sequence in TNC	Applied assay	Disease context	Observation	Refs.
	F16-IL2 plus DOX	FNIII-A1	Toxicology and therapy	Breast cancer patients with metastasis	Tolerance, no immunogenicity	[85] NCT01131364 Phase Ib/II trial terminated
	F16-IL2 plus low-dose cytarabine	FNIII-A1	Therapy PET/CT imaging	Patients with AML	Detection of tumors Transient reduction of AML lesions	[77] [87] NCT0297032 Phase I trial active
	<sup>124</sup> I-F16SIP	FNIII-A1	PET imaging	HNSCC patients	Detection of tumors in all patients within 24 h	[88]
	F16-IL2 plus temozolomide	FNIII-A1	Biodistribution study	U87MG tumor bearing mice	Specific tumor uptake CR, tumor free for over 160 days Tumor size reduction Prolonged survival	[80]
	F16-IL2 plus anti-CD33 antibody BI 836,858	FNIII-A1	Pharmacokinetics	Relapsed post-transplant AML patients	Acceptable safety Activation of NK cells in bone marrow and peripheral blood eliciting ADCC OR in 20% of patients, CR (1/15)	[89]
	F16-IL2 plus Anti-PD1	FNIII-A1	Safety study	Patients with NSCLC	Unknown	NCT05468294
	<sup>124</sup> I-F16SIP	FNIII-A1	PET imaging	HNSCC patients	Tumor specific signal in all patients within 24 h	[88]
	4C8MS	FNIII-B	Proliferation assay Migration assay	TNC knockout GHOM5E cells	Reduced migration and proliferation in absence of TNC	[63]
	A12 (scFv)	FNIII-C	Immunohistochemistry	GBM tissue	High TNC expression	[90]
	<sup>125</sup> I-E10 (scFv)	FNIII-C	Biodistribution	U87MG xenografts	No accumulation in the tumor	[91]
	G11 (scFv)	FNIII-C	Immunohistochemistry	Human lung tumors	High TNC expression	[91]
	<sup>125</sup> I- G11 (scFv)	FNIII-C	Biodistribution study	U87MG tumor bearing mice	Detection of tumors	[91]
	<sup>125</sup> I- G11 (scFv)	FNIII-C	Ex-vivo radioimaging	ApoE knockout atherosclerosis mice	Detection of atherosclerotic plaques	[92]
	<sup>125</sup> I- G11-IL2 (scFv)	FNIII-C	Biodistribution study	U87MG tumor bearing mice	Tumor detection	[91]
	<sup>131</sup> I-G11 (SIP)	FNIII-C	Biodistribution study	Orthotopic brain tumor, C6 glioma bearing rats	Tumor detection	[91]
	Human IgG1 antibody-NIR fluorophore IRDye88CW	FNIII-D	Ex vivo imaging	Colon tissue from DSS mice	High TNC expression in chronically inflamed tissue	[93]
	Human IgG1 antibody	FNIII-D	Immunohistochemistry	IBD patient colon tissue Murine DSS colon tissue	High TNC expression in chronically inflamed tissues	[93]
	P12 (scFv)	FNIII-D	Immunohistochemistry	U87MG, A375 melanomas, F9 teratocarcinoma tumor xenograft tissues	High TNC expression	[21]
	<sup>125</sup> I-P12 (SIP)	FNIII-D	Biodistribution study	U87MG tumor bearing mice	Tumor detection	[21]
	L7D (scFv)	FNIII-D	Immunostaining	Murine (SKRC52, U87, A431, A375) and human (colon 26, C51, SMA540, SMA497) tumor xenografts	High TNC expression	[94]
	R6N-IgG1 (L7D derivative)	FNIII-D	Immunostaining	Tumors in immunocompetent mice (Colon 26, C51, SMA-540 and SMA497), xenograft tumors (SKRC52, U87, A431 and A375)	High TNC expression	[94]
	R6NIgG2a	FNIII-D	Immunostaining	SKRC52 xenograft tumor tissue	High TNC expression	[94]
	R6NIgG1-FITC	FNIII-D	Immunostaining	Human ovarian, breast, uterine tumor tissue	High TNC expression	[94]
	mIL12-R6N	FNIII-D	Immunotherapy	Orthotopic SKRC52 and SMA497 syngenic tumor bearing mice	90% tumor regression in 20% of mice (1/5 CR)	[94]
	NSCT-121	FBG	ELISA Capture : 9F8 Detection: NSCT-121	Serum from patients with RA, psoriatic arthritis, ankylosing spondylitis, SLE, polymyalgia rheumatic, vasculitis Macrophages supernatants RA synovial cells supernatants	Detection of all TNC isoforms	[95]
	C3 (Fab)	FBG	Immunostaining In vivo injection	RA patient synovial biopsies Rat collagen-induced arthritis model Cells from RA synovial membranes exposed to FBG	High TNC expression Reduced clinical score and paw swelling Significant reduction of TNF release upon antibody treatment	[96]

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Table 1 (continued)

Targeting molecule	Compound name	Sequence in TNC	Applied assay	Disease context	Observation	Refs.
	C3 (Fab) plus anti-PD-L1	FBG	<i>In vivo</i> injection	MMTV-NeuNT tumor mice	Reduced lung metastasis in comparison to single treatments	[97]
<b>Nanobodies</b>						
	<sup>64</sup> Cu-NJT6	G23-P625 (EGFL)	Immuno-PET/CT imaging	MDA-MB231 and LM2-TGL xenograft bearing mice	<i>In situ</i> tumor imaging <i>In situ</i> lung metastases detection High signal-to-noise ratio	[98]
	Nb3, Nb4	FNIII3–5	Immunohistochemistry Cell culture assays	Human OSCC tissue GBC liver metastasis tissue U87MG xenograft tissue KRIB osteosarcoma cells Human mesangial cells Murine DC2.4 dendritic cells	High TNC expression High TNC expression High TNC expression Inhibition of cell rounding by TNC Inhibition of cell rounding by TNC Inhibition of TIL matrix retention by TNC/CCL21	[13]

**Abbreviations:** ADA, anti-drug antibodies; ADCC, antibody dependent cell cytotoxicity; AML, acute myeloid leukemia; ApoE knockout, apolipoprotein E-deficient mice; CAIA, collagen antibody-induced arthritis; CHC, chronic hepatitis C; CR, complete response; DOTA, 1,4,7,10-tetraazacyclododecane tetraacetic acid; SS, Dextran sulfate sodium; DOX, Doxorubicin; DTPA, diethylenetriamine pentaacetic acid; EGFL, TNC EGF like domains; FBG, TNC fibronectin related domain; FNIII, TNC fibronectin type III domain; GBM, glioblastoma multiforme; HL, Hodgkin lymphoma; HNSCC, head and neck squamous cell carcinoma; IBD, inflammatory bowel disease; IRDye, infrared fluorescent dye; MMTV, mouse mammary tumor virus; MRSA, Methicillin-resistant *Staphylococcus aureus*; NCS, N-Chlorosuccinimide; NHL, Non Hodgkin lymphoma; NHS, N-Hydroxysuccinimide; NIR, Near-infrared fluorophore; NN, unknown; OR, objective response; OS, overall survival; PAGRIT, pre-targeted antibody-guided radio immunotherapy; PET/CT, positron emission tomography and computed tomography; PXT, Paclitaxel; RA, rheumatoid arthritis; PR, partial response; SCRC, surgically created resection cavity; SD, stable disease; SPR, surface plasmon resonance spectroscopy; SLE, systemic lupus erythematosus.

So far, only a few antibodies against diverse epitopes have been developed as immunocytokines with some being tested in clinical trials, including TNC (F16). In the majority of these antibody-cytokine conjugates, IL2 was used (reviewed in [42,106]). For targeting TNC, recombinant fully humanized single chain scFv antibodies “F16” and “P12”, recognizing TNC *FNIII-A1* and *FNIII-D*, respectively, were engineered and largely improved TNC binding (two-digit nanomolar range) [21], (Table 1). <sup>125</sup>I-F16 and <sup>125</sup>I-P12 selectively accumulated in the tumors with rapid clearance from other organs [21]. Upon fusion to IL2 giving rise to the immunocytokine F16-IL2, named *Teleukin* selective enrichment in the breast MDA-MB231 xenografted tumors was seen [86], (Fig. 2A, Table 1). Moreover, a case report of an AML patient treated with F16-IL2 revealed a remarkable transient reduction of the AML lesions [77]. F16-IL2 may induce an anticancer action by enhancing recruitment and infiltration of immune cells, in particular natural killer cells (NK) suggesting TNC as excellent tumor delivery address for IL2 [19,86,107]. This effect was even further potentiated by co-administration of chemotherapeutic agents such as doxorubicin and paclitaxel, [77] (Fig. 2A, Table 1). This combination treatment induced apoptosis, proliferation inhibition, infiltration of NK cells and macrophages, and reduced tumor volume in the large majority of the intracranial U87MG GBM xenografts. Also, F16-IL2 potentiated the action of temozolomide even further, leading to an amazing complete tumor eradication of the subcutaneous GBM in all five treated mice with no tumor recurrence over the following 160 days. Finally, the F16-IL2 uptake was 10-times higher inside the U87MG tumors than in liver and kidney [80].

These promising results fostered a combined clinical phase I/II study in AML patients (frequently relapsing after allogeneic hematopoietic stem cell transplantation (HSCT)) with F16-IL2 together with an anti-CD33 antibody (BI 836858) to further activate NK cells. The Maximum Tolerated Dose (MTD) was not reached and adverse effects were all manageable, transient, and of lower range and, a stronger cytotoxic NK cell phenotype and potential ADCC were observed. These results may explain 20% of an objective response, one patient each with CR, incomplete count recovery (CRi), PR and, SD in the remaining 4 patients [89]. F16-IL2 was also used in with low dose cytarabine in AML patients showing transient remission of AML lesions. However, an

extensive infiltration of immune effector cells in the bone marrow may have caused termination of the Phase I clinical trial (NCT02957032) [87,108].

Also, other combinations of F16-IL2 have been explored in clinical trials such as F16-IL2 together with doxorubicin (NCT01131364) or paclitaxel (NCT01134250) in solid tumors comprising breast cancer, metastatic melanoma and non-small cell lung carcinoma (NSCLC). This caused an objective response and long-lasting SD [109]. A phase I study (NCT05468294) exploring F16-IL2 in combination with the immune checkpoint inhibitor anti-PD-1 antibody nivolumab in advanced NSCLC patients, is currently ongoing.

As the scFv format of F16 shows some solubility and stability issues, the “F16” antibody was further engineered into a Small ImmunoProtein (SIP) or F16SIP “mini-antibody” (80 kDa) (Fig. 2C), having superior pharmacokinetic properties *in vivo* [110]. The F16SIP allowed to image TNC on sections from many different malignant tumors which fostered a clinical trial where F16SIP was used in four HNSCC patients as <sup>124</sup>I-labelled molecule proving tumor detection by Positron Emission Tomography (PET) imaging [88], (Fig. 2A, Table 1).

Similarly, <sup>131</sup>I-F16SIP, *Tenarad* was generated and administered intravenously to eight patients with recurrent refractory Hodgkin’s lymphoma (HL), in whom all conventional treatments had failed. Toxicity was acceptable despite the high dose and repeated administrations. At the first response assessment, (4 – 6 weeks after therapy), five of the eight treated patients showed SD with reduced number and/or size of lesions. One patient showed SD which then improved to a PR, three showed clinical benefit while maintaining SD and only one patient showed disease progression. Thus, *Tenarad* is considered as a promising radio-conjugate that could potentially be used for radioimmunotherapy approaches in the future [83,84], (Fig. 2A, Table 1).

Another promising targeting sequence in TNC is the *FBG* domain that has a unique structural epitope which is essential for binding to and activating TLR4 [30]. This domain is recognized by mAb DB7 [17], the rabbit polyclonal EPR4219 antibody (Sigma) and the Fab fragment of the NSCT-121 antibody to name some examples. Recently, NSCT-121 was used to establish an ELISA where the mAb 9F8 (MAB1911, MERCK, Millipore, recognizing the TNC FNIII1–3 sequence) was used to capture all TNC isoforms and NSCT-121 to recognize bound TNC. Since all TNC

isoforms are recognized by this assay higher TNC levels were detected in sera from patients with different chronic inflammatory pathologies (such as RA, SLE) than with other commercial ELISA assays specific for the TNC FNIII-C or FNIII-D domains, thus likely better revealing the overall TNC levels in a given tissue [95], (Fig. 2A, Table 1).

The monoclonal Fab antibody designated “Clone C3” is also recognizing the TNC *FBG* domain of human and rat TNC (with a Kd of 1.2 nM) and was found to detect TNC in synovial biopsies of patients at different stages of RA development and, most importantly blocked TLR4 activation by TNC, leading to reduced expression of IL6, IL17 and TNF- $\alpha$ . Moreover, Clone C3 significantly reduced the clinical score and paw swelling in rats with CAIA [30,96]. These studies demonstrate that blocking inflammatory signals induced by the TNC/TLR4 interaction can prevent joint damage and RA disease progression. The same Clone C3 antibody has later been shown to reduce lung metastasis in the MMTV-NeuNT breast cancer model when applied together with anti-PD-L1, extending the potential of this antibody for future anti-cancer immunotherapies [97], (Fig. 2A, Table 1).

Altogether, these results are highly promising confirming that TNC can serve as a valid zip code to direct immuno-radiotherapeutic and immunity activating agents inside malignant tumors with some but tolerable side effects. As TNC was shown to orchestrate the immune suppressive properties of the TME [14,97,111–114], it would be interesting to know whether any of these antibodies relieves TNC-associated immune suppression which so far has not been addressed. Moreover, many more TNC specific mAbs have been generated that cannot be described here which also may be suitable for the delivery of toxic payloads into TNC-rich tissues (reviewed in [39,40,95], Fig. 2A) or for engineering TNC targeting CAR T cells (see below).

### Nanobodies specific for TNC

Recombinant nanobodies (Nbs), only 12–15 kDa in size, are single-domain variable region antibody fragments (VHHs) that are derived from camelid heavy-chain-only antibodies (HCABs) (Fig. 2C). They harbor remarkable characteristics such as high stability, solubility and specificity and low immunogenicity [115,116]. Nbs may also overcome some of the limitations of antibodies such as the difficulty to reach the epitope and to recognize the antigen in formalin fixed tumor tissues, routinely used in the clinical practice. A potential drawback for a clinical application of Nbs particularly in a radionuclide formulation is renal toxicity (as Nbs are largely eliminated through the kidney) which can be overcome by several means that have been reported elsewhere [117–119].

Two recent studies have reported the development of TNC-specific Nbs. Jailkhani and colleagues (2023) isolated the matrisome from liver metastases of two patients with colon cancer and used this material to immunize alpacas to generate the phage display nanobody library “B”. By panning against the recombinant EGFL (Gly23 – Pro625) domain of hTNC three Nbs (NJT3, NJT4, NJT6) were isolated that showed high specificity for hTNC with a binding affinity in the picomolar range (Fig. 2A, Table 1). These Nbs recognized TNC by western blot and by staining of formalin fixed tissue from MDA-MB231 and LM2 (LM2-TGL) xenograft tumors and also lung metastasis. Upon coupling,  $^{64}\text{Cu}$ -NJT6 recognized the primary mammary gland injected tumor cells and importantly the lung metastasis by immuno-PET/CT scan with good clearance from other organs after 24 h [120].

Dhaouadi and colleagues (2021) used the long isoform of recombinant hTNC (expressed in HEK293 cells [121]) for the immunization of a dromedary that generated anti-TNC-HCABs [122]. Using a phage display library and panning with the same TNC molecule allowed to isolate two Nbs, “Nb3” (Kd of 711 nM) and “Nb4” (Kd of 537 nM) that bind in TNC FNIII-3–5 [13]. Nb3 and Nb4 recognized human and murine TNC by western blot, and tissue staining even in formalin fixed tissues (Fig. 1A, Table 1). Most importantly, Nb3 and Nb4 were shown to

impair TNC-induced cell rounding on a fibronectin/TNC substratum and, to block dendritic cell adhesion on TNC in conjunction with CCL21, thus opening the possibility to overcome TNC functions in immune suppression [13], (Fig. 2A, Table 1).

Altogether, as the small size of the TNC-Nbs allows good penetration of tumors also reaching the metastasis the described Nbs could be valuable tools for so urgently needed early PET/CT detection of metastasis already expressing TNC [123]. Nbs may also be excellent tools for generating future ADC and immunocytokines delivering toxic drugs and immune enhancing factors into cancer/metastasis tissues.

### Peptides binding TNC

Peptides are another group of molecules that with a smaller size, low immunogenicity and easy manufacturing may have some advantages over antibodies. Several TNC specific peptides were selected through screening of peptide libraries by phage display technology. Kim and colleagues (2012) used a tumor-specific large isoform of TNC for panning and selected “Peptide # 1” (FHKHKSPALSPVGGG) leading to the “FHK” peptide (FHKHKSPALSPV) that binds the large TNC isoform (FNIII A1-D) recognizing TNC in tumor xenografts (U118MG and HT29) and tumor tissue from cancer patients by staining with a remarkably high affinity of  $4.58 \pm 1.4 \mu\text{M}$  for TNC (Fig. 2A, Table 2). *In vitro*, the selected peptide also reduced TNC-induced cell rounding and migration of U118MG cells and thus may have additional TNC inhibitory opportunities that have not yet been addressed *in vivo* [124].

Aiming at deep entry into the GBM tissue, Kang and colleagues (2016) synthesized a FHK peptide-based nanoparticle (NP) termed “Ft” where the FHK peptide (binding TNC) and the truncated form of Lyp-1 (CGNKRTR) recognizing NRP-1 (neuropilin-1, a transmembrane protein highly expressed in GBM and in newly formed blood vessels), were coupled via a cysteine at the amino-terminal site of tLyp-1. To enhance stability, a poly (ethyleneglycol) - poly (lactic acid) NP was constructed, functionalized with the Ft peptide (Ft-NP) and paclitaxel, giving rise to Ft-NP-PTX that was found to be internalized in 3D spheroid cultures of U87MG and HUVEC endothelial cells. Moreover, *in situ* imaging showed a strong accumulation in the tumors of U87MG GBM-bearing mice. Ft-NP-PTX induced high apoptosis and cytotoxicity, reduced U87MG cell migration *in vitro* ( $15.1 \pm 4.2\%$ ) and, most importantly prolonged survival of tumor bearing mice, suggesting a potential synergistic toxic effect when targeting TNC and NRP-1 together [126], (Fig. 2A, Table 2). The FHK peptide in formulation with doxorubicin-loaded ultrasonic nanobubbles (FHK-NB-DOX) or Navitoclax (ABT-263)-loaded nanoliposomes (FHK-SSL-Nav) have recently been shown to target carcinoma associated fibroblasts and to enhance the anti-tumor effects of the 7pep (HAIYPRH)-modified liposomal doxorubicin formulation (7pep-SSL-DOX) in a human HepG2 xenograft model [134,145]. Despite these promising results, so far, efficacy and safety in the human patient have not yet been reported.

In addition, the “PL1” peptide (PPRRGLIKLKTS) was isolated through phage biopanning (of the X7 peptide library) on the TNC FNIII-C molecule. Surprisingly, PL1 also recognizes the fibronectin FNIII-EDB molecule that is often found to be coexpressed with TNC [146]. This peptide was investigated as systemic tumor targeting probe, in particular upon stabilization through conjugation with nanoworms (NWs), super paramagnetic iron oxide nanoparticles (with a worm-like shape and dextran coating), that indeed promoted PL1-NWs homing to tumor xenografts and to angiogenic neovessels induced by VEGF (Table 2). Further loading of the PL1-NWs with the pro-apoptotic peptide  $_{\text{D}}$ [KLAKLAK] $_{\text{2}}$  significantly inhibited U87MG tumor growth and, importantly enhanced the median survival of NCH421k tumor bearing mice [127] (Table 2).

Lingasamy and colleagues (2020) also developed an octameric TNC homing “PL3” peptide (1.46 kDa, AGRGRLVR) that interacts with the TNC FNIII-C domain *in vitro* and *in vivo* but also recognizes NRP1. To improve PL3 stability, iron oxide NWs and AgNPs were fused (Kd of  $51 \pm 19 \mu\text{M}$  for PL3/FNIII-C) and found to get enriched in the TNC FNIII-C

**Table 2**

Overview about TNC targeting small molecules

TNC targeting nanobodies, peptides, aptamers and RNA molecules are sorted according to recognition of TNC sequences (N to C terminus). Information about the applied assay, results and reference is provided.

Targeting molecule	Compound name	Sequence in TNC	Applied assay	Disease context	Observation	Refs.	
<b>Peptides</b>	MAREMO peptides MP5: STDSTSAPASNLQPASEYS MP13: STETTSAPASGLQPGTDYS	FNIII3–5	Cell culture assays	Murine NT193 breast cancer cells Human KRIB osteosarcoma cells Human mesangial cells Murine DC2.4 dendritic cells	Inhibition of cell rounding by TNC Inhibition of TGFβ signaling Inhibition of fibronectin and collagen I synthesis Inhibition of TNC-induced CCR7 and CD86 expression Inhibition of TIL matrix retention by TNC/CCL21	[125]	
	Peptide #1 FHKHKSPALSPVGGG	FNIIIA1-D	Immunostaining	U118MG and HT29 xenograft tissue Human lung tumors	High TNC expression	[124]	
	Peptide #1 FHKHKSPALSPVGGG	FNIIIA1-D	Cell culture assays	U118MG cells	Reduced cell rounding Increased migration	[124]	
	Ft (FHK- tLyp-1 fusion) peptide; FHK: FHKHKSPALSPV tLyp-1: CGNKRTR	FNIIIA1-D	Cell culture assays	U87MG, HUVEC	Reduced migration	[126]	
	Ft-poly lactic acid NPs	FNIIIA1-D	Cell culture imaging	Glioma spheroids	NP uptake Spheroid growth inhibition	[126]	
	Ft-Paclitaxel (PTX)-NPs	FNIIIA1-D	Tumorigenesis assay	U87MG bearing tumor mice	NP tumor enrichment Prolonged survival	[126]	
	PL1-D(PRRGLIKLKT)-NWs	FNIII-C FN-EDB	Tumorigenesis assay Immunostaining	GBM, PC3 xenograft bearing mice Human GBM tissue	Reduced tumor size High TNC expression	[127]	
	PL1-D[KLAKLAK] <sub>2</sub> -NW	FNIII-C FN-EDB	Tumorigenesis assay	Human NCH421k orthotopic xenograft bearing mice	Reduced tumor growth Prolonged survival	[127]	
	PL3- (AGRGLVR) coupled to AgNPs	FNIII-C NRP-1	Immunostaining	PPC1, U87MG cells	High TNC expression	[128]	
	PL3- NWs	FNIII-C NRP-1	Immunostaining	U87MG, PC3 xenografts	High TNC expression	[128]	
	PL3-D[KLAKLAK] <sub>2</sub> -NWs	FNIII-C NRP-1	<i>In vivo</i> tumorigenesis	U87MG bearing tumor mice	Reduced tumor growth Prolonged survival	[128]	
	<b>Aptamers</b>	Biotinylated GBI-10	FNIII3–5	ELISA	U251MG cell surface protein extract	Detection of TNC	[129]
		GmLs (GBI-10)	FNIII3–5	CLSM Flow cytometry	MDA-MB-435s cells	Detection of TNC	[130]
		GTLs (GBI-10)	FNIII3–5	Confocal microscopy, Flow cytometry, MRI	C6 glioma cells TNC-knockdown NIH 3T3 cells	Binding to cells No binding to cells in absence of TNC	[131]
		QD-Apt (GBI-10)	FNIII3–5	Laser-scanning confocal microscopy	U251MG glioma cells	Detection of TNC	[132]
d/I-isoNA (GBI-10)		FNIII3–5	Confocal laser microscopy	U251MG glioma cells	Detection of TNC	[133]	
GBI-10 (DGL-ZA) n		FNIII3–5	CLSM Z-scanning <i>In vivo</i> imaging	4T1 spheroids 4T1 xenograft tumor bearing mice	Tumor detection Tumor autophagy induction	[134]	
GBI-10 Apt/ CPP-CPTD NPs		FNIII3–5	Cell culture assay <i>In vivo</i> treatment	PDAC Miapaca spheroids Orthotopic Miapaca-2 pancreatic cancer cell xenograft model	Deep spheroid and tumor penetration Tumor drug delivery	[135]	
<sup>32</sup> P-TTA1 <sup>99</sup> Tc-TTA1 LNA-TTA1		FBG	Plasma stability assay <i>In vivo</i> tumor imaging Biodistribution study	Human plasma U251MG-tumor bearing mice	High plasma stability High tumor uptake Unspecific uptake in all organs except intestine	[136]	
TTA1-AS1411 - RGD peptide «SMART» imaging NP Rhodamine B-isothiocyanate <sup>68</sup> Ga, Cobalt ferrite <sup>18</sup> F-FB <sup>64</sup> C-FB		FBG Nucleolin Integrin α5β1 NN	Immunostaining Immunostaining <i>In vivo</i> PET imaging	C6, NPA, DU145, HeLa, A549 tumor cells U87MG, MDA-MB-435 xenograft tumor bearing mice	Imaging of cancer cells with high specificity and signal sensitivity Tumor detection High TNC expression in tumors	[137] [138]	
<b>Small RNAs</b>		ATN-RNA (406 to 569 bp)	EGFL	Therapy	Local treatment of 46 GBM patients	Prolonged survival	[139]
	ATN-RNA (406 to 569 bp)	EGFL	Therapy	10 high grade and low-grade glioma patients	Local suppression of tumor growth	[140]	
	miRNA 198 (a)	TNC mRNA	Immunohistochemistry, Immunoblot	Human colorectal carcinoma (SW620, CCD18-Co) cells	Correlation of low miRNA with high TNC protein levels	[141]	

(continued on next page)

Table 2 (continued)

Targeting molecule	Compound name	Sequence in TNC	Applied assay	Disease context	Observation	Refs.
	miRNA 335 (b)	TNC mRNA	Immunostaining Cell culture assays	Human breast cancer cells (MDA-MB 231, LM2, CN34-BoM1)	Correlation of low miRNA with high TNC protein levels	[142]
	miRNA 150 (c)	TNC mRNA	Immunohistochemistry Immunoblot Cell culture assay	HNSCC tissues HNCC cells (FaDu, SAS and HSC3)	Correlation of low miRNA with high TNC protein levels	[143]
LncRNA	ET20 Ensembl geneID ENSMUSG0000073821	Antisense direction in TNC locus	Cell culture assays	PyMT-1099 breast cancer cells Mammary gland NMuNG cells	ET20 and TNC protein are required for TGFβ-induced EMT	[144]

Abbreviations: AgNPs, silver nanoparticles; Apt, aptamer; <sup>64</sup>C, copper-64; CCL21, chemokine (C—C) motif ligand 21; CCR7, chemokine receptor 7; CD86, cluster of differentiation 86; CLSM, confocal laser scanning microscopy; CPP-CPTD, cell-penetrating peptide-camptothecin drug; DGL-ZA, (Dendrigrift poly-L-lysines)-zole-dronic acid; d/IsoNA, 2'-deoxyinosine/D-L-isonucleoside; ELISA, enzyme linked immunosorbent assay; EMT, epithelial mesenchymal transition; <sup>18</sup>F, Fludeoxyglucose-18; FN-EDB, fibronectin domain EDB; FNIII, fibronectin type III repeat; FBG, fibrinogen related domain; <sup>68</sup>G, gallium-68; GBM, glioblastoma multiforme; GmLs, modified GBI-10 targeted liposome; GTL, GBI-10 targeted gadolinium-loaded liposomes; HNSCC, head and neck squamous cell carcinoma; HUVEC, human umbilical vein endothelial cells; LNA, locked nucleic acid; LncRNA, long non-coding RNA; MAREMO, matrix regulating motif; MRI, magnetic resonance imaging; NN, unknown; NP, nanoparticle; NRP1, neuropilin-1; NWS, nanoworms; PDAC, pancreatic ductal adenocarcinoma, QD-Apt, quantum dot-labeled aptamer; RGD, arginyl glycol aspartic acid; PyMT, polyoma middle T antigen; <sup>99</sup>Tc, technetium-99; TGFβ, tumor growth factor β; TIL, tumor infiltrating leucocytes; (a) miRNA 198, GGUCCA-GAGGGGAGCUAGG; (b) miRNA 335, UCAAGAGCAAUAACGAAAAUGU; (c) miRNA 150, UCUCCCAACCCUUGUACCAGUG.

positive tumors in contrast to a low uptake in the TNC FNIII-C-negative M21 melanoma cells (Table 2). PL3-NWs were also loaded with the pro-apoptotic D(KLAKLAK)<sub>2</sub> peptide that caused reduced tumor size and less angiogenesis in the U87MG bearing mice [128]. Thus, PL3 coupled NPs may be useful for imaging and growth inhibition of many malignant tumors that frequently express the TNC FNIII-C domain [9–12].

TNC binds to fibronectin (FN) and blocks cell spreading on FN [147]. By searching for potential common amino acid sequences in two TNC binding sites of FN, particularly in the 5th (FN5) and 13th (FN13) FNIII domains, the “MAREMO” (MATRIX REGULATING MOTIF) was identified that is composed of two small stretches of 4 (Loop I) and 8 amino acids (Loop II) forming a common niche in three-dimensional space [125]. The MAREMO sequence mediates interactions of FN5 and FN13 with TNC in the so called MAREMO Binding Site (MBS) of TNC FNIII-3–5. Importantly, the same domains of TNC are also binding immunity regulating molecules such as TGFβ, CCL21 and CXCL12 however far away from the MBS [125,148,149]. Through interaction with these soluble factors TNC (that is usually anti-adhesive) becomes a sticky substratum for several leukocytes (e.g. dendritic cells, macrophages and CD8+ T cells) *in vitro* as well as *in vivo* retaining the tumor infiltrating leukocytes (TIL) in the stroma contributing to the immune exclusion phenotype [14,97,114]. The immune suppressive properties of TNC and TIL-matrix retention in particular was shown to be abolished *in vitro* and *in vivo* by inhibiting key pathways triggered by TNC such as CCR7 (receptor of CCL21) or CXCR4 (receptor of CXCL12) thus restoring anti-tumor immunity, reducing tumor growth and causing less metastasis (thereby phenocopying the TNCKO tumors), altogether suggesting that inhibition of TNC-associated TIL-matrix retention has therapeutic potential [14,114], (Fig. 2A, Table 2). Indeed, this possibility was recently further substantiated by Li, Orend and colleagues (manuscript submitted) who demonstrated a profound and unprecedented broad anti-tumor activity of one of the MAREMO peptides in an autologous breast cancer model.

Altogether, the TNC FNIII-3–5 domain in TNC might be a particular active site of TNC not only in cancer but also in wound healing [125,148,149]. Interestingly, the scFv TN64 recognizing hTNC FNIII-1–5 reduced the RA phenotype in the murine CAIA RA model [70]. Also, the aptamer GBI-10 (see below) and the Nbs (Nb3 and Nb4) bind TNC FNIII-3–5 and impair TNC functions *in vitro* [13,135]. Thus, it will be interesting to know whether targeting the TNC FNIII-3–5 domains with different tools has cancer inhibitory effects as seen for the MAREMO peptide (Li et al., submitted).

### Aptamers binding TNC

Aptamers are short sequences of synthetic DNA or RNA based nucleotides with favorable pharmacokinetic properties such as small size, low immunogenicity, ease of synthesis and high binding affinity in the pico to micromolar range [150–152]. For generating aptamers binding TNC, an iterative cell-SELEX (Systematic Evolution of Ligands by Exponential Enrichment) process of selection and amplification was performed (using a random single-stranded 10<sup>14</sup> - 10<sup>15</sup> oligonucleotide sequence library). The “GBI-10” aptamer, binding to TNC FNIII-3–5, was selected by using the 70-mer single stranded DNA molecules that were applied to cultured human glioblastoma U251MG cells that do highly express TNC. To confirm TNC specificity, the U251MG cell proteins were incubated with the biotinylated GBI-10, where TNC was found in the streptavidin magnetic beads pull-down [129], (Fig. 2A, Table 2). This aptamer has been further modified to enhance stability and to deliver toxic agents in preclinical *in vivo* tumor models as outlined below.

Nuclease sensibility was reduced by integration of D-/L-isonucleosides and 2'-deoxyinosine (2'-dI) phosphoramidites which did not induce any toxicity [133], (Fig. 2A, Table 2). The GBI-10 aptamer was also used to guide gadolinium-loaded liposomes for future diagnosis and tumor therapy and, to guide a NP camptothecin (topoisomerase inhibitor) dimeric prodrug (CPTD) conjugate into the tumors of a PDAC xenograft model, thereby improving tumor-selective targeting with reduced systemic toxicity [135]. Given its small size, high stability and low antigenicity, the GBI-10 aptamer could be an ideal tool for future cancer diagnosis and treatment.

Another promising aptamer is the 39-mer “TTA1” (13.4 kDa) that is binding the FBG domain. Specificity for hTNC was confirmed *in vitro* by ELISA on lysate from TNC expressing U251MG tumor cells and by tumor uptake of human tumor xenografts [153], (Fig. 2A, Table 2). This aptamer was further engineered to enhance stability and targeting with a few examples described below.

Upon labeling, <sup>Tc-99m</sup>TTA1 revealed a high uptake in U251MG tumors at 18 h at a concentration of 1% ID/g [154] which is in a similar range as for <sup>124</sup>I-F16SIP (4–5% ID/g) suggesting conditions that could be suitable for tumor targeting in the human patient [88].

To further increase *in vivo* stability the so called “Locked Nucleic Acids” (LNAs) hexanucleotides were inserted into the non-binding regions giving rise to two derivatives TTA1.1 and TTA1.2 that upon injection into U251MG tumors bearing nude mice showed a high tumor uptake and long

retention in the blood. However, the slower clearance of the modified aptamers and their predominant urinary excretion led to high unspecific uptake in several organs such as kidney and liver, suggesting that these LNA modified TTA1 aptamer molecules are not suitable for radionuclide associated cancer imaging in its current form [136].

However, in combination with AS1411, an aptamer targeting nucleolin, TTA1 found an application in a multimodal Magnetic-Fluorescent-Radioisotope (MFR) based imaging approach that was coined as “SMART” cancer imaging for simultaneous and specific targeting of *nucleolin*, *integrin  $\alpha 5 \beta 1$*  and *TNC*. The aptamer TTA1 and AS1411 were coupled to the RGD peptide (binding to integrin  $\alpha 5 \beta 1$ , that is highly expressed on many tumor cells) and, Rhodamine B isothiocyanate,  $^{68}\text{Ga}$  and a magnetic resonance (MR) imaging compatible cobalt ferrite core to facilitate multimodal imaging in all tested cultured cancer cell lines [137,150,155], (Table 2). Whether this probe is suitable for application *in vivo* and potentially in the human cancer patient has to be evaluated in the future [137,156].

Jacobsen and colleagues (2015) developed even another 70 nucleotides aptamer “FB” that upon coupling was used for PET imaging as  $^{18}\text{F}$ -FB and  $^{64}\text{Cu}$ -FB in subcutaneous MDA-MB435 breast and U87MG xenograft tumor models where a rapid blood clearance and a good tumor uptake with a low accumulation in most other organs was noticed leading to a high tumor-to-background signal ratio [138]. Hence, for *in situ* tumor (and potentially metastasis) imaging by PET, the TNC specific aptamers TTA1, GBI-10 and FB might be useful tools (Table 2).

### Targeting TNC expression

For reducing TNC expression inside the tumor an RNA interference strategy was developed. A total of 46 patients with astrocytic brain tumors (grade II, III and IV) showing the worst Karnofsky Performance Status (KPS) score and strong neurological deficits were included in the study. The ATN-RNA of 160 bp (US Patent no. US 8946.400 B2) that is complementary to the EGF-like domain sequence of the TNC mRNA (406 to 569 bp), caused gene silencing and reduced TNC expression. After a subtotal tumor resection, patients were locally treated with this double stranded ATN-RNA and then received standard radiotherapy which prolonged the survival of the patients by 4.8 (grade II), 13.2 (grade III) and 13.9 weeks (grade IV), respectively. A significant improvement of the patient’s quality of life (determined by the KPS score) as well as the lack of tumor recurrence at the site of ATN-RNA application were also observed, which is highly remarkable [140,157]. Although the application of ATN-RNA has some benefits, the knock-down of TNC expression was transient and only active at the site of injection and would need iterative applications, which likely explains why this approach has not reached the clinical practice in its current form [139]. However, these data suggest that down regulating TNC expression in cancers may counteract tumor growth opening novel avenues for future TNC repression strategies (see below).

A variety of miRNAs, that act as tumor suppressors, were identified to downregulate the TNC transcript such as miRNA-198 in colorectal cancer [141], miRNA-335 in breast cancer [142], miRNA-150 in HNSCC [143], and miRNA-218 in gliomas [158], (Table 2). However, these miRNAs may have also other targets. Future studies have to address whether these miRNAs could be useful for an anti-cancer therapy.

Recently, a long non-coding RNA (ET20) was discovered to be located inside the TNC locus however in opposite orientation. Loss of function approaches showed that downregulating expression of TNC is reducing ET20 expression and on the contrary, reducing ET20 caused repression of TNC, indicating an unknown crosstalk that may have therapeutic potential. Importantly, for TGF $\beta$  to induce EMT in breast cancer cells, which promotes tumor progression, both ET20 and the TNC protein were required [144], (Table 2).

### Employing TNC protein sequences as carrier for siRNA and cell death inducing molecules

So called “Centyrin” or “Tencon” molecules that are based on the consensus sequence of the third FNIII domain of hTNC represent another means to exploit TNC [159,160]. The prototype Tencon has a sequence identity of 40% to the natural FNIII domain of TNC [161] where the Tencon backbone is almost identical to the third FNIII repeat of TNC (FNIII-3, 1TEN) which has a MBS that could have a not yet investigated biological activity [125]. Centyrins/Tencons were engineered to carry a siRNA or cytotoxic payload exemplified by using siRNA against beta-catenin (*CTNNB1*) or Monomethyl auristatin F (MMAF, inhibiting cell division through blocking tubulin polymerization). After injection of the EGFR-Centyrin-coupled siRNA, the targeted *CTNNB1* expression was reduced in A431 xenograft tumors even 72 h after intra-venous injection (up to 75% knockdown with an IC50 of 5 nM), suggesting that the Centyrin might be a useful tool for the delivery of siRNA and divers cytotoxic payloads, which potentially could also be applied to TNC specific ATN-RNAs [161].

TNC could also be exploited to enforce intrinsic cell death by the TNF family members FAS (Fas Cell Surface death receptor) and TRAIL (Tumor necrosis factor-Related Apoptosis-Inducing Ligand) that are under consideration for enforcing tumor cell killing since a long time [162,163]. CD95/Fas is a cell surface receptor that induces apoptotic cell death upon binding to its ligand CD95L where receptor and ligand trimerization is required that can be enforced with a bispecific TNC (BC24) antibody in conjunction with an agonistic antibody specific for CD95L, in a bispecific antibody formulation [164], (Fig. 2A). Indeed, this bispecific antibody induced apoptosis in cultured GBM cells (U87MG, LN18) [164]. However, so far no information about safety or any anti-tumor activity is available.

Berg and colleagues (2007) used a different approach to enforce tumor cell killing by CD95/CD95L or TRAIL/TRAIL-R by fusing the N-terminal trimerization domain of TNC (30 amino acids) to CD95L or TRAIL and, observed that these fusion molecules were highly active in killing several cultured cells [165], (Fig. 2A). Both approaches are powerful means to increase TNF ligand family-induced cell death. The recently described TRAIL binding to the TNC FNIII-3–5 domains, potentially enforcing TRAIL signaling, might also be relevant for enforcing tumor cell death [125], (Li et al., submitted).

### Vaccination against TNC

Compared to the use of mAbs, vaccination against cancer could overcome cost-intensive repetitive injections as the body produces an immune response and the tumor specific antibodies by itself. This can be achieved by therapeutic vaccination with tumor-derived peptides that trigger tumor-specific immune responses with already promising results in glioma patients [166–168].

Dutoit and colleagues (2012) identified a set of human leukocyte antigen (HLA)-A\*02 restricted peptides that eluted from the surface of GBM tissue in association with the respective HLA molecule. Originally eleven, now 20 of these tumor-associated peptides (TUMAP), including a TNC peptide (position 3–11, AMTQLLAGV), were further examined as potential antigens. Indeed, these molecules are overexpressed in the majority of GBM, however poorly or not in healthy tissues and, the peptides were found to be immunogenic *in vitro* and are presented at peptide level on the GBM tissues, indicating that these molecules are potential targets for T cell-mediated immunity [169]. Based on these promising results, the authors formulated vaccine “IMA950” [169] that was investigated in a clinical trial (Cancer Research UK IMA950–101, NCT01222221) using granulocyte macrophage colony-stimulating factor (GM-CSF) as adjuvant either before or after concomitant radiochemotherapy which revealed that this protocol is safe and immunogenic [170].

These promising results further fostered a recently published clinical

study by Migliorini and colleagues (2019) where the authors investigated different protocols of co-administration now in conjunction with the poly-ICLC adjuvant. Indeed, a high number of the enrolled 19 GBM patients developed CD8+ T cell responses against one (63%) or multiple peptides (31%) including TNC. The median OS of the GBM patients in the IMA950/poly-ICLC trial was 19 months. In comparison, OS survival was only 15.3 months when the IMA950 vaccination was combined with recombinant GM-CSF which could be explained by a better adjuvant function of poly-ICLC [171].

Subsequently, the vaccination protocol was combined after a treatment with an anti-angiogenic anti-VEGFA antibody (Bevacizumab) which did not provide an overall nor median survival benefit for the GBM patients [172]. Possibly activation of the complement system by Bevacizumab may have negatively impacted the vaccination. Altogether, the results are considered as promising since the vaccination triggered CD8+ T cells to recognize all antigens of the IMA950 formulation and in context with the better adjuvant poly-ICLC also activated T helper cells. Based on these encouraging results two additional clinical trials using IMA950 together with Varilumab (against CDX-1127, anti-CD27) in grade II glioma (NCT02924038) and IMA950 together with Pembrolizumab (anti-PD1 antibody) in relapsing GBM patients (NCT03665545) have been launched without information about study outcome yet.

Vaccination against tumor specific neopeptides can also be achieved by injection of mRNA molecules encoding suitable tumor peptides in a personalized mRNA vaccination approach that so far has not been published for TNC yet [173–175]. Some amino acid sequences of TNC could indeed be recognized as antigens as suggested in human GBM (e.g. from the EGFL domain, [176]) and in RA patients (e.g. citrullinated FBG, reviewed in [177]). This idea is intriguing and is even further inspired by the observation that in human cancer tissues the TNC sequence is experiencing several mutations leading to amino acid exchanges potentially generating neoantigen epitopes, whereas this is negligible in TNC expressed by PBMCs [15]. Thus, more information about naturally occurring TNC sequences with an antigenic potential could open the opportunity for novel tailored anti-cancer immunization strategies.

### Some considerations about TNC as diagnostic and theranostic tool

That ECM, as a prominent part of the “hardware” of malignant tumors, does not only form “pretty fibrils” [178] but also plays an active role in tumor progression, is slowly receiving recognition. Given that the highly expressed TNC (together with other ECM molecules) is generating tumor specific immune suppressive niches (compromising tumor immunity and anti-cancer therapy), the tumor specific ECM should be considered as a cancer hallmark, even further expanding the tumor targeting opportunities [1,2,179].

Pioneering work from several laboratories has provided a high number of domain-specific anti-TNC antibodies that with their smaller formulation as scFv, Fab', Fab or SIP have the potential to improve anti-cancer therapy in the future by delivering toxic molecules or radionuclides or to activate the immune system. Numerous clinical trials using TNC as zip code have already been performed (reviewed in [39,40,43]) but faced obstacles impossible to anticipate when using preclinical xenograft tumor models often lacking a proper immune system. Moreover, as these experimental tumors develop over a short period of time and most often ectopically underneath the skin, they are largely devoid of a relevant TME seen in human tumors where the ECM-dense TME often is a confounding factor in therapies. One lesson we learned from the many preclinical studies that do not translate into the same responses in the cancer patient is that more attention should be paid to the choice of immune competent preclinical tumor model. Finally, in the clinical trials often cancer patients for which no cure exists have been

included, where even some responses, as seen, have to be considered as remarkable.

Since more often it is not the primary tumor but the metastasis that kills the cancer patients, it is important to develop metastasis targeting strategies. Groundbreaking work from the Richard Hynes/Alexandra Naba laboratories has generated a protein inventory of all ECM and associated molecules (the so called matrisome) of several tumors revealing that the primary tumor and the investigated human lung and liver metastases have many matrisome molecules in common. TNC is one of them [120,180]. Interestingly, the tumor cells already prime TNC deposition in the future metastasis site rendering TNC as a valid zip code for metastasis imaging [123,181]. *In situ* metastasis imaging with radiolabeled nanobody <sup>64</sup>Cu-NJT6 indeed detected metastasis site-specific TNC expression which could offer the opportunity for future metastasis detection by non-invasive imaging.

Finally, TNC is acting in conjunction with many other ECM molecules by forming common and dense networks promoting inflammation and representing an obstacle in cancer therapy [1,5,179,182]. Interestingly, loss of function approaches suggest that TNC acts as an orchestrator of these so far poorly understood networks. This was shown by investigating tumors lacking TNC (TNCKO) in Rip1Tag2 endocrine insulinoma [183], 4NQO-induced tongue OSCC [114] and NeuNT breast cancer models [14] where many matrisomal molecules and in particular many collagens and matrix degrading enzymes were largely down-regulated in comparison to the TNC expressing control tumors. Importantly, this coincided with a less immune suppressive TME, altogether suggesting that eliminating TNC can change the entire stromal network towards therapy promoting conditions. If we understood how TNC impacted the formation of the fibrotic immune suppressive TME, we could develop more and better tumor tailored targeting strategies.

### Outlook: towards future TNC targeting in cancer

Since its discovery over 40 years ago, extensive research has provided evidence that TNC is a major tumor promoter and an excellent zip code for the delivery of various agents. How could we make better use of this information? A molecular inventory of the tumor would inform which TNC isoforms are expressed by which cells and along tumor progression. The many excellent TNC specific monoclonal antibodies (and their smaller variants), nanobodies, peptides and aptamers, could be used for tumor imaging and for delivery of toxic and immune stimulating therapeutics. Also, reducing TNC expression by ATN-RNA molecules delivered by mAbs (particularly in their Fab or SIP formats), Centyrin/Tencon, Nbs, peptides or aptamers, or through vaccination may cause matrix remodeling towards a less immune suppressive TME as seen in the murine TNC KO models.

Malignant tumors are highly adaptive systems that react towards anti-cancer treatments with compensatory strategies, but we often seem to treat them as static entities. We must better understand how each treatment is changing the TME and in particular the expression and particular biochemical properties of highly abundant ECM molecules such as TNC to use this information for developing a tailored targeting strategy for the next line of therapy. We already know that cytotoxic drugs as well as ionizing irradiation can induce expression of collagens, TNC and other pro-fibrotic ECM molecules, generating an even more immune suppressive TME [184–186]. Moreover, we need more information about the matrisome and the immune cell infiltrate upon any given anti-cancer treatment to be faster in developing a new strategy than the tumor, preferably by using non-invasive TNC imaging technologies or TNC ELISA-based assays on liquid biopsies (such as blood and urine) where it is important to gain information about all TNC isoforms including those with extra domains suitable for specific targeting [95]. As many cancer targeting drugs have to be used over long periods of time, they pose the risk to induce cardiac and kidney toxicity

(reviewed in [187,188]). Here, TNC expression that gets induced in chronically damaged tissues, could serve as a disease indicator molecule.

Information about other targetable factors (e.g. integrin  $\alpha 5\beta 1$ , NRP1) would allow to develop SMART-like combination approaches or appropriate TNC-targeted immunocytokines, to boost anti-tumor immunity where immune checkpoint inhibitors could be suitable. In support, targeting TNC/TLR4 signaling in combination with anti-PD-L1 was more efficient in reducing lung metastasis than anti-PD-L1 alone [97].

Ideally, one would like to “normalize” the tissue by e.g. abolishing matrix stiffness. However, many cytotoxic drugs are even further inducing expression of collagens and TNC thus enforcing tumor stiffness [189,190]. Here, the MAREMO peptides may be useful as they were shown to inhibit TGF $\beta$  signaling and expression of fibronectin, collagen and other pro-fibrotic molecules ([125], Li et al., *submitted*). To improve immune checkpoint therapy, one would also like to abolish TIL-matrix retention and the immune exclusion phenotype, where the MAREMO peptides and the nanobodies Nb3 and Nb4 showed such activities *in vitro* [13,125].

Empowering anti-tumor defense by Chimeric Antigen Receptor (CAR) T cell therapy represents another highly promising anti-cancer targeting strategy with over 500 clinical trials performed already [191]. CAR T cells specific for the extra domain A (EDA) splice variant of fibronectin that is highly abundant (along with TNC) in the stoma of many cancers were generated, recognizing FN-EDA in an antigen-dependent manner and inducing anti-tumor activity in several murine tumor models with a long-lasting immunity [192]. Furthermore, these CAR T cells induced an anti-angiogenic effect and significantly reduced gene signatures associated with plasticity, collagen synthesis, ECM organization as well as the IL-6/STAT5 and KRAS pathways [192], altogether proving that targeting the tumor specific ECM by CAR T cells is a powerful novel approach that could also be applied to TNC, given the many excellent TNC binders (Tables 1 and 2).

In summary, despite promising case reports with remarkable responses in end-stage cancer patients and numerous tenacious experimental approaches, none of the many TNC targeting strategies have yet reached any application in the clinical practice. However, new strategies are still tested in clinical trials potentially providing novel avenues for future therapies. Importantly, understanding how TNC is generating an immune suppressive TME may open novel opportunities to abolish these TNC functions. We have never been in a better position to combine our versatile arsenal of TNC targeting and imaging tools to tackle anti-tumor immunity which may allow to largely improve anti-cancer therapy in the not so far future.

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## Declaration of competing interest

The authors declare no conflict of interest.

## Data availability

No data was used for the research described in the article.

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