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Title

Neuroendocrine tumours and fibrosis: An unsolved mystery?

Running title: Neuroendocrine tumours and fibrosis

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Keywords: neuroendocrine tumour, fibrosis, carcinoid heart disease, carcinoid tumour, mesenteric desmoplasia

Abstract

Neuroendocrine tumours are a heterogeneous group of slow-growing neoplasms arising mainly from the enterochromaffin cells of the digestive and respiratory tract. Although they are relatively rare, their incidence is rising. It has long been observed that they are often associated with the development of fibrosis both locally and distant. Fibrotic complications, such as carcinoid heart disease and mesenteric desmoplasia, may lead to considerable morbidity or even affect prognosis. The elucidation of the pathophysiology of fibrosis would be of critical importance for the development of targeted therapeutic strategies. In this article we review the available evidence on the biological basis of fibrosis in neuroendocrine tumours. We explore the role of the tumour microenvironment and the interplay between tumour cells and fibroblasts as a key factor in fibrogenesis and tumour development/progression. We also review the role of serotonin, growth factors and other peptides in the development of carcinoid-related fibrotic reactions.

Introduction

Neuroendocrine tumours (NETs) are rare neoplasms with an incidence between 2 and 9.3 per 100,000 persons per year.[1] They represent a heterogeneous group of tumours arising from neuroendocrine cells of the diffuse endocrine system, which occur most frequently in the gastrointestinal tract and bronchopulmonary system.[2] Neuroendocrine neoplasms are often associated with fibrosis, which may develop locally or at distant sites.

Carcinoid heart disease (CHD) affects up to 40% of patients with carcinoid syndrome (classically defined as the triad of cutaneous flushing, diarrhoea and bronchospasm) and is characterised by the development of fibrotic endocardial plaques and typically right-sided heart valve dysfunction, although approximately 10% of patients may also have left-sided valvular involvement.[3] CHD can progress to heart failure which is associated with considerable morbidity and may be the cause of death in up to 40% of affected individuals if left untreated.[4] In a series from Mayo clinic 3-year survival for patients with CHD was 31% compared with 68% for those without echocardiographic evidence of cardiac involvement.[5]

Mesenteric fibrosis is a hallmark of small bowel NETs and may occur in up to 50% of cases.[6,7] It is commonly detected radiologically and often has a characteristic appearance of a mesenteric mass with linear soft tissue opacities radiating outward in a ‘spoke-wheel’ pattern associated with distortion of the surrounding tissues. Calcification can be present in the mesenteric mass.[6] The number and thickness of radiating strands detected on

imaging has been shown to correlate with the degree of fibrosis detected histologically.[8] Mesenteric fibrosis may lead to bowel ischaemia due to impingement of the mesenteric vessels in about 10% of affected patients and occasionally kinking of the small intestine resulting in bowel obstruction.[7] It can also cause venous ischaemia due to superior mesenteric vein involvement, ultimately resulting in malnutrition and cachexia. Compromise of the mesenteric vasculature can lead to ascites and extensive involvement may often render surgical resection technically challenging or impossible.[9] The heavy mass of mesenteric lymph nodes may also predispose to volvulus.[7] Apart from being associated with considerable morbidity, mesenteric fibrosis is also a poor prognostic factor for survival.[7,10]

Other less commonly encountered fibrotic reactions can occur in patients with NETs, such as retroperitoneal fibrosis which can lead to obstructive uropathy[7,11], scleroderma[12], pleural disease[13], cryptogenic fibrosing alveolitis[14], fibrosis of the bladder[15] and elastic vascular sclerosis causing intestinal infarction.[16]

Although the association of NETs with fibrosis has been recognised for a long time, the underlying biology of this relationship remains poorly understood.[15, 17] The elucidation of the pathogenesis of fibrosis is of critical importance for the identification of appropriate therapeutic targets and the development of specific strategies for the prevention and treatment of its complications. This article reviews the existing literature on the pathophysiology of fibrosis in NETs.

Methods

We conducted a literature search of the MEDLINE database from its inception to June 18, 2017 for relevant articles on the pathophysiology of fibrosis in NETs using the keywords ‘carcinoid’ or ‘neuroendocrine tumour’ AND ‘fibrosis’, ‘serotonin’, ‘growth factor’ or ‘desmoplasia’. We included original research articles (basic research, animal and clinical studies), as well as review articles and case reports/case series relevant to the topic. We excluded: (1) articles which did not focus on NETs, (2) articles related to NETs but without a substantial focus on fibrosis, (3) articles related to NETs with some relevance to fibrosis but no considerable focus on its pathogenesis and (4) articles not written or translated in English. Reference lists from studies selected by the electronic search were manually reviewed to identify further relevant articles. The quality and strength of the results were considered with more emphasis placed on systematic reviews and well-designed original research studies. Case reports and case series were included if clinically relevant.

Results

A large number of results were returned for each of our searches, leading to a total of 20,523 records (in more detail, '*carcinoid and fibrosis*' led to 182 records, '*carcinoid and growth factor'* to 728, '*carcinoid and serotonin'* to 1,331, '*carcinoid and desmoplasia'* to 187, '*neuroendocrine tumour and fibrosis*' to 718, '*neuroendocrine tumour and growth factor'* to 14,946, '*neuroendocrine tumour and serotonin'* to 1,673 and '*neuroendocrine tumour and desmoplasia'* to 758). After removing duplicates 17,334 records were screened by title and abstract based on our selection criteria, leaving 98 articles for full-text assessment, of which 81 were included in the final review. A manual search of bibliographies of the selected articles was performed and 61 additional articles were also considered relevant resulting in a total of 142 pertinent articles (31 basic research studies, 16 animal studies, 46 clinical studies, 7 case reports/case series, 2 letters and 40 reviews) (**Figure 1**). Of these, 10 articles were considered for the '*tumour microenvironment*' section, 82 for the '*serotonin'* section, 45 for the '*growth factor'* section and 12 for the '*kinins and other peptides'* section (7 articles were referenced twice in different sections).

A. Tumour microenvironment

The tumour microenvironment has been recently recognised as a critical determinant of neoplastic evolution due to the realisation that tumours are not simply masses of neoplastic cells, but instead represent complex tissues consisting of extracellular matrix (ECM) and a plethora of cells (neoplastic cells, cancer-associated fibroblasts/myofibroblasts, inflammatory cells and capillary-associated cells) with functional interactions.[18] This dynamic microenvironment may influence tumour cell growth, survival and metastatic potential, thus playing a key role in tumour progression.[19] It can also be involved in the development of tumour heterogeneity in certain cancers.[20] The complex interplay between tumour cells and cancer-associated fibroblasts is largely responsible for the pathological ECM remodelling and desmoplasia observed in various types of cancer.[21,22] Tumour desmoplasia may facilitate tumour cell migration and invasion in certain types of malignancy, such as breast cancer and squamous cell carcinoma, and can promote tumour proliferation and chemotherapeutic resistance in malignant tumours, such as hepatocellular carcinoma. It is therefore considered a poor prognostic factor in some cancers. [21,22]

The elucidation of these complex and dynamic interactions between tumour cells and stroma ideally requires functional studies of the tumour microenvironment. There are few such studies in the field of NETs. Svedja *et al*. investigated the cross-talk between tumour cells and fibroblasts in a co-culture model using the KRJ-I (small intestinal NET cell line[23,24]) and HEK293 cells (human embryonic kidney cell line), an experimental model

essentially simulating the microenvironment of a small intestinal NET associated with fibrosis (**Figure 2**). In this Transwell co-culture system (which is representative of a non-direct co-culture system to evaluate paracrine effects) the addition of a specific 5-HT_{2B} receptor antagonist in the tumour cell-containing compartment inhibited the proliferation of KRJ-I cells and significantly reduced serotonin (5-hydroxytryptamine, 5-HT) release and profibrotic/angiogenetic factor (Transforming Growth Factor β1 [TGF-β1], Connective Tissue Growth Factor [CTGF], Fibroblast Growth Factor 2 [FGF2]) synthesis. Interestingly, cell proliferation and transcripts of these factors also decreased in co-cultured HEK293 cells, despite the absence of a 5-HT_{2B} receptor on this cell line. The authors suggested that the KRJ-I-derived 5-HT in this co-culture model was responsible for the cellular cross-talk between tumour cells and HEK293 cells (which are known to express 5-$HT_{2A/C}$ receptors).[25]

In a separate study Kidd *et al*. isolated intestinal stellate cells from a single patient with a small intestinal NET associated with peritoneal fibrosis. These cells were cultured for 5-7 days. Stimulating these cells with TGF-β1 (a growth factor known to be expressed in many gastrointestinal NETs[26]) significantly increased CTGF mRNA expression compared to unstimulated cells. CTGF is a known mediator of fibrosis and this experiment therefore demonstrated that the interaction between tumour and stellate cells plays a crucial role in fibrogenesis.[27]

B. Serotonin

Serotonin (5-HT) is synthesised mainly in the enterochromaffin cells of the gastrointestinal tract and in enteric neurons from the essential amino-acid tryptophan.[28] Platelets become loaded with serotonin as they pass through the intestinal circulation with up to 95% of circulating serotonin stored in platelets. Serotonin in tissues is rapidly metabolised to 5-hydroxyindole acetic acid (5-HIAA) by monoamine oxidase and excreted in the urine. The gut and central nervous system also possess an inactivating mechanism, the serotonin reuptake transporter, which plays an important role in terminating transmitter action. 5-HT effects are mediated through its binding to 5-HT receptors and seven types or families with multiple subtypes have now been identified by a combination of pharmacological techniques and molecular cloning (termed 5-HT1 through 5-HT7). With the exception of 5-HT3 receptor, a ligand-gated ion channel, serotonin receptors are a group of membrane-bound G-protein coupled receptors, which activate various intracellular pathways.[29] These receptors show variability in expression and a different distribution in various organs.[28] 5-HT is a key mediator of gastrointestinal function and motility and plays a major role in the pathophysiology of many gastrointestinal diseases[30,31]. In addition, there is a strong evidence base implicating the activation of 5-HT_{2B} receptors in the pathogenesis of carcinoid

heart disease, but 5-HT appears to also play a role in the development of other fibrotic conditions associated with neuroendocrine tumours. There are several lines of evidence suggesting that 5-HT is involved in carcinoid-related fibrosis:

1. *Experiments in Cell cultures*

5-HT has mitogenic effects in fibroblasts[32-35], mesangial cells[36], smooth muscle cells[37,38], endothelial cells[39] and osteoblasts.[40, 41] It is also mitogenic in carcinoid tumour cells, as demonstrated in experimental studies using *Mastomys natalensis* carcinoid tumour cells, bronchopulmonary (NCI-H720 and NCI-H727) and small intestinal (KRJ-I) neuroendocrine tumour cell lines.[35, 42] It also seems to be an important mediator in the cross-talk of tumour and stromal cells in a co-culture model simulating the microenvironment of a fibrotic small intestinal NET.[25] In addition, *in vitro* experiments suggest that 5-HT contributes to fibrin resistance to fibrinolysis providing further evidence for the implication of 5-HT in fibrogenesis.[43]

2. *Role in mesenteric fibrosis*

The nidus of tumour growth within the mesentery is thought to be a lymph node but haematogenous spread directly to the mesentery has also been suggested as a separate mechanism of metastasis.[8, 44] A fibroblastic reaction is known to occasionally develop around the metastatic deposit and the previously described co-culture model of KRJ-I and HEK293 cells suggests a role of serotonin in the pathophysiology of this process.[25] These findings are also supported by a clinical study of 52 patients with midgut NETs, which demonstrated that elevated platelet 5-HT correlated with the presence of a mesenteric mass.[45] In addition, a recent study from Charité showed a significant association of mesenteric fibrosis with elevated urinary 5-HIAA levels suggesting a potential causal relationship. [46]

However, a smaller study of 31 patients (17 with and 14 without intra-abdominal fibrosis) failed to demonstrate a correlation between fibrosis and mean or peak urine 5-HIAA levels, but the authors acknowledged the small study size and the lack of data on 'area under the curve' for urine 5-HIAA (reflecting levels in relation to time) as limitations.[6]

3. *Role in pancreatic fibrosis*

A small number of clinicopathological studies have described an association between prominent stromal fibrosis in pancreatic neuroendocrine tumours (PNETs) and 5-HT expression. The so-called 'sclerosing variant' PNET is rare accounting for less than 15% of PNETs and the associated fibrotic reaction usually involves the main pancreatic duct, resulting in ductal stenosis and upstream duct dilatation and/or pancreatic atrophy. This variant has distinct pathologic features and biomarker expression profiles and a significantly higher proportion of these tumours are serotonin-producing neoplasms compared to non-fibrotic PNETs.[47-51] The sclerosing PNETs usually demonstrate infiltrative growth patterns and are associated with more advanced stages of disease compared to non-sclerosing tumours.[48]

4. Role in cardiac fibrosis

Several experimental animal studies have demonstrated that 5-HT can induce cardiac fibrosis and valvulopathy, therefore suggesting that it may play a critical role in the development of CHD.[52-61] A summary of these studies is provided in **table 1**.

In addition, a combination of prospective and retrospective clinical studies have implicated 5-HT and its metabolite 5-HIAA in the pathogenesis of CHD and a threshold effect has been suggested with urinary 5-HIAA levels greater than 100 mg.[5, 62-74] A summary of the most important studies is provided in **table 2**.

Further evidence for the role of serotoninergic pathways in the development of CHD comes from the elucidation of the pathophysiology of drug-induced valvular heart disease. Antimigraine ergot alkaloid agents (ergotamine and methysergide), appetite suppressants (fenfluramine and dexfenfluramine) and ergot-derived dopamine agonists used for the treatment of Parkinson's disease and restless leg syndrome (pergolide and cabergoline) have been associated with the development of mainly left-sided valvular abnormalities that closely resemble carcinoid-related valvulopathies, raising the suspicion for a common pathophysiologic mechanism with CHD.[3,75-83] Methysergide and its active metabolite methylergonovine are also known to induce retroperitoneal, myocardial, aortic and pleuro-pulmonary fibrosis.[84-86] All the implicated drugs (or their metabolites) have been shown to have high affinity for and to be partial or full agonists of the 5-HT receptor subtype 5-HT_{2B} (5-HT_{2B} R).[87-89] Although the precise signalling pathways leading to valvulopathy are unknown, 5-HT_{2B} Rs are known to activate various mitogenic pathways, such as the phosphorylation of Src kinase and extracellular regulated kinases (ERK) with a final common pathway that may include phosphorylation of retinoblastoma protein

leading to cell division (**Figure 3**).[90,91] In fact, 5-HT_{2B} R agonists can mediate fibroblast mitogenesis *in vitro* suggesting that circulating serotonin may directly stimulate proliferation of valve interstitial cells and induce heart valve fibroplasia.[92,93] The 5-HT_{2B} R also mediates important trophic functions in cardiovascular morphogenesis.[94,95] Overexpression of 5-HT_{2B} Rs in transgenic mice induced ventricular hypertrophy[94], while ablation of 5-HT_{2B} Rs led to severe cardiac hypoplasia and ventricular dilatation associated histologically with loss of myocardial organisation, cardiomyocyte degeneration and myofibrillar disarray.[95, 96] In an *in vivo* model of drug-induced valvular heart disease, the administration of intraperitoneal injections of pergolide induced aortic, mitral and pulmonary regurgitation in rats with histological evidence of diffusely thickened and myxoid valves.[97] Cyproheptadine, a 5-HT_{2B} antagonist, effectively prevented toxic valvulopathy in this animal model and this therapeutic benefit was associated with downregulation of 5-HT_{2B} Rs in interstitial and endothelial valvular cells. This study provided further evidence for the involvement of 5-HT_{2B} R in the pathogenesis of serotoninergic drug-induced valvulopathy with potentially important clinical implications for its prevention.[98]

Furthermore, recently a link was found between serotoninergic pathways and oxidative stress which could act as a mediator of valvular fibrosis. Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS) and the ability of cells to produce an effective antioxidant response. Oxidative stress has been implicated in the development of many fibrotic diseases, including idiopathic pulmonary fibrosis[99], liver fibrosis[100], kidney fibrosis[101], skin fibrosis[102] and tissue remodelling after myocardial infarction.[102] Evidence showed that ROS could also be involved in the pathogenesis of CHD. Incubation of homogenates of human heart valves and proximal segments of pulmonary artery with serotonin significantly increased levels of superoxide ($O_2^{\cdot-}$). ROS were released as a byproduct during metabolism of serotonin by monoamine oxidase A (MAO), which is normally present in a variety of tissues, including heart, blood vessels, brain, kidney and liver.[103] Other experimental studies have shown that oxidative stress is involved in the development of valvular heart disease (calcific aortic valvular stenosis) in humans.[104] Therefore, serotonin could contribute to the pathogenesis of CHD by inducing MAO-dependent oxidative stress in human heart valves which may cause valvulopathy.

C. Growth factors

Several growth factors have been investigated for their potential role in the development of fibrosis in NETs and a summary of the most important studies is provided in **table 3**.

TGF-β

The Transforming Growth Factor β (TGF-β) family is divided into two general branches (the BMP/GDF and TGF-β1, β2, β3 and their latent forms/activin/nodal branches), whose members regulate multiple cellular processes, including cell division, differentiation, migration and programmed cell death and therefore have key roles in development and carcinogenesis.[105-108] Mutations or alterations in the TGF-β signalling pathway may have a substantial role in carcinoid tumour development, which has been shown in experimental studies using small intestinal (KRJ-I) and pancreatic (BON) NET cell lines.[109, 110]

Chaudhry *et al* demonstrated that in a series of 30 gastroenteropancreatic (GEP) NETs the majority of tumour cells expressed all 3 isoforms of TGF-β and stromal cells significantly expressed TGF-β2 and latent TGF-β binding protein (LTBP). TGF-β type 2 receptor immunoreactivity was observed mostly in the stromal cells, suggesting that TGF-β might play an important role in the interaction of tumour and stromal cells and possibly stimulate matrix production in these tumours.[26] TGF-β may have a critical role in fibrogenesis due to its known ability to stimulate collagen synthesis.[17, 111,112] Beauchamp *et al* demonstrated that the culture medium of BON cells (derived from a peripancreatic lymph node that contained a metastatic deposit of a PNET) contained all 3 mammalian types of TGF-β and that it could stimulate the proliferation of mouse (AKR-2B) fibroblasts.[113]

TGF-β has also been implicated in the development of CHD. In a small clinico-pathological study of 9 patients with CHD, TGF-β1 and TGF-β3 were immunohistochemically present in the fibroblasts of carcinoid plaques, whereas LTBP was present mainly in the extracellular space. In contrast, sections from unaffected control heart tissue contained few fibroblasts with only weak or no immunostaining for TGF-β1, β2, β3 and LTBP.[114] However, in another study plasma levels of TGF-β were not associated with the presence of CHD, but the investigators acknowledged that the levels of growth factors in the systemic circulation may not be an accurate reflection of local levels within the carcinoid plaques.[71] A link has been suggested between serotonin and TGF-β signalling pathways which may also have a key role in the pathophysiology of CHD. Experimental studies using cultured sheep aortic valve interstitial cells (SAVICs) demonstrated that serotonin induced TGF-β1 mRNA production and increased TGF-β1 activity via 5-HT_{2A} receptor activation. Serotonin also increased collagen biosynthesis by SAVICs, which was inhibited by an anti-TGF-β antibody, thus indicating the specificity of serotonin stimulation by TGF-β1.[115,116] In addition, clinico-pathological investigation of human carcinoid and

normal valve cusps using immunohistochemical techniques demonstrated that staining for the latent form of TGF-β1 (LAP TGF-β1) and α-SMA (a marker of activated myofibroblasts) was more prominent in carcinoid compared to control valves. Conceivably, the greater amounts of LAP-TGF-β1 found in carcinoid cusps compared to controls are likely to reflect upregulation of TGF-β because of serotonin.[116]

Another member of the TGF family, activin A, has been shown to be an independent predictor of CHD, although there was no significant difference in activin A levels between early and advanced disease. Activin A ≥0.34ng/ml had 87% sensitivity and 57% specificity for detecting CHD. Immunohistochemically activin A was strongly expressed in tumour tissue, as well as fibrotic plaques, although no control tissue was used for comparison.[117]

BMP4, another member of the TGF family, may be implicated in the pathogenesis of mesenteric angiopathy (defined as vascular sclerosing changes involving all layers of the vessels) in midgut carcinoids. In a small study BMP4 expression was found to be significantly higher in midgut tumours with angiopathy than in those without, independent of the presence or not of mesenteric sclerosis.[118]

CTGF

Connective Tissue Growth Factor (CTGF/CCN2) is a downstream mediator of TGF-β1 in fibroblastic cells and plays a substantial role in wound repair, tumourigenesis and fibrosis.[2, 119-121] CTGF acts as a mitogenic growth factor that regulates ECL cell proliferation in the Mastomys animal model and can also promote the development of gastrin-autonomous gastric carcinoids.[122]

In a study of a heterogeneous group of NETs, CTGF was more prevalent in small intestinal NETs (>50% of tumour cells demonstrated intense CTGF immunoreactivity) compared with pancreatic, rectal and bronchial NETs. Immunoreactive cells were adjacent to areas with increased fibrovascular stroma that expressed α-SMA, suggesting that CTGF may play a critical role in myofibroblast-mediated fibrosis associated with ileal carcinoids.[123] These findings were supported by another study which demonstrated significantly higher mRNA levels of CTGF and TGF-β1 in small intestinal carcinoid tumours compared to normal mucosa and gastric (non-fibrotic) carcinoids.[27] Similarly, higher levels of CTGF immunostaining were observed in small intestinal NETs with clinically and histologically documented evidence of peritoneal fibrosis compared with non-fibrotic gastrointestinal carcinoids and normal mucosal tissue. Serum CTGF levels were higher in patients with small intestinal NETs compared to patients with gastric carcinoids and control patients.[27] In another study of 69

patients with midgut NETs elevated plasma CTGF levels were associated with right ventricular dysfunction and right-sided valvular regurgitation, although there was no significant association with the presence of CHD using defined criteria. Plasma CTGF≥77 μg/L was predictive of right ventricular dysfunction with 88% sensitivity and 69% specificity.[124]

CTGF may be a promising therapeutic target. Anti-CTGF single-chain variable fragment antibodies attenuated bleomycin-induced pulmonary fibrosis in mice and neutralising monoclonal antibodies to CTGF attenuated ventricular remodelling in animal models of dilated cardiomyopathy and pressure overload-induced heart failure. [125,126] However, there are no therapeutic trials assessing the antifibrotic effect of antibodies against CTGF in NETs.

PDGF

Platelet Derived Growth Factor (PDGF) is a major mitogen for mesenchymal derived cells and is composed of four isoforms (PDGF-A, -B, -C and –D) which may form homo- and heterodimers (PDGF-AA, -AB, -BB, -CC and –DD). Two distinct receptor types, α- and β-receptors, have been characterised. The classical targets for PDGF effects (e.g. fibroblasts and smooth muscle cells) have both α- and β- receptors, whereas other cell types (e.g. oligodendrocyte progenitor cells) have only α- receptors and others (e.g. rat brain capillary endothelial cells) have only β-receptors.[127] PDGF production by malignant cells is known to lead to recruitment of cancer-associated fibroblasts in skin cancer, thereby promoting the formation of a thicker fibrous capsule surrounding the tumour[128] and has been implicated in the development of fibrosis in systemic scleroderma, chronic liver disease and renal disorders. The elucidation of the extra- and intra-cellular signalling events involved in PDGF pathways in these conditions has led to the development of PDGF antagonists which act as effective antifibrotic agents in culture and in some animal models, although their clinical use has been limited by their lack of specificity and side effect profile.[129-131]

There is evidence that PDGF is involved in fibrogenesis associated with NETs, although clinical research in this area has lagged behind other fibrotic conditions, such as liver fibrosis and scleroderma. Chaudhry *et al* demonstrated that in a heterogeneous group of GEP NETs PDGF was expressed on tumour cells and stroma in 70% of cases. PDGF α-receptors were present on tumour cells and occasionally on stromal cells, whereas PDGF β-receptors were only seen in the stroma. The authors hypothesised that PDGF may be involved in the stimulation of stromal cell growth through a paracrine and possibly autocrine mechanism, suggesting a role in

fibrosis. Additionally, PDGF synthesised by the surrounding stromal component of these tumours may stimulate tumour growth in a paracrine manner.[127, 132]

In another study of a heterogeneous group of mostly midgut and pancreatic NETs, stromal cells adjacent to tumour cells stained more strongly positive for PDGF β-receptors than stromal cells which were distant from tumour cell clusters. The authors suggested that carcinoid tumour cells may induce expression of PDGF β-receptors on adjacent stromal cells, which may contribute to the pronounced fibrosis surrounding the tumour tissue.[133]

IGF

Insulin-like Growth Factors (IGFs) (IGF-I and II) have structural homology with proinsulin and have biological effects similar to insulin. Their effects are mediated via specific receptors (type I and type II) and they are considered to play an important role in growth regulation of NETs.[134-136]

Nilsson *et al* demonstrated the presence of IGF-I in 11 consecutive cases of midgut carcinoids and IGF-I receptors in approximately half of the tumours. Stimulation of cultured tumour cells with IGF-I induced proliferation suggesting that this growth factor may act as an autocrine stimulator of carcinoid tumour growth.[137] IGF-I and II are also known to stimulate cell replication in primary chick embryo fibroblast cultures, passaged human fibroblasts and fibroblast lines of mammals, although they are less potent than PDGF.[138] Therefore, they may have a role in the development of fibrosis in NETs.

TGFα/EGFR

Transforming Growth Factor a (TGFα), a peptide structurally related to Epithelial Growth Factor (EGF), is overexpressed in NETs and mediates its effects by binding to the EGF receptor (EGFR). It is known to act as a mediator of angiogenesis[139], tumour cell proliferation[140, 141] and fibrosis in NETs. In a study of a heterogeneous group of GEP NETs, TGFα was expressed in approximately 90% of cases, but EGFR expression was significantly higher in the tumour and stromal component of midgut compared to pancreatic neuroendocrine neoplasms. The authors suggested that this difference may account for the more pronounced fibrosis observed in midgut carcinoids.[142] They also hypothesized that TGFα produced by tumour cells could bind to EGFR expressed by stromal and tumour cells and contribute to the associated desmoplastic reaction and neovascularisation perhaps by a paracrine mechanism and to tumour cell growth by an autocrine mechanism.

FGF

The Fibroblast Growth Factor (FGF) family includes a large number of growth factors which regulate multiple developmental processes[143] and have been implicated in the development of fibrosis in gastrointestinal NETs. In a small study of patients with gastric NETs, 10 of 17 patients with a type 3 gastric NET demonstrated positive immunostaining for basic FGF (bFGF) and some of those were also associated with diffuse stromal fibrosis, suggesting a possible functional role for bFGF secreted by tumour cells in the proliferation of stromal fibroblasts or other mesenchymal cell types.[144] In another study of a heterogeneous group of 41 gastrointestinal NETs, a positive correlation was found between acidic FGF (aFGF) and the amount of fibrous stroma, suggesting that aFGF might be involved in the proliferation and activity of stromal fibroblasts.[145] However, in a small study of 37 patients with carcinoid syndrome, 9 of which had CHD, circulating levels of FGF were not associated with cardiac fibrosis.[71]

VEGF

Vascular Endothelial Growth Factor (VEGF) has 2 receptors (VEGF-R1 and VEGF-R2) and is an important pro-angiogenic factor. VEGF-R2 is responsible for the proliferative response of endothelial cells to VEGF, whereas VEGF-R1 is responsible for vascular tabulation and maturation. In a small series of 12 patients with pulmonary tumourlets and neuroendocrine cell hyperplasia, tumourlets were located in hypervascularised areas associated with fibrosis, which was ranging in severity from mild to intense. These patients demonstrated significantly higher expression of VEGF and VEGF-R2 expression in endothelial cells and significantly increased VEGF-R1 expression in bronchial epithelial and endothelial cells, as compared with a control group of normal lungs. This may indicate an involvement of VEGF in the development of fibrosis around tumourlets. However, the majority of subjects in the patient group had pulmonary diseases, such as emphysema, bronchiectasis and tuberculosis, which may be associated with pulmonary fibrosis and act as a confounding factor.[146] A case report of pulmonary carcinoid tumourlet associated with fibrosis also demonstrated strong production of TGF-β1 and VEGF by tumour cells suggesting possible involvement of these growth factors in the fibrotic process.[147]

D. Kinins and other peptides

Kinin peptides are known to be released into the circulation during flushing episodes in patients with carcinoid syndrome and may act as fibrotic mediators.[148, 149] Bradykinins are potent in altering endothelial permeability

and may play a role in the production of carcinoid heart lesions.[150] Tachykinins (substance P, substance K) are mitogenic in arterial smooth muscle cells, human skin and mouse (3T3) fibroblasts.[151, 152]

In a Swedish study of 68 patients with midgut NETs and carcinoid syndrome the plasma levels of tachykinins neuropeptide K and substance P were significantly higher in those subjects with severe right heart disease compared to those with mild or no changes.[67] Similarly in a large multicentre UK study of 187 patients with metastatic NETs evaluating a panel of biomarkers as predictors of CHD, neurokinin A levels were significantly higher in patients with CHD compared to those without cardiac involvement.[74]

In a small series of 28 cases of medullary thyroid carcinoma (MTC) (which is derived from the thyroid parafollicular calcitonin-secreting cells or C-cells), there was a significant correlation between the degree of desmoplasia determined histologically and expression of Fibroblast Activating Protein α (FAPα) (a type II serine protease capable of degrading gelatin and type I collagen) and Tenascin-C (Tn-C) (an extracellular matrix glycoprotein). The extent of this desmoplastic stromal reaction was also shown to correlate with the incidence of lymph node metastases. FAPα is known to function as an active serine protease and may be involved in direct remodelling of the ECM during cancer invasion. Tn-C is upregulated in many human cancers and this increased expression has also shown correlation with increased invasiness.[153] In a larger study of 100 cases of MTC, the degree of desmoplasia correlated significantly with expression of Tn-C, as well as hypoxia-associated proteins HIF1α and CAIX, suggesting that focal tumour hypoxia may trigger remodelling of the ECM.[154]

Another peptide which has been implicated in the development of dense fibrosis around tumourlets is the gastrin releasing peptide (GRP). In a case report of diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH) with a central and peripheral typical bronchial NET and multiple tumourlets associated with fibrosis, the pre-operative serum proGRP level was high but returned to normal after lobectomy, indicating that GRP was produced and secreted by carcinoids, tumourlets or neuroendocrine cell hyperplasia (NECH). Immunohistochemical analysis of 17 neuropeptides and a trophoblastic peptide (human gonadotrophin-alpha) demonstrated intense expression of GRP in carcinoids, tumourlets and NECH lesions, suggesting that this peptide could be involved in the development of local fibrosis in a paracrine manner.[155]

Finally, neuroendocrine tumour cells can produce many other amines and polypeptides, including prostaglandins and cytokines.[91] Although their role in the development of NET-related fibrosis has not been investigated, prostaglandins and interleukins are known to play a role in the development and maintenance of chronic inflammation and to act as pro-fibrotic molecules. [156,157]

E. Pathway crosstalk and other considerations

The role of the various factors known to be involved in NET-related fibrogenesis has been described in separate sections to allow a more structured presentation and summary of the literature. However, it would be naïve to suppose that these factors act independently via discrete signalling pathways. In contrast, there is a growing body of evidence that these factors interact with each other and the complex crosstalk between signalling pathways is largely responsible for the complex nature of fibrosis and the difficulty to establish effective antifibrotic therapies. Growth factor signalling pathways share many downstream signalling molecules and may act synergistically or antagonistically to each other. The crosstalk between pathways may also occur at the level of receptor-ligand interactions.[158, 159] For example, CTGF binds its receptor CTGFR but may also bind IGF receptors. In addition, activation of CTGFR augments both IGF-R and EGF-R signalling pathways, which in turn induce collagen production and cell proliferation.[141] It is also intriguing that patients with NETs often have very similar clinical presentations in terms of tumour site, functionality and stage but only some of them develop fibrotic complications during the course of their illness. In addition, despite the known central role of serotonin in the development of CHD, more than 50% of patients with elevated circulating serotonin levels paradoxically do not develop cardiac fibrosis.[90] It may be that the development of fibrosis depends on the balance between pro-fibrotic and antifibrotic factors and the crosstalk among multiple signalling pathways rather than a single or a few factors considered in isolation.

It is also apparent from our review that although many studies have focused on the pathogenesis of CHD, less emphasis has been placed on the pathophysiology of mesenteric fibrosis, which at present is poorly understood. The limited evidence that is available suggests that 5-HT, TGF-β and CTGF signalling pathways appear to play a role in the development of mesenteric fibrosis[25,26,45,46]. However, these factors are probably produced by the tumour cells that metastasise to the mesentery and possibly cancer-associated fibroblasts and seem to act locally to produce a fibrotic reaction, unlike CHD where circulating levels of these factors are more important. Further research is needed in this area to improve our understanding of the underlying mechanisms of mesenteric fibrogenesis.

Many factors have been investigated in terms of their potential involvement in NET-related fibrogenesis, but not to the same extent or with the same scrutiny. Serotonin, TGF-β and CTGF have certainly been explored in more depth and their association with NET-related fibrosis has additionally been demonstrated in functional (dynamic) studies of the tumour microenvironment. The remaining growth factors have generally been

investigated in older studies mostly using 'static' techniques (immunohistochemistry) to establish their possible association with fibrosis, but the lack of functional investigations means that a causal relationship cannot be proved with this methodology.

Conclusion

Neuroendocrine tumours are often associated with diverse fibrotic complications, which can lead to devastating clinical sequelae and cause considerable morbidity and mortality. However, our knowledge of the biologic basis of this relationship is relatively limited. As there are no established medical therapies for the prevention or regression of fibrosis in NETs, there is an unmet need for meaningful investigations into the pathophysiology of this association. Research in this area has lagged behind in comparison to other chronic fibrotic conditions, such as liver disease and there is undoubtedly a lot to be learnt from developments in the study of fibrogenesis in other disorders. Unfortunately, neuroendocrine tumour cell lines that can serve as a model for human cancer have several limitations, may not be widely available or well characterised, and this poses significant challenges in basic science in NETs.[161] In addition, compared to other disciplines, there is a lack of suitable animal models, which are generally limited to a few *in vivo* xenograft mouse models using neuroendocrine tumour cell lines.[161] Therefore it is difficult to study mechanisms of fibrogenesis and more importantly to investigate the effect of antifibrotic agents in pre-clinical animal studies. Despite these challenges, there are undoubtedly many exciting opportunities for future research in the field of fibrosis in NETs. The evaluation and development of non-invasive biomarkers of fibrosis could have important clinical implications. Such biomarkers could potentially predict the development of fibrosis at an early pre-clinical stage or help stratify patients in different categories of risk of fibrotic complications, perhaps alongside other clinical information. This would be important not only for prognostication purposes, but also for targeting patients at highest risk of poor outcome with experimental antifibrotic therapies. The investigation of epigenetic changes in NETs as a driver of fibrogenesis is another promising area for future research. Common epigenetic alterations (DNA methylation, post-transcriptional modifications of histones and non-coding RNAs) are known to contribute to the pathogenesis of fibrosis in many chronic fibrotic disorders, such as systemic scleroderma, cardiac, kidney and pulmonary fibrosis.[162] The measurement of cell-free DNA methylation modifications in the blood has also been suggested as a potentially useful novel biomarker for the stratification of liver fibrosis associated with non-alcoholic steatohepatitis, although its role as a liquid biopsy requires further validation.[163] This may be an exciting avenue for future research in NET-related fibrosis. The role of oxidative stress in the development of fibrosis in NETs is another

interesting research area that requires further exploration. Although suggestions for study design are beyond the scope of this review, the investigation of variations in gene/protein expression and epigenetic changes in NET patients with presence and absence (or with varying degrees) of fibrosis may be an appropriate research strategy. Other important areas for future study would include functional and dynamic experiments of the tumour microenvironment using appropriate NET cell lines or cancer-associated fibroblasts isolated from human tissue, in order to unravel the complex mechanisms of fibrogenesis at a cellular level. Certainly, the precise details of research protocols will need to vary depending on the specific area of interest in NET fibrogenesis. Finally, therapeutic studies assessing the effect of antifibrotic agents with appropriate co-culture models of the tumour microenvironment and available animal models would be of considerable value for translating advances in basic research into clinical practice.

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Author contributions:

FL wrote the first draft of the manuscript. FL and DM performed the literature search. All authors agreed on the original concept and design of the manuscript, reviewed the draft, provided input and contributed to the final manuscript.

Conflicts of interest:

FL, KR, DM, CT– none declared

CT, MC - Honoraria for lectures, advisory boards, educational grants for the Royal Free NET unit (NOVARTIS, IPSEN), advisory board (Lexicon)

Funding: None

Acknowledgements: None