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REVIEW ARTICLE

Extracellular nicotinamide phosphoribosyltransferase, a new cancer *metabokine*

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In this review, we focus on the secreted form of nicotinamide phosphoribosyltransferase (NAMPT); extracellular NAMPT (eNAMPT), also known as pre-B cell colony-enhancing factor or visfatin. Although intracellular NAMPT is a key enzyme in controlling NAD metabolism, eNAMPT has been reported to function as a cytokine, with many roles in physiology and pathology. Circulating eNAMPT has been associated with several metabolic and inflammatory disorders, including cancer. Because cytokines produced in the tumour micro-environment play an important role in cancer pathogenesis, in part by reprogramming cellular metabolism, future improvements in cancer immunotherapy will require a better understanding of the crosstalk between cytokine action and tumour biology. In this review, the knowledge of eNAMPT in cancer will be discussed, focusing on its immunometabolic function as a *metabokine*, its secretion, its mechanism of action and possible roles in the cancer micro-environment.

Abbreviations

eNAMPT, extracellular NAMPT; FK866, daporinad, (E)-*N*-[4-[1-(benzoyl)piperidin-4-yl]butyl]-3-pyridin-3-ylprop-2-enamide; iNAMPT, intracellular NAMPT; NAMPT, nicotinamide phosphoribosyltransferase (EC 2.4.2.12).; PBEF, pre-B cell colony-enhancing factor

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Tables of Links

TARGETS	
Other protein targets[a]	**Enzymes**[e]
CD28	Akt (PKB)
CD38	COX-2
Notch receptors	DDAH2, N^G,N^G-dimethylarginine dimethylaminohydrolase 2
GPCRs[b]	ERK
CCR2	FAS, fatty acid synthase
CCR5	HDAC
CXCR4	Indoleamine 2,3-dioxygenase 1
CXCR7	iNOS
Ligand-gated ion channels[c]	MMP2
P2X7 channels	MMP9
Catalytic receptors[d]	JAK2
Insulin receptor	JNK
TLR-4	p38
	PKA
	PARPs
	SIRT1

LIGANDS	
ATP	Insulin
CCL2	IL-7
Chloroquine	IL-10
CXCL8, IL-8	LPS
CXCL12, SDF-1	Sivelestat
FK866, daporinad	Trichostatin A
G-CSF	TNF-α
IL-1ß	VEGF
IL-6	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d,e]Alexander *et al.*, 2015a,b,c,d,e).

Introduction

Nicotinamide phosphoribosyltransferase (NAMPT) is a pleiotropic protein that exists in two distinct forms: (i) an intracellular form (iNAMPT) and (ii) an extracellular form (eNAMPT) (Shackelford *et al.*, 2013). Intracellular NAMPT is a homodimeric class type II phosphoribosyltransferase (EC 2.4.2.12) that catalyses the production of nicotinamide mononucleotide (NMN) from nicotinamide (vitamin B3 or vitamin PP) and phosphoribosyl pyrophosphate (PRPP), the rate-limiting reaction in the salvage pathway for NAD biosynthesis (Wang *et al.*, 2006). It is through this enzymic activity that iNAMPT regulates the intracellular levels of NAD and indirectly affects both cellular energetics and NAD-dependent enzymes [such as sirtuins (SIRTs) and PARPs]. Figure 1 depicts the main pathways leading to NAD synthesis.

NAMPT is involved in different immunometabolic disorders, including cancer (Bi and Che, 2010), and selective inhibitors of this enzyme, such as FK866/APO866 and CHS828/GMX1777, have been entered into phase I/II clinical trials for solid and non-solid tumours (http://www.clinicaltrials.gov) (Galli *et al.*, 2013).

In sharp contrast to our understanding of iNAMPT, the roles of the eNAMPT are poorly understood, despite the number of articles published, which are mostly descriptive. While it is likely that eNAMPT represents a secreted form of iNAMPT (i.e. part of the protein used for NAD synthesis is released), this has not been formally demonstrated. In this review, we use the terms eNAMPT or iNAMPT to refer to the cellular location of the same protein. Moreover, because cleavage of eNAMPT has never been reported, we also assume that both forms have the same amino acid sequence and share a common protein pool.

Our poor understanding of the extracellular protein is well represented by the names other than eNAMPT still found in the literature: pre-B cell colony-enhancing factor (PBEF) and visfatin (Jieyu *et al.*, 2012). These two names historically defined their roles in the immune system and as an adipokine (Sethi and Vidal-Puig, 2005; Sethi, 2007). Given the seemingly ubiquitous nature of its production and in the interest of clarity in accordance with the HGNC nomenclature, in the present review, we have opted to drop the use of the names visfatin and PBEF for iNAMPT and eNAMPT. This review discusses the possible role of eNAMPT as a 'metabokine' in cancer, namely, a cytokine-like protein involved in immune and metabolic functions, and the different targeting strategies that may benefit future therapeutics. It must be acknowledged that there are reviews concentrating on the effects of eNAMPT in other disorders (Peiro *et al.*, 2010; Montecucco *et al.*, 2013; Wang and Miao, 2015), and therefore, this review will not cover reports on this enzyme in other disease states.

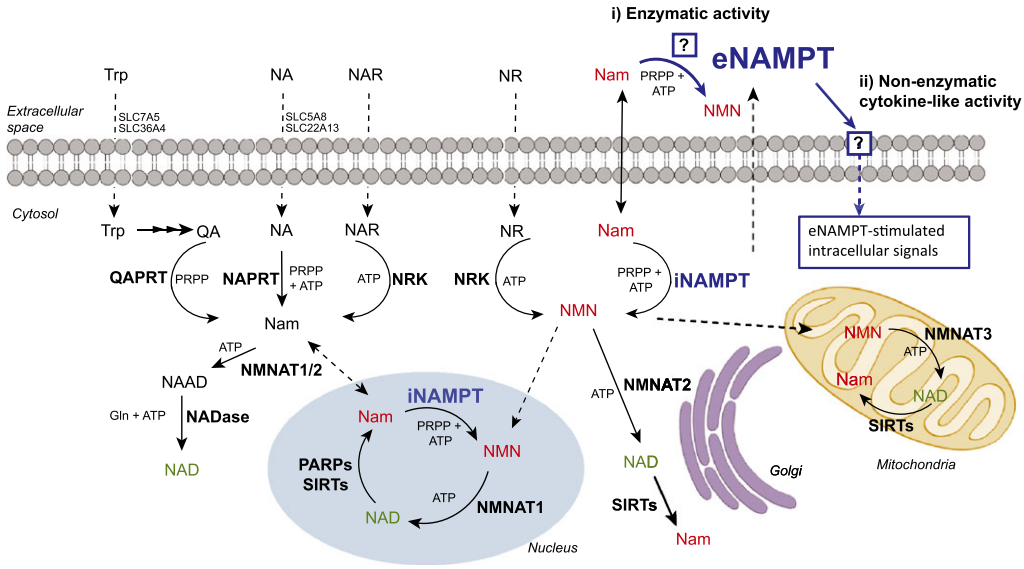

Figure 1

The role of cellularly localized NAMPT in NAD synthesis. Both iNAMPT and eNAMPT (blue text) arise from the same protein pool but differ in their cellular localization. Key enzymes located in specific cellular compartments are indicated in bold: QAPRT, quinolinic acid phosphoribosyltransferase; NAPRT, nicotinic acid phosphoribosyltransferase; NRK, nicotinamide riboside kinase; NMNAT, NMN adenylyltransferase. NAD metabolites or substrates: Trp, tryptophan; NA, nicotinic acid; NAR, nicotinic acid riboside; NR, nicotinamide riboside; Nam, nicotinamide; QA, quinolinic acid.

Cellular sources and stimuli of eNAMPT secretion

Samal *et al*. first described eNAMPT as an active protein in the extracellular space in 1994. They reported its secretion from pre-B cells and its ability to synergize with stem cell factor and IL-7 to promote colony formation (Samal *et al*., 1994). Indeed, this was the basis of its classification as a cytokine and demonstrated its biological potential as a putative paracrine and autocrine factor. It was not until 2005 that eNAMPT was identified as an adipokine. It was called visfatin because it was thought to be secreted preferentially by visceral adipose tissue in obese patients (Fukuhara *et al*., 2005; Sethi and Vidal-Puig, 2005). Twenty years on, it is now clear that eNAMPT is not only produced by pre-B cells and adipocytes but also readily detectable in conditioned media from cultures of most cell types (Table 1). As is clear from Table 1, the studies of eNAMPT release from tumour cells are relatively recent and show that it can be secreted by most, if not all, cancer cell lines investigated. Intriguingly, it is noticeable that conditioned media from tumour cells appears to contain substantially more eNAMPT when compared with non-tumour cultures. However, this has not been systematically studied. Nonetheless, the higher levels produced by tumour cells compared with other cells are entirely consistent with the constitutive up-regulation of iNAMPT also reported in the majority of cancer cell lines and in tumour tissues (Galli *et al*., 2013; Sampath *et al*., 2015).

There is limited information on the relative amounts of eNAMPT found in the media compared with the amount of iNAMPT found intracellularly. The only group that has attempted to measure it suggested that, in unstimulated melanoma cell lines, the relative abundance of the extracellular form is approximately 1% of the total NAMPT (Grolla *et al*., 2015). However, this remains to be confirmed by others in other models, and also after stimulation, as it would set a quantitative link between intracellular bioenergetics and extracellular signalling. It should be noted that antibodies and assays for NAMPT can recognize iNAMPT and eNAMPT similarly and, therefore, these experiments are relatively easy to perform.

The stimuli that promote eNAMPT release have also been subject to a few studies and some notable patterns are beginning to emerge. First, it is clear that eNAMPT release occurs in the absence of cell death and therefore appears to be a true and specific phenomenon. However, whether it is released under basal, unstimulated conditions remains unclear. Indeed, it must be acknowledged that, for practical reasons to facilitate immunodetection, most experiments investigating eNAMPT release are performed in serum-free conditions (Garten *et al*., 2010; Ghaemmaghami *et al*., 2013; Pillai *et al*., 2013). While most reports have confirmed that such conditions do not lead to cell death, it still represents a strong form of nutritional/metabolic stress, which may be a *bona fide* stimulus to trigger eNAMPT release for many cell types. Indeed, this hypothesis is strengthened by observations that similar stress conditions, including ischaemia (Jing *et al*., 2014) and oxygen–glucose deprivation (OGD), strongly increase eNAMPT release in neurons and glial cells (Zhao *et al*., 2014) as well as hypoxia in melanoma cells (Grolla *et al*., 2015). Similarly, oxidative and endoplasmic reticulum (ER) stress also appear to increase eNAMPT release (Huh-7 cells) (Pillai *et al*., 2013; Lin *et al*., 2015). These observations also remain to be systematically investigated. Nonetheless, these conditions appear to be highly relevant in cancer as nutrient deprivation, hypoxia and oxidative stress are all features of the tumour micro-environment.

Table 1

Summary of cell types that secrete eNAMPT

	Cell type	Modulation of eNAMPT release	Reference
Adipocytes	• 3T3-L1 adipocytes	Increased by Ox-LDL, CTRP3, glucose, rosiglitazone, adipocyte differentiation	(Fukuhara *et al.*, 2005)
			(Tanaka *et al.*, 2007)
	• SGBS adipocytes		(Chen *et al.*, 2013)
			(Derdemezis *et al.*, 2011)
	• Adipocytes derived from healthy donors	Decreased by insulin, PI3K and AKT inhibitors, quercetin	(Li *et al.*, 2014a)
			(Haider *et al.*, 2006a, b)
	• HIB-1B adipocytes		(Haider *et al.*, 2006b)
			(Revollo *et al.*, 2007)
Immune cells	• LPS-activated monocytes	Increased by ATP, LPS	(Schilling and Hauschildt, 2012)
	• Macrophages in visceral adipose tissue		(Curat *et al.*, 2006)
	• Leucocytes		(Friebe *et al.*, 2011)
	• Peripheral blood lymphocytes		(Samal *et al.*, 1994)
Brain cells	• PC12 cells	Increased by $CoCl_2$, ischaemia, OGD	(Kang *et al.*, 2011)
	• Primary neurons		(Jing *et al.*, 2014)
	• Primary glial cells		(Zhao *et al.*, 2014)
Cancer cells	• Hepatoma cells (HepG2, Huh-7)	Increased by anti-CD38 and differentiation in CCL, oxidative stress (H_2O_2), hypoxia	(Samal *et al.*, 1994)
			(Garten *et al.*, 2010)
			(Soncini *et al.*, 2014)
	• Colorectal cancer cells (HCT-116, LS180)		(Ghaemmaghami *et al.*, 2013)
	• Breast cancer cells (MCF10A, MCF7, T47D, MDA-MB-231, BT549, MDA-MB-468)		(Audrito *et al.*, 2015)
	• Melanoma cells (B16, MeWo, HMCB, SkMel28, LB24)		(Lin *et al.*, 2015)
	• Neuroblastoma and glioma cells (SH-SY5Y, SK-N-Be, U87)		
	• Mesothelioma (MSTO)		
	• Prostate cancer cells (DU-145)		(Grolla *et al.*, 2015)
	• Cervical cancer cells (HeLa)		
	• Chronic lymphocytic leukemia lymphocytes		
Other cells	• Fibroblast (COS-7, PA317, CHO)	Increased by LPS, TNF-α, IL-1β, starvation and oxidative stress (H_2O_2), differentiation, glucose, C-peptide	(Samal *et al.*, 1994; Jia *et al.*, 2004)
	• Amniotic epithelial cells		(Ognjanovic *et al.*, 2005)
	• Inflamed HUVECs		(Romacho *et al.*, 2013)
	• Neonatal rat cardiomyocytes		(Pillai *et al.*, 2013)
	• Pancreatic beta cells		(Revollo *et al.*, 2007)
	• Isolated human islets		(Kover *et al.*, 2013)
	• Sebocytes		(Kovacs *et al.*, 2016)
	• Melanocytes		(Grolla *et al.*, 2015)

PubMed was used to retrieve the evidence using 'visfatin' or 'PBEF' as a search string. Only cultured cell lines were considered for this table.

Alongside conditions of cellular stress, nutritional cues have also been shown to promote eNAMPT release. Indeed, other than the first (albeit controversial and eventually retracted) report that linked increased eNAMPT levels to obesity-linked diabetes (Fukuhara *et al.*, 2005), secretion of eNAMPT promoted by glucose or high insulin has been reproduced by others (Haider *et al.*, 2006b; Unluturk *et al.*, 2010).

Last, as is the case for many cytokines, eNAMPT is also secreted in response to inflammatory stimuli. For example, treatment of LPS-activated monocytes with ATP induces a five-fold increase in eNAMPT levels. Similarly, stimulation of leukocytes with LPS increases eNAMPT release as does the treatment of amniotic epithelial cells with pro-inflammatory TNF-α (Ognjanovic *et al.*, 2005) or cardiomyocytes with IL-1β

(Pillai *et al.*, 2013). Recent data in sebocytes seem in line with this, showing that eNAMPT release increased, as did that of IL-6, upon treatment with toll-like receptor-4 (TLR-4) activators (Kovacs *et al.*, 2016). Moreover, the *NAMPT* gene contains several glucocorticoid regulatory elements (Ognjanovic *et al.*, 2001) in its promoter, and therefore, it would be interesting to ascertain whether corticosteroids act on eNAMPT as they do on other inflammatory cytokines.

In theory, eNAMPT secretion reduces the iNAMPT pool devoted to NMN synthesis, and therefore, an excessive secretion could create a significant metabolic strain on the cell. Schilling *et al.* have hypothesized that a safety net for this may be represented by the requirement of two separate stimuli to trigger secretion. They have postulated that activation of P2X7 receptors and the subsequent K^+ flux might be one of these stimuli (Schilling and Hauschildt, 2012). This possibility should therefore be taken into account when investigating eNAMPT release.

Overall, a wide range of stimuli have been used to modulate eNAMPT release both *in vitro* and *in vivo*. These can be classified into three categories: (i) cellular stress; (ii) nutritional cues; and (ii) inflammatory signals. This may indicate that eNAMPT secretion is recruited to function under specific circumstances and as such may have physiological roles in addition to (or similar to) those associated with cancer pathology.

Mechanisms of eNAMPT secretion

Unlike many secreted proteins, newly translated eNAMPT lacks a signal peptide. This suggests that it is released through a non-classical pathway. Indeed, most reports suggest that its release is insensitive to brefeldin-A and monensin, that is, inhibitors of the classical ER–Golgi-dependent pathway (Revollo *et al.*, 2007; Tanaka *et al.*, 2007; Garten *et al.*, 2010). However, this was not observed in melanoma cells (Grolla *et al.*, 2015). Also, in this latter system, eNAMPT release was augmented in the presence of chloroquine, suggesting that lysosomal trafficking might be involved. The possibility that eNAMPT is present in exosomes or micro-vesicles has also been explored, but this mechanism appears to account only for a very small proportion of all eNAMPT released, if any (Tanaka *et al.*, 2007; Grolla *et al.*, 2015).

Independently of the mechanism of secretion, an important question is whether eNAMPT requires post-translational modifications (PTMs) prior to its secretion. Surprisingly, PTMs have not been systematically evaluated on this protein, although a number of PTMs have been predicted using bioinformatic tools. The NAMPT peptide sequence encodes two putative sites for asparagine glycosylation (a characteristic modification that occurs in many secreted proteins) and at least 24 phosphorylation sites (14 serines, 3 threonines and 7 tyrosine), but whether these predispose to secretion has yet to be experimentally ascertained.

In contrast, acetylation of NAMPT has been connected to its release. Pillai *et al.* (2013) treated cardiomyocytes with trichostatin A (a histone deacetylase inhibitor) or with high concentrations of nicotinamide (a non-specific SIRT inhibitor, among other activities) and found that both treatments reduced the intracellular levels of iNAMPT and completely blocked eNAMPT release after stress, suggesting that an acetylation-dependent mechanism is likely to participate in the release of this protein. More recently, Imai and colleagues

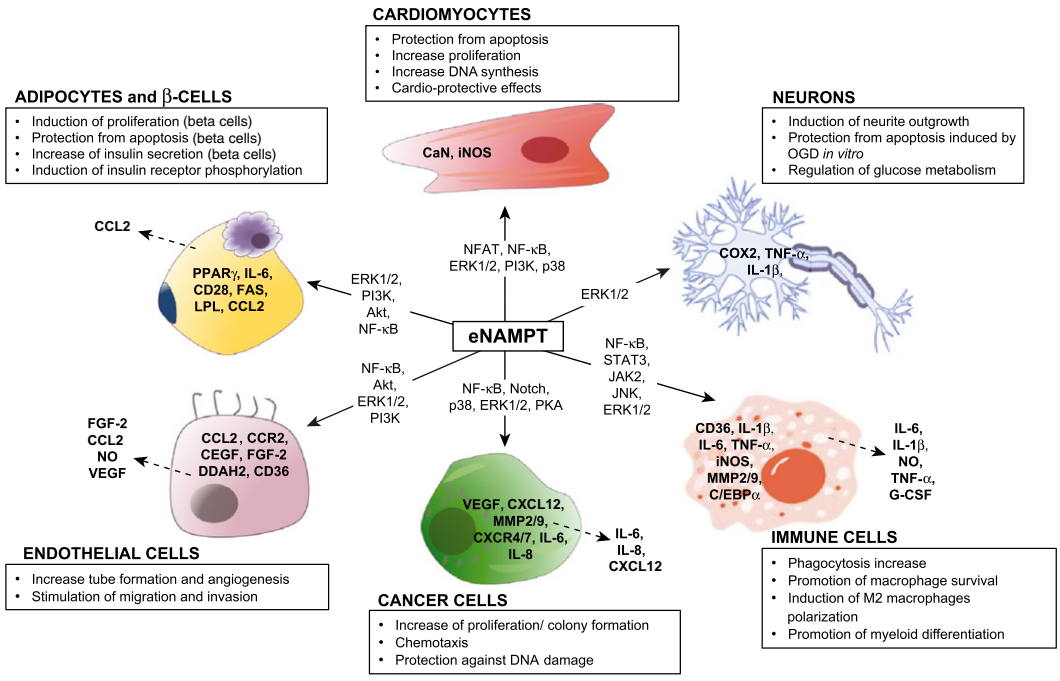

Figure 2

Autocrine and paracrine effects of eNAMPT. eNAMPT is released by most of the cell types and acts as a cytokine on a wide range of cells. It is able to activate downstream intracellular pathways by stimulating a subsequent release of other cytokines or by favouring a variety of physiological and pathological effects. PubMed was used to retrieve the evidence using 'visfatin', 'PBEF' or 'eNAMPT' as a search string.

demonstrated that deacetylation of iNAMPT by the mammalian NAD-dependent deacetylase SIRT1 predisposes the protein to secretion in adipocytes. Furthermore, mutagenesis studies of NAMPT suggest that SIRT1 deacetylates lysine K53 and thereby enhances eNAMPT activity and secretion (Yoon *et al.*, 2015).

In summary, the mechanisms by which eNAMPT is secreted are at present ill defined, although it would appear that deacetylation might be a required step and that several mechanisms might be involved, a feature reported also for other cytokines such as IL-1β.

The mechanisms of action of eNAMPT

There is ample evidence that eNAMPT triggers numerous intracellular signalling pathways with varying temporal dynamics and can also stimulate many biological effects in a variety of cell types (Figure 2). However, the molecular understanding of how such events are triggered remains largely unknown. Given that eNAMPT has potential to remain enzymically active upon release, these effects can be either attributed to (i) its extracellular enzymic activity and/or to (ii) the binding and activation of a cell surface receptor (Figure 1). Currently, there is evidence for and against both these possibilities, which are not mutually exclusive.

Mechanisms of action of eNAMPT: a cell surface receptor for eNAMPT

The fact that treatment with ng·mL^{-1} concentrations of exogenous eNAMPT is sufficient to activate specific intracellular signalling pathways, particularly within minutes, may be evidence that eNAMPT has cytokine-like properties and binds to and activates a cell surface receptor. Although the identity of this receptor remains unknown, a few candidates have been proposed. The first to be proposed was the insulin receptor, based on the insulin-mimetic properties of eNAMPT (Fukuhara *et al.*, 2005). However, lack of reproducibility of this phenomenon led to the retraction of this report (Fukuhara *et al.*, 2007), and the molecular basis of the insulin-like properties of eNAMPT is likely to be indirect.

A number of molecular screening studies (e.g. two-hybrid assays, affinity capture and co-fractionation) have identified eNAMPT as a putative protein-binding partner (Zhang *et al.*, 2008; Perez-Hernandez *et al.*, 2013; Huttlin *et al.*, 2015; Wan *et al.*, 2015), although these also remain to be validated. Nonetheless, more robust data exist for at least two other putative eNAMPT receptors. In 2012, eNAMPT was reported to selectively inhibit infection of macrophages by HIV and this activity was linked, using surface plasmon resonance, to a direct interaction with the chemokine receptor CCR5 (Van den Bergh *et al.*, 2012). Surprisingly, this report has gone unnoticed and has not been replicated or disputed by others. More recently, eNAMPT induced, in lung, NF-kB transcriptional activities and inflammatory injury via direct binding to the TLR-4. This was also confirmed by surface plasmon resonance analysis (Camp *et al.*, 2015). Moreover, computational analysis revealed similarities in binding surface between eNAMPT and MD-2, an essential LPS cofactor involved in TLR-4 activation. While these data are highly promising, they do require further validation. However, such validation is not necessarily straightforward as recombinant eNAMPT is often contaminated with endotoxins, natural ligands of TLR-4, a condition that was controlled for in the original paper but that requires accurate monitoring by others. The commercial availability of highly pure preparations of recombinant forms of eNAMPT should facilitate such studies, although the presence of endotoxins in such preparations should nonetheless be monitored.

Mechanisms of action of eNAMPT: eNAMPTas a metabolic ectoenzyme

It is possible that the biological actions of eNAMPT may also be attributed to its enzymic activity. Indeed, using size exclusion chromatography, it has been demonstrated that secreted eNAMPT can exist as a dimer in conditioned media of HIB-1B adipocytes (Revollo *et al.*, 2007). Given that dimerization is required for enzymic activity of NAMPT (Wang *et al.*, 2006), this presents the possibility that eNAMPT is active in the extracellular space. Obviously, the availability of substrates is crucial for this hypothesis, and the presence of sufficient concentrations of phosphoribosyl pyrophosphate (PRPP), nicotinamide or ATP has been disputed (Hara *et al.*, 2011). It should be noted that in this respect, the cancer microenvironment may favour conditions to promote the enzymic activity of eNAMPT. Indeed, the substantial increase of necrosis may allow eNAMPT substrates to be present at sufficient concentrations, creating a niche for immunometabolism, and the acidic pH in hypoxic areas of tumours may increase or interfere with its activity. It could be argued that, if eNAMPT functions as an active enzyme, its product, NMN, should be able to mimic the effects of eNAMPT exposure. While a minority of experiments have confirmed this, many others have failed to demonstrate an effect of NMN, leading to further uncertainty (Revollo *et al.*, 2007; Li *et al.*, 2008).

Another approach to evaluate the role of the enzymic activity of eNAMPT relies on the use of either enzyme inhibitors or mutant forms of recombinant eNAMPT that lack enzymic activity. The biggest limitation of the former approach is that NAMPT inhibitors are cell permeable and therefore are unable to discriminate between the intracellular and extracellular form. Hence, any effects of treatment with FK866 cannot be attributed solely to the extracellular form. Nonetheless, a lack of effect can support the hypothesis that the enzymic activity is irrelevant to a particular effect. Indeed, some reports, but not others, have shown no effect of FK866 on eNAMPT function, demonstrating that at least part of its effects are independent of enzymic activity (Li *et al.*, 2008; Audrito *et al.*, 2015).

To attempt to unravel the controversy, a number of point mutants with different features, such as loss of function, switch of substrate affinity and loss of dimer conformation, have been described (Table 2) and most of the mutants retained all the effects of wild-type protein. For example, H247E, R392A, R311A and S200D mutants retain the same ability as wild-type eNAMPT in STAT-3 activation, IL-6 secretion, macrophage survival following ER stress (Li *et al.*, 2008) and M2-polarization (Audrito *et al.*, 2015). This has been also shown in melanoma cells, in which both wild-type eNAMPT and mutants induced p42/44 ERK activation (Grolla *et al.*, 2015). On the contrary, only one study so far has found that

Table 2

Functional effects of point mutations in eNAMPT

Feature	Mutant	Reference
Loss of activity	H247E, H247A, S199D, S200D, R392A R311D, G384A, K342R, D219A	(Khan *et al.*, 2006) (Wang *et al.*, 2006) (Olesen *et al.*, 2010) (Burgos and Schramm, 2008)
Switch the substrate affinity	D219S	(Khan *et al.*, 2006)
Prevent inhibitor binding	H191R, G217R, A244M	(Olesen *et al.*, 2010) (Watson *et al.*, 2009)
Prevent dimerization	Q388R, D93del, S199D, S200D	(Olesen *et al.*, 2010) (Revollo *et al.*, 2007)

treatment of wild-type eNAMPT, but not the H247A enzymically inactive mutant, attenuated the detrimental effect of OGD on cell viability of neuronal and glial cells (Zhao *et al.*, 2014).

Circulating eNAMPT in cancer

The link between iNAMPT and cancer is well documented (Garten *et al.*, 2009; Galli *et al.*, 2010; Burgos, 2011; Sampath *et al.*, 2015); indeed, it is essential for restoration of the NAD pool following over activation of PARPs and SIRTs, in cancer cells (Wang *et al.*, 2011; Chan *et al.*, 2014). It is therefore not surprising that iNAMPT is overexpressed and overactive in cancer and represents an exciting new therapeutic target (Wang *et al.*, 2014; Zak *et al.*, 2015).

Like the intracellular form, eNAMPT also seems to be linked to cancer, and this has potential to influence novel therapeutic strategies. Current literature largely confirms that serum and plasma levels of eNAMPT are increased in almost all cancer patients analysed, with solid or non-solid tumours, compared with healthy subjects (Table 3). In the majority of cases reported, eNAMPT levels positively correlated with the stage of progression. As summarized in Table 3, in colon, breast, gastric, bladder and endometrial cancer, eNAMPT levels correlated with increased risk of developing the pathology. Moreover, in oral squamous cell carcinoma, in breast cancer, in glioblastoma/astrocytoma and in gastric cancer, eNAMPT levels correlate with overall survival and metastasis formation. It is noteworthy that only three separate studies reported a lack of correlation with eNAMPT levels (Table 3), although this may also be influenced by the bias of not publishing negative data.

An example of an area in which conflicting data exist is in oesophageal cancer. Here, two opposing observations have been described. Nakajima *et al.* found that eNAMPT was not elevated in 117 patients with oesophageal squamous cell carcinoma (Nakajima *et al.*, 2010). In contrast, Takahashi *et al.* demonstrated that circulating eNAMPT levels are elevated in plasma of 27 pre-oesophagectomy and post-oesophagectomy patients. Despite having a small cohort, this study did also demonstrate that therapeutic treatment with sivelestat, a neutrophil elastase inhibitor, resulted in decreased eNAMPT levels (Takahashi *et al.*, 2010). The disparate findings of these two independent studies may be explained in part by the methodology used to detect plasma eNAMPT. Indeed, different commercial kits (RIA, EIA and ELISA) exist that do not yield quantitatively superimposable results and may contribute to some discrepancies (Korner *et al.*, 2007).

While the results above are encouraging, a number of issues remain to be resolved. First, the current literature is unable to determine a threshold of physiological versus pathological eNAMPT plasma levels and what magnitude (how many-fold) of change represents a true pathological eNAMPT rise. These limitations are in part attributable to the presence on the market of diagnostic kits that do not yield overlapping results. Second, eNAMPT is elevated in both metabolic and inflammatory disorders (Moschen *et al.*, 2010), and this represents an important confounder that can be bypassed only by very large datasets and accurate monitoring. Third, while it is possible that patients with different cancer types will exhibit different levels of plasma eNAMPT, at present this cannot be confirmed, given that the different groups have used different methods to determine eNAMPT levels. Fourth, most studies to date are retrospective, providing a strong methodological limitation.

An elegant manuscript investigated eNAMPT levels in healthy subjects and in patients with colorectal cancer, colonic polyps or inflammatory bowel diseases. The cancer patients displayed the highest levels followed by those with benign lesions and chronic inflammatory states (Neubauer *et al.*, 2015). However, larger independent datasets standardized for assay used are required to prove eNAMPT as a *bona fide* cancer biomarker, determine its specificity and selectivity (both of which at present would appear low) and to understand its place in diagnosis and whether it may represent a diagnostic or prognostic biomarker.

Given the wide array of cells that can release NAMPT, a fundamental question is the source of eNAMPT in cancer. While it is likely that a number of cell types (e.g. inflammatory cells, endothelial cells and adipocytes) release eNAMPT in cancer, our group has recently demonstrated that at least part of the circulating protein derives from the tumour itself (Grolla *et al.*, 2015). This was shown by performing allografts in mice with cells that overexpressed a tagged NAMPT, which

Table 3

Evidence that eNAMPT is involved in cancer

Cancer types	eNAMPT levels	Clinical significance	Main correlations	References
Breast cancer	↑ EIA	Independent predictor of mortality	Lymph node invasion CA 15-3 CEA	(Dalamaga *et al.*, 2012) (Dalamaga *et al.*, 2011) (Li *et al.*, 2014b)
Gastric cancer	↑ EIA ELISA	Predictor of 5-year mortality	Stage progression Distant metastasis Tumour size Overall survival Resistin	(Nakajima *et al.*, 2009) (Lu *et al.*, 2014)
Colorectal cancer	EIA ↑ ELISA	Risk factor for early and advanced CRC	Resistin Omentin-1 Vaspin	(Fazeli *et al.*, 2013) (Chen *et al.*, 2016)
+ Chemotherapy (5-FU, oxaliplatin and irinotecan)	↓ ELISA		Resistin Insulin	(Słomian *et al.*, 2014)
+ Surgery (radical resection of tumour, stage II-III)	↔ ELISA		—	(Kosova *et al.*, 2013)
Astrocytoma/ glioblastoma	↑ EIA	Serum marker and prognostic indicator of Glioblastoma multiform (circulating levels: GMBs > AAs > ADs)	iNAMPT	(Reddy *et al.*, 2008)
Oesophageal cancer	↑ ELISA Serum mRNA	eNAMPT serum mRNA is an independent factor of mortality in the first year follow-up. Sivelestat affects eNAMPT expression	MUC1	(Takahashi *et al.*, 2010) (Nakajima *et al.*, 2010)
Bladder cancer	↑ ELISA	Independent prognostic marker of non-muscle invasive bladder cancer High eNAMPT levels indicate shorter recurrence-free survival rate	—	(Zhang *et al.*, 2014a) (Zhang *et al.*, 2014b)
Endometrial cancer	↑ ELISA	eNAMPT levels are associated with over-all survival; risk factor of endometrial cancer	iNAMPT	(Luhn *et al.*, 2013) (Tian *et al.*, 2013)
Oral squamous cell carcinoma	↑ EIA	eNAMPT correlates with stage progression	White blood cells Neutrophils count Hematocrit	(Yu-Duan *et al.*, 2013)
Pancreatic cancer	↔ EIA	—	—	(Gasiorowska *et al.*, 2013)

PubMed was used to retrieve the evidence using 'visfatin' or 'PBEF' or 'eNAMPT' and 'cancer' as a research string.

was then found in significant levels in the plasma of the grafted mice and was dependent on the size of the tumour. This is the first preclinical study therefore to suggest that part of eNAMPT found in serum of cancer patients may be traced to the tumour itself.

The actions of eNAMPT in cancer

The secretion of eNAMPT from tumours and the higher plasma levels in cancer patients opens the possibility that eNAMPT may be involved in autocrine, paracrine and endocrine signalling in cancer. Indeed, it has been shown that eNAMPT influences many of the hallmarks of cancer (Hanahan and Weinberg, 2011). First of all, eNAMPT affects the most fundamental trait of cancer cells: sustained chronic proliferation. For example, eNAMPT stimulation of prostate cancer PC3 cells androgen-insensitive cell line (Patel *et al.*, 2010), melanoma cells (Buldak et al., 2013b) and breast cancer (MCF7) cells (Park *et al.*, 2014) significantly increased cellular proliferation. Recently, it has been reported that eNAMPT also affects telomerase gene expression and proliferation of a gastric cancer cell line (Mohammadi *et al.*, 2015). Furthermore, even where a proliferative burst was not observed, as is the case of melanoma cells, colony formation was enhanced (Grolla *et al.*, 2015).

Along with sustained chronic proliferation, cancer cells developed the ability to avoid programmed cell death. In this context, one report has described that eNAMPT protects Me45 melanoma cells from cell death triggered by DNA

damage (Buldak et al., 2013a). Furthermore, eNAMPT is involved in the epithelial–mesenchymal transition (EMT), a process in which epithelial cells can acquire the abilities to resist cell death, become invasive and disperse. Indeed, eNAMPT promotes osteosarcoma cell migration and invasion in a NF-kB-dependent manner (Cheng *et al.*, 2015). Similarly, eNAMPT stimulated the EMT in breast cancer cells, by inducing fibroblast-like morphology, accompanied by a striking reduction in E-cadherin expression and by a consistent up-regulation of N-cadherin, vimentin and ZEB1 (Soncini *et al.*, 2014). These data lead us to conclude that eNAMPT can act as a tumour-promoting cytokine by increasing proliferation, decreasing cell death and promoting EMT.

Cells other than the cancer cells in the tumour process appear to be affected by eNAMPT as well. Indeed, eNAMPT increases capillary tube formation and angiogenesis via up-regulation of CCR2 and up-regulation and secretion of CCL2 and FGF-2 in human mammary epithelial cells (Adya *et al.*, 2009; Bae *et al.*, 2009). Similar data have been obtained in HUVECs, in which eNAMPT leads to NF-kB activation and then VEGF secretion following by migration and angiogenesis (Kim *et al.*, 2007; Lovren *et al.*, 2009). Moreover, in human aortic endothelial cells, eNAMPT extends the lifespan and enhances the angiogenic capacity in a high-glucose environment (Borradaile and Pickering, 2009). These *in vitro* findings were reproduced *in vivo*, where eNAMPT induced angiogenesis in chorioallantoic membrane assay and in Matrigel plug assays in mice (Kim *et al.*, 2007).

It is now clear that every neoplastic lesion contains immune cells of both the innate and adaptive immune systems and a role of eNAMPT in this field has also been documented (Colotta *et al.*, 2009). Indeed, in inflammatory cells, such as human monocytes, mouse peritoneal macrophages and bone marrow-derived macrophages, eNAMPT promotes cell survival upon ER stress, up-regulates MPP-9 and MPP-2 and increases activated morphology (Li *et al.*, 2008; Kang *et al.*, 2013). Furthermore, treatment with eNAMPT increases $CD34^+$ cell proliferation and granulocytic and monocytic differentiation (Skokowa *et al.*, 2009). Moreover, stimulation of resting monocytes isolated from chronic lymphocytic leukemia with eNAMPT polarizes them towards tumour-supporting M2-macrophages (Audrito *et al.*, 2015). Specifically, eNAMPT increases expression of CD163, CD206 and indoleamine 2,3-dioxygenase and secretion of immunosuppressive (IL-10) and tumour promoting cytokines (IL-6 and IL-8). These data suggest an immunosuppressive role of eNAMPT in cancer-related inflammation.

In conclusion, while the current literature of eNAMPT suffers from a lack of systematic exploration and has been gathered from tumours of different origins, they have prompted the initial definition of eNAMPT as a tumour-promoting cytokine.

Therapeutic targeting of eNAMPT

Despite the evidence discussed in this review, it is clear that more studies are warranted to improve our understanding of the physiological actions of eNAMPT as well as its role(s) in cancer. An elaboration of pharmacological strategies would serve both to further our understanding and to unravel its possible role as a candidate for therapeutic targeting. Yet devising such strategies is not straightforward. The use of enzyme inhibitors, such as those used in clinical trials (http://www.clinicaltrials.gov) (Zak *et al.*, 2015), will not target eNAMPT specifically, as it will also abolish intracellular activity, leading to both beneficial and detrimental side effects from this former target. Furthermore, limitations of global enzyme inhibition are likely to include systemic toxicity due to additional lack of tissue and cell specificity (Chiarugi *et al.*, 2012; Zabka *et al.*, 2015). Moreover, given the uncertainty over the requirement of the enzymic activity for eNAMPT, it might not target this pathway altogether. Modulating its activity via genetic manipulation, gene therapy or RNA strategies (e.g. shRNA and siRNA) will also not allow iNAMPT to be distinguished from eNAMPT, as the same gene encodes these proteins.

A possibility for selective pharmacological intervention would be represented by development of a soluble receptor for eNAMPT, analogous to those successfully used against VEGF (aflibercept; Ciombor and Berlin, 2014) and TNF-α (etanercept; Suffredini *et al.*, 1995), thereby sequestering eNAMPT in the extracellular space. However, such a strategy is, at present, crucially hampered by the fact that we do not know the structure of the receptor. The possibility to interfere with the acetylation/deacetylation of the protein, thereby reducing its release, is a possibility, although this approach would obviously lack specificity and therefore cannot be used to determine the potential of this target for pharmacological intervention. Nevertheless, assessment of the effects of deacetylase inhibitors, aimed at HDAC or SIRT, in cancer should also include the possibility that part of these effects may be mediated by the modulation of eNAMPT.

Last, a possibility would be to raise neutralizing antibodies against eNAMPT. Indeed, this strategy has already been reported in a different setting with very promising results (Hong *et al.*, 2008; Audrito *et al.*, 2015; Yoon *et al.*, 2015). Garcia and collaborators have developed a neutralizing polyclonal NAMPT antibody (Camp *et al.*, 2015; produced by Lampire Biological Laboratories Inc., Pipersville, PA) and have demonstrated its ability to protect against ventilator-induced lung injury (Hong *et al.*, 2008). Moreover, a humanized form of this antibody (P-BEFizumab) has been developed and is under preclinical investigation in acute lung injury (Hong *et al.*, 2008). The first report of the anti-tumour potential of the neutralizing polyclonal antibody has recently been presented by Audrito *et al.* (2015), who demonstrated that eNAMPT neutralization reversed M2-polarization of chronic lymphatic leukemia-derived monocytes. Based on these observations, the neutralization of eNAMPT seems to be a promising therapeutic strategy, and future investigations are warranted to determine the efficacy of this approach in the range of cancers, linked to elevated eNAMPT.

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Conflict of interest

The authors declare no conflicts of interest.

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