April 30, 2021

PharmacologyOnLine

Special issue • 2021 • vol.1 • 11-28

TUMOR RECEPTOR IMAGING: OVERVIEW AND CORRELATION WITH NETs

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Abstract

Nuclear medicine in NENs covers a pivotal role, both in diagnostics and in therapy, outlining the concept of theragnostic approach, bases on radiopharmaceuticals. The whole theme is based on the overexpression of somatostatin receptors (SSTRs), membrane molecule that allow the feasibility of this techniques, mediating the interaction between the drug and the targeted neoplastic cells, in order to depict it or to kill it, depending on the radiopharmaceutical's purpose: this molecular background will be the main focus of this paper, from native somatostatin and receptors to the concept of internalization. Today, the gold standard in nuclear medicine in this context is represented by 68Ga-DOTA-peptides in association with PET/CT, but other important techniques and radiotracers will also be presented. Finally, some clinical condition, in which these techniques are employed, will be presented, such as GEP-NETs, lung NETs, unknown primary NETs (CUP lesions) and metastases.

Keywords: *somatostatin receptor, neuroendocrine tumor, neuroendocrine neoplasia, peptides, DOTA-peptides, , 68Ga-DOTA- peptides, octreotide, OctreoScan®*

INTRODUCTION

Nuclear medicine (NM) is a relatively young branch of diagnostics, but since its birth it showed a rapid capability of progress, allowing better and better results often in relatively short period of time. This was the case of nuclear imaging applied to neuroendocrine neoplasms (also known as NENs or NETs, the latter mainly used to identify low-graded and well-differentiated tumors), especially thanks to NM's "functional nature": this kind of techniques focuses primarily on the pathophysiological aspects of the organism and diseases, enlightening information not otherwise possible to obtain with different procedures, in particular with traditional radiology. For this reason, the involvement and the interest in nuclear procedures became more and more intense in this field, allowing the results that today dictate the gold standard in diagnostics and, even, therapy (an approach called peptide receptor radionuclide therapy, or PRRT) of neuroendocrine tumors, made possible by the deep knowledge of these neoplasms, in particular from a biological and anatomopathological perspective. (1)

NENs are a particular group of tumors, originating from neuroendocrine cells dispersed in the entire organism, also known as part of the APUD system (Amine Precursor Uptake and Decarboxylation). They tend to arise mainly in the gastroenteropancreatic tract (the most common lesion is a pancreatic tumor, that can be functional or non-functional depending on its capability of determining an endocrine syndrome related to the overproduction of a particular type of tumor) and, secondarily, in the lung (especially in the form of borderline bronchial carcinoids, distinguished in typical and atypical ones, whereas the more famous lung microcytoma or small cell lung carcinoma or SCLC has a far more aggressive behavior), but there are also other sites than can host a NEN, let's think for example to thyroid (medullary thyroid carcinoma) or adrenal glands and paraganglia (pheochromocytoma and paraganglioma). (2)

Since these neoplasms have always represented a medical challenge, they have frequently been considered interesting by the scientific medical community, promoting several efforts and studies to allow better clinical results.

This kind of work has traced the path to an even more stimulating scenario, where the exact knowledge of receptor expression on the surface of neoplastic cells plays a determinant role in the success of two
fundamental steps in medical approach: diagnosis and therapy of NENs. Firstly, these discoveries have allowed the diagnosis and localization of primary and eventual metastatic (secondary) lesions, even the smaller ones, thanks to a different approach in comparison to traditional radiology: in this field, nuclear medicine plays a key role, because it has a completely different rationale in the way it studies a lesion, in fact it changed the amount and quality of information the investigation is able to provide, enlightening a functional, biological and pathophysiological account of the neoplasms themselves. Secondarily, the aforementioned "biomolecular profile" of the neoplastic cells plays a pivotal role also in therapy: the strategy adopted by nuclear physicians is to utilize particular drugs called "radiopharmaceutical", in other words a radionuclide attached to a peptide that is able to bind a membrane receptor (acting like its physiological ligand), whose composition is fine tuned in respect of the specific purpose of the medical act (i.e. diagnostic or therapeutic), because different radionuclides provide better performance when implied in a determined task or another one. (3)

In this paper, we are going to discuss the molecular basis of Somatostatin Receptors (or SSTRs) as a pivotal element of study that has allowed important progresses in the diagnosis and even treatment of NENs. We will start with the presentation of SSTRs, then we will dive in-depth into their subtypes and specific functions and, finally, how they have been intercalated into nuclear medicine techniques and clinical application.

SOMATOSTATIN RECEPTORS

INTRODUCTION AND OVERVIEW

One of the most studied receptors regarding NETs, and PNETs more precisely, is SSTR (Somatostatin Receptor).

During last decades, several studies have been performed studying SSTR and other membrane receptors, in searching of molecular targets that could be bound from a radiopharmaceutical. In this way, designing a specific peptide that is able to act as a ligand for a certain receptor, it is possible to label it with a diagnostic-related or a therapeutic-related radionuclide.
In other words, there are different radionuclides used in nuclear imaging, each one with peculiar properties: they can be α, β or γ-emitters and each one is indicated better for diagnostic or therapeutic purposes. This is the core of a theragnostic approach.
Let's go more deeply, but in synthesis: β^+ or γ-emitters radionuclides are aimed to diagnostic imaging, while β^- or α-emitters are employed in therapeutic strategies, i.e. PRRT (Peptide Receptor Radionuclides Therapy). (4)

SOMATOSTATIN

Somatostatin is a small peptide, which exists in two different active biological forms: one is constituted of 14 and the other of 28 amino acids. It is synthetized starting from prepro-somatostatin and pro-somatostatin, that represent its precursors.
Somatostatin is physiologically secreted by:

- endocrine D cells, located in glands in the antrum of stomach;
- neurons, in gastrointestinal tract;
- pancreas: one of the hormones produced by the endocrine component of this organ.

From a pharmacokinetic perspective, somatostatin has a half-life only of less than 3 minutes and the reason of its rapid degradation lies in suitable hydrolytic enzymes, located both in plasma and tissues, with the function to control its concentration levels. (5)

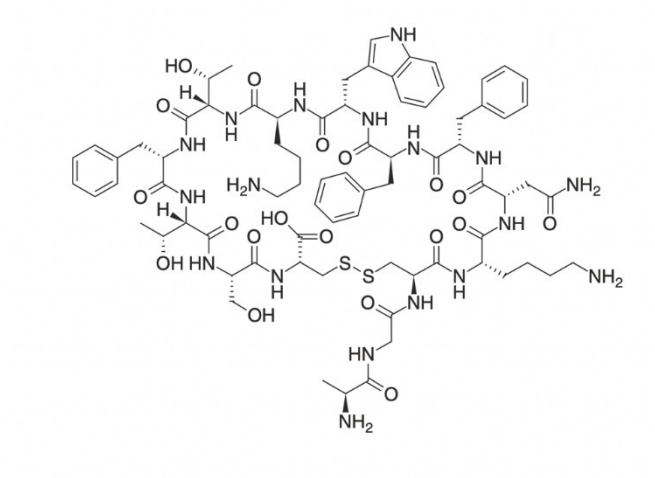

Figura 1 Somatostatin-14 chemical structure

SOMATOSTATIN RECEPTORS

Somatostatin can bind specific receptors, called SSTRs (i.e. SomatoSTatin Receptors). These receptors are expressed both in physiological and malady situations. As an example of the first category, several organs (such as anterior pituitary gland, pancreas, thyroid gland) and some cells display SSTRs, like normal neuroendocrine cells and immunological cells (such as activated lymphocytes, monocytes, epithelioid cells).
That is not all: further, some neoplasms, like NETs (NeuroEndocrine Tumors), can show these receptors on their membrane surface. (6)

SSTR are included in GPCR (G-Protein Coupled Receptor) category, a type of receptor that combines a 7-domains transmembrane protein and another protein bound to the intracellular portion of the receptor itself: when the physiological ligand binds the extracellular portion of the receptor, it changes its conformation, representing a signal for the downstream G protein, which results in an activation for the latter, starting an intracellular cascade of reactions that ends in a change of previous cellular function, metabolism or specific behavior. In the case of SSTRs, these receptors control crucial functions and, among them, even cellular proliferation. This is why these receptors are deeply involved in NETs overexpressing them, but this is also the reason of this rooted interest in diagnosis and therapy promoted by medical nuclear imaging. (7)

Let's have a quick focus on G proteins: these are a particular type of enzymes that function only during the time when they bind a molecule of GTP (Guanosine Tri-Phosphate): they have an intrinsic GTPase activity, which results (after a determined period of time) in the hydrolysis of GTP in GDP (Guanosine Di-Phosphate) and a phosphate group. When a G protein binds GDP, automatically it stops working. Only when the reinstatement of a GTP is operated, the new G protein-GTP complex restarts to function. This is an example of signal transduction in a cell. (8)

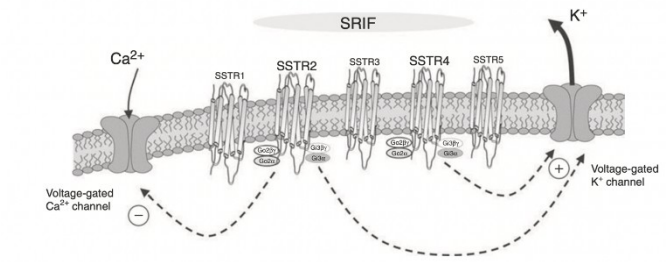

Figura 2 7-domain structure of a SSTR

SSTRs are currently classified into five different subtypes (SSTR-1 to SSTR-5). They are not equal to each other, this is why they explicate different functions in different cell types, depending on the specific subtype activated each time and/or the specific combination of different subtypes simultaneously activated. These variables can alter both the response derived from the ligand activation and also the internalization of the ligand-receptor complex, that occurs with the formation of an endosome (endocytosis process) including it. (9)

Focusing on NETs, these neoplasms tend to overexpress SSTRs, especially SSTR-2, the most frequently one observed: GEP-NETs, pheochromocytomas, paragangliomas, medullary thyroid carcinomas (MTC), SCLC (Small Cell Lung Carcinoma), pituitary adenomas, paragangliomas, but even breast cancer and malignant lymphomas (that are not neuroendocrine neoplasms), are all examples of tumors overexpressing this exact receptor. So, it was thought about a possible way to take advantage of SSTRs and other similar receptors, developing diagnostic and/or therapeutic approaches, and this is when theragnostic comes into the scene. (10)

RECEPTOR INTERNALIZATION AND DIMERIZATION

The pivotal event in the efficacy of diagnosis and (especially) therapy based on radiopharmaceuticals, is the cellular *internalization* of the drug-receptor complex: in this way, the radionuclide (that is the radiation-emitting component of the drug) can work properly, especially from a therapeutic perspective. The drug is able to damage the neoplastic cell only by entering it, since the accumulation of radionuclides inside the cell itself is necessary to provide the toxic effect.

From a diagnostic perspective, instead, the internalization process is relatively marginal, because the simple bond of the radiotracer to the targeted molecule is effective enough in order to report the location of the targets themselves, so the ligand affinity is far more important than the internalization process itself. (11)

But there is a but: regarding the internalization ability, not all receptors neither all drugs perform all the same way, providing different results basing on the specific receptors they are able to activate. To be clearer, it has been discovered that SSTR-2, SSTR-3 and SSTR-5 show the higher results in terms of diagnostic and therapeutic employ, in contrast to SSTR-1 and SSTR-4 that appear the weakest from this perspective.

This phenomenon is fundamentally depending by the internalization of the drug performed by the receptor itself. The better the internalization capacity, the better the diagnostic/therapeutic effect of the drug: it is as simple as that.

Obviously, this started to create interest in this topic, so more studies were conducted (even if nowadays there is still margin of improvement).

In particular, an important discovery was represented by the concept that SSTRs subtypes are able to combine themselves in order to realize both *homo-* and *hetero-dimerization*: this phenomenon has consequences on the response to ligand activation and ligand-receptor complex internalization. (12)

Further, other cell functions are regulated by this mechanism, such as agonist-dependent desensitization and internalization processes: concerning receptors in general, their expression is fine-tuned by the cell in response to several signals, like the rate of activation due to ligand-receptor

bonds. Every receptor is interspersed in a very complex molecular signal transduction that is crucial for the good functioning of the cell *in toto*, so when receptors hyper- or hypo-function inopportunely they create disturbance in metabolic and functional cell equilibrium.
As a reaction, a cell can hyper- or hypo-express a certain type of receptors in order to decrease or increase a certain molecular signaling: these phenomena are called respectively *up-regulation* and *down-regulation*. This is why, when SSAs are used (such as in therapy of PNETs), it can happen that the hyper-stimulation of SSTRs results in a down-regulation of the latter, so the effects of cold SSAs decrease because of this "desensitization". (13)

Hence, a new level of complexity was added. Following, some examples of this topic.
SSTR2/SSTR3 hetero-dimerization has been well demonstrated to be a subtype combination with important consequences in terms of desensitization, which appeared modified in comparison to their individual forms.
Another case is the SSTR1/SSTR5 hetero-dimerization, performing better internalization properties than receptor in themselves: the internalization of SSTR-1 is improved when co-expressed with SSTR-5. A little focus on SSTR-5: it has a C-tail that acts as a multifunctional domain, mediating both ligand binding, internalization and desensitization processes.
Recent studies focused on this topic and in particular on the relation between receptor subtype's ligand (SSAs, in this case) affinity and internalization capability: experts concluded that these two properties are not directly linked each other. For example, the specific combination of SSTR-2 and SSTR-5 subtypes make ^{90}Y-DOTA-NOC or ^{177}Lu-DOTA-BOC-ATE bonds possible, but the two subtypes receptors display different internalization profiles with a high rate one for SSTR2, but the internalization mediated by SSTR5 is absent.
There is more: there are different factors other than ligand binding implicated in receptor internalization. SSTR-2 and SSTR-3 are a good example, because when ligand bond takes place and activates them, they undergo to a phosphorylation operated by GPCR kinases: this event allows the recruitment of β-arrestins (i.e. proteins involved into the endosome formation), meaning the ligand-receptor-arrestin complex triggers the endocytosis process, resulting in the internalization of the latter; then, the receptor itself goes for two possible fates: *in primis* it is separated from its ligand, later the receptor can be either recycled (and re-exposed on the surface of cell membrane) or be degraded by lysosomes (in this way, it has to be newly synthetized). In this context, SSTR-2 and SSTR-3 internalization properties are influenced by the specific subtypes of GPCR kinases and β-arrestins involved in the process, but it is not excluded that even other SSTR subtypes can show similar phenomena. (14)

It was found also that SSTR subtypes can bind other completely different GPCR, such as μ-opioid receptors and D2 (a subtype of dopamine receptors): obviously, these combinations also affect internalization and desensitization properties. In particular, O'Toole et al., in a study involving 35 P-NETs patients, described the presence of SSTR-2 and D2 receptors in 100% of cases, while SSTR-5 was expressed in 89% of cases. This finding induced them to suggest future developments, like the design of new chimeric drugs able to bind, simultaneously, both somatostatin and dopamine receptors (SSTR-2/D2R) in order to influence hormone secretion, angiogenesis and cell proliferation, since these receptors are involved in above-mentioned processes.

SOMATOSTATIN ANALOGUES

Somatostatin Analogues (SSAs) are molecules that mimic native somatostatin. In other words, SSA can bind SSTRs and when this happens the latter reacts in the same way it would have done with native somatostatin, its physiological ligand.
As somatostatin does, also SSAs are able to act on secretion, proliferation and neurotransmission of neuroendocrine cells.
Because of its very short half-life (estimated to be between 1-3 minutes), native somatostatin is not suitable for medical imaging or therapeutic purposes. SSA, instead, benefit of longer half-life, an essential pharmacokinetic property that allows it to stay bound to SSTRs for a sufficient period of time that makes possible acquiring images through nuclear procedures.

SSTRs, as mentioned, are not only expressed by neuroendocrine neoplastic cells, but there are also other physiological sites that capture somatostatin, so they appear in imaging and it is important to avoid pitfalls. (15)
In this context, thanks to the capability of inflammatory cells of binding somatostatin and SSAs, these radiopharmaceuticals have found application also in other benign diseases, like thyroid associated ophthalmopathy, rheumatoid arthritis, idiopathic pulmonary fibrosis, sarcoidosis and histiocytosis, so also in all the disease where an inflammatory component is relevant, they could cover an important role. (16)

The first SSA developed was octreotide (also known with its commercial name Sandostatin®).
Octreotide was labelled to ^{111}In using DTPA (diethylene-triamine-pentaacetic acid, a bifunctional chelator), realizing ^{111}In-DTPA-octreotide (also known as ^{111}In-Pentetreotide or OctreoScan® or OCT): the drug was approved in 1994 by FDA in USA as a radiopharmaceutical indicated in imaging in somatostatin receptor-positive NENs.

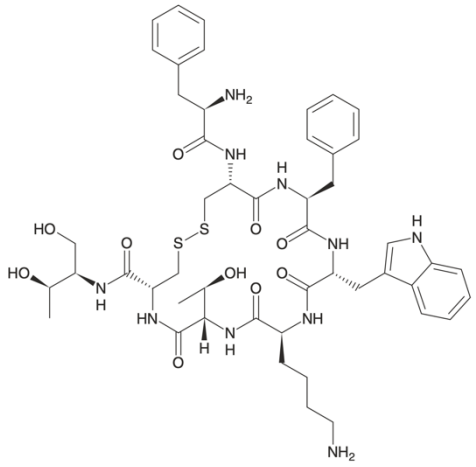

Figura 3 Chemical structure of octreotide, the first somatostatin analogue (SSA)

NUCLEAR MEDICINE TECHNQIEUS IN NENs

^{111}In-DTPA-OCTREOTIDE

^{111}In-DTPA-octreotide (also known as ^{111}In-pentetreotide or OctreoScan®, i.e. its commercial name) is a highly employed radiotracer since long time, in fact it was the first one to gain a large diffusion in clinical practice concerning NETs. The tracer has a high affinity especially for SSTR-2 and SSTR-5 (identifiable as its main targets), making it a good choice for the majority of NENs, especially P-NETs, the most frequent histotype, even if there is an important exception, that we will later discuss more in-depth: the insulinoma, a functional P-NET, because of its lack of somatostatin receptors' expression, making OctreoScan® useless in this scenario. (17)
^{111}In-DTPA-octreotide was firstly employed in SRS (Somatostatin Receptor Scintigraphy), a nuclear imaging technique still in use today: it can provide good imaging results if the TBR (Tumor/Background Ratio) is sufficient and with a lesion dimension of at least 1 cm, succeeding in detecting both primary and secondary lesions. When associated to SPECT/CT and employed with P-NETs, the tracer can reach even 90% and 80% of sensitivity, respectively, with secondary and primary lesions. (18)
From a pharmacokinetic perspective, the drug is administered intravenously, then it distributes to its physiological and, eventually, pathological uptake locations: spleen, liver, thyroid, kidneys, bladder and pituitary gland belong to the first category, while every other uptake site is suspicious. The excretion is mainly through the urinary tract, even if a small amount is eliminated via hepatobiliary tract, therefore it can be difficult the evaluation of abdominal lesion, since the abdomen itself will depict because of the radiotracer accumulation in the enteric lumen. (19) To solve this problem, the employment of SPECT or even SPECT/CT (that is a hybrid technique, improving significantly the quality and quantity of information) can provide better results, thanks to an early scan performed a short time after (i.e. 4 hours) radiotracer's administration, in this way a better TBR in tumor-specific and non-tumor-specific areas is obtained, allowing a higher sensibility and specificity outcome. Another scan is performed 24 hours after the intravenous injection and thanks to pharmacokinetic changes it is possible to better understand findings: areas where the TBR has increased suggest the presence of SSTR-2 expressing neoplasms, while areas where TBR decreased or remained stationary are considered as non-specific uptake sites. (20)

The real misunderstanding can arise with NENs not overexpressing SSTRs, like insulinoma: it is a functional pancreatic tumor, usually well-differentiated, that does not express SSTR-2 or SSTR-5, therefore depicting it with ^{111}In-DTPA-octreotide can be very difficult and not effective; in other cases, secondary neoplasm can undergo to some mutations, dedifferentiated in respect to the primary one, loosing SSTR overexpression (this is a typical situation of malignant transformance of the neoplasia). (21) These situations represent the so-called "escape phenomenon", caused by the lack of somatostatin receptors on the neoplastic cell's surface, making the tracer useless. Especially in insulinoma, the sensitivity of OctreoScan® reaches only maximum value of 50%, due to the small size of the lesion and its SSTR-2 low expression. As an alternative, great results are achieved with the employment of ^{18}F-DOPA PET/CT: its mechanism of actions lies in the catecholamine metabolism of the neoplasia, making it effective to depict the insulinoma. (22)

Current indications for ^{111}In-DTPA-octreotide SRS are staging and restaging P-NET patients and recruiting suitable subjects for PRRT or in non-oncologic diseases (because the latter have shown their accumulation capability of somatostatin-based radiopharmaceuticals thanks to the immunological cells' SSTR expression, reporting the presence of characteristic inflammatory areas of aforementioned maladies).

Obviously, ^{111}In-DTPA-octreotide SRS shows some downsides, mainly related to the low spatial resolution of the techniques, which necessitates of a TBR of at least 2:1, in addition to an unsatisfactory capability in quantifying receptor density; regarding ^{111}In, it is a radionuclide obtained by a cyclotron, so its production process is both pricey and Industries-dependent, in addition to high and multiple energy peaks, requiring high dosimetry; finally, the procedure requires multiple scans, acquired (as aforementioned) 4 hours and 24 hours after the tracer injection, implying a prolonged time of assessment of the patient, ending in costs and discomfort for the latter. (23)

Researcher's efforts and the increased interest in NENs imaging has led to great progresses in this field, especially with the introduction of ^{68}Ga-DOTA-peptide associated with PET/CT (*in primis*) and ^{99M}Tc-EDDA/HYNIC-TOC SPECT (more recently). (24)

^{99M}Tc-EDDA/HYNIC-TOC

^{99M}Tc (Tecnetium-99M) is the current most used radionuclide in worldwide NM. It is a product of nuclear generators, letting it to be cheap and easily available. From a physical perspective, it has a 6-hour half-life emitting γ-rays (141 keV of single peak energy). Additionally, it is easily labelable to other molecules, so the production process of radiopharmaceuticals is made easy thanks to the presence of several kits on the market, allowing to do this operation onsite for most radiotracers; finally, nuclear machineries are also calibrated in order to obtain the best imaging results from the employment of ^{99M}Tc in procedures.

Nowadays, ^{99M}Tc is usually bound to TOC (an SSA). As aforementioned, a radiopharmaceutical is typically constituted by three main parts: a radionuclide (^{99M}Tc), a binding molecule (in this case the peptide is a somatostatin analogue, TOC, an abbreviation of "Tyr3-octreotide") and, finally, a BFC (BiFunctional Chelator). The path to the synthesis of ^{99M}Tc-EDDA/HYNIC-TOC required several attempts, especially in studying the BFCs themselves. From several experiments, it came out HYNIC (6-hydrazinonicotinamide) provided the best outcomes, resulting in the production of stable complexes together with ^{99M}Tc and TOC. In the first phase, with the purpose of perfecting the compound, tricine [also known as N-(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine] was chosen to be the co-ligand of ^{99M}Tc-HYNIC, but chromatography analysis reported unsatisfactory data, especially because of the significant changes in compound's stability and the high number of isomers derived from the production process. Later, the co-ligand was substituted with another one: EDDA (Ethylenediamine-N,N'-Diacetic Acid), that improved also the compound's pharmacokinetics, because changing the structure of a radiopharmaceutical implies also the alteration of other parameters, like its lipophilicity and biodistribution: in this case, the substation of tricine with EDDA allowed the synthesis of stabler complexes and a lower number of isomers. In this way, ^{99M}Tc-EDDA/HYNIC-TOC (also named ^{99M}Tc-Tektrotyd®) gained more and more

consensus, to the point that today it is highly employed as tracer in somatostatin-based SPECT imaging in NETs' context in a large number of countries (especially in Europe). If we can compare it to OctreoScan®, ^{99M}Tc-EDDA/HYNIC-TOC is able to provide better results both in sensitivity and image quality, in addition to a lower exposure to ionizing radiations, all these resulting in a big progress in neuroendocrine neoplasms' imaging. (25)

SSAs AND ^{68}Ga-DOTA-PEPTIDES: THE GOLD STANDARD IN NET DIAGNOSIS

Today, ^{68}Ga-DOTA-peptides PET/CT represents the gold standard in nuclear diagnostics for well-differentiated neuroendocrine neoplasms, especially of the gastroenteropancreatic tract. (26) This technique is based has a fundamental premise: it can work only if there is SSTRs' overexpression on NET's membrane surface, especially SSTR-2 and SSTR-5 subtypes, since they are the most frequent ones; additionally, radiotracers based on somatostatin show the highest affinity to these receptors, representing an exceptional instrument in diagnostics applied to neuroendocrine tumors. (27)

Let's analyze more in-depth this category of radiopharmaceuticals, starting from its structure and single components.

^{68}Ga (Gallium-68) is a β^+ emitting radionuclide, deriving from ^{68}Ge (a generator's product, making it very affordable and cost-effective), with a half-life of 68 minutes. DOTA (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid) is a bifunctional chelator (BFC), capable of mediating the connection between ^{68}Ga and an SSA. The latter can vary from a radiopharmaceutical to another, representing the molecule binding the targeted receptor (SSTR). Several SSAs have been studied during time, but today the most used ones are **NOC** (1-Nal3-octreotide), **TOC** (Tyr3-octreotide) and **TATE** (Tyr3-octreotate). (28) They are not equal to each other and every compound has specific advantages in terms of receptor subtype affinity: DOTA-NOC has a high affinity to SSTR-2, SSTR-3 and SSTR-5, displaying the widest affinity in comparison to the other two peptides; DOTA-TOC has the highest affinity to SSTR-5, together with a good SSTR-2 one; finally, DOTA-TATE is the compound with a tenfold binding affinity towards SSTR-2, if compared with all other DOTA-peptides. (29)

As aforementioned, the pivotal assumption is the presence of SSTRs on neoplastic cells' surface, but sometimes this condition is not satisfied: this is the case of the so-called "escape phenomenon", as previously described. (30) In these situations, a better outcome in terms of sensibility and accuracy in primary and secondary lesions detection is provided by ^{18}F-FDG PET/CT, reaching far better results in insulinoma or in malignant NENs in comparison to techniques based on somatostatin receptors' expression: this is promoted by the increased metabolic activity and glucose uptake shown by these lesions, letting the accumulation of radiomarked glucose into the targeted cells and depicting them with the machinery. (31) In synthesis, this is the reason why they represent the gold standard procedure only in well-differentiated NENs (with proven expression of SSTRs). (32)

^{68}Ga-DOTA-peptides PET/CT has also another important role: when a patient is investigated with a lesion suspected to be a NEN, the evidence of SSTRs' expression represents also an important prognostic factor, not only refereeing to the plausible low grade and benign behavior of the neoplasm, but also because if DOTA-peptides can bind it, it also possible to employ cold SSAs and PRRT in therapeutic approach, because the lack of somatostatin receptor would have a huge negative impact also in the adoption of these treatments. (33-35)

SOMATOSTATIN ANTAGONISTs

Somatostatin receptors' overexpression is, as established, a pivotal premise to allow somatostatin-based radiopharmaceuticals correctly functioning. Later, researchers succeeded in developing drugs with an intrinsic agonist activity, that resulted in SSAs and, especially, ^{68}Ga-DOTA-peptides. As its name suggests, these radiopharmaceuticals are able to mimic somatostatin physiological effects once the bond to the SSTR occurs, inducing the ligand-receptor complex internalization and making possible the intracytoplasmic collection of the drug and, therefore, radiations: we already discussed the importance of this event, both for diagnostics and

even more for the radionuclide therapeutic approach (PRRT). But is it possible to use other types of ligand molecules, perhaps an antagonist molecule in respect to somatostatin? However, recent studies demonstrated the even higher efficiency of SS-ANTs (SomatoStatin ANTagonists), displaying their higher capability both in binding receptors and being internalized by targeted cells.

Ginji et al., in a preclinical study going back to 2005, showed how SS-ANTs could not only bind a larger variety of SSTRs, but also their higher internalization capability, if compared to SSAs, independently of their internalization properties (the study was led *in vivo* using animal neoplastic cells). The higher receptor binding capability of SS-ANTs (in respect to SSAs) lies into their ability to recognize more binding sites in comparison to somatostatin agonists, even when the neoplastic cell shows a lower SSTR density on its membrane surface. (36)

In addition to the aforementioned preclinical considerations, the first consecutive clinical study provided great results that confirmed what previously enlightened.

It was studied the feasibility of somatostatin receptor scintigraphy (SRS) in association to SS-ANTs. Results confirmed a higher TBR due to a more intense ^{111}In-DOTA-SSTR2-ANT uptake [the SS-ANT chosen for this assessment is also known as p-NO2-Phe-cyclo(D-Cys-Tyr-D-Trp-Lys-Thr-Cys)D-Tyr-NH2] by the tumor, then compared to ^{111}In-DTPA-octreotide outcomes: the paragon was firmly favorable to the antagonist radiotracer. Further evaluations confirmed data, leading to the development of β^+ emitting radiopharmaceuticals based on somatostatin antagonists (using ^{68}Ga and ^{64}Cu as labelled radionuclides), employing them with PET/CT imaging. (37)

Other molecules have been developed, leading to ^{68}Ga-OPS202 and, subsequently, ^{177}Lu-OPS202, that are, respectively, β^+ and β^- emitting radionuclides labelled to a somatostatin antagonist, acting as a diagnostic radiotracer (the first one), and then as a receptor-based therapeutic radiopharmaceutical (the second one). Both of them provided better results when compared to other already employed radiotracers: as an example, ^{68}Ga-OPS202 shows far better outcomes in comparison with ^{68}Ga-DOTA-TOC, being capable of increased depict activity in respect of liver metastases. But that is not all: OPS202 has demonstrated also a better efficiency from a PRRT perspective, because its higher internalization properties make it better for intracellular accumulation of radiations, emphasizing its toxic activity against targeted neoplastic cells. A preclinical study compared ^{177}Lu-OPS202 with ^{177}Lu-DOTA-TATE, showing the superiority of the first one when analyzing tumor uptake and residence time: simply, the antagonist allowed a better intracellular radiation retention, making the PRRT with this drug far more efficient.

Cuccurullo et al. suggested it is not needed to interrupt SSA therapy (intended as cold SSAs administration, that provides benefits controlling tumor growth and clinical manifestations of the neoplasm): this consideration derives from the observation of a wider receptor binding range of antagonists, not being influenced significantly by cold SSAs administration, because it was plausible to hypnotize a binding interference to the same targets, i.e. SSTRs on neoplastic cells' membrane. (38)

However, although SS-ANTs represent a solid and promising future alternative in NEN's imaging (potentially substituting agonists), further studies are needed to exactly outline their pharmacokinetics, especially tracers' uptake and their distribution. (39)

CLINICAL APPLICATION OF TECHNIQUES

A. GEP-NETs

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are the most frequently diagnosed phenotype among NETs (60-65%)., immediately followed by lung NETs.

Originating from neuroendocrine cells, these neoplasms are quite rare if overall compared to other oncologic diseases. They tend to be well-differentiated tumors, with a low grading and mitotic index <3%, but there are several exceptional cases presenting as high-graded disease, with a more aggressive behavior.

GEP-NETs are also distinguished in functional and non-functional, depending on their capability of hormone secretion and if this condition is able to determine an endocrine syndrome clinically evaluable. The most frequent functional NET is the insulinoma,

immediately followed by the gastrinoma (responsible of the Zollinger-Ellison syndrome as a clinical manifestation), but overall GEP-NETs arise as asymptomatic, slow-growing neoplasms: this is the main reason why they are usually diagnosed only in an advanced state, together with compression and infiltrating symptoms, typically defining a poor prognosis. (40)

A multidisciplinary approach combining morphologic and functional imaging modalities is important for accurate staging and treatment. Contrast-enhanced CT and MRI provide detailed, anatomic information on the primary-tumor location and identify regional and distant metastases—information that is needed for optimal surgical intervention, treatment selection, and identification of persistent or recurrent disease (41-45)

Nuclear medicine and molecular imaging are playing an increasingly important role in patient care, medical research, and pharmaceutical development. Today, nuclear and molecular diagnostic imaging studies are available for virtually every major organ system in the body. (46-49) The number of nuclear medicine–based therapies for cancer and other disorders is also expanding. (50) Nuclear medicine and molecular imaging are integral to the care of patients with cancer, heart disease and brain disorders. (51-53)
Nuclear imaging based on SSAs has found its really major application with GEP-NETs, since they are the most frequent and most studied neuroendocrine tumors. (54)
Overall, GEP-NETs are usually well-differentiated tumors, that is to say they tend to overexpress SSTRs, especially SSTR-2 and SSTR-5 subtypes: as aforementioned, these receptor clones are the ones most targeted by SSAs. (55)

Although nuclear imaging of gastroenteric NETs started with SRS (OctreoScan® associated to scintigraphy), the introduction of ^{68}Ga-DOTA-peptides represented a real revolution in this field, as several studies concluded, when they began comparing radiotracers in order to deep analyze their clinical performance.
In 2001, Hofmann et al. (56) compared SRS with OctreoScan® (^{111}In-Penteteotride) with ^{68}Ga-DOTA-TOC PET in a group of 8 patients that already received a carcinoid tumor diagnosis, so they were already known to be affected by the neoplasm: in conclusion, the overall superiority of the technique with DOTA-peptide was largely demonstrated, which succeeded in recognize 100% of the lesions versus only 85% reported by SRS with OctreoScan®. This was not the only example, of course: other groups of study reported absolutely similar outcomes, exploring the capabilities of ^{68}Ga-DOTA-TOC PET compared to scintigraphy and SPECT, with the additional advantage of a low renal accumulation, since one of the major downsides of previous radiotracers was the risk of renal toxicity.

We already discussed about the differences among ^{68}Ga-DOTA-peptides themselves and the possibilities offered by several β^+ emitting radionuclides, in the previous paragraph.
To remark the concept, here there are some examples of studies that reported these results.
In 2013, Wild et al. (57) put in comparison ^{68}Ga-DOTA-TATE and ^{68}Ga-DOTA-NOC associated to PET/CT in a group of 18 patients with previously known (confirmed by histopathological examinations) gastroenteropancreatic NETs. DOTA-NOC obtained better results (detecting of 93.5% vs 85.5% of the lesions) in respect to DOTA-TATE, proving that the first was more accurate: in fact, as aforementioned, the first one is capable of binding a wider variety of SSTR subtypes (SSTR-2, SSTR-3, SSTR-5), in particular it was a better performer in detecting liver metastases.

B. INSULINOMA: AN EXCEPTION

Among functional PNETs, insulinoma it the most frequent one. This tumor can arise with a benign (as it happens in the majority of cases) or a malignant behavior, with important implications in biological terms, especially regarding the receptor overexpression on its cell surface, although their frequent benign nature: in fact,

the peculiar aspect of these pancreatic tumors is the lack of SSTRs' overexpression, more precisely they show a low density of SSTR-2, the SSAs' main targeted receptor subtype. This is why they are "an exception" in pancreatic NENs' context. (58)
We already discussed the pivotal role of somatostatin-based radiotracers in NEN's nuclear imaging, but this time the biological premises are not well respected in order to consider these drugs as efficient as they are with other neuroendocrine tumors.

Insulinomas can be considered an optimal example of the so-called "escape phenomenon", as previously explained, meaning they do not properly overexpress SSTRs: in this way, they resemble "escaping" from the drug identification, providing false negative results to the investigations, negatively influencing the prognosis. (59) Given that, DOTA-peptides with PET/CT do not represent the best choice possible in this context and this is the reason that led to the development of other and more appropriate radiotracers. For this reason, new molecules were developed. (60)

Let's start with GLP-1R-based imaging: in well differentiated insulinomas, it was enlightened the overexpression of glucagon-like peptide-1 receptor (GLP-1R), meaning that the use of glucagon-like molecules (mimicking this ligand) could provide a biochemical bond and acting, so, as radiopharmaceuticals. With this premise in mind, new drugs were synthetized, all of them based on **exendin-4** and **SPECT/CT** scans: [Lys40(Ahx-DOTA-^{111}In)NH2] exendin-4, [Lys40(Ahx-DTPA-^{111}In)NH2] exendin-4 and [Lys40(Ahx-HYNIC-^{99m}Tc/EDDA)NH2] exendin-4. These compounds reported excellent results, because of their very higher sensitivity and non-invasive capability in detecting benign insulinoma in comparison to other techniques. Later, other radiopharmaceuticals based on exendin-4 were proposed, allowing the development of even better performing radiotracers: **^{68}Ga-DOTA-exendin-4 PET/CT** represents the greatest procedure in this context, soon defined as the best choice for well-differentiated insulinomas, especially thanks to the higher spatial resolution and image quality of this technique, considering that it shows also a better dosimetry and quantification possibility; even compared to ^{111}In-DOTA-exendin-4 SPECT/CT, the former radiotracer shows better outcomes. (61)

The excellent results reached by exendin-4 compounds are not replicable in malignant insulinomas, due to their GLP-1R overexpression loss, providing mostly false negative results and making these radiotracers useless. In this case, it is needed to focus on another aspect of this type of neoplasia: the typical increased glycolytic activity of aggressive and malignant tumors. For this reason, 18F-FDG PET/CT (the most used nuclear imaging technique in oncologic field) represents a better choice, together with ^{68}Ga-DOTA-peptides PET/CT, even if it is important to remark that the first procedure provides better results (higher sensitivity) than the latter, in this condition. (62)

C. **LUNG NETs**

Lungs represent the second most frequently diagnosed neuroendocrine tumor localization (about 30%), as a primary lesion, mainly including white female patients.

Lung NETs can arise in two main forms: a benign phenotype, known as bronchial carcinoid, distinguished in typical (TC) and atypical (AC), and, on the other hand, LCNEC (Large Cell NeuroEndocrine Cancer) and SCLC (i.e. Small Cell Lung Cancer, also known as lung microcytoma) defining a malignant phenotype, usually associated to a bad prognosis because of their aggressive behavior and tendency to early produce metastatic lesions, especially micro-metastases in the context of the brain. (63) From a therapeutic perspective, carcinoids are usually treatable with a surgical approach, providing good results in terms of prognosis; instead, microcytomas tend to provide a good response at the first treatment attempt, showing a significant involution of the clinical picture, then they arise as chemoresistant

neoplasms not responding to the previous pharmaceutical strategy, deeply worsening the survival rate. (64)

From a molecular and anatomopathological study leaded by Reubi and Weser, they enlightened that lung NETs are mostly bronchial carcinoids overexpressing SSTR-1 and SSTR-2 subtypes (70% of cases), while only in about 20% of cases they display the second most frequently SSTR clone, i.e. SSTR-5. This finding implicates the better indication of ^{68}Ga-DOTA-TATE over other DOTA-peptides, since it has the higher specificity and binding affinity for SSTR-2. (65)

Kayani et al., in 2009 (66) compared the latter with ^{18}F-FDG in a group of 18 patients affected by lung NETs (already diagnosed), with the purpose of correlate histological tumor grade and tracer uptake. The overall better performance has been DOTA-peptide's one (sensitivity 72%), but they made a more in-depth comparison: the authors examined typical carcinoids, observing how ^{68}Ga-DOTA-TATE showed its superiority when compared to ^{18}F-FDG, whose uptake was either negative or low; regarding high-graded tumors or atypical carcinoids, on the other hand, ^{18}F-FDG overperformed ^{68}Ga-DOTA-TATE by far, because of the increased metabolic activity (especially glycolytic pathway) of these more aggressive neoplasms; finally, in diffuse idiopathic pulmonary neuroendocrine cell hyperplasia, no one of the tracer gained an instrumentally significant uptake.

In 2015, Ambrosini et al. (67) started a new study in a group of 11 patients, including ten cases of pathologically proven lung NETs (well-differentiated bronchial carcinoids) and one case of highly suspicious CT scan-based lesion. Employing ^{68}Ga-DOTA-NOC, nine patients (with well differentiated and typical carcinoids) resulted positive to the investigation, detecting at least one lesion: in three out of nine patients, there was also a clinical management change thanks to the detection of unknown metastases.

So, we can conclude that well differentiated typical carcinoids are able to uptake DOTA-NOC in an appropriate way, thanks to the specific overexpression of somatostatin receptors (especially SSTR-1 and SSTR-2), while high grading is a characteristic that allows a stronger indication for ^{18}F-FDG.

D. **UKNOWN PRIMARY LESIONS**

From a clinical perspective, usually NET patients show metastases at first diagnosis, especially localized in the liver, but the primary lesion is unknown: these cases are called "CUP-NETs" (Carcinoma of Unknown Primary Neuroendocrine Tumors).

This condition is associated to a 10-years survival rate of 22%, meaning that the prognosis is not that good.

Finding and resecting the primary tumor is an important and difficult challenge, with a significant improvement of disease-free and overall survival. In addition, some changes in therapeutic strategies may occur when the localization and histopathology of the primary tumor is discovered. (68)

Several studies regarding the application of DOTA-peptides in CUP-NETs have shown some results, even if not reaching an excellent level of sensitivity, although they displayed far better performance in respect to other techniques.

Prasad et al., in 2010, compared the results obtained from ^{68}Ga-DOTA-NOC and CT scans alone studying of a group of 59 patients with CUP-NETs: respectively, they reported a sensibility of 59% (35 patients out of 59) from DOTA-NOC and 20% (12 patients out of 59) from the tomographic technique, an overall better performance even compared with OctreoScan® (that did not exceed a sensitivity of 39% according to studies from medical literature). (69)

In 2011, Naswa et al. led a new study with 20 patients, affected by CUP-NETs, regarding ^{68}Ga-DOTA-NOC, providing promising results because of a more accurate detection of metastases: not only the tracer was able to find additional metastases in 8 patients and further secondary tumors mostly in lymph nodes and bones (inducing a change in clinical management in 15% of cases undergone to surgery), but it also

allowed the localization of primary lesions in 60% of cases (12 patients out of 20), also reporting an important correlation in respect of SUV_{max} values of both the primary and metastatic tumors. (70)
Lapińska et al., also in 2011, studied a group of 14 patients with CUP-NETs (presenting metastases localized in liver and lung) extrapolated by a larger population of 97 patients with NET (already confirmed with a histopathological examination or highly suspected): using ^{68}Ga-DOTA-TATE, they concluded that in 3 patients out of 14 the PET/CT procedure was negative, a result successively confirmed by a negative histopathological examination, not confirming the preliminary diagnosis; on the other hand, in 5 patients out of 14 they were able to detect the primary tumor (45.5% of cases). (71)

E. **METASTASES**
Metastases of NET tumors are usually localized in liver, lymph nodes, peritoneum, lung and bone, representing the most frequent first clinical presentation of the disease (defining a CUP-NET case). (72) As studies report, nowadays ^{68}Ga-DOTA-peptide PET/CT represents a pivotal instrument in clinical approach to the disease, because it acts as a primary tool in the identification of primary and secondary lesions, showing far better results if compared to CT alone or SRS, providing also important information about the functional and molecular profile of the neoplasm, beyond allowing an earlier identification of those lesions that are too small to be detected by traditional radiology procedures. (73)

Bone metastasis occurs in 4-30% of cases of NETs and is related to the histology of the neoplasm: from a diagnostical perspective, there is discordance regarding the best sensitivity rate between SRS and bone scintigraphy in detecting skeletal lesions; on the other hand, a higher positive predicting value is provided by OctreoScan®. Also, using SRS to differentiate bone metastasis and soft tissue lesions can be very difficult, because of the low detail of this type of image. (74)

Other studies have enlightened a correlation between high levels of 5-HIAA (5-hydroxyindoleacetic acid) and CgA (Chromogranin A, a NETs' biomarker) with a positive result to Somatostatin Receptor Scintigraphy (SRS).
Given the importance of functional, molecular and biological definition of the neoplasm in respect to the prognosis, the choice of the right diagnostic procedure is pivotal, in order to correctly study and staging the patient with the purpose of pursuing the best therapeutic approach, especially when facing liver metastasis (that, as aforementioned, can have a significant impact on the overall prognosis), thanks to several alternatives (from surgery to radionuclide therapy and liver transplant) that today are available. (75)

^{68}Ga-DOTA-TATE and ^{68}Ga-DOTA-NOC were compared by Wild et al. in 2013 (57) , studying a group of 18 patients with histologically proven GEP-NETs, respectively with a sensitivity in detecting lesions of 85.5% and 93.5% (P = 0.005), with a particularly brilliant performance of DOTA-NOC in depicting liver metastases. Tumor differentiation grade did not implicate significant differences.
Depending on the site of the metastases, some differences were shown comparing several SSA-based radiopharmaceuticals, because of the specific physiological uptake of the latter: DOTA-NOC is more sensitive to liver metastases than DOTA-TATE (in addition of a tumor-to-background ratio, or "TBR", of 2.7 and 2.0, respectively) as aforementioned; on the other side, in bone metastasis detection ^{68}Ga-DOTA-TATE showed its superiority in comparison with ^{68}Ga-DOTA-NOC, depending by a significantly higher tumor-to-background ratio due to a lower bone marrow activity.
Putzer et al., in 2009, (76) investigated 51 patients (with already histologically diagnosed NETs) with ^{68}Ga-DOTA-TOC and conventional bone scan (CT and bone scintigraphy), since the latter were the reference and standard procedures. In conclusion, DOTA-TOC provided more accurate results than both the other two conventional techniques, given that its

sensitivity of 97% and specificity of 92% were far better values compared to them, with these test results: 37 true positive, 1 false positive, 12 true negative, and 1 false negative patient.

CONCLUSIONS

Nuclear medicine provides an important tool for an integrative approach to medical radiation science. Th e synergies between nuclear medicine and both radiography and radiation therapy are today part of current best practice.

SSTRs and SSAs, with particular emphasis on DOTA-peptides, together with the development and progressive application of nuclear medicine techniques, have represented a successful combination of knowledge and procedures with the purpose of reaching new goals in respect of neuroendocrine tumors, allowing a significant improvement both in diagnostic and in therapeutic scenario. This has deeply changed the survival rate of patients, showing also the potential of this branch of diagnostics, also capable of completely change the therapeutic strategy firstly thought for a certain patient and then completely rethought thanks to the new information provided by these investigations. (77)

The Authors hope that at the end of this journey into SSTRs and NETs the renovated interest in this field was satisfactorily shown, because more and more progress have been and will be made, thanks to the efforts of the research and the power of science.

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