

Review

Prospective of ^{68}Ga -Radiopharmaceutical Development

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Abstract

Positron Emission Tomography (PET) experienced accelerated development and has become an established method for medical research and clinical routine diagnostics on patient individualized basis. Development and availability of new radiopharmaceuticals specific for particular diseases is one of the driving forces of the expansion of clinical PET. The future development of the ^{68}Ga -radiopharmaceuticals must be put in the context of several aspects such as role of PET in nuclear medicine, unmet medical needs, identification of new biomarkers, targets and corresponding ligands, production and availability of ^{68}Ga , automation of the radiopharmaceutical production, progress of positron emission tomography technologies and image analysis methodologies for improved quantitation accuracy, PET radiopharmaceutical regulations as well as advances in radiopharmaceutical chemistry. The review presents the prospects of the ^{68}Ga -based radiopharmaceutical development on the basis of the current status of these aspects as well as wide range and variety of imaging agents.

Key words: positron emission tomography, ^{68}Ga , radiochemistry, coordination chemistry, conjugation chemistry.

Introduction

The number of publications devoted to ^{68}Ga -radiopharmaceutical basic and clinical research has increased drastically during last two years. Rough estimation demonstrates that the number of ^{68}Ga -related scientific articles published during 2011-2012 stands for over 45% of all publications since 1956 (Figure 1). Rather crucial changes and progress occurred during this short period and they influenced the basic perceptions and thus speculation about the nearest 5-10 year future of the field. Nuclear medicine applications and in particular positron emission tomography (PET) have experienced accelerated development.

The major advantage of PET is that it is not only enables in vivo visualization of physiological processes on molecular level in real time, but it also

quantifies them by measuring regional concentration of the radiation source. PET employs imaging agents comprising positron-emitting radionuclides (Figure 2A), and scanners detecting radiation (Figure 2B). Positron scan registration is based on the $180\pm 0.25^\circ$ correlation of the 511 keV photons arising from the annihilation of positrons with electrons and detection by means of two opposing counters recording only coincident events [1]. The registered events are reconstructed into images representing spatial distribution of the radioactivity in a subject. Positron emission is an attribute of neutron deficient nuclides requiring artificial production generally by cyclotrons. However ^{68}Ga is obtained from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator system which is simple in use and relatively inexpensive.

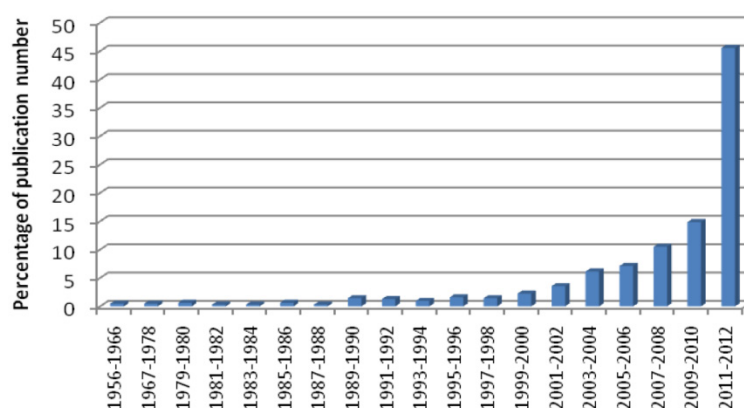


Fig 1. The fraction (%) of ^{68}Ga -related publications per two successive years (except for the periods of 1956-1966 and 1967-1978) appeared from 1956 through the end of 2012. PubMed, Scopus, SciFinder Scholar, Web of Science, and Beilstein databases as well as references from published articles were used for the literature search and assay.

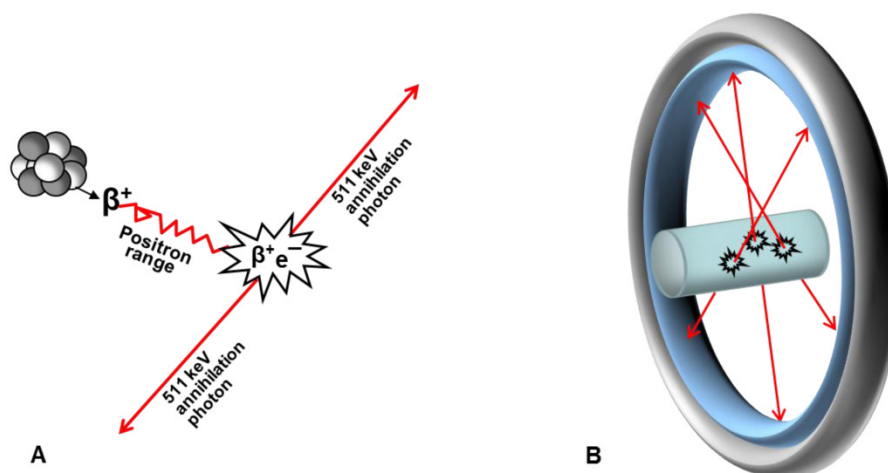


Fig 2. Schematic representation of PET principle. **(A)** After travelling in tissue (positron range) positron loses energy and annihilates with electron resulting in two 511 keV annihilation photons travelling in opposite directions; **(B)** Annihilation photons are registered externally by radiodetectors consisting of scintillation crystals and photomultiplier tubes and assembled in a ring. Only photons that are registered in coincidence are used for image reconstruction.

PET has become an established method for medical research and clinical routine diagnostics. Development and availability of new radiopharmaceuticals specific for particular diseases is one of the driving forces of the expansion of clinical nuclear medicine providing early personalized diagnosis and efficient therapy. The future development of ^{68}Ga -radiopharmaceuticals must be put in the context of several aspects such as role of PET in nuclear medicine, unmet medical needs, identification of new biomarkers, targets and corresponding ligands, production and availability of ^{68}Ga , automation of the radiopharmaceutical production, progress of PET technologies and image analysis methodologies for improved quantitation accuracy, PET radiopharmaceutical regulations as well as radiopharmaceutical chemistry advances. This review presents the prospects of the ^{68}Ga -based radiopharmaceutical development on the basis of the current status of these aspects. ^{68}Ga has demonstrated its applicability for the labelling of small compounds, biological macromol-

ecules as well as nano- and micro-particles promoting the growth of PET field [2]. The major application domain is oncology; however, potential has been demonstrated for imaging of myocardial perfusion, pulmonary perfusion and ventilation as well as inflammation and infection. Imaging of general biologic properties and processes such as proliferation, apoptosis, hypoxia, glycolysis, and angiogenesis have also been investigated. These prerequisites may trigger an explosive progress and introduction of new ^{68}Ga -radiopharmaceuticals into clinics in the nearest future of 5-10 years.

The focus of this critical review resides on the publications from year 2008-2012 reflecting latest achievements as well as selected earlier references that give the background and support the foundation for the future development of basic research and clinical applications. The achievements in gallium radiopharmaceutical chemistry and imaging agent development from 1956 through January 2011 were addressed in a comprehensive and exhaustive review

published earlier [2] and references therein. The explosive growth of publications reflecting the success of ^{68}Ga applications is remarkable. Another strong indication of the worldwide growth of ^{68}Ga studies is the "First World Congress on Ga-68 and peptide receptor radionuclide therapy (PRRT)" that took place in 2011 and attracted participants from all continents [3].

Role of PET in nuclear medicine

Nuclear medicine is a quickly expanding field for the diagnostics and therapy on cellular and molecular level. Molecular imaging techniques such as PET and Single Photon Emission Computed Tomography (SPECT) utilize, respectively, positron and gamma emitting radionuclides for the generation of the signal that results in a whole-body scan in a single examination. They provide fast and non-invasive evaluation of physiology and pathology, and together with external and internal radiotherapy merge into theranostics resulting in personalized medicine [4-7]. Imaging diagnostics enables early detection, staging, therapy selection, planning, and monitoring treatment response thus considerably improving cancer therapy. The most pronounced example is the selection of oncological patients for PRRT.

Radionuclidic properties and detection techniques determine the advantages of PET over SPECT in terms of 100-1000 fold higher sensitivity, higher speed, accurate quantitation, and dynamic image reconstruction. However gamma and SPECT scanners are more accessible and have lower cost. Moreover, a wider range of registered gamma emitting radiopharmaceuticals is available, though their price has enhanced currently due to the reduction of public subsidy [8]. On the other hand, the shorter scanning time and thus higher throughput of PET decreases the cost per patient examination. Nuclear medicine practice is dominated by $^{99\text{m}}\text{Tc}$ /SPECT standing for 85% of all clinical examinations in the USA with 50% in cardiology [9]. Dual-tracer imaging is one more advantage of SPECT, however recent research has demonstrated possibility of multiple PET tracer imaging based on the signal separation according to the differences in radionuclide half-life as well as tracer kinetics and distribution [1].

Although PET was introduced as early as in the 1970s, only recently it has been recognized clinically relevant, to some extent due to the introduction of hybrid PET-CT scanners that are spreading worldwide with acceleration and have become the gold standard of PET imaging. Rather radical question has been raised if there is a role in oncology for SPECT considering current advantages of PET and respective radiopharmaceuticals [8]. The authors suggest that

PET provides more accurate and quantitative diagnosis enabling individualized cancer therapy planning resulting in the efficient and cost saving treatment. The propagation of PET technique is greatly stimulated by the widespread use of [^{18}F]-fluorodeoxyglucose ([^{18}F]FDG/PET-CT) for many indications in oncology as well as centralized production and distribution of [^{18}F]FDG to satellite clinical centres. Another factor influencing the expansion of PET is the need for imaging agents with disease specific action, and ^{68}Ga has a significant contribution to make. The reports on such category of radiopharmaceuticals from molecular imaging and contrast agent database (MICAD) state that PET and SPECT imaging agents stand respectively for 42% and 31% [10]. However, PET still has to overcome the major hurdles such as regulatory barriers for introducing new imaging agents and restriction of reimbursement. The substitution of SPECT with PET might be just a part of evolutionary process until another more advanced technology appears. Just like rectilinear scanners were substituted with gamma cameras.

PET provides more accurate staging than other conventional diagnostic means and thus it is rational to use it independently and as the first diagnostic choice [11]. Dramatic impact of PET-CT on patient management has been recognized by Medicare and Medicaid Services [12]. This can be illustrated by the evidence that the course of considerable number of patient treatments (50-60 %) was changed or adjusted on the basis of ^{68}Ga /PET-CT examinations using somatostatin (SST) ligand analogues in diagnosis and staging of neuroendocrine tumours (NETs) [2, 13]. Somatostatin receptor scintigraphy will most likely be replaced with ^{68}Ga /PET-CT in the nearest future [14].

The widespread opinion is that PET is a costly technique, but in fact the total cost of the otherwise required examinations, hospitalization and the risk associated with biopsy as well as false diagnosis leading to futile surgery cost and patient distress greatly overweighs. In particular, the accuracy of diagnostic methods related to the heterogeneity of primary tumour and between a primary tumour and metastases should be addressed. The biopsy results might be misleading since the tumour diagnosis and staging is conducted *ex vivo* on invasively collected tissue. The sampled tissue is commonly heterogenic and thus might not be representative, while PET may provide more detailed and accurate information on heterogeneity of the whole tumour non-invasively in a single examination. The cost of [^{68}Ga]Ga-DOTA-TOC/PET-CT including material and personnel was found lower as compared to that of ^{111}In -DTPA-octreotide/SPECT [15]. Moreover, when using the

former fewer additional examinations were required. In addition, the cost-effectiveness and cost-saving of PET-CT was demonstrated by [^{18}F]FDG/PET-CT used for staging of lung cancer [8, 16, 17]. Currently PET stands for only ~1.5% of Medicare (USA) cancer care expenses, nevertheless the total imaging costs are increasing fast [18], and the reimbursement of [^{18}F]FDG/PET-CT of lung cancer by public health care system might become reality in the nearest future in industrialized countries, and also will propagate into other cancer groups. This in turn will stimulate the investments and development of new imaging agents. The dramatic growth in PET-CT during the last decade has already been accompanied with reducing costs and introduction of new specific PET tracers.

Unmet medical needs

Early diagnosis is important in order to identify functional abnormalities which precede morphological changes, and in particular for cancer, molecular imaging may contribute to the reduction of morbidity and mortality [19]. Over 90% of clinical PET investigations are nowadays performed with [^{18}F]FDG, however interest in specific molecular probes for, e.g. cancer and inflammation/infection is getting stronger among scientists and clinicians [20, 21]. The reason for this is that although [^{18}F]FDG has been successfully used in many cancers as a biomarker of glucose transport, it fails in diagnosis of slowly growing tumours and in differentiation between tumour and such processes as inflammation, infection, reactive lymph nodes, tuberculosis and sarcoidosis [22, 23]. Moreover, the high uptake in normal organs, particularly in brain and gut, results in poor contrast in those areas and potential failure in lesion detection. Thus alternative imaging agents with specific binding capability to e.g. receptors, antigens, enzymes are of strong interest. Specific imaging agents providing information on the molecular and cellular background of various diseases and in particular cancer would allow improvement in patient management and outcome. Generator produced ^{68}Ga may not only enable PET examinations at remote hospitals distant from [^{18}F]FDG distribution, but would also enrich the radiopharmaceutical arsenal at the medical centers both with and without accelerators.

Various ^{68}Ga -based imaging agents have already been tested in humans [7]. The basic research and development of new ^{68}Ga -based agents for targeted imaging of specific protein expression products, pre-targeted imaging as well as non-targeted imaging of pulmonary and myocardial perfusion and pulmonary ventilation is expanding steadily. Imaging of cell proliferation, hypoxia, glycolysis, angiogenesis as well as inflammation and infection has also been consid-

ered [2]. Radiochemistry plays a central role in the development and ^{68}Ga in particular has demonstrated powerful potential. The prevalent area of ^{68}Ga clinical applications in the nearest future will most probably be oncology. More research is required for the development of agents for cardiology. On the other hand, neurology will still be dominated by ^{11}C - and ^{18}F -based imaging agents since common requirements to specific binding, small size, lipophilicity and charge might be difficult to meet when using bulky radio-metal-chelator complex to be attached to a small biologically active molecule.

Theranostics in nuclear medicine has manifested in pre-therapeutic diagnosis for PRRT. In this context the diagnosis and radiotherapy are conducted with the same ligand labelled respectively with imaging radionuclide, e.g. ^{68}Ga and therapeutic one, e.g. ^{177}Lu (Figure 3B). The radiopharmaceuticals bind to tumour-type specific receptors enabling personalized approach of accurate quantitative diagnosis and staging for subsequent selection and planning of therapeutic means as well as monitoring response to the treatment. The dosimetry estimation prior to PRRT is one of the most important prerequisites of successful treatment, and the higher spatial resolution and inherent quantitative accuracy of ^{68}Ga /PET are of paramount importance for the dosimetry precision and treatment response evaluation. The common perception is that ^{68}Ga is not applicable for the pre-therapeutic dosimetry due to the short half-life of ^{68}Ga (68 min) mismatching that of therapeutic radionuclides ^{90}Y (64 h) and ^{177}Lu (6.71 d), and consequently resulting in different time windows of the pharmacokinetics. However, e.g. in the case of somatostatin analogues their fast pharmacokinetics may enable the utilization of kinetic analysis of ^{68}Ga -labelled peptide for the prediction of radiation doses during PRRT with the ^{177}Lu -labelled analogue. This might be accomplished by the combination of the respective agents, namely by either co-injection or separate use of ^{68}Ga - and ^{177}Lu -labelled peptides. The high accuracy at early time points could be achieved by ^{68}Ga /PET quantitation with an error of less than 5% since the tracer accumulation in the organs at risk reaches its maximum within 2-3 half-lives of ^{68}Ga [24]. The kinetics at later time points could be obtained by either blood sampling during the radiotherapy with ^{177}Lu -labelled analogue or by imaging gammas emitted by ^{177}Lu . The dual-tracer examination is possible since the gamma energy of ^{177}Lu is outside the PET detection window and thus would not interfere with ^{68}Ga /PET-CT quantitation. Such combination of the tracers would provide highly improved internal dosimetry.

Specific radioactivity (SRA) of an imaging agent

is another parameter influencing the organ distribution of the agent and subsequent dosimetry. In the case of macromolecular agents, e.g. based on peptides, SRA is calculated as radioactivity per total amount of the agent. The targeted and pre-targeted imaging might require relatively high SRA in order to avoid saturation of binding sites by non-labeled counterpart and possible pharmacological side effects. In this context the total amount of the injected agent, e.g. peptide, should be optimized prior to the radiotherapy providing the highest possible uptake in the lesions and lowest possible uptake in the healthy tissue and organs with physiological expression of the target [2, 24, 25].

Development of scanner hardware and software technologies

The technology developed from PET scanners to hybrid PET-CT and PET-MRI [26, 27]. The morphologic information from CT and MRI is crucial for the accurate determination of location of the lesions. New hardware, detector, and image reconstruction algorithm technologies (e.g. time-of-flight (ToF-PET), point-spread-function (PSF-PET)) continuously improve image contrast, spatial and temporal resolution as well as quantitation accuracy [1, 28]. Moreover, dual-tracer imaging might be possible distinguishing signals on the basis of differences in radionuclide half-lives, tracer kinetics and distribution, however dynamic scanning and multivariate software tools such as principle component analysis would be required [1]. PET-MRI combining the high soft tissue contrast of PET and high spatial resolution of MRI has become reality although it is used at present mainly for research in neurology, cardiology, and oncology. It considerably decreases the radiation dose to the patient as compared to PET-CT. The special resolution of PET might also be improved since the positron range can be shortened by the influence of strong magnetic field. PET-MRI with [⁶⁸Ga]Ga-DOTA-TOC was found superior to MRI alone or PET-CT in terms of detection rate and specificity of liver lesions in patients with NETs [29]. It has also been found to improve the accuracy of target volume delineation in intensity modulated radiotherapy (IMRT) treatment planning and delivery [30]. Moreover, it reduced the number of pretreatment imaging sessions for meningioma patients.

The spatial and temporal resolution of instrumentation, radionuclide decay mode and energy as well as organ movement and size influence the accuracy of PET quantitation which is especially crucial when monitoring the treatment response with marginal changes. Scanning technology and data reconstruction technique have been improved during last

10-15 years in order to meet the requirements. These advances will definitely stimulate development of ⁶⁸Ga-based imaging agents, empowered by the relation to the therapy selection and planning. It should be stressed that the full potential of the imaging technology can only be realized if new imaging agents for specific indications emerge. Improved clinical outcomes of molecular imaging-guided radiation therapy have already been demonstrated [23]. PET radiopharmaceuticals induce adverse reactions extremely seldom with no serious or life-threatening events [31]. While hybrid imaging, PET-CT and PET-MRI, may require high dose of CT and MRI contrast agents thus enhancing the probability of adverse events.

Overview of common clinical imaging radionuclides and ⁶⁸Ga

The most common radionuclides relevant for the labelling synthesis of radiopharmaceuticals for PET, SPECT, and radiotherapy are presented in Table 1. The majority are metals typically involved in coordination labelling chemistry. Only few of the radionuclides are produced in generator systems. Such factors as production mode as well as physical and chemical characteristics determine the choice of a radionuclide.

Table 1. Selected radionuclides used in clinical PET, SPECT and radiotherapy, their production mode and decay properties. Radionuclides are marked in grey.

Radionuclide	Half-life	E _{max} (keV)	Radiation	Production
Positron emitters				
¹¹ C	20.3 min	961	β ⁺ (100%)	Cyclotron
¹⁸ F	110 min	634	β ⁺ (97%)	Cyclotron
⁶⁴ Cu	12.8 h	656	β ⁺ (19%)	Cyclotron
⁶⁶ Ga	9.5 h	4153	β ⁺ (56%)	Cyclotron
⁶⁸ Ga	67.6 min	1899, 770	β ⁺ (89%)	Generator
⁸⁹ Zr	78.4 h	900	β ⁺ (23%)	Cyclotron
¹²⁴ I	4.17 d	2100	β ⁺ (23%)	Cyclotron
Gamma emitters				
⁶⁷ Ga	78.26 h	91, 93, 185, 296, 388	γ	Cyclotron
^{99m} Tc	6.0 h	141	γ	Generator
¹¹¹ In	67.9 h	245, 172	γ	Cyclotron
Therapeutic radionuclides				
⁹⁰ Y	64.0 h	2270	β ⁻	Generator
¹²⁵ I	60 d	350	Auger electrons	Reactor
¹³¹ I	8.0 d	1810	β ⁻	Fission
¹⁷⁷ Lu	6.71 d	500	β ⁻	Reactor

Two gallium isotopes, ⁶⁶Ga (t_{1/2} = 9.5 h) and ⁶⁸Ga (t_{1/2} = 68 min), decay by β⁺-emission and are therefore suitable for PET imaging, ⁶⁷Ga (t_{1/2} = 78 h) decays by electron capture with concomitant γ-emission and is

used for SPECT imaging, examples of clinically used radionuclides include ^{11}C , ^{18}F , ^{64}Cu , ^{89}Zr , $^{99\text{m}}\text{Tc}$, ^{111}In , ^{124}I (Table 1). MICAD has reported that ~41%, 31% and 28% of PET agents is labelled, respectively with ^{18}F , ^{11}C , and ^{64}Cu , ^{68}Ga , ^{89}Zr , ^{124}I taken together [10]. Amongst SPECT agents the leading position belongs to $^{99\text{m}}\text{Tc}$ (42%) followed by ^{111}In (29%). The major advantage of $^{99\text{m}}\text{Tc}$ and ^{68}Ga is that they are obtained from generators that are cost-effective alternative to reactors and cyclotrons. They are also cheaper as compared to commercial radionuclides, for example ^{111}In and ^{124}I . ^{68}Ga /PET-CT provides advantage over $^{99\text{m}}\text{Tc}$ /SPECT not only due to the inherent benefits of the technology, but also due to the resemblance of coordination chemistry of ^{68}Ga , ^{90}Y and ^{177}Lu and, thus possibility to use the same vector molecule for the subsequent radiotherapy. As a result, the diagnostic agent is directed to the same molecular target as radiotherapeutic one allowing the prediction of the treatment efficacy and selection of patients within the frame of theranostics.

With regard to the decay mode, the advantage of positron emitting radionuclides over gamma emitting ones is inherent and related to the benefits of PET technique such as higher sensitivity, resolution, quantitation, and dynamic scanning. The energy and the content of the emitted particles influence the resolution. ^{68}Ga with rather high positron energy could be expected to perform with lower resolution as compared to ^{18}F , however both computational analysis and experimental measurements demonstrated equally high quality images for these two radionuclides assuming the scanner detector resolution of 3 mm [2]. The disadvantage with ^{64}Cu , ^{89}Zr , and ^{124}I as compared to ^{68}Ga include low positron abundance (^{64}Cu , ^{89}Zr), higher positron energy (^{124}I), as well as simultaneous gamma emission (^{124}I) resulting in poorer image quality, longer scanning duration, and additional radiation dose. However, their longer half-lives allow development of agents based on vectors with slow pharmacokinetics such as, for example antibodies that require 2-4 days post injection for blood clearance and optimal image contrast. Though, it is worth mentioning that the relatively short half-life of ^{68}Ga allows repetitive examinations on the same day [24].

One of the most rapidly expanding areas is the development of peptide-based agents for targeted imaging. Metal and halogen radionuclides such as ^{64}Cu , ^{89}Zr , $^{99\text{m}}\text{Tc}$, ^{111}In , ^{18}F , and ^{124}I are most commonly used for the design of the agents. Typically, the metal ion coordination chemistry is straightforward and mild. However, it requires attachment of a rather bulky chelator to the vector molecule what may deteriorate the biological activity. Simple direct iodination

has been used for decades introducing minor modification at tyrosine amino acid residue though demonstrating poor residualizing of the radioactive iodine catabolites in the cell. On the contrary hydrophilic complexes of radiometals stay trapped in the cell after the degradation of the internalized imaging agent. The incorporation of ^{18}F can be accomplished by the conventional nucleophilic or electrophilic addition, or via prosthetic groups [32]. However, the synthesis might be rather complex with harsh conditions and high macromolecule concentration. A novel method utilizing similarity of $\text{Al}^{18}\text{F}^{2+}$ with metal cations and thus possibility of coordination labelling chemistry has potential for kit type production like radiometals [33]. The relatively long half-life (110 min) of ^{18}F enables centralized production and distribution of either the radionuclide or prepared imaging agents to the satellite clinical centres. Most of the PET agents used in clinical studies are ^{18}F -based. Nevertheless, the simpler labelling chemistry of ^{68}Ga and its availability from the generator system which can be used even at distant and isolated medical centres make ^{68}Ga more preferable.

Only ^{11}C amongst over mentioned radionuclides provides true tracers since it is an endogenous element and the radioactive compounds are chemically identical to the stable counterparts. It is perfect radionuclide for the labelling of small bioactive organic molecules. This field is rather challenging for metal radionuclides including ^{68}Ga , and ^{11}C together with ^{18}F will most probably dominate in the field of neurology and metabolic tracers in oncology employing small molecules [34].

As mentioned earlier the advantages of ^{68}Ga over ^{111}In are multiple and have already been demonstrated in patients affected by NETs in terms of diagnostic output and logistics, superior sensitivity and accuracy, higher detection rate and specificity, faster acquisition and shorter examination time, lower radiation exposure as well as cost, and ^{68}Ga -labelled somatostatin analogues are predicted to replace [^{111}In]In-DTPA-octreotide (Octreoscan®) in the nearest future [14, 15, 35, 36].

Another advantage of ^{68}Ga over ^{18}F , $^{99\text{m}}\text{Tc}$, and ^{111}In is comparable or lower level of effective dose (Table 2) [37, 38]. The latter was calculated considering administration of commonly used levels of radioactivity of 100, 200, 370, and 570 MBq respectively for [^{68}Ga]Ga-DOTA-TOC, [^{111}In]In-DTPA-octreotide, [^{18}F]FDG, and [$^{99\text{m}}\text{Tc}$]-MDP. It is worth mentioning that the examination time is considerably shorter for ^{68}Ga -agents as compared to SPECT agents and somewhat shorter compared to [^{18}F]FDG.

The benefits of ^{68}Ga can be summarized as follows. It is produced from a long shelf-life and

cost-effective generator. The half-life of ^{68}Ga permits production and application of resultant agents, and the labelling synthesis is amenable to automation and kit type preparation. It provides sufficient levels of radioactivity for high quality images, short scanning time (fast patient examination) while minimizing the radiation dose to the patient and personnel, and allows fast dischargement of the patient. It also allows repetitive examinations within the same day [24]. The majority of the therapeutic radionuclides is also metals and might allow for the theranostic development.

Table 2. Effective doses for some PET and SPECT imaging agents.

Agent	Examination time	Effective dose, [mSv]
^{111}In]In-DTPA-octreotide/SPECT	24-48 h	10.8
^{68}Ga]Ga-DOTA-TOC/PET	30-60 min	2.3
^{18}F]FDG/PET	60-120 min	5.6
$^{99\text{m}}\text{Tc}$]BPAMD/SPECT	2-6 h	6
$^{99\text{m}}\text{Tc}$]MDP/SPECT	2-6 h	3-4
^{68}Ga]Ga-BPAMD/PET	30-60 min	3-4

In comparison with the other radionuclides mentioned above there are a number of advantages and disadvantages, and they all complement each other to make PET imaging applicable to a broad range of diseases. $^{99\text{m}}\text{Tc}$ /SPECT has been holding the leading position in nuclear medicine for decades due to such factors as favorable gamma energy, availability from a generator system and kit type radiopharmaceutical production. The future of ^{68}Ga is predicted as PET analogue of $^{99\text{m}}\text{Tc}$ /SPECT with added value of higher sensitivity, resolution as well as accurate quantitation and personalized medicine. However, even if the vision of ^{68}Ga becoming as immense as $^{99\text{m}}\text{Tc}$ will not get fulfilled, ^{68}Ga has definitely strong contribution to make in the improvement of personalized patient management and there is a niche for ^{68}Ga -based agents in nuclear medicine. Moreover, ^{68}Ga -agents have the prerequisites to substitute also ^{111}In -based radiopharmaceuticals.

Biomarkers, targets, and ligands

Development of agents for imaging and radiotherapy involves identification of biological process and target underlying the pathology as well as respective lead compound. Then the radioactive lead compound counterpart must be designed, chemically characterized as well as preclinically and clinically validated. In particular, the specific targeting and pre-targeted imaging require information on biomarkers and is dependent on their discovery. Thus, advances in biological research and biotechnology are crucial. Proteomics and genomics considerably con-

tribute to the expansion due to the increasing knowledge and access to the vectors and targets such as receptors, enzymes, antigens as well as their ligands and substrates. Proteins demonstrate remarkable capability of molecular recognition. Advances in genetic and biochemical techniques resulted in a large number of antibody radioimmunotherapeutics. That in turn triggered further development with the objective to overcome the drawbacks related to antibody high molecular weight, slow pharmacokinetics and clearance that cause high radiation dose to normal tissue and poor image contrast. Thus, large libraries of high affinity small proteins have been created using combinatorial engineering and phage display techniques that provide efficient screening of ligands as well as identification and selection of antibodies and receptors for drug discovery and therapy. A number of non-immunoglobulin scaffolds has also been studied that resulted in libraries as, for example Affibody[®] molecules originating from Z domain scaffold of staphylococcal protein A. Thirteen surface-exposed residues of the scaffold were randomized resulting in a library (3×10^9 members) of high-affinity binders to various targets [39]. Radiolabelled Affibody molecules have extensively been investigated preclinically, and ^{68}Ga - and ^{111}In -labelled analogues with high affinity to HER2 receptors up-regulated in breast cancer has also been studied in patients [40]. This is a strong evidence for the future fruitful development of engineered high affinity proteins and abundant source of ligands to various receptors expressed in diseased tissues.

G-protein and G-protein coupled receptor discoveries, both awarded with Nobel Prize, respectively in 1994 and 2012, provided basis for the important and most explored class of imaging agents comprising small regulatory peptides that are involved in many metabolic processes in almost all organs [41-43]. It should be mentioned that one of the factors that tremendously contributed to the progress of ^{68}Ga applications was the advent of agonist somatostatin ligand analogue (DOTA-D-Phe¹-Tyr³-Octreotide, (DOTA-TOC)). The current development is directed to pansomatostatin and antagonist analogues [44]. Other examples of peptide receptor targets in various cancer types are: gastrin-releasing peptide (GRP) receptors in prostate, ovarian and urinary tract cancers; neuropeptide Y receptors in breast cancers; glucagon-like peptide 1 receptors (GLP-1) in benign insulinomas and islet cells; vasoactive intestinal peptide receptor (VIPAC-1) in breast, prostate cancer; cholecystokinin 2 receptors (CCK) in medullary thyroid cancers; neurokinin-1 receptor in glioblastoma, breast cancer; melanocortin-1 receptor in melanomas; chemokine receptor 4 (CXCR4) in lung, breast, pros-

tate cancer; neurotensin (NT) receptor in SCLC, colon, breast, prostate cancer. Corresponding peptide ligands such as bombesin, CCK/gastrin, exendin, α -MSH, neurotensin, substance P labelled with ^{18}F , ^{68}Ga , $^{99\text{m}}\text{Tc}$ or ^{111}In have been studied clinically [45-50]. Even though just few of them were labelled with ^{68}Ga , it is a solid background and springboard for ^{68}Ga -based analogues to come in the nearest future. Many more studies have been conducted preclinically and it is worth mentioning that the total critical mass of basic knowledge and experience gained until now will lead to the explosively growing clinical studies with ^{68}Ga . The design of an imaging agent is a complex process, and although the major factors that influence the biological function of a probe such as peptide/protein sequence and 3D structure, pharmacokinetic modifiers, chelators, metal cations are known, it is not that straightforward to predict the pharmacokinetics and pharmacodynamics of the candidate agent [51].

Thus fundamental research in biology of surface receptors and antigens, enzyme activity, transport systems, proliferation, apoptosis, hypoxia, glycolysis, and angiogenesis provides invaluable information and lays a basis for imaging agent development.

PET radiopharmaceutical regulations

The absence of legislation and regulations specific to PET radiopharmaceuticals made it difficult to conduct clinical trials and introduce new imaging agents into clinical routine however the situation has improved during last years. It required considerable effort from academic, clinical and patient communities and societies, and the hard work has started giving results. Recent developments indicate possibility of regulatory specific solutions that may allow the clinical use of small scale preparation radiopharmaceuticals without obligation to apply for manufacturing authorization or clinical trial [52].

One of the major difficulties was that regulatory bodies evaluated therapeutic and imaging agents by the same process and put forward the same requirements, however there has been an improvement such as the recognition of the microdosing concept [53-55] by EMEA and FDA, and introduction of the Exploratory Investigational New Drug (eIND) guidelines that reduces the demand on toxicity studies and respective cost burden [56, 57]. This is possible because of the high sensitivity of PET and consequently use of non-pharmacological radiopharmaceutical doses of picomoles (nanograms-micrograms). It should also be mentioned that commonly the adverse reactions to PET radiopharmaceuticals are extremely rare and with no serious or life-threatening events [31].

The recommendations on compliance with reg-

ulatory requirements for radiopharmaceutical production in clinical trials have been discussed for Europe and the USA [58, 59]. A comprehensive overview of relevant current EU documentation concerning requirements to the quality of starting materials and final drug products/radiopharmaceuticals as well as to the preparation procedure has been published with references to specific directives and regulations [52, 58, 60]. Another guidance is more specific and covers Part B of the EANM "Guidelines on Good Radiopharmacy Practice (GRPP)" for small-scale "in house" production of radiopharmaceuticals that are not intended for industrial production, sale or distribution [61]. European pharmacopoeia monograph on compounding of radiopharmaceuticals that includes chapter dealing with standards of the extemporaneous preparation of radiopharmaceuticals has been prepared and submitted to the authorities.

Recommendations on the patient examination protocols, interpretation and reporting of the results have been summarized as guidelines for the assistance to nuclear medicine physicians [62, 63]. Two European Pharmacopoeia monographs are in the preparation and publication, "Gallium (^{68}Ga) edotreotide injection" [64] and "Gallium (^{68}Ga) chloride solution for radiolabelling". One of the critical quality parameters is the breakthrough from the $^{68}\text{Ge}/^{68}\text{Ga}$ generator of long-lived parent radionuclide, ^{68}Ge ($t_{1/2}=270.8$ d) and its content in the radiopharmaceutical preparation of not more than 0.001% may restricts the use of the generators and limit clinical applications of ^{68}Ga specifically with regard to kit type preparation. The limit defined in the European Pharmacopoeia monographs was based on a hypothetical assumption of total accumulation of $^{68}\text{Ge}(\text{IV})$ radioactivity in the bone marrow with an infinite retention. However, $^{68}\text{Ge}(\text{IV})$ biodistribution studies conducted currently in rats with extrapolation to the human organ and whole-body radiation dosimetry demonstrated that the limit defined in the monograph could be increased at least 100 times without compromising patient safety [65]. If the ^{68}Ge limit is increased, then together with the labelling methods of high selectivity towards ^{68}Ga at room temperature, it will open possibility for kit type production at radiopharmacy practice and consequently even wider use of ^{68}Ga -based agents. There are no labelling kits for the preparation of ^{68}Ga -based radiopharmaceuticals with marketing authorization available currently, and it is a matter of years before they appear on the market.

One more crucial reason postponing the clinical introduction of ^{68}Ga -agents was the scarcity of generators, their quality, and absence of corresponding regulations. However currently at least four different generators are commercially available and more are

under development. Some of the manufacturers (Eckert & Ziegler; iThemba Labs) hold license for good manufacturing practice (GMP) production and the generators of pharmaceutical grade are on the way (Eckert & Ziegler). The draft monograph, "Gallium (^{68}Ga) chloride solution for radiolabelling", regulates the requirements to the quality and safety.

In order to accelerate the use of PET for assessing treatment responses, standardization and consolidation of image acquisition, interpretation, and quantitation criteria that have been considered in Europe [52, 66, 67] and the USA [68] are required to be introduced into practice [18]. International Atomic Energy Agency conducted survey of the status of nuclear medicine in developing countries and localized the areas requiring international cooperation and help in order to improve the level of use and the quality as well as promote global standardization, growth, and dissemination [69].

There is an ongoing dispute on the benefits of PET-CT in patient management conducted by national authorities. The validity of such review judgments of health technology assessment (HTA) agencies was questioned [70]. The authors investigated HTA reviews (1996-2010) and found them misleading and that in many countries they unfortunately discredited evidence of PET and PET-CT capability to improve patient outcomes. However, original conclusions of clinicians have proven the opposite, and access to this technology though was delayed it could not be prevented.

In summary, the regulation and legislation of the manufacturing of PET radiopharmaceuticals and in particularly ^{68}Ga -related documentation has been defined during current years reflecting the acceptance of obvious benefits of ^{68}Ga /PET-CT to the patient management. Interestingly the analysis of various reasons causing performance inefficiency of nuclear medicine centres in developing countries revealed that regulatory hinder was the major problem only at 5% of the centres [69]. The USA had a 10 y delay in implementing human studies with ^{68}Ga -labelled somatostatin analogues as compared to Europe due to mainly poor availability of generators, uncertainty about intellectual property of the ligands and regulation of manufacturing; however regulatory uncertainty and generators are less of a problem currently in the USA [71]. There are at least three sites, University of Iowa, Vanderbilt University, and Excel Diagnostics & Nuclear Oncology Center holding approval from FDA for the use of ^{68}Ga -labelled somatostatin analogues in the management of patients with NETs. Peptide based precursors of GMP grade for labelling with ^{68}Ga are becoming available commercially which will most likely accelerate the introduction of respective imag-

ing agents into clinical environment. Nevertheless currently the facilitation of the entry of novel radiopharmaceuticals into clinical practice relies mostly on magisterial preparation/prescription and compassionate use under responsibility of the prescribing physician.

Automation of the labelling procedures

Automated synthesis reduces the radiation exposure to the operator, improves robustness of the production as well as provides on-line documentation of the manufacturing process thus improving GMP compliance. Considerable number of semi-automated and fully automated devices with either stationary tubing system or disposable cassettes for the automated production of radioactive probes is available on the market as well as built "in house" [72-85]. They offer application of three most common methods: fractionation and preconcentration/prepurification of the generator eluate using either cation or anion exchange resins. Synthesizers based on the disposable sterile cassettes and devoted to the production of ^{68}Ga -comprising imaging agents are most preferred in routine clinical setup even though the cost is higher. They exclude the risk of cross-contamination, improve the reproducibility and reduce the radiation burden upon the operator. Both hardware and software should comply with GMP and good automated manufacturing practice 5 (GAMP5). The automation may provide possibility for the harmonized and standardized multicentre clinical studies that in turn would accelerate the introduction of new radiopharmaceuticals as well as their regulatory approval. This in turn will motivate investments into the research and development of novel ^{68}Ga -based radiopharmaceuticals.

Chemistry of ^{68}Ga -based imaging agents

Above mentioned interrelated aspects are critical and prepare soil for the justification and stimulation of the development of new imaging agents which is an expensive and time consuming process. However, the influence is two way and the development of these aspects is in turn motivated by the advent of new agents. Chemistry is a driving force of the development of nuclear medicine and hundreds of various imaging agents have been synthesized and preclinically evaluated.

The labelling with radiometals can be direct or chelator-mediated (tagged). The direct ^{68}Ga -labelling of macromolecules is limited and applies to proteins (e.g. lactoferrin, transferrin, ferritin) designed by nature for iron binding thus utilizing chemistry similarity of Ga(III) and Fe(III) [86]. The direct ^{68}Ga -labelling and formation of low molecular weight complexes is

commonly employed for the development of imaging agents for perfusion or for imaging of biological processes where the agent uptake is defined by its charge, lipophilicity, and size. Particulate agents can also be produced by the direct ^{68}Ga -labelling either by co-precipitation (e.g. macroaggregated albumin) or by co-condensation (e.g. ^{68}Ga -carbon nanoparticles) [87-92]. The chelator mediated ^{68}Ga -labelling, requiring first synthesis of a bioconjugate comprising vector molecule and chelate moiety for the coordination of the radiometal ion, is the most common pathway of imaging agent design. The principle components of such agents are targeting vector, chelator, and radionuclide (Figure 3A). The modulation of pharmacokinetics, biodistribution, and stability can be achieved by the introduction of pharmacokinetic modifiers (PKM) such as hydrocarbon chain, polyethylene glycol (PEG), carbohydrate, and polypeptide chain. PKM may also serve as linker/spacer between the bulky chelate moiety and the active site of the vector mole-

cule. Thermodynamic and kinetic stability, geometry and lipophilicity of a chelator-metal ion complex are important parameters in the development of radiometal based radiopharmaceuticals.

These design principles are valid for the ^{68}Ga -labelling of small biologically active organic molecules, biological macromolecules, complexes of variable charge and lipophilicity as well as particles. Thus the chemistry considered in the development of ^{68}Ga -based imaging agents has several cornerstones: aqueous chemistry of ^{68}Ga , coordination chemistry of Ga(III), design of mono- and bifunctional chelators, and bioconjugate chemistry. As mentioned above most of ^{68}Ga imaging agents known by now are tagged which means that their biodistribution is defined by the vector molecule and ^{68}Ga may or may not influence biodistribution or affinity to the target. Such are agents for targeting and pre-targeted imaging of receptors, enzymes, antigens, and transporters.

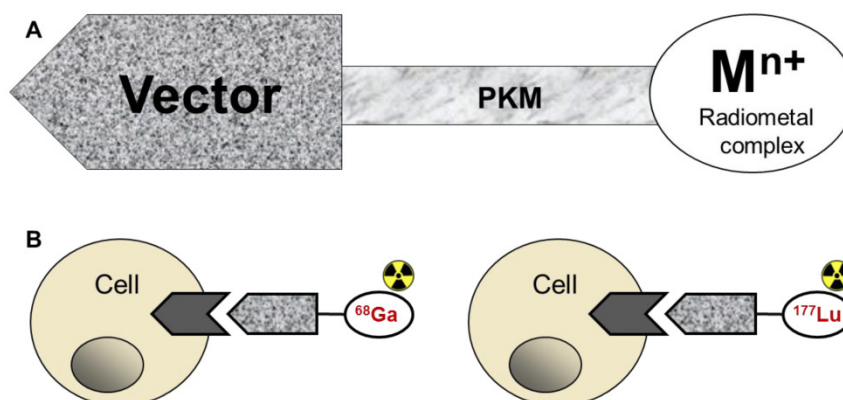


Fig 3. A) Depiction of the basic components of an imaging agent comprising a vector for specific binding, pharmacokinetic modifier, and the complex of the chelator moiety with a radiometal; B) Drawing of the interaction of the agent, either imaging if labelled with ^{68}Ga (left) or radiotherapeutic if labelled with ^{177}Lu (right), with the cell receptor.

Production, availability and quality of ^{68}Ga

^{68}Ga is artificially produced one of twenty nine isotopes of gallium. It is a product of ^{68}Ge decay ($t_{1/2} = 270.8$ d) and is obtained from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator system where ^{68}Ge is absorbed on a chromatographic column with either inorganic or organic matrix [2, 93]. The parent radionuclide decays to a shorter-lived daughter ^{68}Ga that can be simply eluted from the column. It is not only a reliable source of ^{68}Ga at clinical centres remote from the cyclotron and distribution sites but it also enhances imaging agent assortment at centres possessing cyclotrons. Although first generators were introduced in late 1950s early 1960s and ^{68}Ga -based imaging agents were available already from 1970s [2], the development of the respective radiopharmaceuticals was precluded by the absence of reliable sources of the generators as well as their

properties and quality. The first generators produced ^{68}Ga in a complex with EDTA that could be directly used for the clinical patient imaging of blood flow of brain tumours. However, [^{68}Ga]Ga-EDTA complex prevented the development of other radiopharmaceuticals since the chemistry was complicated by prior need of the complex decomposition. The situation in the recent years has changed considerably. Several different generators manufactured in the USA (IGG100, Eckert & Ziegler), Germany (ITG Isotope Technologies), Russia (Obninsk, Cyclotron Co, Eckert & Ziegler) and South Africa (iThemba Labs) are commercially available and more are under development in Europe, North America and Asia [76, 94-97]. The generators are eluted with hydrochloric acid of various molarity (0.05M to 5.5M, 5-7 ml) offering ^{68}Ga in cationic form suitable for the subsequent labelling. The generators have been thoroughly

characterized by various research groups and methods for the efficient and GMP compatible labelling synthesis have been developed [2, 98-101]. The generators can be eluted repeatedly during the day. Fifty percent of the maximum available ^{68}Ga -radioactivity can be obtained every consecutive hour. Four hour accumulation provides respectively over 94%. The long half-life of ^{68}Ge (271 d) allows 1-2 year shelf-life in the clinical setting dependent on the loaded radioactivity amount. The amount of ^{68}Ga and the shelf-life of the generators can be increased by the elution of several sequentially coupled generators.

The long shelf-life of the generator triggered concerns regarding microbiological safety even though the acidic eluent (e.g. 0.1M HCl) is highly unfavourable environment for the microbial growth. However it was demonstrated in a study with a $^{68}\text{Ge}/^{68}\text{Ga}$ generator column intentionally loaded with various bacteria and fungi in exhaustive amounts that the risk of incidental microbial contamination was very low [102]. Another concern was the breakthrough of parent long-lived ^{68}Ge and its content in the ^{68}Ga eluate. However, germanium is not pharmacologically active and in vivo it acts as a non-toxic foreign material which is readily eliminated and thus as a chemical presents low risk to man [65]. Thus with regard to radioactive $^{68}\text{Ge(IV)}$ where the amounts of the element are negligible, the safety issue is reduced to ionizing radiation and, in particular the buildup of $^{68}\text{Ga(III)}$ at the sites of deposition of the $^{68}\text{Ge(IV)}$. However, $^{68}\text{Ge(IV)}$ biodistribution study in rat demonstrated fast excretion ($t_{1/2}=0.6$ h) and no deposition in any organ [65]. Dosimetry calculations revealed that as much as 645 MBq (female) and 935 MBq (male) could be administered before reaching effective dose of 10 mSv which is a general limit to healthy volunteers to which most authorities adhere. Moreover, it was shown that $^{68}\text{Ge(IV)}$ was not chelated by DOTA-TOC and thus accumulation in the sites of DOTA-based imaging agents was also excluded.

Three major methods for the quality improvement of the generator eluate such as fractionation, cation exchange chromatography, and anion exchange chromatography are currently in common use [2]. In particular, reduction of the generator eluate volume as well as purification from metallic impurities (Al(III), In(III), Ti(IV), Zn(II), Ti(IV)) and long-lived parent $^{68}\text{Ge(IV)}$ were achieved to certain extent. However, it should be mentioned that the reduction of the concentration of contaminant metal ions including Zn(II) being product of ^{68}Ga decay in the generator eluate can be achieved simply by regular elution of the generator [99]. The shorter elution time interval is the less contaminant cations are accumulated. Unfortunately, none of these methods

fully eliminates Fe(III) which is the strongest competitor to $^{68}\text{Ga(III)}$ since its chemistry is very similar to that of Ga(III). The anion exchange prepurification and preconcentration method was further modified in order to remove the excess of $[\text{H}^+]$ and ensure high reproducibility of pH which is crucial for the successful labelling [103]. The stabilization of pH and omission of strong sodium hydroxide use was achieved by the addition of a cartridge washing step using 5 M sodium chloride solution. The content and type of gallium chemical species under this conditions was investigated and shown to be prevailed by $[\text{GaCl}]^{2+}$ and Ga^{3+} , respectively before and after the buffering (pH 3) [104]. Another advantage of the anion exchange method is that the highly concentrated ^{68}Ga in small volume can directly be used for production of ^{68}Ga -carbon nanoparticle aerosol for the examination of acute pulmonary emboli [91, 105]. Furthermore the anion exchange method is applicable to any generator type eluted with HCl independent on the molarity of the latter. It can also be used for the preconcentration of several generator eluates independent on the total volume. High amount of radioactivity would provide high SRA however it should be taken into consideration that it also may induce radiolysis of sensitive active substances. In such case radical scavengers, e.g. ethanol or ascorbic acid can be added directly to the reaction mixture. In combination with microwave heating ^{68}Ga preconcentration method allowed production of agents with enhanced specific radioactivity [99], and was successfully used for the labelling of DOTA conjugates with macromolecules and small molecules [2]. The method was automated [84].

The cationic method for the preconcentration and purification of the generator eluate using cation exchange resins and resulting in 400 μL solution of acetone/HCl mixture [101] has also been improved. It offers possibility for the labelling in non-aqueous solution via $^{68}\text{Ga}(\text{acac})_3$ precursor formation [106]. However, precautions for the storage of acetone/HCl mixture should be taken since acetone in the acidic pH under light forms mesityloxide [107]. Methods to avoid usage of the acetone/HCl mixture has been developed based on sodium chloride [108].

In addition the number of patient administration doses from one production would depend on the initially available radioactivity (strength and age of the generator), labelling duration and efficiency as well as the number of scanners for examinations. The maximized utilization would require simultaneous multiple patient examinations immediately after the tracer production since the short half-life of the ^{68}Ga limits the consumption duration. However, this drawback is compensated by the fact that the injected dose can be

as low as 100-150 MBq still resulting in high quality images. Another advantage is that the generator can be eluted repeatedly providing imaging agent production with 1-2 h (50-70% of maximum eluted ^{68}Ga) interval.

The $^{99\text{m}}\text{Tc}$ -based radiopharmaceuticals have globally been used for decades, partly due to the readily availability from a generator system. However, production of the parent ^{99}Mo was drastically decreased due to the closure of the reactor in Canada, ^{99}Mo waste and security issues related to the weapon grade ^{235}U in the nuclear reactor target all together rendering increase in generator price and limited availability [109]. Consequently, the number of clinical examinations using $^{99\text{m}}\text{Tc}$ -radiopharmaceuticals decreased during last years. Even though parent ^{68}Ge is produced in high energy accelerators the latter are more available and affordable than reactors [93]. The shortage of ^{99}Mo has also motivated development of ^{68}Ga -based alternatives to $^{99\text{m}}\text{Tc}$ -radiopharmaceuticals. The authors of [110] have wisely noticed that the solution of the supply shortage is not only in the investment and extension of ^{99}Mo production, but in creating diversity of radiopharmaceuticals by introducing alternative medically relevant isotopes with ^{68}Ga as an obvious candidate.

The current development of the generator market, research on the quality of eluted ^{68}Ga as well as establishment of regulations indicate future diversity and high quality of generators with low ^{68}Ge breakthrough, narrow elution profile, and low metal ion contamination.

Coordination chemistry and design of chelators

The hard acid Ga(III) can form four-, five-, and six-coordinated complexes. The latter are the most stable with octahedral coordination sphere. Oxygen, nitrogen, and sulfur donor atoms form stable coordinate bonds with Ga(III). The examples of the most common functional groups are amine, carboxylate, hydroxamate, phenolate [2]. The coordination reaction requires buffering for two reasons: first, to ensure right pH for the deprotonation of electron donor atoms; second, to weakly coordinate and maintain Ga(III) in solution that might otherwise form $\text{Ga}(\text{OH})_3$ and precipitate at pH 3-7 [2]. Various buffers such as sodium acetate, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer, succinate, formate, tris, glutamate, lactate, oxalate and tartrate have been investigated [75, 99]. Although HEPES is biologically compatible buffer [24, 25] and provides high radioactivity incorporation (>95%) and specific radioactivity [75, 99, 111], it is not listed in pharmacopoeia and thus prior to clinical use the radiophar-

maceutical should be purified from it and additional quality control must be conducted to ensure that HEPES does not exceed the limit. The use of sodium acetate has the advantage of being eligible for human use possibly simplifying the regulatory approval of the radiopharmaceutical as well as feasibility to omit additional quality control tests [75].

As mentioned above coordination chemistry is one of the cornerstones of the development of ^{68}Ga -based imaging agents and thus advances in mono- and bifunctional chelator design is of paramount importance. The topic has recently been reviewed elsewhere [2, 112] and here only the fundamental and recent advances that shape the future of the field are discussed. The basic structures that have been most thoroughly studied are polyaminopolycarboxylate, hydroxyaromatic, macrocyclic and amine-thiol type ligands. The principle requirements are that they should form stable complexes with Ga(III) favourably of octahedral geometry in order to yield stable complexes. The association kinetics must be fast and reaction desirably taking place at room temperature while dissociation kinetics must be very slow [2]. Charge and lipophilicity can be adjusted dependent of the application. Two classes of chelators namely open chain and macrocyclic have been considered. Although the former commonly formed insufficiently stable complexes the interest towards them still remains with the desire for fast complexation kinetics at room temperature that would like NOTA [113] provide possibility for kit type preparation of radiopharmaceuticals and labelling of temperature labile molecules.

Several open-chain chelators such as hydroxyaromatic diamines like *N,N'*-di(2-hydroxybenzyl) ethylenediamine-*N,N'*-diacetic acid (HBED), pyridoxylethylenediamines such as *N,N'*-dipyridoxyethylenediamine-*N,N'*-diacetic acid (PLED), mono- and bifunctional open chain chelators based on [tris(aminomethyl)ethane] (TAME) formed complexes with improved stability [2]. However, neither of them or four-, five- and six-coordinate amino-thiol (N_2S_2 , S_3N , 4SS, 5SS, 6SS, N_3S_3) based open chain chelators [2] have found further application. There have been just few publications reporting on open-chain chelators and not all of them demonstrated desired properties. ^{67}Ga -labelled tripodal 3-hydroxy-4-pyridinone (1, NTP (PrHP) $_3$, Figure 4) showed *in vivo* stability and fast renal excretion in healthy rats [114]. Similar chelator, tripodaltris(hydroxypyridinone) (YM103), was functionalized with maleimide for the conjugation to proteins via Cys [115]. The efficient and stable ^{68}Ga -labelling occurred at room temperature. H_2dedpa (N_4O_2), and its bifunctional derivatives containing amine, pyridine and carboxyl moieties

(H₂dedpa (3), dedpa-1 (4), dedpa-2 (5), Figure 4) were stably labelled with ^{67/68}Ga at room temperature (SRA~360 MBq/nmol) using 0.1 μM chelate [116]. While HBED possessing hydroxybenzyl and amine groups [117] showed low labelling efficiency and slow blood clearance. The isothiocyanato derivatives of the H₂dedpa (H₂dp-bb-NCS (6), H₂dp-N-NCS (7)) have been synthesized and conjugated to c(RGDyK) resulting in monomer and dimer [118]. The uptake of monomer was higher than that of dimer in RAG2M xenografts. However, very slow clearance from blood requires further improvement of pharmacokinetic properties.

An exhaustive number of triazacyclononane (TACN (8)) and tetraazacyclododecane (TACD (9)) (Figure 5) derivatives have been synthesized. The backbone and pendant arms were functionalized for the conjugation to vector molecules and in order to modulate complexation kinetics, charge, lipophilicity and stability of the complex as well as biodistribution, pharmacokinetics, excretion pathways and blood clearance rate. The pendant arm modifications include such functional groups as carboxylic acid, phosphinic acid, α-haloacetyl, alkoxy, alkyl- and arylamine, alkyl- and aryl sulphide, phenol, hydroxamate. A number of TACN based molecules functionalized with 3-hydroxy-4-pyrone pendant arms (H₃NOKA (10)), with carboxylic pendant arms

(NOTA (11)) and its various derivatives, namely, 1,4,7-tris(2-mercaptoethyl)-1,4,7-triazacyclononane (TACN-TM (12)), 1,4,7-triazacyclononane-1-succinic acid-4,7-deacetic acid (NODASA (13)), 1,4,7-triazacyclononane-*N,N,N'*-tris(methylenephosphonic) acid (NOTP (14)), 1,4,7-triazacyclononane-*N,N,N'*-tris(methylenephosphonate-monoethylester) (NOTPME (15)) (Figure 5) demonstrated similar plasma and *in vivo* stability. NOTA and its bioconjugates showed efficient chelation (>95%) of ⁶⁸Ga at pH 3.5 and room temperature within 10 min [113, 119, 120]. Mechanistic studies of the unexpectedly fast complexation kinetics at such low pH suggested that the transchelation step from the buffer to NOTA involved protonation of the buffer and decoordination that lead to the final Ga-NOTA product [121]. The room temperature is advantageous for the labelling of fragile molecules as well as “shake and shoot” type kit production. Triazacyclononane with either hydroxybenzyl or hydroxypyridyl pendant arms at the nitrogens (TACN-meHP (16), TACN-TX (17), TACN-HP (18), TACN-HB (19), TACN-TM-Bn (20) Figure 5) were synthesized in order to increase the lipophilicity of gallium complexes and enable the blood brain barrier penetration [2]. The resultant complexes were stable however did not serve the purpose.

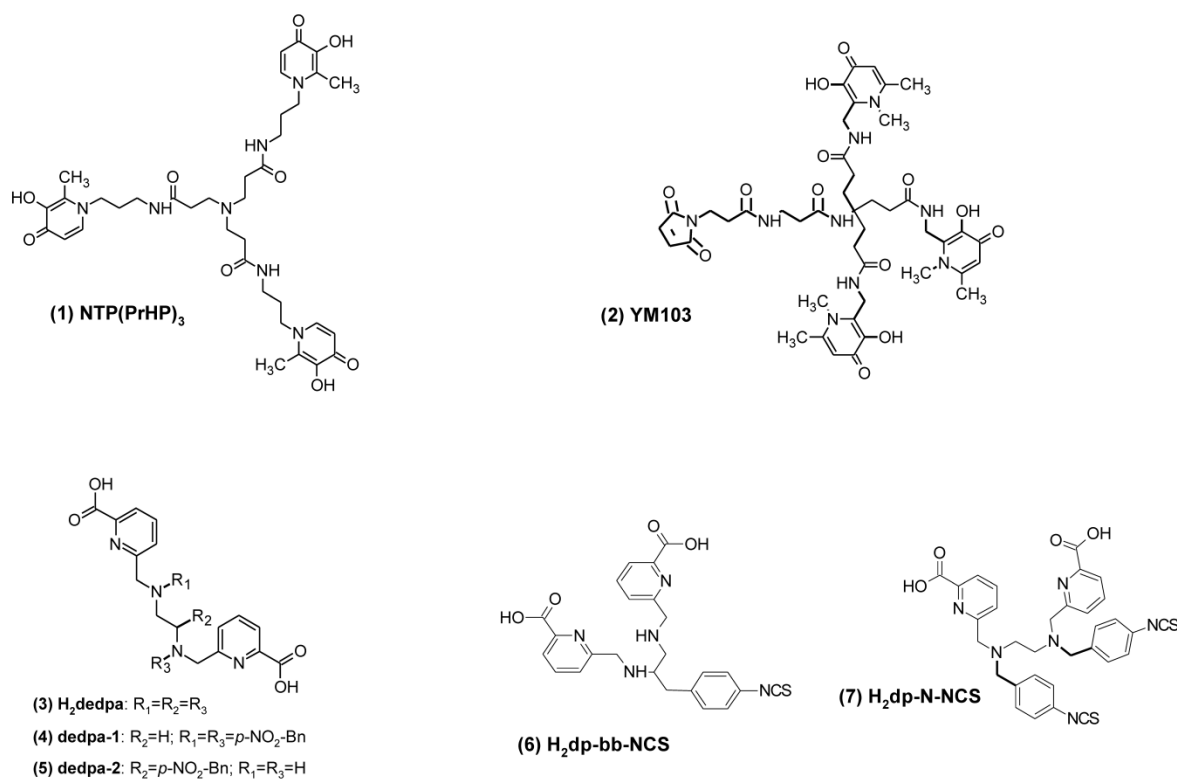


Fig 4. Basic structures of open chain mono- and bifunctional chelators.

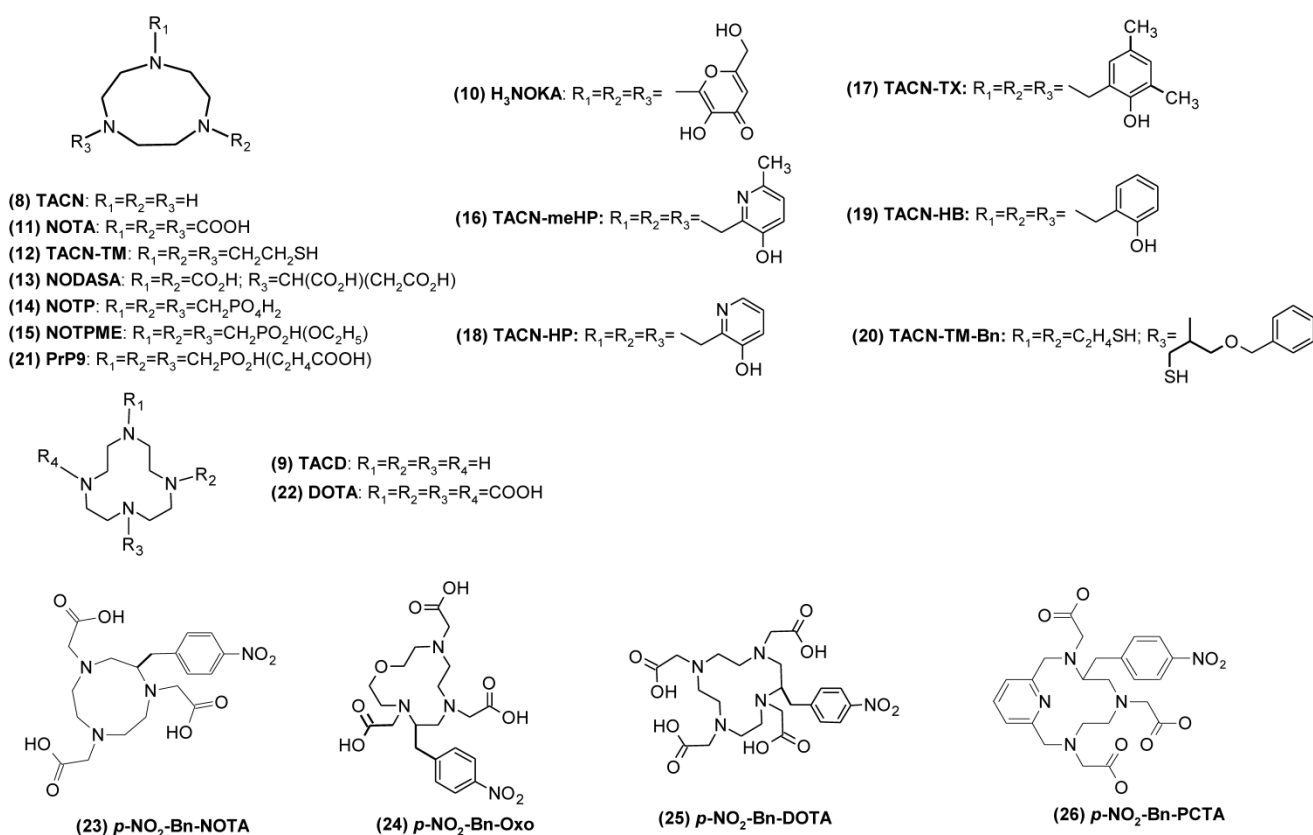


Fig 5. Examples of TACN and TACD based mono- and bifunctional chelators.

The most promising and thoroughly investigated group of chelators is based on TACN and functionalized with phosphinic acid pendant arms. In particular, chelates with basic structure of *N,N',N''*-trisubstituted triazacyclononane with methyl(2-carboxyethyl)phosphinic acid pendant arms (PrP9 or TRAP-Pr (21)) were synthesized for the fast complexation with ⁶⁸Ga [122]. The labelling was possible at ambient temperature and as extreme pH as 1. The complexes were thermodynamically stable ($\log K=26.24$) and kinetically inert. TRAP-Pr was activated with propargylamine for the further conjugation with cyclo(RGDfK) at each pendant arm of the chelate resulting in RGD-trimer [123]. The *in vivo* performance of [⁶⁸Ga]Ga-TRAP-(RGD)₃ was evaluated in athymic nude mice bearing M21 ($\alpha_v\beta_3$ -positive) and M21L (low expression of $\alpha_v\beta_3$) human melanoma xenografts. The uptake could be fully blocked by excess of unlabelled precursor. Another derivative of TRAP possessing just one site for bioconjugation (NOPO) was conjugated with cyclo(RGDfK) and NOC and labelled with ⁶⁸Ga with as high SRA as 5.6 GBq/nmol [124]. The uptake of [⁶⁸Ga]Ga-NOPO-RGD and [⁶⁸Ga]Ga-NOPO-NOC respectively in M21 and AR42J mouse xenografts was specific. NOPO demonstrated highly selective complexation with Ga(III) as compared to Fe(III) and Zn(II) [124]. The higher com-

plexation selectivity of TACD derivatives for Ga(III) as compared to In(III) and Al(III) has also been demonstrated using DOTA-TOC [25]. A comprehensive and thorough investigation has been conducted on the influence of Zn(II), Cu(II), Fe(III), Al(II), Ti(IV), and Sn(IV) on the incorporation of ⁶⁸Ga(III) into NOTA, DOTA (22), TRAP, TRAP-Pr, and NOPO as well as their peptide conjugates [125]. The selectivity of TRAP, TRAP-Pr, and NOPO towards Ga(III) was considerably improved as compared to NOTA and DOTA. This is particularly very critical with regard to Fe(III) and Zn(II). The structural investigation of the underlying mechanism of the coordination revealed formation of various complex diastereoisomers [126]. The presence of phosphinic acid pendant arms improved the ligand coordination ability and resulted in fast complexation in acidic media. The complexes were kinetically inert in acidic as well as basic solutions. The broad range of pH at which the complexation reaction can take place would allow avoiding the pH 3-7 when the Ga(III) is insoluble and thus omit the use of buffers that contribute to cation contamination. This might improve the complexation efficiency and thus SRA. In addition the stability constant for [⁶⁸Ga]Ga-DOTA was recently revised and demonstrated to be even higher ($\log K = 26$) than previously known ($\log K = 21.33$) [127].

Four bifunctional macrocyclic chelators have been investigated with the objective to compare their labelling chemistry and *in vivo* stability and clearance [128]. Nine and twelve member rings were considered, namely 1,4,7-triazacyclononane-1,4,7-triacetic acid (*p*-NO₂-Bn-NOTA (23)), 1-oxa-4,7,10-triazacyclododecane-4,7,10-triacetic acid (*p*-NO₂-Bn-Oxo (24)), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (*p*-NO₂-Bn-DOTA (25)) and 3,6,9,15-tetraazabicyclo [9.3.1]pentadeca-1(15), 11,13-triene-3,6,9-triacetic acid (*p*-NO₂-Bn-PCTA (26)) (Figure 5). The labelling was more efficient for *p*-NO₂-Bn-NOTA, *p*-NO₂-Bn-PCTA, and *p*-NO₂-Bn-Oxo as compared to *p*-NO₂-Bn-DOTA. However, *p*-NO₂-Bn-Oxo was unstable *in vivo*. *p*-NO₂-Bn-NOTA cleared rapidly from the blood and muscle but had 5-fold higher uptake in kidneys.

A novel approach providing a chelator, protoporphyrinIX (PPIX), that can be used both for *in vitro* fluorescence microscopy and *in vivo* PET or SPECT imaging has an advantage of using exactly the same molecule [129]. PPIX was conjugated to RGD peptide sequence and ⁶⁸Ga-labelled under microwave heating. The resulting probe was evaluated in MDA-MB-435 cancer cell line expressing integrin receptors, and demonstrated binding specificity. Porphyrins specifically accumulate in tumour tissue and five various ⁶⁸Ga-labelled analogues were suggested as imaging agents [130]. Preliminary results indicated accumulation in DS sarcoma tumour in rat, however the mechanism of the uptake requires further investigation [130].

Another class of macrocyclic chelators is bridged or macrobicyclic [131]. The structure of [Ga-(1-NH₃-8-NH₂-sar)]⁴⁺ (where sar =3,6,10,13,16,19-hexaazabicyclo[6.6.6.]icosane) was determined by X-ray crystallography as distorted octahedral with six nitrogen atoms. The chelator was coupled to two RGDfK moieties. The resulting conjugate was labelled with ⁶⁸Ga at elevated temperature (85 °C). The specific uptake was observed *in vivo* in 66c14β3 xenografts in mice.

In summary, even though open chain chelators serve the aim of rapid chelation at room temperature they have it difficult to compete with macrocyclic chelators based on TACN and TACD that provide stability, selectivity, fast association kinetics and extremely slow dissociation kinetics as well as high thermodynamic stability. It should also be mentioned that the advent of macrocyclic chelators was one of the critical factors influencing the broader demand for ⁶⁸Ga and its versatile applications. The current improvement in macrocyclic design is substantial, and mechanistic as well as structural investigation of coordination chemistry provides knowledge for more

efficient and diverse imaging agent development and radiopharmaceutical routine production. The development of mono- and bifunctional chelators will direct towards fast complexation and room temperature, broad working pH range and high SRA. Although TACN derived chelators offer an advantage over TACD with respect to fast complexation kinetics at room temperature and higher selectivity there are more aspects to take into consideration such as theranostics applications where it is desirable that both diagnostic and therapeutic radionuclides can be coordinated by the same chelator moiety.

Conjugation chemistry

The most common and commercially available BFC comprise esters (*p*-nitrophenyl, pentafluorophenyl, *N*-hydroxysuccinimide, sulfo-*N*-hydroxysuccinimide), isothiocyanates, maleimides, hydrazides, α -haloamides for the reaction with nucleophilic functional groups (-NH₂, -SH, -OH) of vector molecules and formation of amide, urea, thiourea, Schiff-base, or thioester bond [2]. Methods have been developed for the conjugation with peptides by solid-phase peptide synthesis (SPPS) resulting in defined position and number of chelate moieties. The outcome of the conjugation in solution wherein peptides and proteins comprise several reactive sites is very often a mixture of bioconjugate molecules with various content of the chelator. Such heterogeneity may cause the interpretation ambiguity of the performance of such imaging agents. Regioselective conjugation to antibodies was achieved by enzymatic reaction with lysine and glutamine residues using bacterial and human tissue transglutaminase as catalysts [132].

Milder conditions, faster reaction, and lower concentration of macromolecules were achieved by 6π-Azaelectrocyclization using unsaturated (E)-ester aldehydes with 1,2-dihydropyridines [133] and Cu^I-catalyzed azide-alkyne cycloaddition [134-136]. The “click chemistry” using *N*- ϵ -azido derivatives of the peptides (mono-, di-, tetrameric-cyclo(Arg-Gly-Asp-D-Phe-Lys)) allowed efficient production of the respective bioconjugates [135]. The alkyne derivative of DOTA-tris-*tert*-butyl ester was coupled to folate - γ -(4-azido)butane amide by similar chemistry [137]. A versatile two stage click ligation method based on Cu^I-catalyzed 1,3 dipolar addition and a thio acid/sulfonylazideamidation (“sulfo-click”) was developed for an efficient synthesis of dimeric peptide agents with various spacer lengths between the peptide units [138]. A novel Cu-free click chemistry method has been developed for the conjugation of chelators functionalised with monofluorocyclooctyne to azide-modified peptide in aqueous solution at room temperature [139]. Divalent bifunc-

tional chelators based on DO2A and functionalized with 4-aminobenzyl, 4-isothiocyanobenzyl and 4-maleimidobenzyl groups were developed for the synthesis of bivalent imaging agents [140].

In summary, a new technique, so called “click chemistry”, has been developed for the synthesis of precursors for chelator-mediated radiometallabelling. The reactions are fast, regioselective, require small concentration of reagents and take place under mild conditions often in aqueous solution at room temperature.

Current status and directions of ^{68}Ga -based imaging agent development

^{68}Ga -based imaging agents comprising small molecules, large biomolecules, and particles have been explored for the imaging and quantitation of various physiological disorders and biological functions. The molecular imaging of oncological diseases have been investigated most extensively targeting receptors (e.g. G-protein coupled receptor family and human epidermal growth factor receptor (HER) family, folate and urokinase receptors), enzymes, antigens as well as visualizing downstream biological processes such as angiogenesis, hypoxia, proliferation, apoptosis, glycolysis. Probes for non-targeted imaging of pulmonary and myocardial perfusion and ventilation as well as targeting imaging of inflammation, infection, and mRNA have also been considered and fundamental exploration is ongoing.

Oncological application

Various approaches have been developed for the imaging of certain biological processes involved in cancer diseases with receptor imaging being most thoroughly investigated. There are currently few ^{68}Ga -based imaging agents in routine clinical practice and clinical studies however the number of applications is increasing with acceleration. Somatostatin analogues are already established in clinical routine. Imaging feasibility of GRPR, GLP-1R, MSH, HER2 as well as prostate-specific membrane antigen, osteoblastic bone metastases, amino acid uptake, glucose transport, angiogenesis has been clinically demonstrated in patients [7, 40, 141-145]. Extensive basic research is conducted on the development of ^{68}Ga -based probes for the imaging of CCK, CXCR, MSH, gonadotropin releasing hormone (GnRH), NT, folate, integrin, vascular endothelial growth factor (VEGF), urokinase receptors as well as enzymes, antigens, and multidrug resistance (MDR1) P-glycoprotein (Pgp). Imaging probes for visualizing such processes in tumours as proliferation, apoptosis, hypoxia, glycolysis, and angiogenesis as well as bone metastases have also been investigated. A number of

particulate agents have been developed for the delivery of imaging reporters or therapeutic agents to the tumour target.

Imaging of G-protein coupled receptor family

Somatostatin receptor imaging

The expression of SSTRs has been found in neuroendocrine tumours, small cell lung cancer, renal cell carcinoma, malignant lymphoma, breast cancer, and prostate cancer. PET-CT using somatostatin ligand analogues labelled with ^{68}Ga has become a new golden standard in imaging of NETs with specificity and sensitivity well above 90% and advantages over conventional radiologic and scintigraphic imaging [14, 146-149]. It is the most pronounced example of theranostics [6, 7, 150].

Extensive basic research has been conducted on the development and biological validation of analogues varied in peptide sequence, size and number of peptide rings, chelator (DFO, DTPA, DOTA, NOTA, and their derivatives) and radiometal (Ga, Y, Tc, In, Lu) type. Their receptor binding affinity, internalization and biodistribution have been shown to be dependent on the chemical modifications [2]. Fast tumour localization, blood clearance, and renal excretion are typical characteristics of clinically used [^{68}Ga]Ga-DOTA-TATE, [^{68}Ga]Ga-DOTA-TOC, [^{68}Ga]Ga-DOTA-1-Nal³-octreotide ([^{68}Ga]Ga-DOTA-NOC). Structure activity relation studies allowed fine tuning for the agent properties such as receptor affinity, in vivo stability, biodistribution, pharmacokinetics, excretion pathway, and kidney uptake, and pharmacological activity [138, 151-155].

Consistently higher uptake of antagonist SSTR ligand as compared to agonist counterpart was found in forty-eight SST2-positive human tumour frozen sections in vitro [156]. Similar peptide sequences, p-Cl-Phe-cyclo(D-Cys-Tyr-D-Aph(Cbm)-Lys-Thr-Cys)D-Tyr-NH₂ (where d-Aph(Cbm) is d-4-aminocarbonyl-phenylalanine) (LM3), p-NO₂-Phe-cyclo(D-Cys-Tyr-D-Aph(Cbm)-Lys-Thr-Cys)D-Tyr-NH₂ (JR10), and Cpa-cyclo(D-Cys-Tyr-D-Aph(Cbm)-Lys-Thr-Cys)D-Tyr-NH₂ were conjugated to DOTA and NODAGA [157, 158]. The agents demonstrated antagonistic properties with higher affinity of NODAGA counterparts. Clinical case report with ¹¹¹In-labelled SST analogues demonstrated higher detection rate for the antagonist counterpart [159]. This fact opens possibility for the use of SST analogues not only for the diagnosis of neuroendocrine tumours but also breast carcinomas, renal cell carcinomas, non-Hodgkin lymphomas that express SSTR2 to lesser extent.

The feasibility of quantitation of SSTR density has been demonstrated both preclinically [25, 160] and clinically [24] using [^{68}Ga]Ga-DOTA-TOC. Although a ten-fold higher affinity for the SSTR₂ had been demonstrated for DOTA-TATE as compared to DOTA-TOC *in vitro* in transfected cell cultures [161], no statistically significant difference between [^{68}Ga]Ga-DOTA-TOC and [^{68}Ga]Ga-DOTA-TATE uptake could be observed *in vitro* in monkey brain tissue sections or *in vivo* in rat organs expressing SSTRs (pituitary, adrenal, pancreas) [103]. Moreover, clinical study involving 40 patients did not verify the 10-fold higher affinity for the SSTR₂ of [^{68}Ga]Ga-DOTA-TATE, on the contrary, standardized uptake value (SUV_{max}) of [^{68}Ga]Ga-DOTA-TOC tended to be higher [162]. Another aspect investigated preclinically is the influence of treatment with octreotide and interferon- α (IFN α) used for biotherapy of NETs on the uptake of [^{68}Ga]Ga-DOTA-TATE. The exposure of the animals to the cold octreotide did not enhance the uptake of the tracer while IFN α did, however the mechanism of the observation was not clear [163, 164]. Gene therapy protocols were optimized on the basis of *in vivo* imaging ([^{68}Ga]Ga-DOTA-TATE) of gene expression and quantitative monitoring of gene transfer [165].

[^{68}Ga]Ga-DOTA-TOC (27), [^{68}Ga]Ga-DOTA-TATE (28) and [^{68}Ga]Ga-DOTA-NOC (29) (Figure 6) are the most commonly used analogues in clinical studies [2, 166, 167]. Their pharmacokinetics, blood clearance and target localization rate are compatible with half-life of ^{68}Ga . Renal excretion, short scanning time, high sensitivity and resolution assure high contrast and quality images over organs of interest as well as accurate quantitation. Relatively low radiation

dose is one more advantage that should be mentioned. They served for diagnosis, staging, prognosis, therapy selection and response monitoring of NETs and other types of cancers and diseases. [^{68}Ga]Ga-DOTA-TATE was compared with [^{68}Ga]Ga-DOTA-NOC in 20 patients in terms of detection rate and SUVs [168]. The agents had comparable diagnostic accuracy with higher SUV_{max} for the former. One more analogue, [^{68}Ga]Ga-DOTA-2-Nal, Tyr³, ThrNH₂⁸-octreotide (DOTA-lanreotide, DOTA-LAN) was successfully used for lung and thyroid tumour detection [169].

The individualized diagnosis has been practiced in the selection of patients for PRRT, target definition for fractionated stereotactic radiotherapy (FSRT) planning, target volume delineation for intensity modulated radiotherapy [170-174]. The diagnosis on the cellular and molecular level and determination of the disease associated biomarkers provides basis for the treatment optimization and efficacy for a particular patient [175]. The personalized therapy planning necessity was demonstrated in retrospective study of ten patients examined with [^{68}Ga]Ga-DOTA-TATE [176]. It was concluded that the radiotherapeutical dose should be determined by the tumour burden since the latter influences the radioactivity distribution to the healthy organs, and in particular the higher burden decreases the radiation accumulation in the kidney. The starting point of PRRT after preceding cold octreotide therapy in patients with NETs could be determined [177]. These imaging agents improved the detection rate and diagnostic accuracy [178, 179]. They were used for therapy planning and monitoring response to treatment [180], as well as influenced and changed the therapeutic course [181-184].

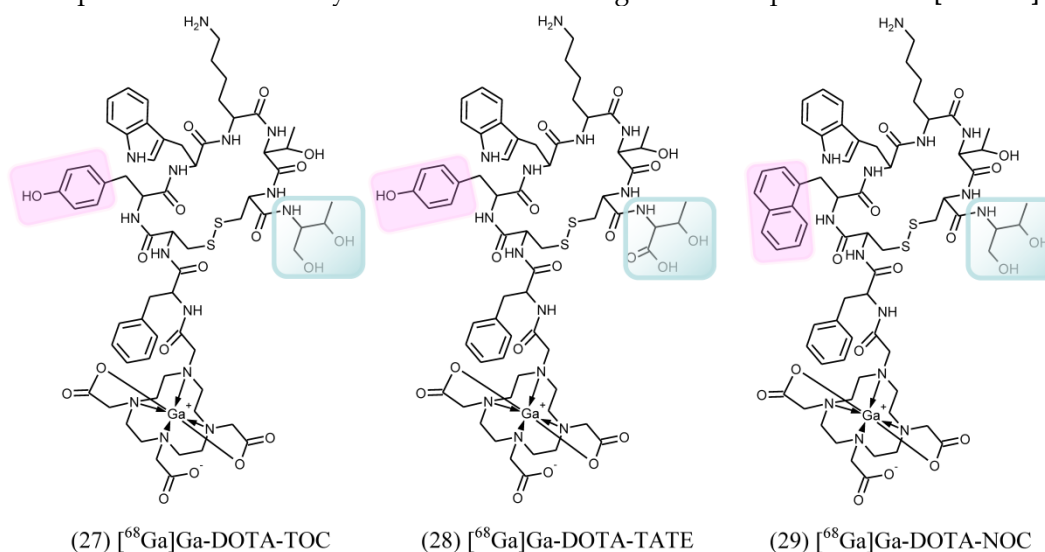


Fig 6. Structural formulae of the clinically used somatostatin analogue imaging agents. TOC stands for D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr(OH); TATE stands for D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr; and NOC stands for D-Phe-Cys-Nal-D-Trp-Lys-Thr-Cys-Thr(OH). The differences in structures are highlighted.

The majority of the clinical studies have been conducted in the field of GEP NETs however considerable number of other indications has been reported demonstrating wider application possibilities. [⁶⁸Ga]Ga-DOTA-NOC was successfully used for the assessment of NETs [185], well-differentiated medullary thyroid carcinoma (MTC) [186], bronchial carcinoid (BC) and Von-HippelLindau (VHL) disease [187], alveolar rhabdomyosarcoma with neuroendocrine differentiation [188], carotid body chemodectomas (CBCs) [189], idiopathic pulmonary fibrosis [190]. [⁶⁸Ga]Ga-DOTA-TOC was found valuable for therapy planning and accurate diagnosis of multiple endocrine neoplasia [184], sudden onset of vision problems [183], duodenopancreatic NET [182], autoimmune thyroid disease like Graves' disease and Hashimoto's disease [191]. Patients affected by prostate cancer with bone metastases were examined with [⁶⁸Ga]Ga-DOTA-TOC [192]. However, due to the low expression of SSTR₂ and SSTR₅ the uptake in the lesions was low and the signal was weak. Even broader areas were covered by [⁶⁸Ga]Ga-DOTA-TATE. Benign and malignant thrombi were distinguished in a patient affected by pancreatic NET tumour with hepatic and regional lymph node metastases [193, 194]. It was found relevant for the diagnosis and therapy planning for meningiomas [195], recurrent medullary carcinoma [196], plaque imaging and characterization in the coronary arteries [197]. In addition, the usefulness of ⁶⁸Ga-labelled somatostatin analogues has been discussed in the context of other available imaging techniques for staging of lung neuroendocrine cancer and therapy selection [147] as well as for the evaluation of Hurthle cell thyroid carcinoma [198], and neuroectodermaltumours [167].

The large number of clinical applications of somatostatin ligand analogues triggers investigations that refine the examination protocols and interpretation of results. Somatostatin receptors are expressed also physiologically in healthy organs and the awareness of that is very crucial for correct interpretation of examination results, and possible causes for false negative or positive findings should be kept in mind. Distribution of [⁶⁸Ga]Ga-DOTA-TATE and organ uptake (SUV) in healthy volunteers has been studied and compared with *in vitro* findings [199]. The organs with physiological expression of SSTR₂ as well as metabolic and excretion sites (pituitary, adrenals, pancreas, spleen, thyroid, stomach wall, liver, salivary glands, bowel, pelvic/colic system, kidneys, urinary bladder) demonstrated distinguished uptake. Forty-three patients with NETs were examined with [⁶⁸Ga]Ga-DOTA-TOC in order to distinguish pathological and physiological uptake of the head of pancreas [200]. The uptake that was similar to that of liver

was considered physiological. The findings stressed importance of accurate quantitation of the uptake for the avoidance of false-positive diagnosis. The uptake in the head of pancreas was also considered physiological in the retrospective study of 100 patients [201] and specifically designed study of 96 patients [202] since the accumulation was stable over time regardless of the uptake intensity or shape. [⁶⁸Ga]Ga-DOTA-TOC scans of 165 patients with various thyroid pathologies were analyzed [203] and in 8 cases of normal thyroids with increased uptake, follow-up examination after 6-14 months did not show any thyroid pathology. Another case study using [⁶⁸Ga]Ga-DOTA-TOC reported on the uptake in wandering spleen which could mistakenly be interpreted as malignant process instead of physiological expression of SSTR₂ in spleen in an unusual position [204].

The option to quantify the disease underlying processes and provide personalized diagnosis and therapy is one of the crucial advantages of PET-CT. Various methodologies and approaches are being considered with the aim to assure accuracy and unambiguity of the quantitation. The uptake of [⁶⁸Ga]Ga-DOTA-NOC measured in SUV_{max} correlated with pathologic features and was suggested as a prognostic tool characterizing stable disease with SUV_{max} cutoff value ranging from 17.9 to 19.3 [205]. The quantitation of diagnostic procedures is getting refined. The correlation between [⁶⁸Ga]Ga-DOTA-TOC (SUV) and SST₂ mRNA was investigated in 120 patients and a novel normative database was created for the accurate quantitative evaluation. It may improve diagnostics, treatment monitoring and therapy of SST expressing tumours or inflammation on a molecular basis [206]. In another study the evaluation of the response to the radiotherapeutic treatment was conducted using tumour-to-spleen SUV ratio which was found more accurate measure than solely SUV_{max} [180]. Treatment with cold somatostatin analogue did not reduce the binding of [⁶⁸Ga]Ga-DOTA-TATE, but improved the tumour-to-background ratio [207]. The detection rate of pre-therapeutic [⁶⁸Ga]Ga-DOTA-TATE/PET-CT was found higher as compared to sequential planar scans with [¹⁷⁷Lu]Lu-DOTA-TATE (9% of the lesions not detected) [208]. Most importantly the highest detection concordance was observed at later time point (72h) thus indicating the potential of [⁶⁸Ga]Ga-DOTA-TATE for pre-therapeutic dosimetry and dose planning.

The superiority of ⁶⁸Ga-labelled somatostatin analogues in terms of specificity, sensitivity, staging accuracy, detection rate, quantitation, acquisition time to [¹⁸F]-FDG [210], [¹⁸F]F-DOPA [211],

^{123}I -metaiodobenzylguanidine (^{123}I -MIBG) [212, 213], and [^{111}In]In-octreotide (Octreoscan®) (Figure 7) [209], [^{18}F]-NaF and [$^{99\text{m}}\text{Tc}$]-dicarboxypropanediphosphonate [214] has been demonstrated. Advantage of ^{68}Ga /PET-CT in terms of detection rate over ultrasonography, CT, MRI, and skeletal scintigraphy was confirmed in over 5800 clinical cases [7].

Further unique application is the use of hand-held gamma probe in combination with either [^{68}Ga]Ga-DOTA-TATE or [^{68}Ga]Ga-DOTA-NOC intraoperatively in order to localize metastases and primary tumours [215]. The surgery was conducted 30 min after agent injection. One of the most significant advantages of the radioguided surgery (RGS) was the differentiation of scar tissue from small tumour metastases. RGS resulted in change in the operative procedure in 56%. In addition, overexpression of SSTR has been found in patients with inflammatory bowel diseases, pulmonary diseases, rheumatoid arthritis, Sjogren's syndrome, Grave's disease [43, 216].

Imaging gastrin releasing peptide receptors

Breast, prostate, gastrointestinal, and small cell lung cancers overexpress bombesin receptors, specifically gastrin releasing peptide (GRP) receptors. An efficient diagnostic tool for the staging of prostate cancer specifically metastasis spreading to soft tissue is required in order to avoid the understaging or overtreatment of prostate cancer. Around 300 studies

on the development of corresponding ligands labelled with ^{68}Ga [74, 217-219], ^{18}F , ^{64}Cu , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{111}In , ^{125}I , $^{185/187}\text{Re}$, ^{177}Lu have been conducted preclinically as well as clinically in patients. Twelve clinical studies several of which utilizing ^{68}Ga were performed using analogues with agonistic function [143]. [^{68}Ga]Ga-DOTA-PEG₂-[D-Tyr⁶,βAla¹¹,Thi¹³,Nle¹⁴]bombesin (6-14) ([^{68}Ga]Ga-BZH₃) demonstrated high *in vivo* stability and uptake in a pancreatic carcinoma model [217, 220] and gastrointestinal stromal tumour (GIST) [142] in patients, however detection rate was higher for [^{18}F]-FDG. Nonetheless the quantitation of [^{68}Ga]Ga-BZH₃/PET-CT uptake in patients with recurrent gliomas allowed differentiation between low- and high-grade gliomas thus demonstrating the advantage over [^{18}F]-FDG [221]. Although tumours with smallest ones of 5 mm were detected in patients affected by prostate cancer using [^{68}Ga]Ga-DOTA-BOM already 15-25 minutes post injection, the nonspecific radioactivity accumulation in the upper abdomen presumably in pancreas deteriorated the tumour detection in that area [222]. Comparative study of [^{68}Ga]Ga-DOTA-CHCO-Gly-4-aminobenzyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH ([^{68}Ga]Ga-AMBA) and [^{18}F]FCH in PC xenograft-bearing mice revealed superior performance of the former in terms of tumour uptake and image contrast [223].

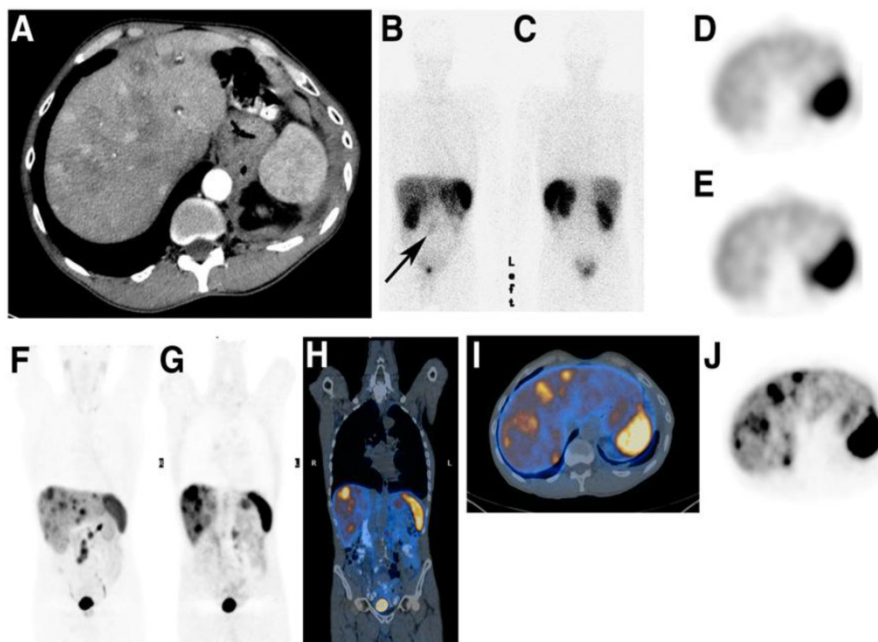


Fig 7. A 69-y-old man with low grade metastatic midgut NET. (A) Arterial-phase CT shows multiple arterially enhancing and low-attenuation liver metastases. (B and C) Anterior and posterior whole body ^{111}In -DTPA-octreotide scintigraphy shows low grade (Krenning score, 1) mesenteric metastases (arrow) but no liver metastases. (D and E) Axial ^{111}In -DTPA-octreotide SPECT at level of spleen shows heterogeneous liver uptake with no discernible liver deposits. (F) Maximum-intensity-projection [^{68}Ga]Ga-DOTA-TATE PET shows multiple liver deposits in liver and mesentery. (G) Coronal [^{68}Ga]Ga-DOTA-TATE PET anterior to kidney shows multiple liver metastases. (H) [^{68}Ga]Ga-DOTA-TATE PET of G. (I) Axial [^{68}Ga]Ga-DOTA-TATE PET at level of spleen shows multiple liver metastases. (J) Axial [^{68}Ga]Ga-DOTA-TATE PET at level of spleen shows multiple liver metastases. Reproduced by permission of SNMMI from [209].

Although bombesin-based tracer agonists have demonstrated high binding affinity to GRPRs and good radioligand internalization, they have strong potency and mitogenicity even in trace amount as well as high and maintained uptake in the normal GRPR positive organs that results in poor contrast of images and radiation dose to normal tissue. In order to exclude the agonistic function and related side effects, a bombesin antagonist was developed and showed higher uptake compared to the agonist counterpart [224]. The biodistribution in PC-3 and LNCap tumour-bearing nude mice of ^{68}Ga -labelled DOTA-4-amino-1-carboxymethyl-piperidine-D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH₂ was studied in order to investigate the difference between androgen-dependent and androgen-independent tumours. The pharmacokinetics was similar however the uptake in PC-3 tumour was higher. In the further development

DOTA-4-amino-1-carboxymethyl-piperidine was substituted with NODAGA and ^{68}Ga -labelled counterpart demonstrated high contrast tumour accumulation in PC3 tumour-bearing nude mice, however uptake in abdomen organs was also high at 1 h time point [225]. The excretion pathway of these series of analogues was modulated and shifted from the liver by introducing NOTA bifunctional chelator via PEG₂ linker [226]. One carboxylic arm of NOTA was used for the conjugation thus resulting in positively charged [^{68}Ga]-NOTA complex moiety. The modification resulted in improved biodistribution pattern and image contrast. Heterodimeric peptide ligand for imaging of breast cancer comprising RGD and bombesin motifs (RGD-BBN) was designed in order to compensate for the variable GRPR and $\alpha_v\beta_3$ receptor density in estrogen dependent and estrogen independent breast cancers and demonstrated higher tumour uptake in mice than monomeric BBN did [227]. In another report the performance of the heterodimeric [^{68}Ga]-Ga-NOTA-RGD-BBN was compared to both [^{68}Ga]-Ga-NOTA-RGD and [^{68}Ga]-Ga-NOTA-BBN in a PC3 tumour model revealing higher tumour uptake [228].

Bombesin receptor ligand analogues have been used in clinical studies however the high potency of agonists brings up challenges requiring synthesis with high specific radioactivity in order to exclude pharmacological effects. Antagonists even though they do not internalize have shown improved imaging capabilities. Given recent achievements in the design of antagonists and experience with SST analogues demonstrating advantages of antagonists, the future development will most likely move in the direction of bombesin antagonists.

Imaging GLP-1R, CCK, CXCR, MSH, GnRHR, NT receptors

The development of new peptide based agents is expanding with novel targets of interest. High incidence and density expression of GLP-1R has been found in insulinomas and pancreatic beta cells. Metabolically stable agonists of GLP-1R, exendin-3 and exendin-4, have been labelled with ^{68}Ga via DOTA moiety and demonstrated specific uptake in mouse tumour models as well as in pancreatic beta cells in healthy non-human primate [229-231]. Moreover, examination of a patient affected by insulinoma revealed not only multiple small liver metastases but also paraaortal lymph node lesions that could not be conclusively detected by computed tomography, ultrasound, [^{18}F]FDG/PET-CT or [^{11}C]HTP/PET-CT (unpublished data). The CCK-2 is upregulated in NETs and could complement the diagnostic means of NETs with low expression of SSTRs such as stromal tumours, medullary thyroid carcinoma (MTC), and neuroendocrine gut tumours. Linear and cyclic peptide analogues were designed and preclinically evaluated [232, 233]. Several cyclic monomeric and dimeric peptide ligands targeting chemokine receptor subtype CXCR4 which is up-regulated in human solid tumours as, for example mammary cancer, prostate cancer, melanoma, glioma, lymphoma, cervical adenocarcinoma demonstrated specific binding [234-237]. A number of ^{68}Ga -labelled α -MSH analogues have been constructed and preclinically evaluated for melanoma imaging and staging. The content, lipophilicity and charge were varied in order to optimize the retention in tumour and kidneys [139, 238-241]. Non-peptidic probe comprising benzamide derivative that specifically binds to melanin demonstrated uptake in B16F10 tumour model [242]. GnRHR expressed on the reproductive system [243, 244] and NT receptor subtype 1 involved in neoplastic processes and up-regulated lung, prostate, colon, pancreatic and breast cancers [245] were targeted by ^{68}Ga -based peptide and peptoid-peptide hybrid agents.

Human epidermal growth factor receptor (HER) family

HER family receptors are overexpressed in head-and-neck, lung, breast, colorectal, ovary, and urothelial cancer cells. A number of imaging agents labelled with ^{68}Ga has been preclinically evaluated for the visualization of the receptors: natural peptide EGFR ligand (DOTA-hEGF) [246], Affibody[®] molecules [247-249], 2-helix Affibody derivative [250], F(ab')₂ fragment of trastuzumab [251], as well as tyrosine kinase inhibitor [252]. An innovative approach where various affibody molecules were

pre-administered and allowed saturation of physiological EGFR uptake prior to the injection of [$^{67/68}\text{Ga}$]Ga-DOTA-hEGF was successfully proved with the aim to improve the image contrast [253]. Fast target localization, blood clearance and normal tissue wash out providing high image contrast as well as high kidney uptake are characteristic to various Affibody molecule based agents. A clinical imaging of metastatic breast cancer using [^{68}Ga]Ga-ABY-002 proved to be an efficient method for the non-invasive determination of HER2 status of metastases not amenable to biopsy [40]. Two-helix Affibody derivative ([^{68}Ga]Ga-DOTA-MUT-DS) [250], Herceptin fragment ([^{68}Ga]Ga-DOTA-F(ab')₂) [254], camelid heavy-chain antibody variable domain based ligand ([^{68}Ga]Ga-Df-Bz-NCS-7D12) [255] were developed from a larger molecules in order to accelerate pharmacokinetics and demonstrated compatibility with ^{68}Ga half-life time frame.

Pre-targeted imaging

Although the number of antibody based radio-immunotherapeutic agents is considerable, their use is limited by the slow pharmacokinetics and blood clearance that cause high radiation dose to the normal organs [256]. In the case of imaging the achievement of optimal image contrast might require as long as 2-4

d. In order to improve the tissue penetration and facilitate clearance and excretion, the size of an antibody was reduced to antibody fragments (F(ab')₂, Fab'), medium-sized proteins and small peptides with high affinity for the same target [2, 257]. Another method that has attracted strong interest is pre-targeting. It uses bispecific antibody (bsmAb) with two binding sites for the interaction with a cell target and a hapten molecule carrying radionuclide. First bsmAb is administered and after its localization at the target, clearance from the blood, and washout from the normal tissue, the radiolabelled hapten molecule is administered. The latter has fast pharmacokinetics so that high contrast imaging is possible within short time. Hapten molecules comprising histamine-succinyl-glycine (HSG) motif and chelate moiety (30) (Figure 8) [258-261] have been used for the imaging of carcinoembryonic antigen (CEA) pre-targeted with anti-CEA bsmAb [262-264].

Transplantation of islets of Langerhans is a promising treatment for type 1 diabetes mellitus. Avidin-covered agarose resin microbeads were used as a model for islet transplantation in mice [265, 266]. After intraportal transplantation they were visualized in hepatic volume by [^{68}Ga]Ga-DOTA-(PEG)₂-biotin (31) (Figure 9).

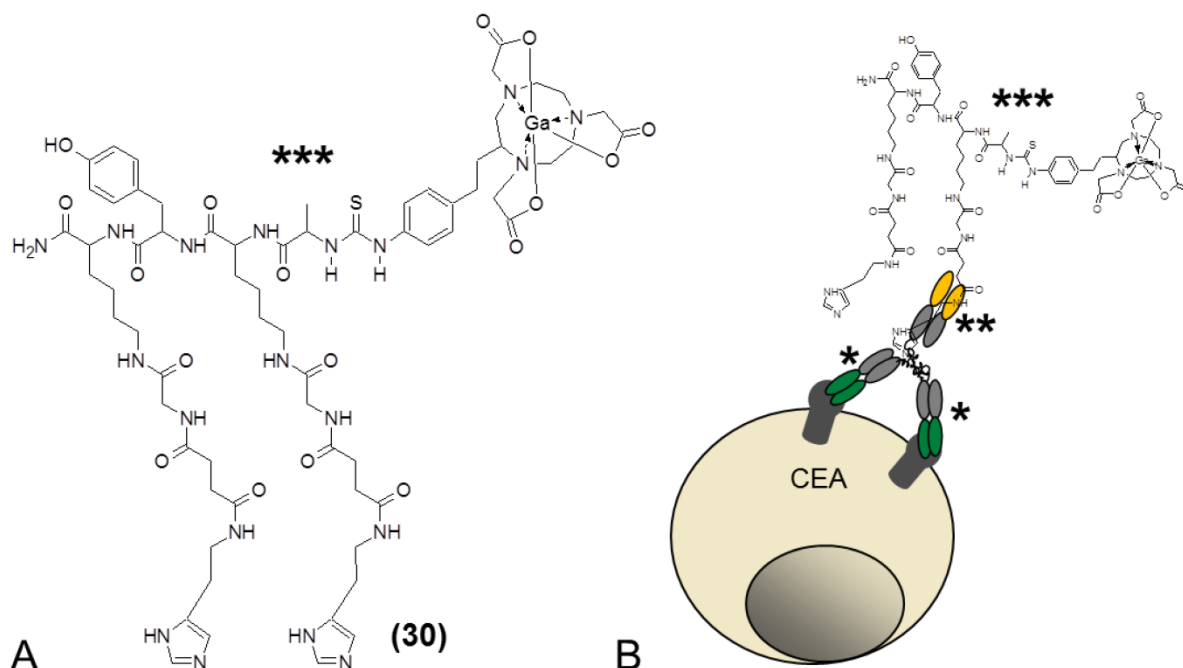


Fig 8. The principle of pre-targeting: A) An example structure of peptide molecule containing histamine-succinyl-glycine residues and coupled to NOTA chelate moiety complexed with ^{68}Ga (***) ; B) Bispecific antibody with two antigen binding F(ab')₂ arms (*) and one anti-hapten binding F(ab) arm (***) interacts with the target antigen (CEA). ^{68}Ga -labelled hapten molecule (***) binds to the bispecific antibody (***) on the next step.

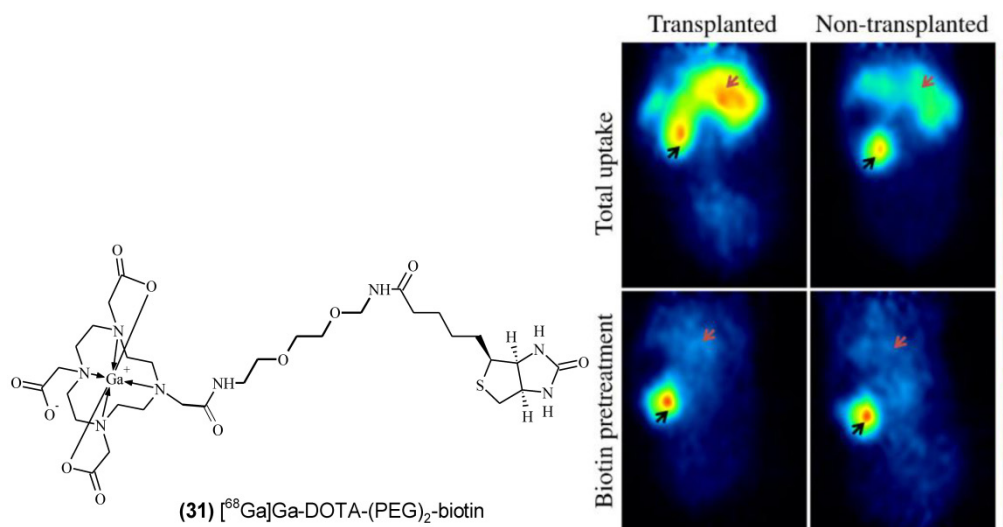


Fig 9. Left) Structure of $[^{68}\text{Ga}]\text{Ga-DOTA-(PEG)}_2\text{-biotin}$ (31) analogue used for the imaging of avidin-covered agarose resins transplanted in mice. Right) Coronal views showing total tracer uptake in left kidney (black arrows) and liver (red arrows) in transplanted (top left) and non-transplanted mice (top right). Pretreatment with unlabelled biotin displaced a majority of the tracer uptake in both transplanted (bottom left) and non-transplanted mice (bottom right). The color bar indicates SUV ranging from 0 to 6. All images are summations of the initial 20 minutes following tracer administration. Reproduced from [265].

Imaging angiogenesis

Growth of cancer cells and healing of ischemic injuries require formation of new capillaries which is known as angiogenesis process. Monitoring of angiogenetic activity would allow validation of antiangiogenic drug efficacy on early stage and on personalized basis. Molecular targets such as integrin receptors, vascular endothelial growth factor (VEGF) receptors, and matrix metalloproteinases 9 (MMP9) have been identified and the respective imaging agents were developed [2].

The most widely studied is the imaging of integrins using radiolabelled peptide ligands comprising arginine-glycine-aspartic acid (RGD) sequence for binding to $\alpha_v\beta_3$ integrin receptors [267]. The influence of the major components of the agent structure such as DOTA and NOTA chelator moiety, PEG linker as well as multivalency on the pharmacokinetics has been studied preclinically in mice with various cancer xenografts or ischemic or atherosclerotic models [80, 99, 120, 268-274]. The enhancement of the valency from mono- to di- to tetrameric cyclic RGD commonly results in increased uptake, however the mechanism is unclear [275-278]. Further optimization is required in order to find fine balance between the tumour uptake on one hand and blood clearance and kidney accumulation on the other hand. The performance of ^{68}Ga -based agents has been compared with clinically used $[^{18}\text{F}]\text{Galacto-RGD}$ and demonstrated comparable or better pharmacokinetics. Another advantage of ^{68}Ga -based agents is more efficient and cost-effective as well as simpler production chemistry [80].

Single chain VEGF (scVEGF, MW \approx 28 kDa) which is a functionally active single-chain version of VEGF [279, 280] was modified with PEG linkers in order to modulate pharmacokinetics. ^{68}Ga -labelling was accomplished via HBED or NOTA chelate moiety [117, 281]. The agents accumulated in the tumour xenografts in mice, and the further development is focused on the improvement of tumour-to-background ratio and addressing high kidney uptake.

The first studies in the development of peptide-based ligands to MMP9 proved the concept however further fundamental research is required [282]. A peptide (HWGF) probe developed for dual-labelling with ^{68}Ga and IRDye 800CW, respectively for PET and near infrared imaging, demonstrated specific binding to the metalloproteinases-2 and -9 (MMP-2/-9) [283]. The dual-labelling strategy provides the advantage of using identical agent for both optical and nuclear imaging.

Imaging hypoxia, bone metastases, proliferation, glycolysis

Small biologically active molecules have also been tagged with ^{68}Ga for the visualization of hypoxia, glucose transport, cell proliferation, and bone metastases. Although most of them are currently on pre-clinical level however these examples demonstrate potential of ^{68}Ga for the diversity of imaging agents like $^{99\text{m}}\text{Tc}$ -based radiopharmaceuticals. Biphosphonates [144, 284-287] and ^{68}Ga -labelled ethylene diamino- N,N,N',N' -tetrakis-methylene phosphoric acid [288, 289] were found useful for early diagnosis of

bone metastases. An encouraging illustration is ^{68}Ga -labelled bisphosphonate probe (BPAMD (32), Figure 10) that successfully proved the concept in a clinical examination of a patient with prostate cancer. Osteoblastic bone metastases were visualized with high contrast and detection rate (Figure 11) [144]. Analogues comprising nitroimidazole, mercapto-benzynamine or 5-nitroimidazole derivatives coupled to DOTA have been investigated for the imaging of hypoxia which is an important parameter in tumour

and myocardial ischemia physiology [290-293]. The monitoring of hypoxia could contribute to improved diagnosis, prognosis, treatment planning and validation of response to therapy. DOTA- and NOTA-coupled alanine, lysine and tyrosine analogues targeting transporters for the cell proliferation visualization showed uptake in animal tumour models [294-296]. ^{68}Ga -DOTA-2-deoxy-D-glucosamine demonstrated higher tumour-to-organ ratio as compared to ^{18}F FDG [297].

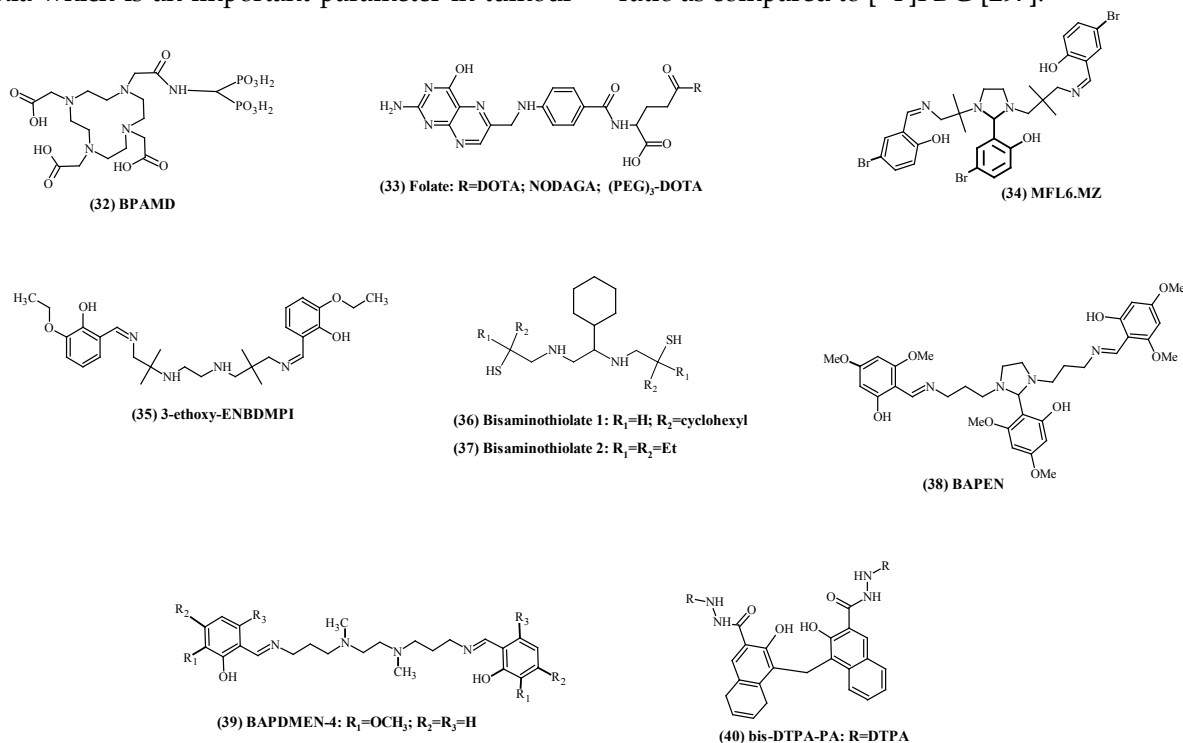


Fig 10. Low molecular weight vectors for the labelling with ^{68}Ga and subsequent imaging of bone metastases (32), folate receptors (33), MDR1 Pgp (34-35), myocardial perfusion (36-39), and necrosis (40).

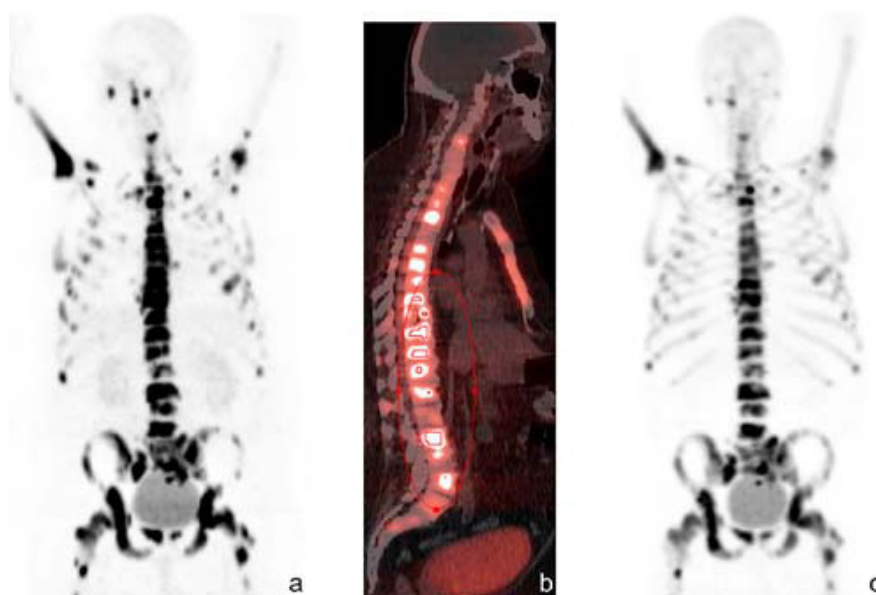


Fig 11. ^{68}Ga BPAMD was injected i.v. into a patient with known extensive bone metastases of prostate cancer, and revealed intense accumulation in multiple osteoblastic lesions in the central skeleton, ribs and proximal extremities: a) coronal PET; b) sagittal PET/CT; c) ^{18}F -fluoride PET (sagittal). Reproduced from [144].

Imaging of tumour with particulate agents

Nano- and microparticles are used as vectors to deliver imaging reporters or therapeutic agents to the target. Radiolabelled nanoparticles such as ^{68}Ga -labelled albumin nanoparticles can be used for the imaging and localization of sentinel lymph nodes in breast and melanoma cancer not only for diagnosis and prognosis but also for therapy and surgery planning [298]. Commercially available albumin nanoparticle kit ($^{99\text{m}}\text{Tc}$ Nanocoll) was labelled with ^{68}Ga , however, the biodistribution of the particles in rat revealed unexpected accumulation in lung [88]. A novel mannosylated human serum albumin (MSA) was synthesized by coupling α -D-mannopyranosyl-phenylisothiocyanate to HSA and then to NOTA active ester (NOTA-MSA) [299]. High uptake was observed in liver, spleen and femur with RES content after intravenous injection to mice, and after subcutaneous injection into mice footpads it migrated to lymph nodes. Novel particles amenable to functionalization and labelling with ^{68}Ga , e.g. organic CdSe/ZnS quantum dots (QDs) encapsulated by polysorbate 60 [300], nano-graphene oxide sheets (GOs) [301], cobalt-ferrite [302], and bifunctional zeolite Y particles [303], demonstrated promising results in mouse tumour models. GOs were chemically modified and coupled to NOTA for ^{68}Ga -labelling. The imaging agent showed specific uptake in tumour vasculature CD105 [301]. QDs functionalized with such amphiphiles as RGD-C18, NOTA-C18, mannose-C18, lactose-C18, and labelled with ^{68}Ga were specifically accumulated in U87MG human glioma xenografts in mice [300]. Micelle agents were formed by the aggregation of amphiphilic ligands comprising NOTA or DOTA coupled to an α -alkyl chain via acetate pendant arms [304, 305]. Direct proportionality was found between the agent accumulation in rat liver and the length of the pendant α -alkyl chain and thus lipophilicity of the ligands [305].

Miscellaneous imaging targets in oncology

Breast, cervical, ovarian, colorectal, nasopharyngeal, renal, and endometrial cancers express folate receptors (FR). [^{111}In]In-DTPA-folate is clinically used and motivates development of an imaging agent comprising ^{68}Ga . Folic acid has been conjugated to NOTA and DOTA (Folate (33), Figure 10) based chelators and resultant analogues were validated in mouse tumour models [306, 307]. The improvement of tumour-to-kidney ratio and protection of kidneys remains a challenging task.

An agent comprising 9 amino acid residues, DOTA and ^{68}Ga was developed for the detection of the receptors of urokinase-type plasminogen activator (uPAR) upregulated in gastric, colorectal, and breast

cancers [308]. Its uptake in human glioblastoma cancer xenograft mouse model could be observed, though tumour-to-background ratio was low.

Tria- and diabody derivatives of an antibody to epithelial cell adhesion molecule (EpCAM) expressed in tumours were constructed with the goal to accelerate the blood clearance and improve the image contrast [257]. The diabody variant labelled with ^{68}Ga via HBED-CC showed faster blood clearance and normal tissue washout as well as rapid tumour uptake in mice A-431 xenograft.

The response to therapy can be evaluated by monitoring expression of multidrug resistance (MDR1) P-glycoprotein (Pgp). ^{68}Ga -labelled hexadentate Schiff base [309] (MFL6.MZ (34), Figure 10) and [^{68}Ga]Ga-(bis(3-ethoxy-2-hydroxy-benzylidene)-*N,N'*-bis(2,2-dimethyl-3-propyl)ethylenediamine) [^{68}Ga]Ga-ENBDMPI [310] (3-ethoxy-ENBDMPI (35), Figure 10) were used for the imaging of Pgp activity dependent on the extracellular acidosis. [^{68}Ga]Ga-ENBDMPI demonstrated better balance between uptake and inhibition in Pgp-positive tumour cells [310].

Annexin A5 was used for the development of a probe for the imaging and quantitation of apoptosis in the evaluation of therapy response [311]. Regiospecific coupling of the protein to DOTA maleimide was achieved via Cys² and Cys¹⁶⁵ amino acid residues thus maintaining the active binding sites intact. The resultant bioconjugate labelled with ^{68}Ga demonstrated statistically higher uptake after cancer therapy in mouse model of hepatic apoptosis.

Rapid progress has taken place in imaging of prostate cancer reaching clinical studies within short period of time. The urea-based inhibitor of the prostate-specific membrane antigen (PSMA) [312] was modified in order to adjust all components required for the strong interaction with PSMA. For one of the analogues HBED-CC was added as a lipophilic side chain to the hydrophilic pharmacophore Glu-NH-CO-NH-Lys for the interaction with the active binding site of PSMA. This analogue demonstrated enhanced internalization in LNCaP cells in vitro and high accumulation in mice LNCaP tumour xenografts. Thorough study on structure-activity relation has been performed by [313]. [^{68}Ga]Ga-HBED-CC-Lys-NH-CO-NH-Glu was taken further to the clinical study and compared with [^{18}F]FECH [145]. Prostate carcinoma relapses could be clearly visualized only by ^{68}Ga -agent. The biodistribution in 37 patients demonstrated uptake in kidneys, salivary glands, lacrimal glands, liver, spleen, and bowel (Figure 12) [314]. The lesions were detected with excellent contrast already at 1 h p.i. even at low PSMA levels.

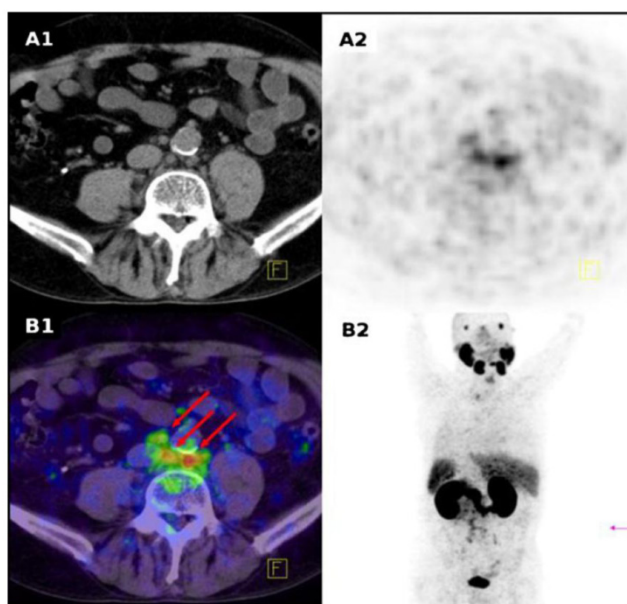


Fig 12. ^{68}Ga -PSMA PET/CT of patient 22 who received the lowest dose of radiotracer (52 MBq). Red arrows point to several small lymph nodes with clearly visible pathological tracer uptake. A1 CT, A2 PET, B1 fusion of PET and CT, B2 MIP. Reproduced from [314].

^{68}Ga -based agents for imaging of thrombosis, atherosclerosis, intracoronary radiation therapy, imaging agent for the diagnosis of hepatobiliary and renal disorders, mitochondrial-targeting imaging of cancer were suggested earlier [2]. However, to my best knowledge no related studies have been published during last five years.

Antisense oligonucleotide based tracers

A number of ^{68}Ga -labelled antisense oligonucleotide agents were developed for *in vivo* imaging of gene expression and tumours that express ras oncogene point mutations [2, 315]. The oligonucleotides varied in size (15-30 mer), sequence and basic structure: phosphodiester, phosphorothioate, 2'-O-methyl phosphodiester, locked nucleic acid (LNA), LNA-DNA mixmer (LNA and DNA nucleotides in alternation along the sequence), and peptide nucleic acid [316-320]. The uptake of the ^{68}Ga -labelled analogues in non-hybridization specific tissue was most likely mediated by scavenger receptors. The rat distribution pattern of ^{68}Ga -labelled oligonucleotides varied and was influenced by sequence and length rather than chelator type [315, 321]. The mechanism of the oligonucleotide agent uptake is complex and not understood, and more fundamental research is required in order to elucidate the applicability of this category of vectors for imaging purposes.

Myocardial perfusion, necrosis, and lung ventilation

Imaging agents for myocardial perfusion were

constructed using either small lipophilic chelators or particles. Lipophilic and cationic gallium complexes with bis- and tris(salicylaldimine) and bisaminothiolate (36, 37) (Figure 10) analogues have shown myocardial uptake and retention in rodents. However, the optimization of the lipophilicity of the agents so that the higher uptake in the heart is not accompanied by the high uptake in the liver is very challenging. Highly lipophilic ^{67}Ga Ga-bisaminothiolate complex was accumulated during the first-pass extraction with high uptake in the healthy heart, but not in the permanently legated coronary artery of infarct Sprague-Dawley rat heart [322]. Still the imaging quality was deteriorated by high liver uptake. Tris(4,6-dimethoxysalicylaldimin-*N,N'*-bis(3-aminopropyl)-*N,N'*-ethylenediamine (BAPEN (38), Figure 10) [323], hexadentate bis(salicylaldimine) ligands (BAPDMEN (39), Figure 10) [324] with variable aromatic ring substituents for lipophilicity modulation have also demonstrated hepatobiliary excretion. The distribution in rat varied dependent on the imaging agent and animal species. Four analogues of BAPEN with varied substituents on the salicyl ring and a number of salicyl rings that were labelled with ^{68}Ga and tested in pigs at rest and under adenosine stress did not reveal correlation with myocardial perfusion measured with ^{15}O water and their myocardial accumulation was not determined by perfusion [325].

Various human serum albumin (HSA) based nano- and microparticles have been developed for the lung function monitoring by measuring pulmonary and myocardial perfusion as well as vascular permeability [2]. The first studies on ^{68}Ga -labelled HSA microspheres were conducted in the 1970s but nowadays the interest is revitalized. The labelling with ^{68}Ga was accomplished either by co-precipitation with the commercially available macroaggregated albumin (MAA) for $^{99\text{m}}\text{Tc}$ -labelling [88, 89] or by complexation with DOTA chelator attached to HSAM via surface lysine amino groups and *p*-SCN-Bn-DOTA [326, 327]. Simultaneous formation of MMA and its ^{68}Ga -labelling under microwave heating resulted in stable agent that was successfully tested in a piglet model of pulmonary embolism [87]. ^{68}Ga Ga-MAA has been used to quantify spatial differences in pulmonary blood flow in rats in different postures [90]. The visualization of pulmonary thromboembolism and perfusion was conducted prior selective intra-arterial therapy using ^{68}Ga Ga-MMA [7].

N,N'-bis(diethylenetriaminepentaacetic acid)-pamoic acid bis-hydrazide (bis-DTPA-PA (40), Figure 10) was labelled with ^{68}Ga for the visualization of necrosis that occurs in acute myocardial infarction, stroke, chronic heart failure, neurodegenerative disorders, allograft rejection, and inflammation [328].

Acute pulmonary emboli requires fast evaluation of lung ventilation distribution for accurate diagnosis. PET provides necessary prerequisites such as high resolution and quantitation. ^{68}Ga -labelled carbon nanoparticles in the form of inhalation aerosol (^{68}Ga]-Ga-GallGas) were tested in piglets with lobar obstruction or diffuse airway obstruction [91]. The same animals were examined with $^{99\text{m}}\text{Tc}$]-Technegas aerosol for comparison (Figure 13). The heterogeneity of the airway response in the case of bronchoconstrictive challenge was more distinguishable in the investigation with ^{68}Ga]-Ga-GallGas. The distribution of

the aerosol in the lung, alveolar space of healthy volunteers was homogeneous and without bronchial deposition [105]. In an intraindividual comparative study ten patients with suspected pulmonary embolism underwent conventional ventilation-perfusion (V/Q) scintigraphy and PET-CT V/Q imaging after inhalation of ^{68}Ga -carbon nanoparticles ("Galligas") and administration of ^{68}Ga -macrosggregated albumin [92]. PET-CT provided images of higher resolution and possibility of lung function quantitation, and thus more accurate diagnosis. The short half-life of ^{68}Ga enabled more flexible acquisition protocols.

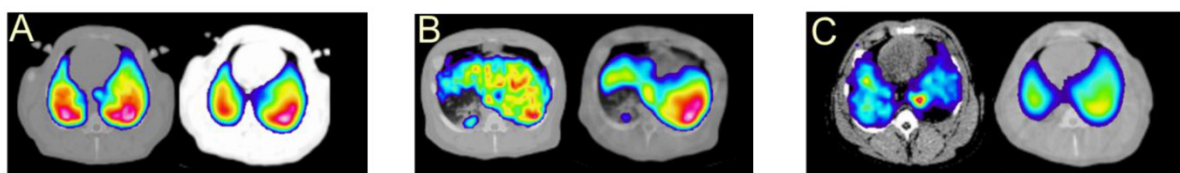


Fig 13. Lung ventilation imaging using carbon particles behaving as a "pseudo gas" for diagnosis of acute pulmonary emboli. Dual study using ^{68}Ga]-Ga-GallGas (left) and $^{99\text{m}}\text{Tc}$]-Technegas (right) in healthy piglets demonstrating similarity in ventilation distribution (A), in piglets with lower lobar obstruction (B), and in the piglets with diffuse airway obstruction induced by infusion of methacholine (C).

Imaging inflammation and infection

The development of ^{68}Ga -based imaging agents of various action mechanisms for the differentiation and diagnosis of inflammation and infection started during last five years [77, 86, 282, 329-338]. Several peptide analogues were designed for the visualization of vascular adhesion protein-1 (VAP-1) expressed on endothelial surface in inflammation and infection. A ligand to VAP-1, nine amino acid residue linear peptide conjugated with DOTA, was labelled and studied in rats with diffuse *Staphylococcus aureus* tibial osteomyelitis (infection), in rats with healing cortical bone defect (inflammation) demonstrating specific binding and capability to differentiate between inflammation and infection [329, 332]. In order to prolong the in vivo stability, peptide was coupled to PEG₄ spacer [333]. The resulting agent demonstrated higher stability and target-to-background ratio. The renal excretion was modulated also by PEG modification introduced to another ligand to VAP-1 (cyclic 17 amino acid residue peptide; CARLSLSWRGLTLP SK) [331, 334]. The accumulation in the site of inflammation induced in rats was clearly observed in the whole body coronal PET image. The important function of such agent must be the differentiation between tumour and inflammation/infection. However, this novel tracer demonstrated also uptake in the melanoma xenografts in mice.

Siderophores that are iron transporters in bacteria, fungi, and some plants were used for the devel-

opment of imaging agent for invasive pulmonary aspergillosis (IPA) caused by *Aspergillus fumigatus* [335]. Desferri-triacetylfulsarinine C (TAFC) and desferri-ferricrocin (FC) siderophores were labelled with ^{68}Ga . ^{68}Ga]-Ga-TAFC demonstrated specific binding, fast blood clearance and high stability in a rat model of *A. fumigatus* infection while ^{68}Ga]-Ga-FC showed prolonged retention in blood and rapid metabolism. TAFC and ferrioxamine E (FOX E) [336] were selected from a group of 7 various siderophores as best candidates for *Aspergillus* infection imaging in terms of ^{68}Ga complex stability, uptake specificity, rapid clearance and elimination. Both agents demonstrated lung focal uptake in invasive aspergillosis rat model correlated with the severity of disease and high image contrast [337].

^{68}Ga]-Ga-citrate was found effective in clinical examination of bone infection such as osteomyelitis and diskitis. The overall accuracy of diagnosis was of 90% [77, 338]. Apo-transferrin was labelled with ^{68}Ga and subsequently used for the bacterial infection imaging in rat model with *Staphylococcus aureus* [86]. Infection site could be clearly visualized within 1 h after intravenous injection.

Future perspective

First generators were introduced in the late 1950s early 1960s and ^{68}Ga -based imaging agents were available already from the 1970s but further progress was precluded by the absence of reliable sources of the generators as well as their properties and quality.

During last decade PET has been recognized as a clinically relevant and valuable diagnostic technique providing quantitative information on the physiological status of a disease and possibility to perform a whole-body scan in a single examination. There are many interrelated factors that contributed to the clinical acceptance of PET in general and blossom of $^{68}\text{Ga}/\text{PET}$ in particular. Technological aspects include achievements in improved sensitivity, resolution, accurate quantitation, and introduction of hybrid systems, PET-CT and PET-MRI; availability and increasing assortment of commercial $^{68}\text{Ge}/^{68}\text{Ga}$ generators; advances in biological research and biotechnology providing identification of biomarkers; accessibility of automated synthesizers for GMP compliant production of imaging agents. The automation may also provide possibility for the harmonized and standardized multicentre clinical studies that in turn will accelerate the introduction of new radiopharmaceuticals as well as their regulatory approval. Steady progress is being observed in PET radiopharmaceutical regulations and multicenter trials. The plausible correction and increase of the parent ^{68}Ge breakthrough limit in the radiopharmaceutical preparation (European Pharmacopoeia) may together with the labelling methods of high selectivity towards $^{68}\text{Ga}(\text{III})$ at room temperature allow for kit type production at radiopharmacy practice and consequently even wider allocation and use of ^{68}Ga -radiopharmaceuticals. The feasibility of PET-CT being cost-effective and cost-saving has been demonstrated, especially in combination with cheaper, as compared to cyclotron, generator produced ^{68}Ga radionuclide. Further technological development might render cost reduction and consequently improve profitability and affordability. This in turn may stimulate investments in radiopharmaceutical research and development.

The above mentioned factors have played prominent roles in accelerating the growth of ^{68}Ga -radiopharmaceutical field and they will stimulate the investments into development of new imaging agents. The growth has been reflected in the explosive increase of related publication number and more ^{68}Ga -agents reported at an ever-increasing rate. However the central role in the expansion of nuclear medicine belongs to identification of unmet medical needs as well as development and introduction of new specific imaging agents and thus achievements in chemistry.

Methods for the enhancement of ^{68}Ga quality have been developed and automated however their further simplification is required. Mono- and bifunctional chelators for fast, efficient and selective complexation with ^{68}Ga are established and further development will be directed towards radiopharmaceu-

ticals comprising chelators that are highly selective for $\text{Ga}(\text{III})$, form efficiently and rapidly complexes at room temperature, broad pH range and low concentration. Conjugation chemistry will further develop towards fast and regioselective coupling reactions at mild conditions and low concentrations.

The ^{68}Ga -applications in oncology will continue growing in harmony with the introduction of new receptor specific peptides and high affinity proteins for tumour-type specific diagnosis and treatment that would allow personalized approach of accurate quantitative diagnosis and staging for subsequent selection and planning of therapeutic means as well as monitoring response to the treatment. The imaging of receptors with peptidic agents is the most explored field. The high clinical value of ^{68}Ga -labelled SST ligand analogues is recognized and clinical routine practice and regulations are getting established. Imaging feasibility of GRPR, GLP-1R, MSH, HER2 as well as prostate-specific membrane antigen, osteoblastic bone metastases, amino acid uptake, glucose transport, angiogenesis using ^{68}Ga -agents has been clinically demonstrated in patients. The experience with a number of ligands specific to G-protein coupled receptors (GRP, CCK, GLP-1, MSH, neuropeptide Y, melanocortin-1, VPAC-1, NT, CXCR4, GnRHR) labelled with $^{99\text{m}}\text{Tc}$, ^{111}In , ^{18}F , ^{64}Cu , ^{90}Y , ^{177}Lu and used in clinical studies provide solid basis and will facilitate advent of ^{68}Ga -labelled analogues in the nearest future. Obviously, oncology will be the major arena for ^{68}Ga -agents, especially those based on regulatory small peptides specific to disease type. This is greatly supported by the robustness, favourable pharmacokinetics and accessibility of the peptides. Immuno-PET with ^{68}Ga will also expand due to the pre-targeted imaging technique. The basic research for the development of agents for myocardial perfusion imaging will most probably persist in order to meet the need for quantitative diagnostic means as well as to compensate for $^{99\text{m}}\text{Tc}$ shortage. Strong clinical need for the specific and quantitative imaging of inflammation and infection will stimulate the development of the corresponding ^{68}Ga -agents, and the vast experience of $^{99\text{m}}\text{Tc}$ -radiopharmaceuticals provides valuable basis [216].

The future of ^{68}Ga is predicted as PET analogue of $^{99\text{m}}\text{Tc}$ with added value of higher sensitivity, resolution, quantitation, dynamic scanning, and personalized medicine. The production of ^{68}Ga -based imaging agents can be accomplished either under GMP or radiopharmacy practice, and it is a cost-effective complement to cyclotron-based tracers. The kit type preparation similar to that of $^{99\text{m}}\text{Tc}$ is feasible. Most importantly it will enable PET-CT investigations globally in remote hospitals without access to accel-

erators and radiopharmaceutical distribution centres thus promoting worldwide PET technique for earlier better diagnostics and further for individualized medicine. The input of ^{68}Ga into oncology and development of theranostics bridging quantitative diagnosis with subsequent therapeutic management has already been proven and will continue to expand. Moreover, it is simply a matter of time when the imaging of bone, sentinel lymph node, lung ventilation/perfusion will be taken over by ^{68}Ga /PET-CT. However, even if the vision of ^{68}Ga becoming as immense as $^{99\text{m}}\text{Tc}$ will not get fulfilled in the nearest future, ^{68}Ga definitely has strong contribution to make in the improvement of personalized patient management and there is a niche for ^{68}Ga -based agents in nuclear medicine. Moreover, ^{68}Ga -agents have the prerequisites to substitute also ^{111}In -based radiopharmaceuticals.

Conflict of Interest

None.

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