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Preclinical Research Highlighting Contemporary Targeting Mechanisms of Radiolabelled Compounds for PET Based Infection Imaging

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It is important to constantly monitor developments in the preclinical imaging arena of infection. Firstly, novel radiopharmaceuticals with the correct characteristics must be identified to funnel into the clinic. Secondly, it must be evaluated if enough innovative research is being done and adequate resources are geared towards the development of radiopharmaceuticals that could feed into the Nuclear Medicine Clinic in the near future. It is proposed that the ideal infection imaging agent will involve PET combined with CT but more ideally MRI. The radiopharmaceuticals currently presented in preclinical literature have a wide selection of vectors and targets. Ionic formulations of PET-radionuclides such $^{64}CuCl_2$ and $^{68}GaCl_2$ are evaluated for bacterial infection imaging. Many small molecule based radiopharmaceuticals are being investigated with the most prominent targets being cell wall synthesis, maltodextrin transport (such as [^{18}F]F-maltotriose), siderophores (bacterial and fungal infections), the folate synthesis pathway (such as [^{18}F]F-PABA) and protein synthesis (radiolabelled puromycin). Mycobacterial specific antibiotics, antifungals and antiviral agents are also under investigation as infection imaging agents. Peptide based radiopharmaceuticals are developed for bacterial, fungal and viral infections. The radiopharmaceutical development could even react quickly enough on a pandemic to develop a SARS-CoV-2 imaging agent in a timely fashion ([^{64}Cu]Cu-NOTA-EK1). New immuno-PET agents for the imaging of viruses have recently been published, specifically for HIV persistence but also for SARS-CoV2. A very promising antifungal immuno-PET agent (hJ5F) is also considered. Future technologies could include the application of aptamers and bacteriophages and even going as far as the design of theranostic infection. Another possibility would be the application of nanobodies for immuno-PET applications. Standardization and optimization of the preclinical evaluation of radiopharmaceuticals could enhance clinical translation and reduce time spent in pursuing less than optimal candidates.

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Introduction

The costs of communicable illness, both in human life and in economic output, is grave. In a study published in 2020 by the Institute of Labour Economics, the economic burden of the eight major infectious diseases (HIV/AIDS, malaria, measles, hepatitis, dengue fever, rabies, tuberculosis, and yellow fever) are an estimated eight trillion US dollars. Even more concerning, a total of 156 million life years were lost to these major infectious diseases in 2016 alone.[1,2] Some of the most important infections that should be the focus of

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preclinical research into bacteria specific diagnostic agents are *Mycobacterium tuberculosis* (MTB), the *Enterobacteriaceae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Clostridium difficile*. These are the major bacterial infections that result in the high burden of disease, but also the rise of drug-resistant infections.[3] Globally, MTB resulted in an estimated 1.6 million deaths in HIV negative patients and another 187,000 deaths in HIV positive patients according to statistics from the WHO (World Health Organization). A clear case is made for better diagnosis and follow-up strategies including identifying persistent loci of MTB disease.[4,5] Sexually transmitted infections are still a major public health issue worldwide with the incidence of common infections being as high as 563.3 million worldwide in 2016.[6] Whilst treatable, these can often present with persistence which could benefit from accurate high resolution diagnostic imaging techniques. As such, the rational for imaging of the human immunodeficiency virus to characterize the whole-body HIV burden is also very tangible.[7] It is estimated that in 2050 drug-resistant infections will be the leading cause of death worldwide.[3]

Non-invasive imaging of infectious patients with PET/CT would offer a time-sensitive and accurate way to not only quantify disease burden, but also to localize areas of infectious foci.[8] PET/CT has the superior advantage that it may allow for a unique, noninvasive evaluation of deep-seated infections.[3] The response of the patient on anti-infectious therapies could also be monitored in an accurate way, making PET/CT a comprehensive tool for the physician. Complications with non-responders could be identified in a timely fashion. In future, imaging of infection may also provide the means to identify antibiotic resistance before treatment.[9]

Continuous reviewing of the development of novel infectious disease targeting radiopharmaceuticals tailored must provide a strong motivation for researchers to push beyond the current infection imaging staples of the Nuclear Medicine Clinic, such as [^{18}F]FDG, $^{68/67}$Ga-Citrate and technetium-99m tagged leukocytes. The drawbacks of utilizing Nuclear Medicine procedures with the commercially available tracers have been extensively reviewed.[10-12] Therefore, recent advances made in preclinical development of novel strategies for infection imaging are reviewed and promising new, more infection-specific radiopharmaceuticals for PET/CT imaging are highlighted.

Radiopharmaceuticals in Preclinical Research

When evaluating infection imaging agents, the rational design of these radiopharmaceuticals is essential. The choice of radionuclide could have an integral impact on the success of the respective targeting vector. The radionuclide may have a big impact on the size and structure of the molecule and the location of the target is key, either being intracellular or extracellular. Northrup et al.[13] comprehensively reviewed this aspect including a critical discussion. Often the PET radionuclides copper-64, gallium-68, and also zirconium-89 are considered; however, a radiometal chelator is required for incorporation. This mostly results in the application of bioconjugated molecules. These strategies could be hampered in infection imaging where the crossing of tightly preserved cellular barriers are necessary for intracellular targeting. Subsequently, for intracellular targets, it is recommended that radiopharmaceuticals should preferably reside to radiolabelling strategies that covalently bind fluorine-18, iodine-124, or even carbon-11 thereby avoiding significant structural changes to the molecule.[13]

The pharmacokinetic behaviour of the vector and the radionuclide should match since the effective in vivo bioavailability is determined by both the biological half-life of the vector and the physical half-life of the radionuclide.[14] For instance, since longer intervals from the point of administration are required, for example, by using radiolabelled antibodies, a radionuclide with a longer half-live (zirconium-89 or copper-64) may provide superior imaging results as it will allow for unaccumulated radiopharmaceutical to clear from the body fist, reducing the background signal. Often this strategy achieves excellent image quality and high target to noise ratios.

The study design featuring PET imaging can be a challenge and should address an appropriate animal age, type and size as the host for the infection. Generally, using larger animals may result in more accurate data for translation into the clinic whereas small animals allow for sufficient study power to characterize any new radiotracer. It is important that the mechanism of action is considered correctly which can attribute what kind of preclinical imaging setting may be performed (ie, testing radiotracer sensitivity versus pharmacokinetic behaviour). Further characterization of the infection imaging agent, controls are equally essential to identify selectivity (sterile inflammation, cancer), nonspecific mechanisms of accumulation (blocking studies) or sensitivity (different concentrations of the infective agent, especially low density, dispersed infection sites).[15,16]

The spatial resolution of the imaging technique responsible for the localization of infectious foci is an important technical consideration. It is well-established that the combination of resolution and preclinical setting options provided by microPET is better suited for research purposes. Though the resolution of clinical PET scanners is less superior, it is also highly recommended for the clinical setting to further establish novel infection imaging agents, above other available modalities.[17] Ideally, microPET imaging would be combined with MRI for optimal sensitivity and accuracy. PET/MRI should also be the modality of choice for the clinical translation of these agents.[18] It is foreseen that high-sensitivity PET/CT afforded by long axial field of view PET could also have a considerable impact on infection imaging in the Nuclear Medicine Clinic.[19,20]

Contrary to the currently available radiopharmaceuticals, infection-specific agents must clearly distinguish between sterile inflammation and infection. It is even plausible that radiopharmaceuticals may be designed to single out an individual organism as the cause of the infection. Other specific questions that is of interest in the clinic are the imaging of biofilms, imaging of HIV persistence and applicability of

Nuclear Medicine Imaging in investigating Long Covid disease.[9,21,22] It is important to note that preclinical imaging of infectious disease could advance both radiopharmaceuticals for infection imaging, but also provide an efficient tool during preclinical development of new antibiotics or other anti-infective agents.[9]

The emerging nuclear imaging strategies are still relatively broad, investigating ionic radionuclide formulations, small molecules, peptides and monoclonal antibodies as radiopharmaceutical vectors in a preclinical setting. This review will briefly highlight each group of molecules.

Ionic Formulations of PET-Radionuclides

Similarly to [^{18}F]NaF used for imaging purposes in oncology, the use of ionic radiometal salts has been considered as a possible strategy to the imaging of infection or inflammation. This strategy allows for ease of production with very little manipulation of the radionuclide to yield a radiopharmaceutical. As an iron analogue, ionic gallium binds to transferrin which is a role-player involved in the response to inflammatory processes. However, in infection specifically, it may bind to bacterial siderophores. A study by Lankinen et al.[23] proposed that there might be some differences in the in vivo behaviour of [^{68}Ga]gallium-chloride salt and [^{68}Ga]gallium-citrate salt. This was investigated in vivo in *Staphylococcus aureus* infected rats. The study found that the SUV_{MAX} of both such tracers were higher in osteomyelitic tibias of rats compared to the uncomplicated healing of operated bone without infection. Similarly, the transport of ionic copper through the copper transport 1 (CTR1) is upregulated in inflammation. This application was also investigated with positive results in in vivo inflammation models with [^{64}Cu] copper chloride salt.[24]

A consensus is yet to be made on the clinical usefulness of ionic metal radionuclides for infection imaging.[25-27] It is clear that clinical applications are subject to careful patient selection and appropriate interpretation, thus preclinical investigations might be merited to better establish the value and roles of the proposed agents. Clinical translation might be more complicated than more complicated targeting systems.

Small Molecules

Small molecules are the targeting vectors responsible for the most prolific area of preclinical research in infection imaging (Fig. 1). These include molecules targeting transport mechanisms like siderophore metal transport and maltodextrin transport. Mechanisms traditionally used for antibiotic or antiviral targeting are also popular and this could also include labelled antibiotics, very popular for SPECT (single photon emission computed tomography) radiopharmaceutical development, but also finding some application in preclinical microPET development.

Over the past decade, bacteria-unique radiolabelled sugar molecules have been reported as radiopharmaceuticals. It is proposed that they would have similar characteristics to [^{18}F]FDG. Although [^{68}Ga]Ga-NODA-aminoglucose was

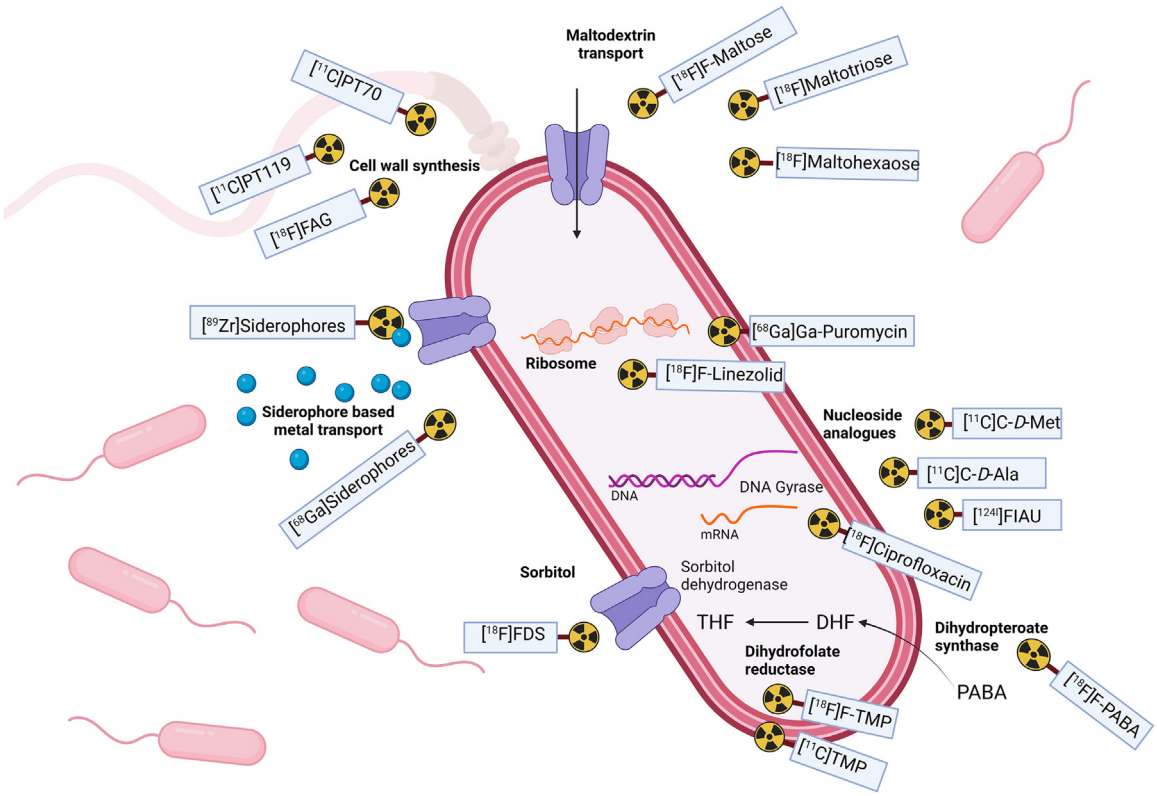

Figure 1 An overview of small molecule radiopharmaceuticals investigated preclinically for bacterial infection specific imaging (Created with BioRender.com).

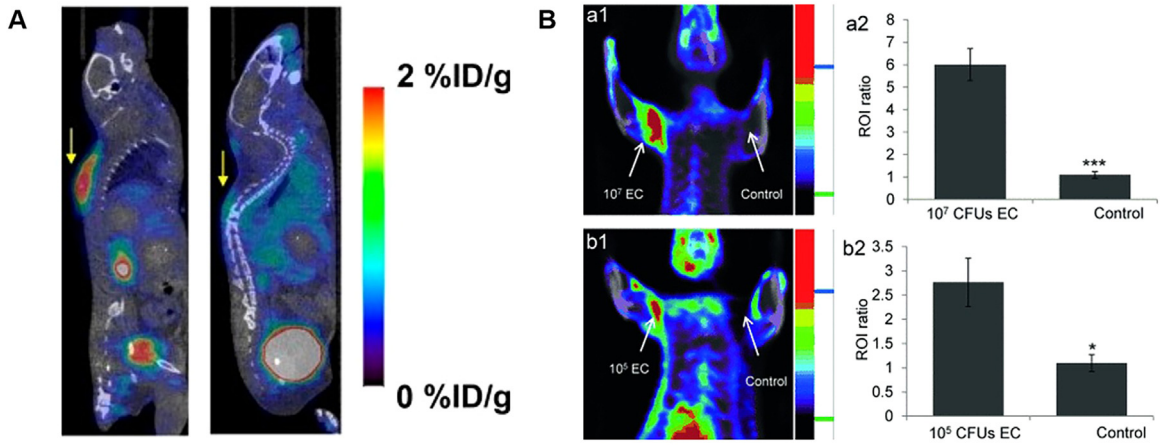

Figure 2 Investigations using the maltodextrin transport system. A) [18F]F-maltotriose imaging of P. Aeruginosa infection (left); control mice (right) (This research was originally published in JNM. Gowrishankar et al. J Nucl Med. 2017;58(10):1679-1684. ©SNMMI) B) [18F]F-Maltohexaose in rats infected with E. Coli (105 CFUs) (reprinted with permission from Ning et al. Angewandte Chemie, 2014: 53(1): 14096-14101 ©Wiley.

evaluated for accumulation in aseptic inflammation and malignant tumour models in vivo it was found to be nonspecific for bacteria.[28] Knowing about the lack of selectivity of [^{18}F]FDG, the metabolic product [^{18}F]FDG-6-P was evaluated for its selectivity to image Gram-positive bacteria (*S. Aureus)* derived infection.[29] [^{18}F]FDG-6-P indeed demonstrated selectivity in vitro, however it was not significantly different in vivo from [^{18}F]FDG.[29] A sorbitol based molecule labelled with fluorine-18, [^{18}F]FDS, has been investigated for selective targeting of Gram-negative bacteria, not targeting Gram-positive bacteria or the human host. Following very encouraging results, [^{18}F]FDS was recently tested exploratory first-in-human and currently undergoes trials for translation to the Nuclear Medicine Clinic (NCT02450942).[30,31] Interestingly, [^{18}F]FDS is also proposed for fungal infections. It was preclinically investigated for *Aspergillus* infection recently, but demonstrated to be more selective for Gram-negative bacteria.[32]

The maltodextrin transport system was promoted in literature as a potential target that may allow for infection-specific imaging using maltodextrin transport system radiopharmaceuticals (Fig. 2). An additional fortuitous characteristic associated with these radiopharmaceuticals is the high in vivo stability they present. These agents might also be able to overcome the challenges connected to imaging of infectious biofilm manifestations. The maltodextrin transporter is expressed solely by Gram-positive and Gram negative bacteria. Furthermore, maltodextrins are cleared by uninfected tissues due to their hydrophilic, neutral nature.[33-35] Radiopharmaceuticals that have been evaluated for this system are fluorine-18 based (maltose, maltotriose, and maltohexaose) all demonstrating selectivity for bacterial infections in vivo in based on rodent models.[17,36,37] An interesting approach for a multimodal fluorescent / PET tracer has been proposed by Takemiya et al.[34] It is envisioned for infection of cardiovascular implantable devices, due to the location nearer to the skin and the near-infrared penetration offered, this could provide an additional modality for intraoperative and routine monitoring purposes.

The *siderophore based metal transport* system has found relevant application in both fungal and bacterial infection imaging. The gallium-68 labelling was reported by Petrik et al.[38]; an elegant and efficient method and very relevant as siderophores are essential to the micro-organisms. Earlier labelling strategies were also published with preclinical evaluations.[39,40] In brief, siderophores are designed to address the lack of iron in microorganisms. The iron in siderophores can be replaced or mimicked by other metal radionuclides, in particular copper-64 and gallium-68 and also zirconium-89. Originally the reporting literature focused on the imaging of *Aspergillus fumigatus fungal* infections, but recently the concept found expansion into the imaging of bacterial infections.[41,42] An example of such a probe is gallium-68 labelled pyoverdine ([68Ga]Ga-PVD-PAO1), a siderophore manufactured by P. Aeruginosa, labelled with gallium-68 obtained from a generator system.[43] Radiolabelled siderophores featuring pyoverdine (PVD), desferrioxamine (DFO) and ferrichromes. Due to convincing results, [^{68}Ga]Ga-DFO-B may be approaching clinical translation (Fig. 3).[44] Recently, a copper-64 based siderophore ([^{64}Cu]Cu-YbT) for the imaging of bacteria was developed that demonstrated selectivity for bacteria over sterile inflammation, but even more specifically, bacteria that express a functional FyuA transporter protein.[45]

Due to their unique ribosome structure, protein synthesis inhibition in bacteria was previously considered for development of antibiotics. Such PET radiopharmaceutical development only showed momentum recently, suggesting [^{18}F]F-linezolid and various radiolabeled derivatives (fluorine-18, carbon-11, scandium-44 and gallium-68) puromycin.[46-51] It should be noted that puromycin is a non-selective antibiotic chimere molecule that never found clinical application and it might suffer from similar non-selectivity for protein synthesis across disease processes.[52] [^{18}F]F-linezolid was recently

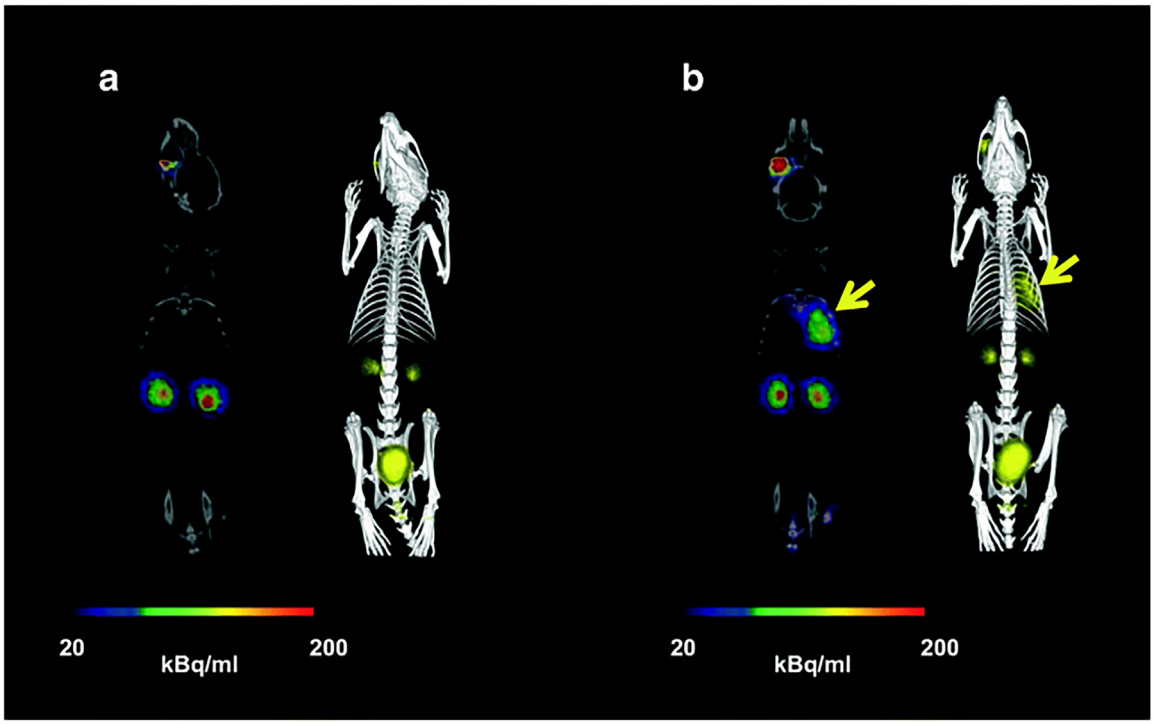

Figure 3 Static PET/CT images of non-infected control rats (A) and P Aeruginosa pulmonary infected Lewis rats (B) with [68Ga]Ga-DFO-B (reprinted under the Creative Commons Attribution 4.0 License from Petrik et al. EJNMMI, 2021, 48(2): 372-382, Copyright, Petrik et al, 2020).[43]

highlighted with excellent pharmacokinetic characteristics for bacterial imaging using microPET/CT; however, *in vitro* analysis reported poor accumulation in mycobacterial cultures.[53] The broad spectrum fluoroquinolone [^{18}F]F-fleroxacin was successfully developed preclinically and was even tested in a first-in-human exploratory study.[54-55] A lack of any further clinical investigation may indicate that this radiopharmaceutical did not gain traction in the Nuclear Medicine Clinic. Instead, eventually some research on the pharmacokinetics of the unlabelled fleroxacin as an antibiotic was reported.[56] Similarly, a fluorine-18 derivative of lomefloxacin was reported in literature, but it was limited to pharmacokinetic profiling studies.[57]

Alternatively, another antibiotic-based mechanism of action targeting cellular folate metabolism targeting was adopted to radiolabel trimethoprim derivatives ([^{11}C]TMP, [^{18}F]F(P)-TMP) and inhibiting dihydrofolate reductase or [^{18}F]F-PABA inhibiting dihydropteroate synthase.[57-59] Excellent preclinical selectivity was proven for bacterial specific imaging with [^{18}F]F-TMP and a healthy biodistribution study in nonhuman primates demonstrated advantageous pharmacokinetics.[60,61] Indeed a clinical trial (NCT04263792) is registered to evaluate this radiopharmaceutical in a first-in-human study.[62]

Targeting the bacterial cell wall synthesis, [^{18}F]FAG was proposed in the 90's.[62] It is a hexosamine which is important in the synthesis of glycoproteins and mucopolysaccharide. It was investigated in vivo and demonstrated the ability to distinguish between bacterial infection and sterile inflammation in vivo in *E. Coli* infected mice.[63] No further development on this tracer is presented in literature. Another strategy pursued for the bacteria specific targeting was the utilization of nucleoside analogues, specifically *D*-amino acids. Bacteria produce and incorporate a significant amount or D-amino acids in the synthesis of their cellular membranes. Neumann et al.[66] presented an adapted synthesis of the carbon-11 labelled methionine to produce asymmetric synthesis of [^{11}C]*D*-Met.[64] This radiopharmaceutical was able to distinguish between live and heat-inactivated *E. Coli* and *S. Aureus*. An initial experience in humans was published recently with promising results.[65] Similarly [^{11}C]D-Alanine demonstrated selective in vivo imaging of life bacteria in rodent models of discitis-osteomyelitis and *P. Aeruginosa* induced pneumonia with clear justification for human translation.

Particular mycobacteria-specific antibiotics were radiolabelled to evaluate them as possible PET imaging agents. These include isoniazid (fluorine-18, carbon-11), pyrazinamide (fluorine-18 and carbon-11) as well as carbon-11 labelled rifampin.[67-69] None of these has found application for imaging of mycobacterial infections. Indeed, in a study on 5-[^{18}F]F-PZA, there was no significant difference in tracer uptake between the infected and uninfected lung tissue.[68] Bacterial cell wall synthesis visualization by isoniazid analogues [^{11}C]PT70 and [^{11}C]PT119 for mycobacterium fatty acid biosynthesis is also presented in literature,[70] but the study design was not adequate to address mycobacterial uptake. Recently, [^{18}F]F-pretomanid, a nitroimidazole antimicrobial with activity against drug-resistant tuberculosis, was reported, but again, PET imaging was used to evaluate the pharmacokinetics of this antibiotic for application as an antimicrobial agent against tuberculosis, not as a radiotracer for PET imaging.[71]

Viral infection imaging agents based on small molecules for PET imaging is an area with few notable developments. The proposal of imaging of viral infections with PET based technology has however often surfaced in literature, with some even going as far to suggest a theranostic approach to detection and treatment of viruses.[72] A perspective on how such an imaging probe would look is provided by Bray and co-authors.[73] Radiolabelled analogues of antivirals exist but are not applied in this capacity. This includes a tenofovir analogue ([^{18}F]PMPA), [^{11}C]oseltamivir and [^{11}C]zanamivir.[74-76] Herpes simplex virus imaging with radiolabelled probes did not show in vivo success. However, these agents are still under consideration for the evaluation of the success of herpesvirus mediated anticancer gene therapy.[73] The ganciclovir analogue [^{18}F]FHPG was evaluated in vivo for the imaging of herpes-mediated human cytomegalovirus encephalitis in rats, but again, poor pharmacokinetics hampered further evaluation.[77] Similarly, the thymidine analogue [^{11}C]FMAU was evaluated for cytomegalovirus but it demonstrated high non-selectivity during its *in vitro* assessment.[78]

Other radiolabelled antifungal agents (beyond siderophores, see earlier) reported for imaging of fungal infections include [^{68}Ga]Ga-AMB and [^{18}F]F-fluconazole. The first *in vitro* evaluations of [^{68}Ga]Ga-AMB was only published recently with no in vivo evaluation available. This radiopharmaceuticals demonstrates a fungal specific mechanism of action, i.e it binds to ergosterol which is a component of fungal cellular membranes. *In vitro* data was very positive demonstrating selectivity for *A. fumigatus*, *R. arrhizus* and other clinically relevant mould pathogens. Selectivity was ascertained, with no AMB accumulation present in bacterial strains as controls.[79] Again, as with radiolabelled antibiotics, the radiolabelling of fluconazole is reported in literature, not as a fungal imaging agent, but for the purpose of evaluating pharmacokinetic behaviours.[80]

Biotin transporters are expressed in certain bacteria (eg, *E coli* and *S Aureus*) and biotin based radiopharmaceuticals incorporating carbon-11, copper-64, fluorine-18, and gallium-68 have been developed.[81] To date, clinical translation for infection imaging is yet to be attempted. Whilst cyanocobalamin has been investigated for infection imaging in technetium-99m based systems, it is yet to be applied to PET.[82]

Radiolabeled Peptides for PET Imaging of Infection

Nowadays, radiolabelled peptide derivatives are well understood molecules and they have a broad, longstanding history in Nuclear Medicine as potential radiopharmaceuticals.[83] Of note is Neuroendocrine imaging agents such as DOTA-TATE/TOC and NOC, and prostate carcinoma imaging agents based on PSMA imaging, both which have been approved by the FDA.[84] Avid research is proposing that naturally occurring (often antimicrobial) peptides (and synthetic pseudopeptides) could offer great diversity and thereby become role-player as target-selective infection imaging agents. Furthermore, it has been shown that some of these peptides could be useful in the imaging of more chronic infections that contain the biofilm barrier that is currently hampering the imaging of these persistent pathologies.[85] Peptides are also considered in research aimed to develop fungal-specific infection imaging agents. The use of antimicrobial peptides for infection-selective molecular imaging has been previously reviewed thereby highlighting the importance of the peptide design and their targeting mechanisms.[86] Many reviews focus on the general process of design of peptide based radiopharmaceuticals.[87-88] A list of radiolabelled peptide derivatives and their properties for future clinical consideration is provided in Table 1.

Peptide-based infection imaging mostly reported successful development using fragments of ubiquicidin (UBI) and for PET imaging, most investigations (clinical success with exploratory studies in infected patients) use the gallium-68 labelled radiopharmaceuticals (abbreviated names include [^{68}Ga]Ga-(DOTA/NOTA/NODAGA)UBI-29-41/-31-38); however, many missing investigations and large cohort patient studies still need to be performed until this peptide can be confidently uses in routine Nuclear Medicine practice.[89]

Another antimicrobial peptide that has been extensively investigated is the human peptide LL-37 and (now) its smaller derivatives. It is based on cathelicidin that is present in mammals and forms part of the innate immune system and is released at sites of infection. It has antimicrobial activities against Gram-negative and Gram-positive bacteria. Literature report the investigation of LL-37 as well as fragments CDP1, GF-17, and CDP1, functionalized with DOTA and NODAGA to allow for gallium-68 radiosynthesis.[93-95] Clear, supportive in vivo results with respect to tracer selectivity and sensitivity during microPET/CT imaging are yet to be published on either of these candidate radiopharmaceuticals. The use of depsipeptides, natural- antimicrobial cyclic peptides, has been proposed by Mokaleng et al.[90] as a possibly more stable peptide version as a targeting vector.[90] In the case of TBIA101, isolated from *S. lavendofoliae*, a gallium-68 radiosynthesis and a preliminary in vivo evaluation for targeting *E.Coli* infection was reported. Other strategies for identifying possible peptides for infection imaging vectors included phase display, use of unnatural peptides with lipid targeting abilities.[91,97] Various endogenous peptides that are targeting the host response instead of the infective agent was also investigated in the past namely fMLP, VAP and FLFLFK.[98,100]

Recently, imaging of non-bacterial infections has also been attempted with peptide based agents, including gallium-68 labelled PAF26 targeting *Aspergillus spp.* derived fungal infections.[92] Antifungal activity was lost during conjugation, however the study proposed that with more research, this technique can provide viable PET imaging tracers. Furthermore it is proposed that this strategy could utilize the theranostic principle based on treatment with unlabelled peptides as therapeutic and labelled peptides as diagnostic.[92]

Also due to the global pandemic, efforts emerged to attempt selective in-vivo imaging of SARS-CoV-2. Notably, the copper-64 radiolabelled peptide EK1 (ACE2 targeting

Table 1 Peptides Currently Investigated Preclinical for Various Applications in Infection Imaging

Peptide and Origin	Sequence	Target Investigated	Radiolabelling Strategy	Ref
Ubiquicidin$_{29-41}$	TGRAKRRMQYNRR	Broad spectrum bacteria	[^{68}GaGa-NODAGA, NOTA, DOTA	89
Ubiquicidin$_{31-38}$	RAKRRMQY	Broad spectrum bacteria	[^{68}GaGa-NODAGA, NOTA	89
TBIA 101	PLPVLTI-GG	Broad spectrum bacteria	[^{68}GaGa-DOTA	90
A9-K Phage display	TDGRRYSSGAMR	S. Aureus (selectivity not investigated)	[^{68}GaGa-DOTA	91
Siderophore labeled PAF26	WFWKKR	Antifungal: *Aspergillus fumigatus*	[68GaGa-DAFC	92
CDP1 (LL37)	LLGDFFRKSKEKIGKEFK RIVQRIKDFLRNLVPRTES	Broad spectrum bacteria	[^{68}GaGa-DOTA/ NODAGA	93,94
GF-17 Fragment of LL37	GFKRIVQRIKDFLRNLV	Broad spectrum bacteria	[^{68}GaGa-DOTA	95
EK1	SLDQINVTFLDLEYEMK-KLEEAIKKL-EESYIDLKEL	SARS-CoV-2	[^{64}CuICu-NOTA	96
AB1 (synthetic peptide)	RYWVAWRNRG	Gram positive (selectivity not determined)	[^{64}CuICu-DOTA	97
fMLP (TP1096)	N-formyl-leucyl-phenylalanine	Broad spectrum bacteria	[^{64}CuICu-DOTA	98
VAP-P1	GGGGKGGGG	Human endothelial protein exposed during inflammation - inflammation specific	[^{68}GaGa-DOTA	99
Synthetic peptide	cFLFLFKNH$_2$	Targets the formyl peptide receptor on leukocytes - non-specific inflammatory response	[^{64}CuICu-PEG- DOTA	100

agent) was reported (Fig. 4) aiming to provide a pan-coronavirus imaging agent more sensitive than [^{18}F]FDG, in order to evaluate the efficacy of therapeutic intervention in SARS-CoV-2 animal models and eventually human subjects.[96]

Considering a peptide-based imaging strategy comes with a few factors that need to be addressed, in particular when designing a (synthetic) peptide. The major challenge remains the in vivo vector stability. Antimicrobial peptides are sensitive to peptidases which could lead to their in vivo degradation and loss of activity. Another problem is the immune system could target these peptides before accumulation at the target (e.g., infected tissue) can occur. Valuable insights together with some design strategies to overcome vulnerabilities has recently been provided in a critical review article by Northrup et al.[13]

Overall, the factors influencing peptide-based radiopharmaceuticals are known and research is vigilant to address the matter which is encouraging towards meeting the objectives of clinical translation. To this end, the success story of using radiolabelled peptides already routinely as imaging agents in oncology should boost efforts and spark momentum in the preclinical research space.[101,102] The gap to achieve more specific, accurate and sensitive detection and monitoring of

Figure 4 [64Cu]Cu-NOTA-EK1 targeting engaged with the S2 subunit of SARS-CoV-2 in HEK293T/ACE2 infection xenograft-bearing mice (Reprinted with permission from Xian et al. Mol. Pharmaceutics, 2022, 19(11):4264-4274. Copyright ©2022 American Chemical Society).[96]

infection using PET/CT must be overcome. Again, a combination with MR imaging procedures should be more considered both in preclinical and clinical imaging.

Immuno-PET

Immuno-PET is a powerful diagnostic tool that combines the targeting specificity associated with monoclonal antibodies (mAb) and the sensitivity and resolution contributed by PET. The possibility exists to not only incorporate complete mAb structures, but also various antibody fragments and antibody-mimetics. Recent publications provide an in-depth coverage of the design strategies of immuno-PET radiopharmaceuticals.[103,104] Table 2 highlights novel Immuno-PET radiopharmaceuticals in preclinical development assessed for their potential applications in infection diagnostics.

With the right target selectivity and correct imaging protocols, immuno-PET could indeed become a powerful infection imaging technique. For instance, it is already understood that if HIV remains concentrated in critical tissues, despite being repressed by antiretroviral therapy, serious health consequences are posed. The affinity of HIV and co-receptor CXCR4 could lead to increased malignancy. Furthermore, anatomical reservoirs like the lungs could lead HIV patients more susceptible to chronic obstructive pulmonary disease and pulmonary arterial hypertension. Various other long-term HIV-associated complications are evident as well.[116] Nowadays, addressing HIV as the whole-body disease that it is, investigations feature immuno-PET strategies with their focus on the localization of HIV infection in both the viraemic population and treated cohorts. Excitingly, [^{89}Zr]Zr-VRC01, a radiolabelled antibody construct that targets the gp120 glycoprotein core as part of the HIV-1 envelope, has successfully surpassed its preclinical development and first-in-human explorations are underway. Clinical translation was encouraged by favourable pharmacokinetics in healthy Balb/C mice and very supportive radiation dosimetry predictions from rhesus macaques. Initial results from [^{89}Zr]Zr-VRC01-PET/CT imaging first-in-human allowed for detection of HIV-harbouring foci in the intestinal mucosa.[106] The mAb 73D labelled with copper-64 presented positive in vivo results in SIV infected primates, but is yet to be translated to HIV imaging in humans.[108] Another first-in-human study on an antibody construct targeting the HIV envelope, [^{64}Cu]Cu-3BNC117, revealed positive results from similar investigations in macaques (using the anti-SIV neutralizing antibody) but not in humans (anti-HIV neutralizing antibody). The authors state that selecting copper-64 as the radionuclide might have been detrimental to the radiopharmaceutical's longitudinal performance - zirconium-89 may be suggested for its further application.[107] A preprint article (not peer-reviewed) also presents the possibility of an immuno-PET probe to monitor SARS-CoV-2 infection.[105]

Nuclear imaging of complex fungal diseases might be supported by novel immuno-PET radiopharmaceuticals in the near future. For functional imaging of such infections caused by fungal species such as *A. Fumigatus* (pulmonary

Table 2 Relevant Immuno-PET Radiopharmaceuticals in Preclinical Development

Antibody	Type	Targeted Infection	Functionalization Strategy for Radiolabelling	Radiometal	Ref
CR3022	Human antibody IgG1	SARS-CoV-2	DOTA at molar ratio 5:1	Copper-64	105
VRC01 (translated to first-in-human)	Human antibody (broadly neutralizing)	HIV persistence	DFO at molar ratio 5:1	Zirconium-89	106
3BNC117 (first-in-human studies, unable to detect HIV)	Human Antibody IgG_K	HIV persistence	MeCOSar 1-3 chelators with average of 1.6 chelators per antibody	Copper-64	107
7D3	Rodent antibody $IgG2_{AK}$	HIV persistence	DOTA-NHS	Copper-64	108
hJ5F & mJ5F	Human Rodent	Aspergillus fumigatus	DOTAGA & NODAGA at molar ratio 20:1	Copper-64	109
hJ5F-DyLight650	Human	Candida Albicans	NODAGA-labeled antibody at 10:1 access with DyLight 650	Copper-64 and DyLight650	110
MC3	Rodent antibody $IgG2b_K$	Candida Albicans	NODAGA at molar ratio 20:1	Copper-64	111
IIIB6	Rodent antibody IgM	Plasmodium Falciparum	DFO (NCS-Bz-DFO) ratio 20:1 or 50:1	Zirconium-89	112
1D9	Rodent antibody IgG1	Staphylococcus Aureus	DFO at a molar ratio 7:1	Zirconium-89	113
YadA	Polyclonal antibody	Yersinia Enterocolitica	NODAGA at a molar ratio 55:1	Copper-64	114
Mab69	Rodent antibody	Enterococcus Faecalis	DOTA at a molar ratio of 20:1	Copper-64	115

aspergillosis) or *C. Albicans*, the humanized and rodent constructs of the mAb J5F were functionalized with copper-64 and tested preclinically with multimodal imaging strategies (mainly using microPET/MRI). Alternately, [^{64}Cu]Cu-mAb-MC3 was tested similarly for its imaging performance of *C. Albicans* infection, preclinically. The latter radiolabelled mAb all demonstrated selective targeting of fungal infections in rodent models.[109,111] Translation into the clinic is yet to be made.

Interestingly, parasitic infections are still very much unexplored, however some preliminary investigations seem to be underway. The characterization of the malaria pan-reactive mAb IIIB6 was reported Duvenhage et al.[112] The successful zirconium-89 radiolabelling allowed for its pharmacokinetic evaluation using [^{89}Zr]Zr-IIIB6-microPET/CT in healthy rodents, but targeting of *Plasmodium spp*. is still outstanding.

Highlights from imaging of bacterial infections using immuno-PET include preclinical studies using the zirconium-89 labelled mAb1D9 which succeeded in selective detection of *S. Aureus* infection in vivo and copper-64 radiolabelled mAb68 demonstrating excellent imaging properties, thereby detecting *E. Faecalis* derived endocarditis in vivo (Fig. 5).[113,115] Wiehr et al.[114] also reported on the remarkable pathogen-specific targeting abilities of [^{64}Cu]Cu-YadA for targeting sole infections caused by *Y. Enterocolitica* and no accumulation in other pathogens.

It is noteworthy that all the currently investigated mAbs are in the preclinical setting and are all full-length antibodies (i.e. no application of antibody fragments). There are some constraints associated with this strategy, some of which could be both negative or advantageous in infection imaging. Firstly, the size of full mAbs result in a longer pharmacological half-life and slow blood clearance. Inevitably, more time after administration should be afforded for radiolabelled mAb constructs to accumulate at infectious foci; however, the selected radionuclides must have a physical half-life to strategically match the biological half-life of these vectors. These radiolabelled mAbs also have a high-cost to produce due to the more expensive radionuclides used as well as the complex production associated with mAbs.[103] There is a lot of room for development in infection immuno-PET as the knowledge of the in vivo behaviour and design parameters of these vectors increase.[104] During the design of an mAb, a critical aspect is how much molar amount of chelating agent is paired with the vector. It is generally assumed that one to two chelators per mAb does not interfere with the normal biodistribution of the vector.[104] The selected chelator should not only take into account the radiometal of choice, but also the heat-sensitivity of the mAb. Evidently, elevated reaction temperatures required for the reactivity DOTA towards efficient radiometal complexation are normally detrimental (i.e., discouraged) to the function of heat-sensitive mAbs, although this strategy has been applied in the past.[104] On this note, the use of less heat vulnerable and smaller antibody fragments such as Fab2, Fab, svFc, affibodies or nanobodies as vectors are encouraged to overcome limitations attributed to the mAb radiolabelling. This will also extend on the plausible list of radionuclides that may be paired which may allow for more elegant radiolabelling strategies.

Critical Comments

Nuclear imaging of infectious diseases is gradually growing in relevance with significant progress made due to the more readily available PET/CT and emerging PET/MRI. It is therefore quite conceivable that efforts should focus towards the

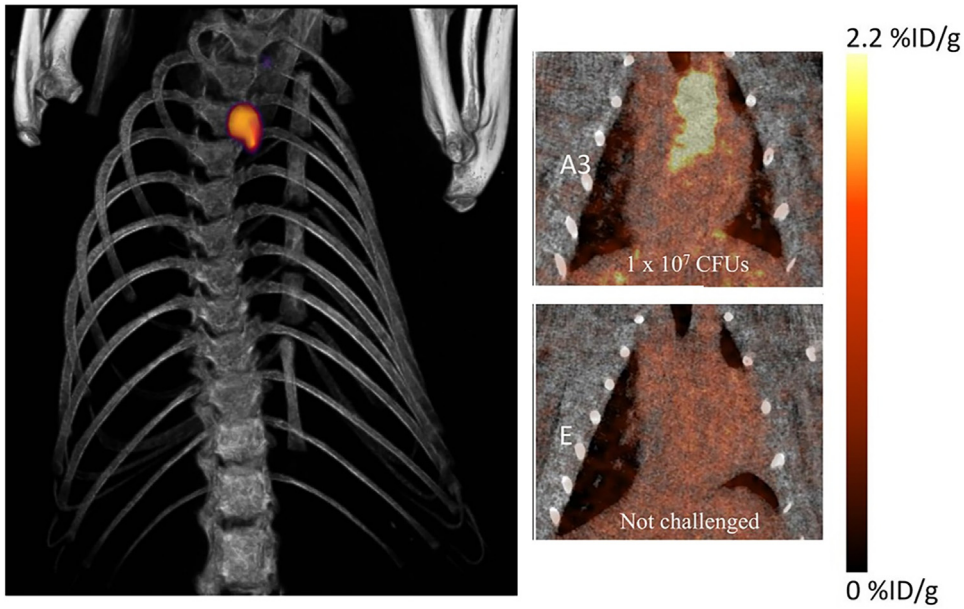

Figure 5 A PET/CT image taken at 24 hours post-injection of [64Cu]Cu-DOTA-MAb 69, 72 hours post-infection with E. faecalis in a rat model of endocarditis (reprinted with permission Pinkston et al., 2014).[115]

development of sensitive and accurate PET radiopharmaceuticals. As highlighted by current literature, even if a radiopharmaceutical is bacteria-specific, the successful visualization of an infectious foci depends on the amount of tracer accumulating at the desired target region as well as the spatial resolution of PET. Current nondigital PET machines have a spatial resolution of around 6 mm and therefore in vivo evaluations should mimic these conditions. This means, for instance, that a more dispersed, low-concentrated bacteria inoculum should be favoured in animal models to mimic a mild-moderate infection for optimal evaluation of feasibility as infection imaging agent. Also, the higher spatial resolution of microPET cameras must be considered during clinical translation.[117] Although still a subject to debate, utilizing SPECT radionuclides may not afford these agents the highest probability for routine use in clinics.

Particularly to infection imaging, key attention must also be given to the type of anatomical imaging modality combined with PET imaging. It is mentioned by Wehrl et al., that MRI could be vastly superior for infection imaging in both clinical and preclinical imaging. An example is made by referring to the imaging of a parasitic infection (*E. multiocularis*) with the superior soft-tissue contrast increasing the selectivity and sensitivity of the radiopharmaceutical marginally.[18]

Due to frequent occurrence of ill-fitted pairing of radionuclides to vectors in literature, the authors would like to re-emphasize the importance of the matching of these during radiopharmaceutical design. Given appropriate knowledge about the vector's targeting mechanisms and pharmacokinetics either the short half-life of that of widely available PET emitting radionuclides such as fluorine-18, gallium-68 and carbon-11 may be suggested or, such as for long-lived monoclonal antibodies, zirconium-89 and copper-64 should be the radionuclides of choice.[13,107]

As observed by Sverin et al.[126] a lack of radionuclidic controls during imaging studies is often limiting the value of the data. For instance, depending on the tissue pH (acidic conditions occur in infection sites) zirconium-89 has a high tendency for accumulation at disease sites in its free form, which is significantly different to its known biodistribution in healthy subjects. Therefore, as a control experiment, infectious disease animals should also be subjected to an injection of a formulated solution of zirconium-89 which can visualize the target contribution of the free radionuclide. This will provide the means to normalize the data of the test compound.[126]

It is well known and a remaining challenge that some agents might suffer from *in vivo* stability issues in the *in vivo* setting — often the radionuclide is either (or both) being freed from the vector, or the formation of additional in vivo metabolites occurs.[125,126] Comprehensive preclinical data is essential to substantiate the latter aspect before first-in-human studies are approached. Radiometabolite testing must be performed.

The animal infection model itself can be a limiting feature of the study, thereby an overall attention must be given to the level of translatability of the tested radiopharmaceutical into the Nuclear Medicine Clinic. Currently, the scientific analysis for the use of infectious animal models for specific diseases pertaining to radiopharmaceutical development is rare. One such analysis presented by Alstrup et al.[125] provides unique insights in the disease modelling for osteomyelitis specifically for radiopharmaceutical evaluations. In this review it is proposed that the size of the animal does indeed matter when applicability of translation into the clinic is evaluated. Furthermore, the animal's age and the origin of the bacterial cultures (human or animal) can become aspects of concern.[125] The scientific community involved in infection imaging should be encouraged to provide a critical justification with respect to the animal models used during these studies. Standardization and optimization of these techniques could realistically increase the translation of these radiopharmaceuticals into the clinic and reduce time spent in pursuing less than optimal candidates.

Despite the avid research field with various compounds suggested as infection imaging agents, some underexplored molecule types should be highlighted—aptamers and bacteriophages (BPh). As a promising class of molecules, aptamers are an interesting targeting vector that has yet to find application in PET based radiopharmaceuticals for infection imaging. A review by Li et al.[118] demonstrated the interesting targeting abilities of aptamers that have been applied in other diagnostic modalities. Aptamers are suitable vectors for PET radionuclides as it was already successfully demonstrated for oncology; the reader is referred to a comprehensive review on aptamer-based radionuclide imaging and therapy for more information.[120] While technetium-99m labeled aptamers have been designed for infection imaging, it is arguable if current SPECT performance can meet the technology demands for this application.[119] BPh are viruses that inherently target bacteria and provide an interesting vector. Similar to siderophores, the specific nature of the BPh's mechanism of action may allow for a pathogen-specific imaging. Radiolabeling of these viruses may not be straightforward and is yet to be attempted with PET radionuclides but has been reported preclinically in the past for technetium-99m labelled SPECT agents.[121-123]

Whilst the option for theranostic applications is already applied in the oncology clinic, it sparked discussions on how feasible, relevant and realistic such a strategy (technically) would be to imaging of infection within Nuclear Medicine. As was the case with oncological applications of theranostic radiopharmaceuticals, the see (diagnostic) what you treat (therapeutic) principle should be the main objective. This option has already been mentioned in recent literature along with the development of a lutetium-177 labelled benzylpenicillin.[124] It was also communicated in 2020 as an approach along with suggesting SARS-CoV-2 targeting theranostic radiopharmaceuticals.[72] Whilst we as authors may support with the latter idea, we believe it should be the complexity of the disease and lack of response to more established forms of therapy should dictate the consideration of a theranostics strategy. On this note, resistant bacteria (TB, MRSA) or some viral diseases would be possibly candid pathologies for a proof of principle approach. For instance, existing TB animal models (Kramnik mouse, infected guinea pigs or rabbits) are readily available for exploration in microPET imaging.

Overall, to better support clinical investigations on infected patients, it is expected that "the lessons to learn" may only get more intricate and even superior candid radiopharmaceuticals may not surpass the final assessment during clinical trials. Again, infectious diseases are expected to be a more and more complicated role player with major global health care burden and financial demands. While infection imaging - it is urgent to reach a stage where microorganism-specific radiopharmaceuticals are fully developed that provide selective and sensitive tools for PET imaging. This could very well be the next frontier for infection targeting Nuclear Medicine.

Conclusion

The recent SARS-CoV-2 pandemic demonstrated how devastating infectious disease still remains and research in this field should be a top priority. The preclinical development of novel more pathogen-specific PET radiopharmaceuticals has been highlighted. This area of research has made substantial progress; however, radiopharmaceuticals with the desired performance and properties only slowly emerge as part of Nuclear Medicine investigations in humans. Further demonstration of results in both smaller and larger animal studies could provide a clearer picture with respect to translation into the clinic. It is our recommendation for researchers to focus more on animal model design and to publish the results of the optimization of infectious imaging animal models, thereby provide better guidance to other researchers in the field. In addition, these infections in the animal models should be well validated and characterized. It should also be proven that the radiopharmaceutical has the ability to target low grade infections, as often these are closer in nature to the infections that need to be visualized in the human subjects. Despite the lack of harmonization of the preclinical evaluation methods for infection imaging radiopharmaceuticals a few can be mentioned as most promising candidates (in no particular order), namely flouride-18 labeled TMP, PABA, FDS as well as [^{68}Ga]Ga-PAF26, [^{89}Zr]Zr-VRC01 and other radiolabeled mAbs. Results from ongoing clinical investigations using [^{68}Ga]Ga-UBI and [^{18}F]FDS and [^{18}F]F-TMP in infected patients will determine their full potential and perhaps allow them to form part of routine clinics in Nuclear Medicine in the near future.

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