A decade of Ubiquicidin development for PET imaging of infection: a systematic review

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Highlights

- Gallium-68 UBI(29-41) is selective and specific for imaging of bacterial infections.
- Radiolabelling procedures are uncomplicated, fast and product is of adequate stability.
- Systematic optimization of labelling conditions and validated QC methods required.
- Larger clinical trials needed to ensure the translation of tracer into the clinic.

Abstract

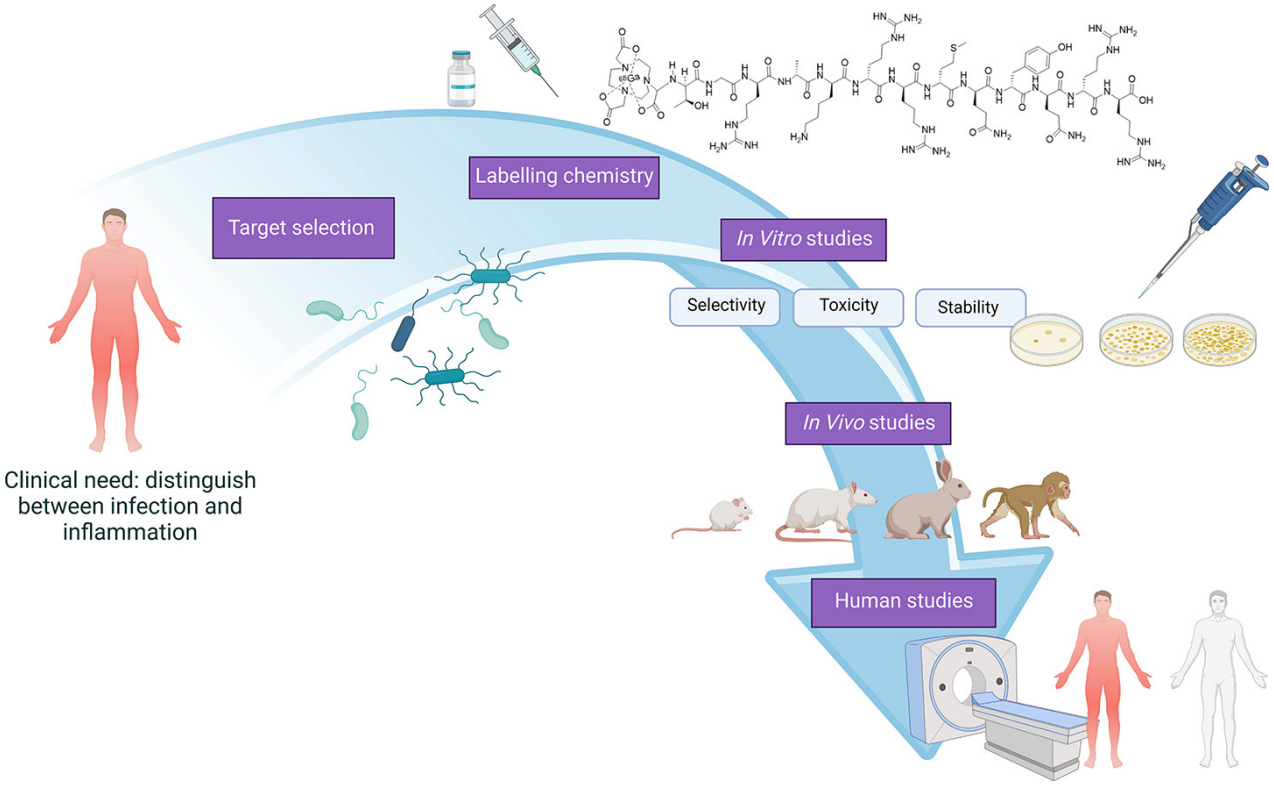
Background: Ubiquicidin is a peptide fragment with selective binding to negatively charged bacterial cell membranes. Besides its earlier labelling with gamma emitting radionuclides, it has been labelled with Positron Emission Tomography (PET) radionuclides in the last decade for imaging infection and distinguishing infectious disease from sterile inflammation. This systematic review aims to evaluate the technology readiness level of PET based ubiquicidin radiopharmaceuticals.

Methods: Two independent researchers reviewed all articles and abstracts pertaining ubiquicidin and PET imaging that are currently available. Scopus, Google Scholar and PubMed/Medline were used in the search. Upon completion of the literature search all articles and abstracts were evaluated and duplicates were excluded. All non-PET articles as well as review articles without new data were deemed ineligible.

Results: From a total of 17 papers and 10 abstracts the studies were grouped into development, preclinical and clinical studies. Development was published in 15/17 (88%) publications and 6/10 (60%) abstracts, preclinical applications in 9/17 (53%) publications and 1/10 (10%) of abstracts. Finally, clinical studies made up 6/17 (35%) of full publications and 4/10 (40%) of the available abstracts. Development results were the most abundant. All the findings in the different areas of development of ubiquicidin as PET radiopharmaceutical are summarized in this paper.

Conclusion: Labelling procedures are generally uncomplicated and relatively fast and there are indications of adequate product stability. The production of PET radiopharmaceuticals based on UBI will therefore not be a barrier for clinical introduction of this technology. Systematization and unification of criteria for preclinical imaging and larger clinical trials are needed to ensure the translation of this radiopharmaceutical into the clinic. Therefore a conclusion with regards to the clinical relevance of ubiquicidin based PET is not yet possible.

Graphical abstract



Keywords

Gallium-68 ubiquicidin, fluorine-18 ubiquicidin, infection imaging, radiochemistry, radioanalysis

1. Introduction

Microbial infections are becoming more challenging to treat making it the ticking time bomb of diseases in the 21st century. Infectious diseases are already the second highest cause of mortality globally and antimicrobial resistance, caused by inconsiderate prescribing practices, prophylactic use of antibiotics or excess usage of antibiotics by the food industry often negates treatment strategies that once offered effective disease management. The antimicrobial resistant ESKAPE organisms (*E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter species*) are bacterial infections of particular concern that have a major impact on human health.

It is estimated per annum that antimicrobial-resistance contributes to more than 29 000 deaths in the United States and more than 33 000 deaths in Europe. It is an underreported, but major threat, in developing nations [1-4]. One of the strategies to combat antibiotic therapy resistance is the early detection and accurate diagnosis of the infective microorganism. It is obvious that an early and accurate detection can reduce the overall residence time of the pathogen, curb pathogenic spreading and mitigate the risk of microbial resistance towards therapy. If prolonged hospital stays are reduced by evidence-based medicine, it will decrease the risk of infection with nosocomial infections in the immune deficient patient. All of these measures will reduce the mortality rate of infectious disease [5]. Currently, only persistent infections are further investigated by culturing or biopsies [6]. Radiological techniques (X-ray, Computed Tomography, Magnetic Resonance Imaging and Ultrasound) have a low sensitivity since gross changes in tissue caused by infection are visible only in a progressed stage of disease [7]. In this area, Nuclear Medicine based imaging techniques such as Positron Emission Tomography (PET) and Single-Photon Emission Computed Tomography (SPECT) have several advantages and great value and therefore may become more of a focus area in applied research. The pathogen can hereby be identified and monitored by non-invasive, often specifically targeted imaging, making a rationalized and personalized medicine approach possible. Currently, the gold standard for imaging of infection in Nuclear Medicine is using radiolabelled leukocytes and SPECT. Other radiopharmaceuticals often applied are [$^{68/67}$Ga]Ga-citrate-SPECT and [^{18}F]FDG-PET [8]. These techniques demonstrate improved sensitivity in comparison to anatomical imaging but are severely hampered with regards to

specificity and selectivity in this application since many pathways investigated are shared with malignancy and inflammation [9]. The novel nuclear imaging-based strategies more often involve pathogen-based targeting mechanisms as recently reviewed by Signore *et al*. [10] and Welling *et al*. [11] highlighting the targeting of the bacterial cell envelope, bacterial metabolism, bacteria-specific receptors or enzymes, intracellular proteins or bind to bacterial DNA/RNA. Aside from this, various synthetic antimicrobial compounds and antimicrobial peptides are radiolabelled and under further investigation [12,13]. However, to accurately evaluate the full potential of the newer bacterial infection imaging agents, the urgent need for a more standardized approach and a set of guidelines for investigation are required. It will potentially allow for the timeous elimination of unsuccessful candidates in early stages. More resources can then be focused on the investigations of viable candidates for infection imaging [14]. To this end, antimicrobial peptides (AMP) have a few unique features by way of interacting with bacteria making them promising candidates for their specific, selective and sensitive detection using real time imaging [15].

As a cation-rich AMP secreted by activated macrophages, ubiquicidin (1-59) was early-on considered to fit the properties required as for an *in vivo* infection imaging agent. Initially, a 12 amino acid-long ubiquicidin (UBI) peptide fragment (UBI_{29-41}) with retained targeting properties was radiolabelled with indium-111 or technetium-99m and therefore became available to clinicians as a radiopharmaceutical for detection of clinical bacterial infection [16,17]. Soon after, the widely investigated UBI_{29-41} was routinely labelled with technetium-99m for SPECT imaging [18,19]. Early-on it was demonstrated that UBI interacts, binds and accumulates in bacteria [18, 20] which paved the path for UBI to become interesting to the nuclear imaging sciences community. Labelled with technetium-99m [11], UBI-SPECT was considered as a technique for non-invasive imaging of complex bacteria manifestation in infected patients. However, further following the development of more advanced PET techniques with higher resolution able to pinpoint smaller lesions and provide better quality of images, investigation using PET-radioisotopes, in particular gallium-68, gained momentum.

This manuscript therefore aims to provide an assessment through a systematic review of the current status and an overview of work done on development of UBI-based PET imaging. The aim is to identify gaps in the current literature that need urgent attention for clinical translation.

2. Methods

2.1 Search Strategy

Two independent researchers systematically reviewed and identified all investigations pertaining to radiolabelled UBI fragments and their applications. The published content was searched by using scientific web search engines, e.g., Google Scholar, Scopus, PubMed, Research Gate, Web of Science, Science Open, *et cetera.* The main search terms were included: ubiquicidin, UBI, UBI peptides, NOTA/NODAGA/DOTA-UBI (-ubiquicidin) as standalone and/or combined with the terms such as PET-radioisotopes/-nuclides/-radiopharmaceuticals/-tracers, carbon-11, gallium-68, copper-64 and fluorine-18, radiometal-isotopes. Duplicate references in cases where more than one search term yielded the same reference and any articles by the same research group providing the same information with no additional insights were removed.

2.2 Inclusion criteria

All publications included were written in the English language. Articles that fulfilled the search terms as previously described, were critically reviewed. Additional records were identified by looking at literature referenced in appropriate review articles. Overall, available data input was divided into research describing UBI PET radiopharmaceutical development, as well as its preclinical and clinical investigations. Papers including data on synthesis of the radiopharmaceuticals, quality control, production methods and stability aspects of the produced product were grouped with data on the *in vitro* testing as the radiopharmaceutical development section. Preclinical content included testing the potential UBI radiopharmaceuticals in animal models whereas publications including studies in human participants were separated and grouped as clinical investigations. If the publication covered multidisciplinary investigations, it was accounted as a contribution to each of the groups. Furthermore, all research included was based on PET radionuclides.

2.3 Exclusion criteria

Review articles were excluded as well as studies on ubiquicidin that focused solely on SPECT radionuclides due to the predefined scope of this analysis. The exclusions of particular abstracts were made based on a consensus decision from all authors.

2.4 Limitations of the literature search

The search and review process were kept as unrestricted as possible to cover all possible search inputs and also find relevant evidence in auxiliary research output (conference proceedings, dissertations or similar academic output; however, limitations do occur. The systematic aspect of the search was hindered concerning reported methods in papers with focus on the abovementioned excluded content and cross-referencing research presented in review articles with a broad scope (but including UBI content). Restrictions were also experienced to access or include research content available in journals that are not indexed in the abovementioned search engines or are presented in other languages.

2.5 Quality of evidence and risk of bias

In order to determine the quality of evidence and risk of bias, certain tools were consulted but not necessarily applied in the traditional application way. The PRISMA guidelines together with the Equator checklist were implemented for systematic reviews [21,22]. For quality assessment of clinical studies, the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) was applied. Overall, the Risk of Bias in Systematic Reviews (ROBIS) tool was applied, albeit with some adjustments, to evaluate the quality of the systematic review results.

2.6 Study heterogeneity and presentation of results

As the selected studies was a very heterogeneous group, results were sub-grouped towards the use of different chelators, and/or different UBI fragments but kept cohesive for comparison for subsequent use of complexing gallium-68 (main PET-radioisotope used). A small set of reports on fluorine-18- and copper-64-radiolabeled UBI was evaluated separately. Due to lack of study standardization preclinical and clinical studies were presented chronologically. All results were collected by two independent searches and summarized in tables with all relevant parameters included. Tables were cross-checked and discrepancies resolved by all authors' consensus.

3. Results

3.1 Systematic review analysis

The outcome from the systematic review process is illustrated in **Figure 1**. with the article selection process following previous guidelines [22]. In this systematic literature review 27 studies were included which can be categorized by the PET-radioisotope-UBI investigation as follows:

- ❖ Fifteen research articles and eight cited abstracts featuring studies on gallium-68-UBI,
- ❖ Two research articles highlighting development of fluorine-18-UBI and preliminary work cited in one abstract on the potential labelling of NOTA-UBI using the aluminium-[^{18}F]fluoride pseudo-complex.

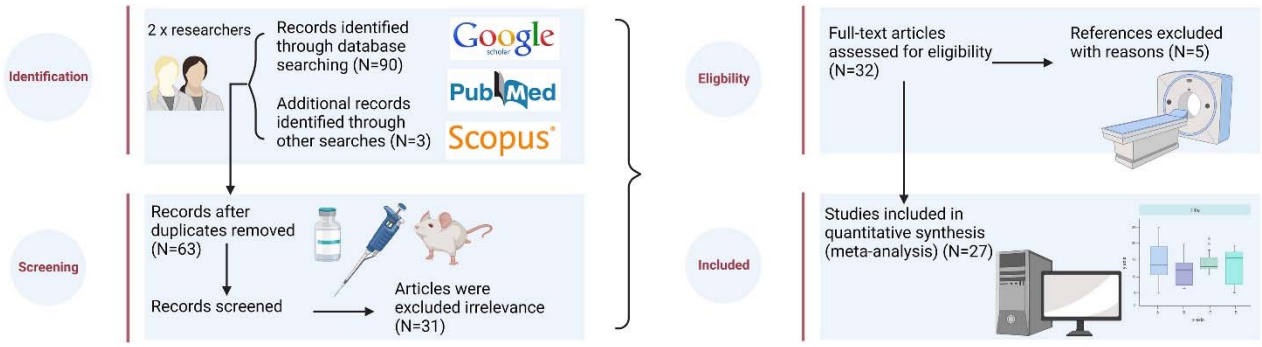


Figure 1: The identification and screening processes. The article selection was performed according to previous strategies followed [22].

Considering the results found in details of these studies, data was grouped on radiopharmaceutical development, preclinical investigation, or clinical application. Identification of the separate bodies of research will firstly provide insight into the readiness level and secondly provide valuable information on how soon UBI-PET is expected to become a routine application in Nuclear Medicine. The outcome discussed in this review will also be of value to identify the common factors (i.e., radiopharmaceutical aspects) that can possibly hamper further clinical translation.

As to be expected, basic (radiopharmaceutical) development on gallium-68-UBI forms the major research efforts evident in 76% (13/17) of research articles and 60% (6/10) of cited abstracts. Preclinical investigations were undertaken in 53% (9/17) of referred articles but only 10% (1/10) of cited abstracts. Finally, evidence for early clinical gallium-68-UBI and PET imaging in humans were found in 30% (8/27) of the total referred research articles and abstracts.
Due to this immense focus on gallium-68-UBI, such results were further tabulated and systematically analysed as described above; however, the other PET radionuclides are discussed, accordingly.

Considering the overall number of abstracts and full publications, 85% research refers to early, basic development followed by 37% preclinical and 37% clinical study content. A large number of the abstracts could be considered as been presented as preliminary work that preceded

the full-sized research articles from the relevant authors. Interestingly, only 18% (3/17) of the full-size research articles and 10% (1/10) of the abstracts report on basic development along with preclinical and clinical applications (i.e., bench-to-bedside research translation) and are counted as separate contributions to each field.

Publications were only excluded from further analysis [23-28] when supported by an overall agreement of the authors. This was applicable to any articles that provided no data , showed an ill-fitted match to the structure of the article [23], provided duplicate information [24-26], and clinical case studies that formed part of review articles that could not be cross-referenced or otherwise attributed to a peer-reviewed clinical study; the bias level was deemed too high [27,28].

3.2 Development of UBI as a PET radiopharmaceutical

Following the discovery of UBI_{1-59} (6600 g/mol) several linear peptide fragments were identified, namely UBI_{18-35}, UBI_{22-35}, and UBI_{31-38} and UBI_{29-41}. All of these fragments retained antimicrobial activity despite the lower molecular weight. However, UBI_{31-38} (RAKRRMQY) and UBI_{29-41} (TGRAKRRMQYNRR) have been mainly suggested as the most viable fragments due to better biological properties during their initial *in vivo* investigations. These two peptides are different from each other in length, size and net charge (UBI_{29-41} = 13-mer, 1692 g/mol, +7 vs. UBI_{31-38} = 8-mer 1107 g/mol; +5) but both show both good water solubility [29]. Following the early-on development of [^{99m}Tc]Tc-UBI for SPECT (utilizing exclusively UBI_{29-41}) research for UBI-based development for PET imaging predominantly focused on UBI_{29-41}, as well.

During the past 5 years, the synthetic UBI peptide fragments or their derivatives became commercially available with preference for radiopharmaceutical use (pharmaceutical grade / good manufacturing practice- certified). The origin of the radiosynthesis precursor material was declared in most publications, one of the following: ABX GmbH, CheMatech, Auspep, GL Biochem Ltd or piChem GmbH. Two publications indicated that the peptide raw material was synthesised in-house. Both UBI_{29-41} and UBI_{31-38} were derivatized to allow radiometal complexation the necessary chelating agent. Predominately, protocols for gallium-68 radiolabelling were developed, including testing the precursor concentrations, labelling conditions, eluate acidity or chelator variations to establish the best (robust) achievable yields and stability. The development also focussed on capable quality control measures to justify safe (potential) applications of radiolabelled UBI-radiopharmaceuticals. The available information on radiolabelling of UBI peptide derivatives with gallium-68 are summarized in **Table 1**.

Table 1: Overview of radiolabelling of UBI peptide derivatives with gallium-68

Radio-pharmaceutical	Ge-68/Ga-68 Generator information			Eluate Pre-process	Radiosynthesis method	Labelling conditions (optimized or routine)				Ref
	Manufacturer	Eluent (HCl)	Matrix			Concentration (nmol/ml)	pH	Incubation Temp/Time	Buffering agent (scavengers)	
NODAGA-UBI$_{29-41}$ NODAGA-UBI$_{31-38}$	iThemba	0.6 M	SnO_2	EF	Manual	25.0 36.0	3.5 - 4.0	90 °C /10 min	0.5 M Sodium acetate	30
NODAGA-UBI$_{29-41}$	iThemba	0.6 M	SnO_2	None	Manual	25.0	4.0	90 °C / 15 min	2.0 M Sodium acetate	31*
NOTA-UBI$_{29-41}$	iThemba	0.6 M	SnO_2	EF/ SCX	Manual Automated ^	10 5.7-14.3	3.5 - 4.0 3.5 - 4.0	90 °C / 10 min 90 °C / 10 min	1.0 M Sodium acetate 1.0 M Ammonium acetate 1.4% Ascorbic acid 1.0 M Sodium acetate Ascorbic acid/ethanol ##	32
NOTA-UBI$_{29-41}$	IGG100^X	0.1 M	TiO_2	SCX	Manual	202.0	3.5 - 4.0	90 °C / 12 min	5.0 M Sodium acetate	33
NOTA-UBI$_{29-41}$	IGG100^X	0.1 M	TiO_2	SAX	Manual	Not provided	3.5 - 4.0	85 °C / 10 min	Sodium acetate ++	34*
NOTA-UBI$_{29-41}$	iThemba	0.6 M	SnO_2	EF/ SCX	Manual Automated ^	10 5.2-9.0	3.5 - 4.0 3.5 - 4.0	90 °C / 10-15 min 90 °C / 10 min	1.0 M Sodium acetate 1.0 HEPES	35
NOTA-UBI$_{31-38}$	iThemba ITG	0.60 M 0.05 M	SnO_2 SiO_2	EF Not provided	Manual Manual	35.0 35.0	3.5 3.5	90 °C / 10 min 90°C / 10 min	4.0 M Sodium acetate 4.0 M Sodium acetate	36
NOTA-UBI$_{29-41}$	ITG	0.05 M	SiO_2	EF	Kit-based preparation	25.0	3.5 - 4.0	90 °C / 10 min	2.0 M Sodium acetate	37
NOTA-UBI$_{29-41}$	ITG	0.05 M	SiO_2	EF	Kit-based preparation	30.0	4.0	90 °C / 15 min	2.0 M Sodium acetate	38
NOTA-UBI$_{29-41}$	ITG	0.05 M	SiO_2	None	Microfluidic system	10.0 – 25.0	4.6	100 °C / 5 min	2.0 M Sodium acetate	39*

NOTA-UBI_{29-41}	E&Z ITG	0.10 M 0.05 M	TiO_2 SiO_2	EF	Manual	10.0 - 25.0	3.5 - 4.0	90 °C / 5 min	5.0 M Sodium acetate	40
NOTA-UBI_{31-38} NOTA-UBI_{29-41}	iThemba E&Z	0.60 M 0.10 M	SnO_2 TiO_2	EF/ SAX	Manual	26.0 25.0	2.5 - 3.0 3.3 - 4.0	80 °C / 15 min 80 °C / 15 min	2.5 M Sodium acetate 2.5 M Sodium acetate	41
NOTA-UBI_{29-41}	iThemba	0.60 M	SnO_2	EF	Manual	25.0	3.5 - 4.0	90 °C / 15 min	2.5 M Sodium acetate	42
DOTA-UBI_{29-41}	$IGG100^X$	0.10 M	TiO_2	SCX	Automated [xx]	Not provided	4.5	95 °C / 10 min	2.5 M Sodium acetate	43
DOTA-UBI_{29-41}	E&Z	0.10 M	TiO_2	EF	Manual	14.4	4.0 - 4.2	100°C / 15 min	1.0 M Sodium acetate [#]	44
DOTA-UBI_{29-41}	$IGG100^X$	0.10 M	TiO_2	Not provided	Manual	24.0	Not provided	95°C / 10 min	Not provided	45*
DFO-UBI_{29-41}	$IGG100^X$	0.10 M	TiO_2	SAX	Manual	Not provided	4.0	RT / 20 min	Sodium acetate [++]	46*

Abbreviated content:

DOTA) 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; NOTA) 1,4,7-triazacylonane- 1,4,7-triacetic acid; NODAGA) 1,4,7-triacyclonone-1-glutaric acid-4,7-acetic acid; DFO) deferoxamine; UBI_{29-41}) ubiquicidin peptide fragment TGRAKRRMQYNRR; UBI_{31-38}) ubiquicidin peptide fragment RAKRRMQY; iThemba) iThemba laboratories for accelerator-based science; E&Z) Eckert & Ziegler Eurotope GmbH - generator for human use; EF) Generator eluate fractionation; SAX) strong anionic exchange chromatography; SCX) strong cationic exchange chromatography; TiO_2) titanium-dioxide; SnO_2) tin-dioxide; RT) room temperature

Footnotes:

*) cited conference abstracts; ++) molarity not provided; [x]) Eckert & Ziegler Eurotope GmbH - generator of chemical grade; [xx]) Eckert & Ziegler Eurotope GmbH - Modular-LabPharm Tracer; [#]) unspecified amounts of ascorbic acid were added; ^) GMS Australia - Scintomics GRP 3V synthesis module, Manual) manual synthesis (by hand).

The two UBI fragments reported both require conjugation to a bifunctional agent at the amino-terminal group to allow for complexation of radiometal isotopes (hereby predominately gallium-68 reported), thereby providing a high degree of chemical and thermodynamic integrity. Although there is a large variety of chelating agents available, published evidence focussed on using the aza-macrocyclic chelators 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,7-triazacylonane- 1,4,7-triacetic acid (NOTA) and 1,4,7-triacyclonone-1-glutaric acid-4,7-acetic acid (NODAGA) [47]. Recently, one literature abstract reported the functionalized deferoxamine (DFO; 4%) conjugated to UBI29-41 exploring the untapped opportunity to use DFO-complexed Gallium-68, but its applications in vitro and in vivo are still pending [46]. Opting for a particular chelator may affect the radiolabelling strategy and parameters to achieve optimal radiolabelling yields and purity may vary significantly (Table 2).

3.3 *UBI radiolabelling strategies*

Commercially available germanium-68/gallium-68 generators from different suppliers were reported using 0.6 M to 0.05 M hydrochloric acid to elute the gallium-68 activity. The iThemba generator was the most used generator. Other generators employed were the ITG generator and the Eckert and Ziegler generators. Only Ebenhan *et al.* [42] and Vilche *et a*l. [40] used two different generator types during the optimization of labelling procedures. Whilst the usage of specific generators is possibly connected to regional availability of generators, it is our recommendation, due to the starting pH resulting from the different concentrations of hydrochloric acid, to develop more universally applicable radiosynthesis methods.

An overview of all available results from publications (**Table 2**) indicated that most of the research groups managed to establish a straightforward manual/direct labelling followed later on by equal efforts to implement [^{68}Ga]Ga-UBI preparation using manual/kits or automated synthesis. Evidence for more regulated production was found in 2 publications using cold kit starting material for reconstitution with gallium-68 (i.e., radiopharmaceutical compounding) and 3 publications reporting a fully remote automated procedure with relevance to GMP production. Interestingly, the automatic synthesis as well as kit labelling procedure was directly compared in 1 research article [32].

Most publications used NOTA as a cyclic radiometal chelator of choice. Except for one publication describing incubation at 100 °C for a short 5-minute period [39] all reports on the radiolabelling refer to incubation of the reaction between 80 to 90 °C for times varying from 5-15 minutes [32-

Table 2 : Quality control parameters

Compound (UBI derivative)	Radiochemical purity				Post-purification	Radiochemical yield	Molar activity MBq/nmol	Activity concentration (MBq/ml)	Reference
	HPLC method A:0.1% TFA/H_2O B:0.1% TFA/ACN	ITLC method	HPLC RCP	ITLC RCP					
NODAGA-UBI_{29-41} NODAGA-UBI_{31-38}	C18 RP - gradient 0.1% TFA in H_2O/0.1 % TFA in ACN	**ITLC-SG** 15% HCL in MeOH 0.1 M NaCitrate (pH 5)	>95%	>95%	Not performed	>95%	5.2 3.6	216	30
NODAGA-UBI_{29-41}	**C18 reverse phase** Water and ACN with 0.1% TFA - gradient	Not performed	>98%	Not per-Formed	Not performed	Not provided	1.5	55	31*
NOTA-UBI_{29-41}	**C18 reverse phase** Water and ACN with 0.1% TFA - gradient	**ITLC-SG** 0.1 M NaCitrate (pH 5)	>95	>95%	C18 Sep-Pak	65.5 ± 22.6% 63.2 ± 1.5% 57.3 ± 3.8%	20.4 ± 11.4 27.6 ± 0.9 11.4 ±1.9	236 345 290	32
NOTA-UBI_{31-38}	**C18 reverse phase** Water and Acetonitrile with 0.1% TFA - gradient	**ITLC-SG** 0.1 M NaCitrate (pH 5)	>96.5	>96.5	Not performed	51 ± 23%	25 ± 12	216 ± 162	52*
NOTA-UBI_{29-41}	**Not performed**	**ITLC-SG** 0.1 M Citric Acid	N/A	87%	C18 Sep-Pak	87.3%	Not provided	Not provided	34*
NOTA-UBI_{29-41}	C18 - gradient 0.1% TFA in H_2O/0.1 % TFA in ACN	**Not performed**	>96%	Not performed	Cationic exchange	72%	1	29 – 36	33
NOTA-UBI_{29-41}	**C18 column** Isocratic 15% acetonitrile and 85% water with 0.1% TFA	**ITLC-SG** 0.1 M NaCitrate (pH 5)	>95%	98.9 ± 0.3 99.3 ± 0.1	C18 Sep-Pak	65.5 ± 22.6% 83.4 ± 6.7% 71.8 ± 3.5% 78.9 ± 3.6%	20.4 ± 11.4% 26.5 ± 0.8% 21.3 ± 2.0% 20.6 ± 0.9%	Not provided	35
NOTA-UBI_{31-38}	**C18 reverse phase** Water and Acetonitrile with 0.1% TFA - gradient	**ITLC-SG*** 15% HCL in MeOH 0.1 M NaCitrate (pH 5)	>90%	>90%	Not performed	>90%	2.1	370	36

NOTA-UBI$_{29-41}$	**C18 RP HiQ Sil** - gradient 0.1% TFA in H_2O/0.1 % TFA in ACN	**ITLC-SG** 15% HCL in MeOH 0.1 M NaCitrate (pH 5)	>90	>90	Not performed	>90	14.8	123	37
NOTA-UBI$_{29-41}$	**Not provided**	**Not performed**	>95%	N/A	Sep Pak C18 Sep Pak C18 Plus	63% 75%	25	49.8	39*
NOTA-UBI$_{29-41}$	**C18 column** Water and Acetonitrile with 0.1% TFA - gradient	**ITLC-SG** 0.1 M Citric Acid	96 ± 3%	96 ± 3%	C18 Sep-Pak light	>90%	0.55	Not provided	40
NOTA-UBI$_{31-38}$ NOTA-UBI$_{29-41}$	**Stable bond C18 column** Water and Acetonitrile with 0.1% TFA - gradient	**ITLC-SG** **1M** 0.1 M NaCitrate (pH 5)	>99%	>99%	Solid phase extraction (3 SPE and 1 online cartridge)	>60%	8.9 ± 0.7 13 ± 0.8	232 ± 80 249 ± 67	41
NOTA-UBI$_{29-41}$	**Performed - not described**	**ITLC-SG** 0.1 M NaCitrate (pH 5)	N/A	99%	C18 Sep-Pak	75 ± 4%	20	168	42
DOTA-UBI$_{29-41}$	Core-shell Polar C18 Water and Acetonitrile with 0.1% TFA - gradient	**Alumina backed ITLC** 0.1 M NaCitrate (pH 5)	>99%	>99%	C18 Sep-Pak	>95%	Not provided	Not provided	43
DOTA-UBI$_{29-41}$	C18 column Water and Acetonitrile with 0.1% TFA - gradient	Not performed	>95%	Not per-Formed	None	Not provided	Not provided	Not provided	44
DFO-UBI$_{29-4}$	C-18 Water and acetonitrile (0.1% TFA) gradient	**ITLC-SG** NH_4OAc (77 g/L) in [1:1] MeOH : H2O	98±2	Not given	None	83±2	Not provided	250	46*

*Conference Abstracts

42]. Heating of reaction mixture with NOTA is not reported as necessary in literature but in such cases, it can be done to allow shortening of labelling time and for achievement of higher labelling yields with respect to the short physical half-life [40-41]. It was reported by Vilche and co-workers [40], that maximum yield and radiochemical purity was reached at room temperature in 10 minutes, but yields were not consistent. When the reaction mixture was heated 90-100 °C and incubated for 5 min, variations of reaction yields were smaller than when labelling was done at room temperature.

NODAGA was used in some publications with all conditions comparable to labelling using NOTA chelator. NODAGA's complexation site (allowing for an additional carboxylic acid moiety to protect the Ga^{3+} ion in the cage) due to its structure is not affected by conjugation which in theory provides and advantage over NOTA and hence perceived improved in vivo stability. This has however not been proven to be established in the studies evaluated during this systematic review as no such a study is published specifically on UBI based radiopharmaceuticals incorporating NOTA, DOTA and NODAGA.

Optimum pH for labelling with gallium-68 is well established within a range of 3.5 to 4.5 for all applications of gallium-68 chelator systems. All publications presented here did keep to this standard pH range; however, in particular clinically oriented publications did not disclose or cross-reference the labelling conditions. Various concentrations of sodium acetate (0.5 M to 5.0 M) were used to adapt the eluate acidity. Le Roux *et al.* explored different buffer types (ammonium formate being recommended) with better suited buffering capacity for a pH range of 2.7-4.7; employing 2-[4-(2-hydrocyethyl)-1-piperazin-1-yl]-ethane-1-sulphonic acid (HEPES) was proven sub-optimal and the authors discouraged its use for [^{68}Ga]Ga-UBI preparations due to a mismatch with the allowable limit in the final product (200 µg/V) [32].

The C18 reverse phase cartridges (Waters or Phenomenex) are most often used in purification of [^{68}Ga]Ga-UBI from non-complexed gallium-68 species and colloids where strongly hydrophobic silica-based bonded phase is used to attract wanted [^{68}Ga]Ga-UBI which will be eluted from cartridge by ethanol later. In 9 publications or abstracts with NOTA-UBI, gallium-68 labelled purification was performed using either a C18 light, plus and tC18 cartridge based on the same chemical principles and properties while a Strata strong cation exchange cartridge with ability of use as reverse phase was reported in 3 publications.

Only two publications focused on the investigation of [^{18}F]F-UBI, published in 2006 and 2008. Ziljstra et al. [48] describe the synthesis method that uses nucleophilic substitution (including kryptofix) ending with N-succinimidyl-4-[^{18}F]fluorobenzoate that is clicked onto the ubiquicidin fragment 29-41 via the lysine group. The fully automated synthesis, with a microsized computer, took 150 minutes to complete. This synthesis method was also followed by Salber et al., (2008) [49]. This method allows for the labelling of UBI at room temperature which could have a positive influence on the stability of UBI. Overall, fluorine-18 labelling has milder reaction conditions compared with gallium-68 labelling. After radiolabelling, the method described, utilizes purification by gradient HPLC which is followed by a second purification step with C18 light cartridges. Radiochemical yields were reported between 9 and 12%. The final molar activity of this radiopharmaceutical was > 35 GBq/μmol. A different approach published by Loppolo *et al.* [50] made use of an AlF^{2+} complexation strategy. Synthesis time was drastically decreased to 20 min, with no further purification necessary. However, stability of the product in plasma remains a concern (RCP decrease to 50 % within 1 h). Studies reported without development parameters of the radiopharmaceutical induce a risk of bias into the analysis [51].

3.4 *Quality control - Radioanalysis*

In 63% of publications on [^{68}Ga]Ga-UBI labelling available HPLC methods are described for assessment of radiochemical purity, while in 38% this information is not available. No validation from nonradioactive construct to HPLC method and finally ITLC development is available. Different types of reverse phase C-18 HPLC columns, functioning on the same separation principle are used. For mobile phase composition a mobile phase A: containing 0.1% TFA/H_2O and B: containing 0.1%TFA/ACN were invariably used. Composition percentage of mobile phases A and B varied from article to article as well as injection volumes. Radiochemical purity should be higher than >95% as is consensus in the field of radiopharmacy and Nuclear Medicine.

Radiochemical reaction yield from publications with DOTA- and NODAGA-chelated UBI, report high values of >95%. NOTA-chelated UBI gave variable yield, early development yields were ≈50 % while later yield improved to 70-90%. The radiochemical yield, despite being an important input parameter for the feasibility of clinical trials, can be compromised by several factors often attributed to the chelator used (pH, temperature and concentration), generator type and variability of elute quality and also method of labelling (multi-step manual radiolabelling or automated radiosynthesis or kit-based radiopharmaceutical preparations).

3.5 In vitro studies

A summary of the input for the in vitro studies along with their principal findings are presented in **Table 3**. Herein, [^{68}Ga]Ga-NOTA-UBI stability in saline was noted to be 1-2 h and serum stability was attested for up to 3 h. Reports provided by Vilche *et al.* showed that at 2 h there was already 10% release of gallium-68 . A change of radiolabelling purity of 3.6% was noted by Ebenhan *et al*., at 3 h. No [^{68}Ga]Ga-DOTA-UBI stability in saline was reported. Stability of [^{68}Ga]Ga-NODAGA-UBI in saline and serum was tested for 1 h and was found to be stable in both.

Bench top stability of [^{68}Ga]Ga-NOTA-UBI was tested in most articles and was found stable for 2-4 h at room temperature. The stability of [^{68}Ga]Ga-DOTA-UBI and [^{68}Ga]Ga-NODAGA was stable up to 3 and 2 h, respectively. Note that the stability testing of [^{68}Ga]Ga-DOTA-UBI and [^{68}Ga]Ga-NODAGA-UBI was only reported by one literature source each. It is noted that often stability is described only by the time tested (influenced by short half-life) and not necessarily by the time when changes and decompositions are noticed.

The partition coefficient estimation (-3.1,-3.6 and -3.8) done on [^{68}Ga]Ga-NOTA-UBI and [^{68}Ga]Ga-NODAGA-UBI demonstrate that they are hydrophilic compounds. This information was not presented for [^{68}Ga]Ga-DOTA-UBI.

Investigations with regards to the *in vitro* behaviour of [^{18}F]F-UBI demonstrated that this radiopharmaceutical had a lower affinity for *S.Aureus* compared to the thoroughly investigated [^{99m}Tc]Tc-UBI derivatives. The binding of [^{99m}Tc]Tc-UBI was reported to be 36% in literature, but Ziljstra *et al.,* (2006) [48] determined only a 15% binding of [^{18}F]F-UBI to *S.Aureus*. Various explanations for this were provided by the author and the radiopharmaceutical was ultimately deemed as a viable candidate for further *in vivo* investigations.

Concerning the quality controls during *in vitro* studies evaluating *S. Aureus* binding affinity, these were not extensive. For the majority of studies, no quality control was reported. In studies where quality control procedures were performed, it was limited to identifying the non-specific binding of the radiopharmaceutical done by including a control group that does not have any *S. Aureus* present.

Table 3: *In vitro* studies

Radiopharma-ceutical	Stability		Biological stability		Partition coefficient	Bacterial uptake (S.Aureus)	Other findings	Reference
	Shelf-life (kit)	Bench-Top	Saline	In vitro				
UBI_{29-41}	N/A	N/A	N/A	N/A	N/A	N/A	No genotoxicity potential.	53
NODAGA-UBI_{29-41} NODAGA-C	N/A	Up to 2 hours	Up to 1 hour	Up to 1 hour	-3.8 ± 0.15 -2.9 ± 0.13	Yes	Differences in uptake between NODAGA UBI_{29-41} and UBI_{29-41} are not statistically significant	30
NODAGA-UBI_{29-41}	N/A	N/A	Stable	Stable	N/A	Yes	MIC > 150 μM for S.Aureus ATCC25923	31*
NOTA-UBI_{29-41}	N/A	Up to 3 hours	N/A	N/A	Not performed	Not performed	None	32
NOTA-UBI_{29-41}	N/A	At room temp & refrigerated up to 1.5 hours	Not performed	Not performed	Not performed	Not performed	Protein binding > 50% up to 1 hour.	34*
NOTA-UBI_{29-41}	N/A	Not performed	Not performed	Not performed	Not performed	Yes	Minimal cellular toxicity against normal human cells. Lack of antimicrobial activity.	54
NOTA-UBI_{31-38}	N/A	Up to 2 hours	Up to 1 hour	Up to 1 hour	-3.1 ± 0.45	Yes	Preincubation of *S.Aureus* ATCC25923 with cold NOTA-UBI_{31-38} demonstrated significant decrease in uptake indicating specific uptake.	36
NOTA-UBI_{29-41}	6 months	up to 2 hours	stable	stable	-3.6	Yes	Preincubation of *S.Aureus* with cold NOTA-UBI_{31-38} demonstrated significant decrease in uptake indicating specific uptake.	37
NOTA-UBI_{29-41}	6 months	Not performed	Not performed	Not performed	Not performed	Not performed	Kit vial was optimized and clinical study was performed	38
NOTA-UBI_{29-41}	N/A	Up to 3 hours	Not performed	Not performed	Not Performed	Yes	Protein binding ranged from 37 - 52% A logarithmic binding demonstrated with increased	40

							concentration of bacteria up to 97 ± 2%	
NOTA-UBI$_{31-38}$ NOTA-UBI$_{29-41}$	N/A	Up to 4 hours	Not performed	Not performed	Not performed	Yes	Preincubation of *S.Aureus* with cold NOTA-UBI$_{31-38}$ demonstrated significant decrease in uptake indicating specific uptake.	41
NOTA-UBI$_{29-41}$	N/A	Up to 4 hours	N/A	Up to 180 min	Not performed	Not performed	The aim of this study was *in vivo* analysis. Not many in vitro analyses were performed.	42
DOTA-UBI$_{29-41}$	N/A	Up to 3 hours	Not performed	Up to 1 hour	Not performed	Yes	Able to bind avidly with *S. Aureus* up to 50% within 30 minutes.	43
DOTA-UBI$_{29-41}$	N/A	Not performed	Not performed	Declined >90% at 4 hours	Not performed	Not performed	Protein binding ranged 50-60%	44
DFO-UBI$_{29-41}$	N/A	Up to 2 hours	Not performed	Not performed	Not performed	Not performed	None	46*

*conference abstracts

Table 4: Preclinical evaluation of [^{68}Ga]Ga-UBI[#]

Radiopharmaceutical	Species	Healthy animals	Infectious model			Control group (Differentiation)	Procedures	Principal findings	Bias	Ref
			Strain	CFU	Inoculation method					
NODAGA-UBI$_{29-41}$ NODAGA-UBI$_{31-38}$	Normal Swiss mice	Yes	N/A	N/A	N/A	N/A	Ex-vivo biodistribution	Pharmacokinetics of NODAGA UBI$_{29-41}$ and UBI$_{31-38}$ the same *in vivo*	High **	30
NOTA-UBI$_{29-41}$	Danish Landrace Yorkshire Pigs	No	S.Aureus	10^5	Femoral artery injection	No	PET/CT Imaging	No accumulation in osteomyelitis lesions	High **	33
NOTA-UBI$_{31-38}$	Balb/c mice	No	S.Aureus	10^7	Injected in thigh muscle	Heat killed S. Aureus (inflammation)	Histology Gram staining Ex-vivo biodistribution µPET/CT Imaging	Target to non-target ration of 3.24 ± 0.7	Medium to low	36
NOTA-UBI$_{29-41}$	Balb/c mice	No	S.Aureus	10^7	Injected in thigh muscle	Heat killed S. Aureus (inflammation)	Infection lesion cultured + Histology Ex-vivo biodistribution	Target to non-target ratio of 2.6 ± 0.7	High **	37
NOTA-UBI$_{29-41}$	Non-Human primates	Yes	N/A	N/A	N/A	N/A	PET/CT Imaging	Extrapolated human biodistribution and radiation dosimetry provided	Low	54
NOTA-UBI$_{29-41}$	Normal Swiss mice	Yes	S.Aureus	1.2 x 10^7	Injected in thigh	Heat killed S. Aureus	Infection lesion cultured	Maximum T/NT ration 4.0 at 60	Low	40

					muscle	(inflammation)	Ex-vivo biodistribution µPET/CT Imaging	minutes Selectivity P<0.002		
NOTA-UBI_{29-41}	New Zealand white rabbits	Yes	S.Aureus	$2x10^8$	Injected in thigh muscle	Turpentine oil (sterile inflammation) Ovalbumin (asthmatic lung condition / inflammation)	Histology Gram staining PET/CT Imaging Urine analysis Serum integrity	Significant uptake in infection, minimal localization in sterile inflammation	Low	41
DOTA-UBI_{29-41}	Male Wistar Rats	Yes	S.Aureus	5×10^4	Intra-muscular	Turpentine oil (sterile inflammation)	µPET/CT Imaging	Fast uptake in target area and fast clearance out of blood pool and soft-tissue.	High	43
DOTA-UBI_{29-41}	Patogen free JcL:ICR male mice	No	S.Aureus	10^8	Injected in thigh muscle	Autoclaved S. Aureus (inflammation)	Ex-vivo biodistribution µPET/CT Imaging Excretion data	Target to non-target ration of infected 4.62 ± 3.44 and inflamed target to non-target ration of 1.53 ± 1.73	High	44
DOTA-UBI_{29-41}	Male Wistar Rats	No	S.Aureus	5×10^5	Intra-muscular	No	µPET/CT Imaging	Target to back-ground ratio increased from 1.6 (6 min) to 4.2 (20 min) to 6.1 (60 min)	High **	45*

*) Conference Abstracts; N/A = information not available or not disclosed.

#) All studies were approved by bodies ascribing to national or local ethics committees (none where performed according to particular harmonized international standards.

**) bias determined due to lack of approving methods (imaging or histopathology or bacterial culturing).

3.6 Preclinical studies

Ten publications (2 abstracts and 8 publications) included preclinical evaluations (**Table 4**). This group of studies is very heterogeneous, yielding in study results that are not suitable for meta-analysis. Four publications report studies in mice (Swiss mice, Balb/c and Pathogen free JcL:ICR) and two publications report rats (Wistar). Only one study (Figure 3) used a larger rodent (New-Zealand White Rabbit). Two studies were performed in pigs and non-human primates for larger animal investigations.

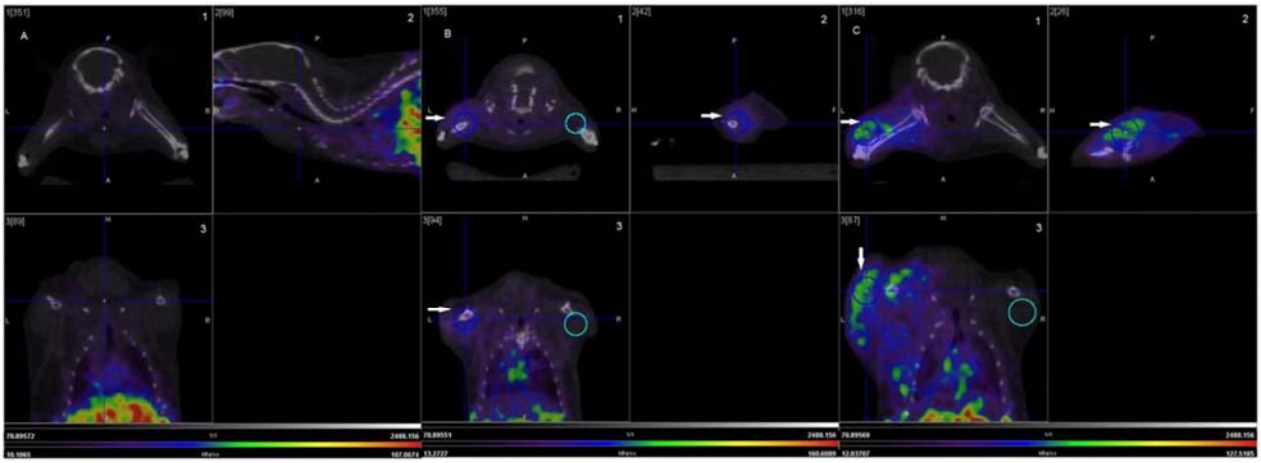


Figure 2: The accumulation of [^{68}Ga]Ga-NOTA-UBI$_{29-41}$ is clearly demonstrated by the differences in accumulation of a healthy mouse (A), a mouse with sterile inflammation (B) and an S.aureus infected mouse (C). This research was originally published in JNM. (Vilche et al., J Nucl Med: 2016:57(4): 622-627 ©SNMMI [40]).

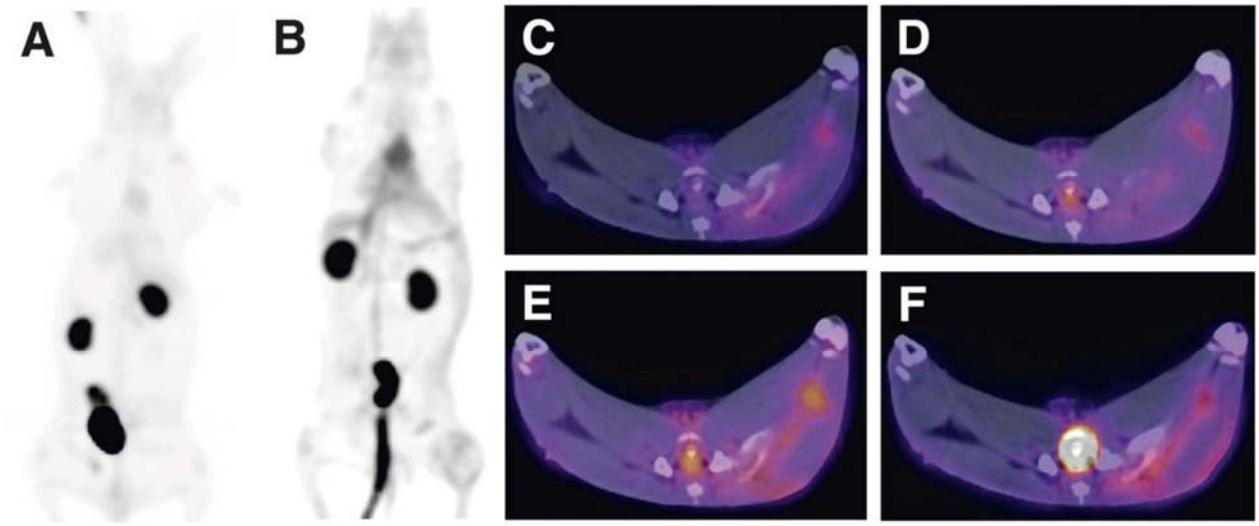


Figure 3: Accumulation of [^{68}Ga]Ga-NOTA-UBI$_{29-41}$ in a healthy animal (A) and a rabbit with muscular infection on the right thigh (B) at 60 minutes after administration. This research was originally published in JNM. (Ebenhan *et al*., J Nucl Med: 2014;55(2):308-314. ©SNMMI [41])

With regards to study design only 50% of studies included healthy animals as a control group. To distinguish between infection and inflammation, an infection control has to be included in the animal model. Most often, this is done in the same animal. If the imaging does not accumulate in the area of the inflammation, it is determined that the radiopharmaceutical is selective for infection. This is provided as a target to non-target ratio. Inflammation was induced in the majority of the studies. The methods used were heat killed *S.Aureus*, turpentine or ovalbumin to induce asthma. Infection was introduced in the relevant studies using S.Aureus as an infectious agent. When used, colony forming unit (CFU) was always reported and the bacteria were injected in the femoral artery or thigh muscle.

The evaluation of the radiopharmaceutical accumulation and biodistribution was done by *ex-vivo* biodistribution. Imaging analysis by PET/CT was performed in 90% of the reported studies. Importantly, four of the ten studies did histology analysis on tissue, either by gram staining or infection culturing. All the small animal studies demonstrated optimal target to non-target ratios. Pharmacokinetic data was gathered. One study stands out with respect to the data gathered and that is the large animal study reported by Afzelius *et al.,* [33]. In this study no accumulation in osteomyelitis lesions was reported.

Salber *et al*., (2008) failed to determine selective *in vivo* binding of [^{18}F]F-UBI to *S. Aureus* [49]. The study animal model was male fisher rats that received injection with *S.Aureus* in the calf muscle (10^7 CFU). It is noteworthy that not [^{18}F]F-UBI nor [^{99m}Tc]Tc-UBI demonstrated accumulation at the target site. No further development of [^{18}F]F-UBI as a radiopharmaceutical to date was found during the literature search.

During this evaluation studies with a lack of imaging (only *ex-vivo* data), lack of histopathology to identify an infection, and lack of the proper controls, was identified as having a high risk of bias. The majority of studies included in this review had a high risk of bias.

3.7 Clinical investigations

Between 2016 and 2022 ten publications emerged containing clinical investigations using [^{68}Ga]Ga-UBI-based radiopharmaceuticals reporting on results from either interesting patient case studies, patient case series and first-in-human studies that supported the proof-of-principle research investigations or research studies featuring diagnostic imaging with retrospective / prospective data analysis. Ethical approval was handled by local (mostly academic) research ethics; patient consent was achieved prior to the enrolment. A summary is presented in **Table 5** featuring results from 61 patients from five different research centres.

Three studies included healthy volunteers that underwent [^{68}Ga]Ga-UBI PET imaging to either approve the expected biodistribution (clinical translation of results from animal studies) or to determine the radiation dosimetry. Most of the studies focussed on research for the preliminary demonstration of clinical utility of [^{68}Ga]Ga-UBI-based radiopharmaceuticals for an accurate diagnosis of infection. For example, intense focal uptake in the ankle joint was demonstrated in a patient with confirmed infection (Figure 4) [54]. Other patients were imaged to ascertain diagnosis of spinal (bone) infection (Fig 5a) or to successfully decipher infectious soft tissue from any bone involvement in the infection (Fig 5b). Three studies also reported true negative PET scans provided for at least 3 patients, for example to confirm postsurgical aseptic condition of a hip prosthesis (Fig. 5c) [37].

Table 5: Summary of current clinical evaluations

Radiopharmaceutical	Study population	Type of clinical investigation	Principal findings	Masking	Bias	Ref
[^{68}Ga]Ga-NOTA-UBI$_{31-38}$	Patient with histologically confirmed foci of infection (n=1) and negative control (n=1)	Case study (as part of proof of principle publication)	Preliminary demonstration of clinical utility with accurate Diagnosis of infection	No	High	36
[^{68}Ga]Ga-NOTA-UBI$_{29-41}$	Patients with suspected infection (n=3)	First-in-Human (as part of proof-of-principle publication)	Preliminary demonstration of clinical utility with accurate diagnosis of infection	No	High	37
[^{68}Ga]Ga-NOTA-UBI$_{31-38}$	Male (n=6) / female (n=8) patients with suspected infection	Diagnostic Imaging - retrospective analysis	Preliminary demonstration of clinical utility with accurate diagnosis of infection	No	High	52*
[^{68}Ga]Ga-NOTA-UBI$_{31-38}$	Patients with suspected infections: Diabetic foot (n=7), cellulitis (n=2) and fracture (n=1)	Diagnostic Imaging (prospective analysis)	Accuracy = 40%	Yes	Medium #	38
[^{68}Ga]Ga-NOTA-UBI$_{31-38}$	Healthy volunteers (male n=1, female n=1) and Patients with known infection (n=3)	First-in-Human investigation (as part of proof-of principle publication)	Preliminary demonstration of clinical utility with accurate diagnosis of infection	ND	High	54
[^{68}Ga]Ga-NOTA-UBI$_{29-4}$	1 patient	Case study	Accurate diagnosis of infection	No	High	55
[^{68}Ga]GaDOTA-UBI$_{29-41}$	Healthy volunteers (male n=2, female n=2)	Radiation Dosimetry	Preliminary demonstration of favourable dosimetry	n/a	Low	56*
[^{68}Ga]GaDOTA-UBI$_{29-41}$	Patient with implant (n=1)	Case study	True negative result with images confirming no implant infection.	No	High	57
[^{68}Ga]Ga-NOTA-UBI$_{29-41}$	Patients with hip implants (n=21)	Diagnostic Imaging (head-to-head comparison with ([^{18}F]FDG)	First comparative imaging with better results for UBI-PET (Sensitivity = 93 % Specificity = 100%) over FDG-PET.	ND	Medium	58*

*) Conference abstract disclosing limited information (information from poster - or oral presentation may not be published); n/a = not applicable; ND = not disclosed; #) Study design issues disclosed (e.g., patients with active antibiotic therapy enrolled for imaging.

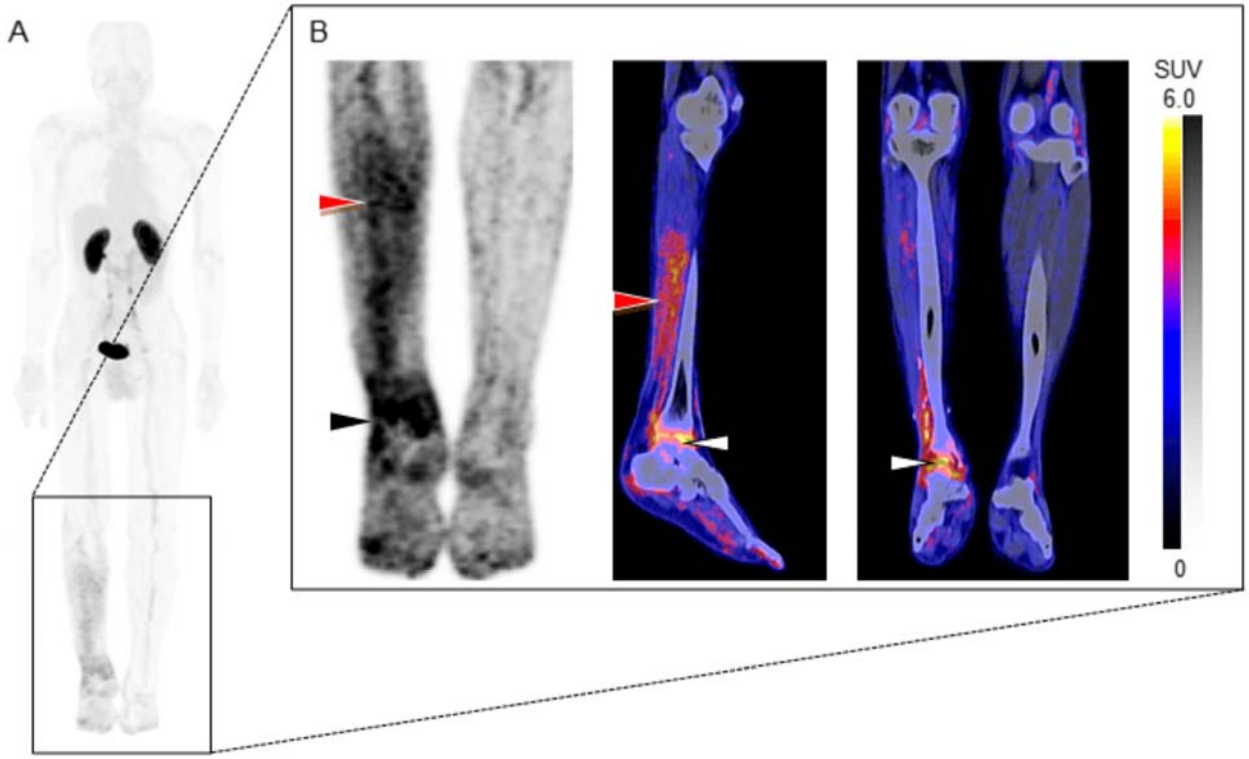


Figure 4: Focally increased radiopharmaceutical uptake in the ankle joint demonstrated by [^{68}Ga]Ga-NOTA-UBI29-41PET/CT obtained 60 minutes after radiopharmaceutical administration. This research was originally published in *JNM*. (Ebenhan *et al*., J Nucl Med: 2018;59(2):334-339. ©SNMMI [54]).

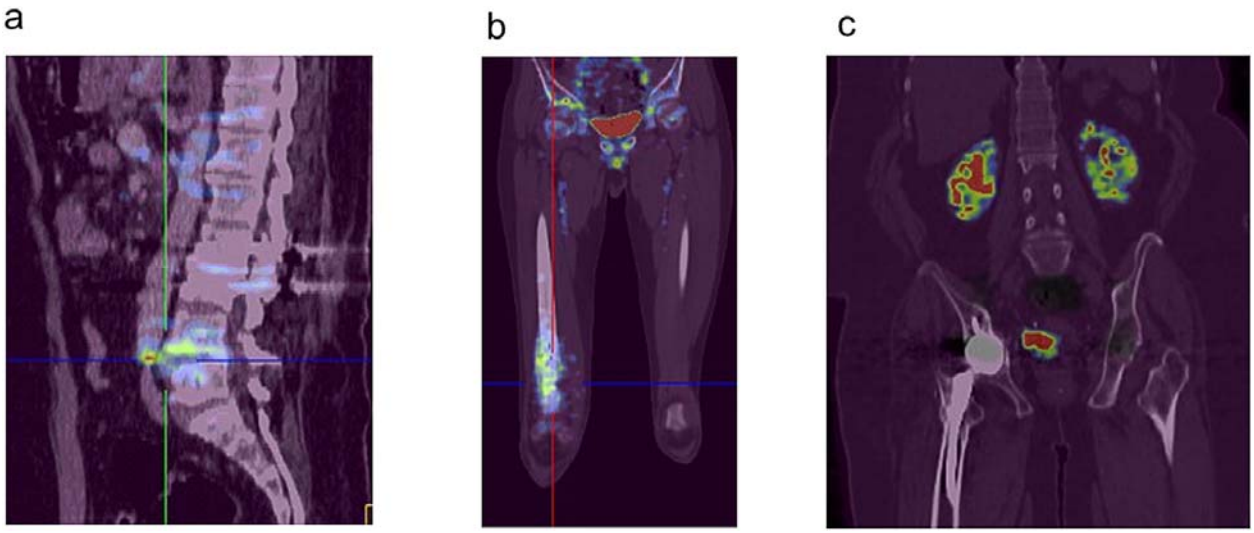


Figure 5: [^{68}Ga]Ga-NOTA-UBI29-41 PET/CT images showing a) true positive scan for TB spine infection b) true positive scan for infection in right thigh and c) true negative scan for suspected prosthesis infection (provided by A. Mukherjee, as previously published in Journal of Pharmaceutical and Biomedical Analysis [37])

4. Discussion and Outlook

Ubiquicidin (UBI) is a 59 amino acid sequence protein, with net charge of +19 at physiological pH and it belongs to a group of antimicrobial peptides. Its high affinity towards bacterial cells and human non-immunogenic nature is considered as suitable for use in diagnostics. It is known that antimicrobial peptides bind to the negatively charged bacterial cell membrane and cause disturbance of the membrane using different mechanisms. This section highlights some progress and shortcomings and also provide some guidance on where the gaps are in the development of UBI based radiopharmaceuticals.

4.1 Gallium-68 based UBI radiopharmaceuticals

The length of peptides and order of amino acids in the peptide chain can influence activity and stability of the peptide. With longer amino acid sequences, peptide chains become more susceptible to the reaction conditions (high pH, high temperature) during radiolabelling. This can result in the breaking of peptide chains and cause loss of original peptide activity. If alternative peptide conformations are formed this can induce the accumulation of the new peptide chains in unwanted organs. There are several mechanisms to improve stability without changing the mechanism of reaction. This includes (but is not limited to) the introduction of new moieties, exchange of labile amino acid bonds with alternative amino acids, or cyclization [59,60]. It is notable that none of these strategies were applied during the design of UBI based radiopharmaceuticals for gallium-68 radiolabelling. However, it was observed by *le Roux et al* (2020) that automated synthesis with a higher starting activity of gallium-68, resulted in impurities, possibly credited to radiolysis. Addition of scavengers increased radiochemical purity and since this issue was not reported in procedures where kits are used, it could be pointed out that stability could be influenced by way of formulation of [^{68}Ga]Ga-UBI. Further characterisation of impurities in Ga labelled UBI 29-41 is not reported.

Reviewing the labelling procedures of ubiquicidin with gallium-68 featured a heterogeneous group of chelators. This makes a meta-analysis difficult, but broad trends can be observed. To our knowledge no study to compare bacterial uptake of [^{68}Ga]Ga-UBI29-41 using different chelators is available nor has any such study looked at the chelator which could lead to the most robust radiolabelling in a clinical environment. The choices of chelators therefore might be made based on past experience with chelators in other radiopharmaceuticals.

The labelling parameters used are standard for gallium-68 based systems. Regardless of the type of chelator, a concentration of the cold peptide of around 25 nmol/ml seems to provide useful yields. Throughout all investigations the pH was kept at 3.5 - 4.0. The eluates from various origins were buffered by either sodium acetate (0.5 - 5 M) or N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer. Various radical scavengers to optimize the radiolabelling efficiency were investigated by Le Roux *et al.,* [32]. For all radio syntheses described, the incubation times varied from 10-15 minutes at temperatures ranging from 85 – 100 °C. Attempts to label NOTA-based chelators lead to unpredictable labelling yields. Simecek *et al*., 2013 [61] emphasized that considerable attention should be paid to the influence of eluate impurities and their concentrations on the gallium-68 incorporation rate to different chelators. A chelator that demonstrates more robust labelling is of course more optimal. The best chelator for gallium-68 ubiquicidin seems to be NOTA, most publications utilize this chelator. However, clear evidence for this choice is not provided.

Manual synthesis is most often used during these investigations, and this indicates that the technology readiness level of these radiopharmaceuticals is definitely not at a stage for commercial production. Thus, studies by Le Roux *et al*, [32,35] and Vilche *et al* [38] deserve special mention since automation was investigated on the Scintomics and Eckhard and Ziegler automated synthesis units. Good Manufacturing Practice (GMP) aspects are also addressed by Vilche *et al.,* [38]. Microfluidics as a synthesis method was also investigated by Vilche *et al*. [39] in a later publication. Contrary to this end, two publications, by Mukherjee *et al*., [37] and Bhusari *et al.,* [38] investigate cold kit-based labelling systems for radiolabelling.

The gallium-68 eluant in the described studies will be obtained from various types of generators. It is of course important to note that the concentration of hydrochloric acid used for each type of generator is different and therefore also the described conditions for labelling with each type of eluant. Methods are therefore not directly translatable to different generators; some adaptation must occur. This further contributed to the heterogeneity of our study sample. One of the biggest issues with generator-based gallium-68 activity elution is a probable occurrence of metal contaminants. These can be formed by decay products (Zn^{2+}), originate from matrix material impurities (Fe^{3+}, Cu^{2+}, Al^{3+}) or by breakthrough of parent radionuclide (germanium-68). These impurities should be carefully monitored and removed or reduced prior to radiolabelling since their concentrations can increase with generator age and improper maintenance of generators. Such

impurities, due to similar chemistry and affinity for NOTA, DOTA and NODAGA can compete with gallium-68 during labelling and result in poor radiochemical yields [61]. Various pre-purification methods have been applied in the studies. There are three methods namely anionic or cationic exchange as chemical-based methods and fractionated elution [30]. However, it has been reported previously [62] that anionic/cationic exchange cartridge-based eluate purifications are more efficient than eluate fractionations, primarily reducing concentrations of co-eluted impurities and elimination of germanium-68 and should be implemented in either manual or automatic procedures of labelling, for reaching higher labelling efficiency and significant reduction of colloids formed. The choice of generator and purification method is however dictated by the facility infrastructure and needs. If the correct circumstances are followed for the unique generator, and the pH is correctly adapted, this should not have a major impact on the radiolabelling of the radiopharmaceuticals.

Post-labelling purification can be useful in environments where radiolabelling parameters are not robust. Most often in these publications the C18 Sep Pak cartridges were used. It is also very important that the post-purification is done for studies that involve *in vivo* and clinical work. We are of the opinion that in order to maintain Good Radiopharmacy Practice, post-labelling purification should always be part of the process for gallium-68 labelling in the clinic.

4.2 *Fluorine-18 based UBI radiopharmaceuticals*

Only two articles and one abstract provided information pertaining to the application of fluorine-18 PET for UBI imaging. The two articles notably found no specificity for infection during the investigation. *In vitro*, affinity was lower for [^{18}F]F-UBI than [^{99m}Tc]Tc-UBI. In the preclinical study neither [^{18}F]F-UBI or [9^{9m}Tc]Tc-UBI accumulated in the target lesion. Application of a different labelling mechanism using AlF $^{2+}$ strategy significantly decreased synthesis time described in the abstract but with relevant plasma stability issues shown. No further development of [^{18}F]F-UBI as a radiopharmaceutical to date was found during the literature search. Clearly no alterations of the UBI fragment, different labelling approaches or radiopharmaceutical design was investigated to optimize fluorine-18 based UBI imaging of infection.

4.3 Quality control parameters

If a radiopharmaceutical is poorly developed (with the emphasis on quality control or compound identification), or preclinical studies have some degree of inconclusiveness then clinical studies are a futile effort leading to wrong conclusions.

Although HPLC methods used in [^{68}Ga]Ga-UBI are similar across all chelators, this agrees with current literature for all gallium-68 based radiopharmaceuticals. However, validation of analytical methods generally helps in understanding the influence of small variations in method parameters on an analytical procedure and possibly on labelling method performance itself. Validation of methods will help to show soundness of method for its intended purpose, robustness and also will provide accurate, reliable, meaningful, trustworthy, and consistent data. This is considered as the starting point of quality assurance. The “harmonisation” of methods and “systematic guideline” approach for analyses and development of quality control procedures for [^{68}Ga]Ga-UBI (and gallium-68 radiopharmaceuticals in general), could eliminate duplications of testing and methods variability, cut costs, and enable scientists in research labs or clinical settings (usually) not so familiar with development of new methods, simply to apply them. It is recommended to use ITLC analyses prior to the determination of radiochemical purity by HPLC. In cases where crude mixture is not post-purified this requirement helps to eliminate the possibility of colloids present blocking and damaging columns or minimizing the risk of colloids being stuck on cartridge. This also decreases the risk of having false positive results with regards to the purity of the injected product. Colloids present in injected products can increase liver uptake. Usually two development systems are used, one a citric acid buffer and the second 15% HCl in methanol where gallium-68, gallium-68 colloids and the [^{68}Ga]Ga-UBI complex are separated.

4.4 In Vitro evaluations

All the *in vitro* evaluations performed are necessary to determine if a product is a viable candidate for further development. Instability of the radiopharmaceuticals causes a change in the biological behaviour/physiological distribution (organ and whole-body radiation dosimetry). With regards to bench-top stability, which indicates how long a radiopharmaceutical in its final formulation is stable, gallium-68 labelled ubiquicidin was deemed very stable. All radiopharmaceuticals tested, remained stable for the time that they were sampled (testing varied from 2 to 4 h depending on the research centre). It is currently not plausible from the existing literature if some degradation occurs (due to radiolysis, colloid formation or trans-chelation) and if radical scavengers should indeed be included in the final formulations. Only one study [34] tested bench-top stability under refrigerated circumstances. The shelf-life of cold kits [37,38] was determined to be more than 6 months. *In vitro* evaluation in serum and saline stability gives a possible view on how the radiopharmaceutical react

in the biological environment. The *in vivo* stability of gallium-68 based ubiquicidin radiopharmaceuticals was appropriate for up to 2 h.

Genotoxicity was determined during an *in vitro* study [53] indicating that ubiquicidin is a safe peptide for further development. When new radiopharmaceuticals enter the research pipeline, partition coefficients (log P) are used to determine and predict how radiopharmaceuticals behave in the human body. It is the measurement of lipophilicity which indicates permeability of components to reach the targeted tissue. It helps to understand how easily components will be absorbed, metabolised and excreted. Log P is usually done as distribution of components between water and octanol and analysed by HPLC [63].The partition coefficient was between -2.9 and -3.6. Protein binding, when tested, was always more than 50%.

Finally, when performed, an adequate uptake in *S.Aureus* cultures was present, showing potential for this application in imaging. It is concerning that more in-depth *in vitro* investigations were not performed before preclinical investigations were launched. An overall lack of standardization evident for these *in vitro* studies is likely being caused by the large heterogeneity in research methodology. For example, although considered essential, very few studies reported the analysis of non-specific binding of the radiopharmaceuticals. Whilst some research articles report on target blocking tests (apply 100-fold excess of unlabelled pharmaceutical) to ascertain specific uptake, concerningly, none of the studies had a non-viable *S. Aureus* control test group. Future studies are therefore urged to apply standardised methodology for *in vitro* assays to address the current shortcoming.

4.5 Preclinical evaluations

Being influenced by several variables and presenting vast differences during its acute or chronic phases imaging of infectious diseases has its unique challenges and limitation of using radiopharmaceuticals. Key aspects of the preclinical study design were recently reviewed by Signore *et al,* [10] herein highlighting the need for aligning the animal model, the type of bacterium, the choices of control measures with the mechanism of action of a candidate radiopharmaceutical to considerably improve bacterial imaging as in the case of radiolabelled UBI.

The preclinical investigations for (mostly) [^{68}Ga]Ga-UBI radiopharmaceuticals in small rodents all demonstrated sufficient-high target-to-background ratios to support further clinical development. Clear evidence for adequate bacteria-selective behaviour was also achieved as proven by significant target-to-non target ratios for the [^{68}Ga]Ga-UBI PET signal to decipher between sterile inflammation and active infectious processes. A study using larger rodents (New Zealand White

Rabbits) also established sufficient evidence to allow for progression with development of UBI-based infection imaging; however, the results in larger animals are limited. Normal biodistribution, radiation dosimetry and pharmacokinetics in healthy, non-human primates contributed valuable insights that relates to the optimal design of the imaging protocol in future clinical studies. In contrast, a study reported by Afzelius *et al*., [33] that used juvenile pigs with S. Aureus biofilm infected bone lesions stands out with respect to the data gathered. In an effort to find a diagnostic radiopharmaceutical a number of radiopharmaceuticals were tested including [^{68}Ga]Ga-DOTA-K-A9, [^{68}Ga]Ga DOTA-GSGK-A11, [^{18}F]NaF, [^{68}Ga]Ga-DOTA-Siglec-9, [^{68}Ga]Ga-NOTA-UBI29-41 and [^{18}F]FDG. In this study the investigators failed to demonstrate the accumulation of [^{68}Ga]Ga-NOTA-UBI29-41 for this indication.

Overall, the preclinical investigations on UBI-based radiopharmaceuticals lacks clear evidence of replicating the findings from small animal studies in larger animal models. Regrettably, if very few aspects of UBI were studied in larger animal models, it may cause more complications during its prospective bench-to-bedside translation. It is well plausible that any knowledge of the exact behaviour of UBI in a larger animals will be better suited for clinical translation – as it occurs under the optimal conditions with regards to patient indications, patient preparation and imaging procedures. On this note, general guidelines including recommendation to develop an appropriate study design for the preclinical assessment of new radiopharmaceuticals have been reported [64]. This addressing the need harmonize the standards for experimentation across centres to avoid clustered findings or a lack of comparability.

4.6 Clinical evaluations

The value of a more bacteria-selective PET imaging agent has been continuously highlighted. Despite using [^{18}F]FDG or radiolabelled blood elements, clinicians could benefit from an imaging agent that provides an accurate (and earlier) diagnosis of complicated, deep seeded infection [7,9,11]. Clinical research using [^{99m}Tc]Tc-UBI-SPECT imaging has paved the way for endeavouring into UBI-based radiopharmaceuticals for PET imaging and the last decade has seen ^{68}Ga-radiolabeled DOTA-/NOTA-UBI29-41/31-38 emerging as candidates for imaging of infection in a clinical setting. It can be stated that clinical UBI-PET is indeed in its early stages – which makes it difficult to make strong conclusions available clinical data. Positively, valuable results - often reporting on patients with similar type of infectious diseases - are available from different centres and the number of patients enrolled per study is gradually increasing. Based on the results

to date it is expected that better designed studies will emerge featuring more diverse patient population and more coherent imaging protocols as well as the radiochemical and radioanalytical support for such large-scale investigations. The current clinical reports mostly fell short in providing detailed instructions with regards to the manufacturing of the radiopharmaceutical as well as the quality control followed. It is important that clinical evaluations should commence with the utmost safety for administration of a radiopharmaceutical.

Therefore, regardless of the research centre where UBI-PET imaging was performed so far, the supporting technology needs to shift to a readiness level that can support large cohort phase I investigations which are required to justify the true clinical power, usefulness and benefit of UBI-based PET radiopharmaceuticals. It may then be foreseeable that for [^{68}Ga]Ga-UBI-PET, this being the furthest developed, well controlled clinical trials are achievable.

Unfortunately, and not unexpected at this stage, the published clinical data is currently presented with various degree of bias which should be alleviated in future by introducing powerful study designs, meaningful control groups and by masking the clinician from the patient's disease history or auxiliary investigations. For example, the study by Bhusari *et al*., [38] made noteworthy efforts by using a masked, prospective study design to evaluate 10 patients with suspected infections. Unfortunately, at this point in the development of [^{68}Ga]Ga-UBI-PET, enrolling patients for imaging whilst being on ongoing antibiotic treatment was a significant compromise to the power of the image analysis. As part of this study, 4/10 patients failed to demonstrate the localization of [^{68}Ga]Ga-UBI in the infectious foci, despite the relevant tissue cultures returning positive for infection. In contrast, an abstract published in 2019 as part of the annual meeting report of the Society of Nuclear Medicine highlighted [^{68}Ga]Ga-NOTA-UBI29-41 PET imaging, showing patients with increased radioactivity uptake along the interface between bone and hip prosthesis. For such patients a definitive diagnosis of infection or aseptic loosening was made from the evaluation of intraoperative specimens (tissues and/or prosthesis) obtained, or from clinical/serological evaluation. However, a full research article with the outcome from a larger patient population is still pending.

It should be mentioned that it remains difficult at this stage to determine what constitutes a positive or negative result when the available data is qualitative and not quantitative in nature. Any future prospects of UBI-based radiopharmaceuticals crucially depend on launching unbiased, (preferably masked), multi-centred, Phase-I clinical trial investigations. To this end, with the new biological

mechanism of action attributed to ubiquicidin, emphasis should be given to determine and perform adequate patient inclusion or exclusion criteria as well as keeping the option for a biopsy to the target site [65]. Given further evidence is produced from more emerging studies, it will be well plausible that PET imaging with UBI-based radiopharmaceuticals will increasingly be utilized as an auxiliary imaging tool to ascertain an (otherwise) inconclusive or complex diagnosis of infection.

5. Summary and Conclusion

The state of development of UBI-based radiopharmaceuticals for PET is presented, using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) concept that is gearing for a strategic, step-by-step data analysis with minimized bias and good transparency when evaluating the body of research. For these radiopharmaceuticals there is currently no systematic approach ascribing to the principal steps to follow in research from basic development through preclinical evaluation to clinical studies.

This review provides strong evidence of gallium-68 $UBI_{(29-41)}$ and its other derivatives being selective and specific imaging agents of bacterial infections. Labelling procedures are uncomplicated and fast and there are sufficing indications of good stability. However, the systematic optimization of labelling conditions and validated quality control methods are necessary as well as the improvement of the relatively small sample size within clinical studies. Additional comprehensive studies for metabolic products of UBI after injection would be of advantage to understand this radiopharmaceutical's performance *in vivo*. However, the *in vivo* environment (presence of different enzymes, higher dilutions than in serum) may result in additional metabolites that were not present in *in vitro* experiments. Although HPLC methods applied for analysis of radiopharmaceuticals are similar, validation of analytical methods would be advantageous in understanding the effect of small adjustments in method parameters on method performance. A validation of the analytical methods will provide assurance the insurance that data is accurate, reliable, and consistent. This is key for eventual GMP compliant preparations needed in the mentioned larger cohort clinical trials.

Many radiopharmaceutical candidates have demonstrated great First-in-Human results in small study populations in the past, only to fail during larger evidence-based trials [66]. One of the methods to combat this trend is to ensure that the order of the steps of the radiopharmaceutical development pipeline is followed rigorously and that at every stage in the investigation, the data

are collected and analysed in a standardised and uniform fashion as prescribed in the IAEA's "Guidance for Preclinical Studies with radiopharmaceuticals" [67]. An fine balance is to be kept between all the development fields (vector development, in vitro evaluation, preclinical studies and clinical application) for any emerging radiopharmaceutical in the drug development pipeline. Every phase builds upon the previous as one progresses to higher technology readiness levels. It is imperative that a technology is reviewed after each stage (sometimes referred to a stage gate) to ensure that all the objectives have been met and it is ready for the next phase. The focus over the entire lifecycle should be streamed towards a clear outcome. It should be an infection specific molecular imaging radiopharmaceutical which should react, reside in or bind with the pathogen. Differentiation between pathogen induced infections and sterile/non-specific inflammation processes is a non-negotiable characteristic for a new agent. A high target-to-background ratio is important and contributing to this is a rapid blood clearance. Ideal radiopharmaceuticals for bacterial infection imaging should be easy, safe and quick to prepare with readily available, inexpensive components.

This review demonstrates that gallium-68 $UBI_{(29-41)}$ is selective and specific for imaging of bacterial infections. Labelling procedures are generally uncomplicated and fast and there are indications of good stability. However systematic optimization of labelling conditions and validated quality control methods are necessary as well as relatively small sample size of clinical studies should be improved upon.

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