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cGMP-compliant high-yield automated production of [^{18}F]AIF-FAPI-74: optimization of quality control and evaluation of molar dose impact

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Abstract

Background Fibroblast activation protein inhibitor (FAPI)-based positron emission tomography (PET) radiopharmaceuticals have shown promise for imaging cancer-associated fibroblasts (CAFs), a key component of the tumor microenvironment. FAPI radiopharmaceuticals offer high tumor-to-background contrast and are not influenced by hyperglycemia. Among these, [^{18}F]AIF-FAPI-74, labeled using the Al ^{18}F -method, offers logistical advantages over ^{68}Ga -labeled radiopharmaceuticals, including a longer physical half-life and suitability for large-scale, centralized production. This study reports a fully automated, efficient, and GMP-compliant synthesis of [^{18}F]AIF-FAPI-74 using the Trasis AllInOne[®] platform, alongside a refined isocratic radio-HPLC method that enhances fluorine-18 recovery and impurity resolution. Additionally, the impact of molar dose on [^{18}F]AIF-FAPI-74 biodistribution is evaluated in a preclinical model and a clinical case-study highlighting the performance of [^{18}F]AIF-FAPI-74 produced at high apparent molar activity is provided.

Results The automated GMP-compliant production was validated in three independent runs, with an average decay-corrected yield and apparent molar activity of $50 \pm 10\%$ and $1124 \pm 254 \text{ GBq}/\mu\text{mol}$ at the end of synthesis, respectively. Total synthesis time was 30 min. Quality control used validated analytical methods, including an optimized radio-HPLC protocol, ensuring regulatory compliance and batch consistency. [^{18}F]AIF-FAPI-74 was produced with a radiochemical purity $\geq 95\%$ and demonstrated excellent radiochemical stability in its final formulation for at least 10 h post-synthesis, at a concentration of $2019 \text{ MBq}/\text{mL}$. The in vitro binding kinetics of [^{18}F]AIF-FAPI-74 were evaluated in a HEK293 cell line stably expressing human FAP, demonstrating rapid, specific uptake and internalization, along with high target affinity. In vivo biodistribution studies revealed a dose-dependent “molar amount effect,” with doses $> 30 \text{ nmol}/\text{kg}$ yielding improved tumor-to-background ratios. This phenomenon

was not observed in clinical imaging, where high molar activity supported excellent image contrast.

Conclusions A robust, fully automated, and GMP-compliant production process for [^{18}F]AIF-FAPI-74 is reported with high yield, purity, and stability, suitable for centralized production and distribution. The improved radio-HPLC method enhances quality control precision by accurately quantifying radiochemical and chemical purity. In vivo experiments confirmed fast tumor uptake of [^{18}F]AIF-FAPI-74. While a clear mass effect on tumor-to-background ratios was observed in preclinical studies, high apparent molar activity resulted in excellent contrast in a clinical setting.

Keywords ^{18}F , PET, AIF-FAPI-74, FAP, molar activity

Background

The non-invasive detection of cancer and its spread within the body through imaging is a cornerstone of modern oncologic care. Molecular imaging with positron emission tomography (PET) plays a significant role in diagnosing, staging, therapy planning, response monitoring and detection of relapse [1]. Currently, the most used radiopharmaceutical in clinical practice is [^{18}F]fluorodeoxyglucose ([^{18}F]FDG). However, it has limitations in detecting cancer types that exhibit a low glycolytic phenotype or present with a low cellular composition [2]. Other challenges associated with [^{18}F]FDG include varying, sometimes high physiological uptake in normal tissue (brain cortex, heart, liver, bowel, muscle, brown fat), dependence on glycemia and false positives in the presence of active inflammation and infection [2].

To overcome these limitations, specific molecular targets have been identified. Recently, novel PET radiopharmaceuticals based on fibroblast activating protein inhibitors (FAPI) have emerged as a new class of clinically applied radiopharmaceuticals. This class of radiopharmaceuticals bind to fibroblast activation protein α (FAP), a type II integral membrane protein and a member of the dipeptidyl peptidase (DPP) 4 family [3, 4], expressed on cancer-associated fibroblasts (CAFs), which are abundant in the tumoral stroma and can make up as much as 90% of the total tumoral volume [3]. FAP is also overexpressed in lung and heart myofibroblasts and can thus be used as a biomarker for fibroblast activity in heart and lung disease. In contrast, in normal fibroblast and in healthy adult tissue no or only insignificant levels of the protein are present [5].

Many ongoing studies regarding FAPI-radiopharmaceuticals show low background activity and high tumor-to-background contrast. Patients can be scanned 10 to 60 min after injection and the biodistribution of the radiopharmaceutical is not influenced by hyperglycemia, with very little patient preparation required [6]. Some tumors with potentially low ^{18}F FDG-avidity show high tumoral FAPI uptake and good detection of metastases. These include gastric cancer [7, 8], pancreatic cancer [8–12], cholangiocarcinoma [8], liver cancer [13], colorectal cancer [14], breast cancer [15], thyroid cancer [16], lung cancer [17] and cancer of unknown primary [18–21], with many more prospective trials ongoing [22]. A recent review by Mori et al., outlined the current potential clinical applications, not only in oncology but also in different reactive and inflammatory processes that are characterized by fibroblast activation such as fibrosis, pathological tissue remodeling and vasculitis [23]. The current clinical consensus is that FAPI PET/CT shows great potential to be established as a high-accuracy tool of reference for many

oncologic or fibroinflammatory investigations, certainly in those where [^{18}F]FDG falls short [4; 24–25].

Most early-stage clinical work was performed using FAPI molecules labeled with gallium-68 (such as [^{68}Ga]Ga-FAPI-04) as a PET radionuclide. However, fluorine-18 offers several advantages over gallium-68, including a longer half-life (109.7 min for ^{18}F compared to 67.7 min for ^{68}Ga), the ability for large-scale production via cyclotron (in contrast to ^{68}Ga , which is primarily produced using costly $^{68}\text{Ge}/^{68}\text{Ga}$ generators), and a lower maximum positron energy (0.63 MeV for ^{18}F versus 1.89 MeV for ^{68}Ga), resulting in a lower positron annihilation range. These characteristics facilitate the widespread delivery of fluorine-18 radiopharmaceuticals to decentralized PET centers, enable high-volume production, and support high-resolution PET imaging [26, 27].

Although direct radiofluorination of FAP-targeting small molecules has been successfully demonstrated by several research groups, these compounds have not yet advanced to clinical application [28, 29]. A NOTA-containing molecule, FAPI-74 (Fig. 1), was developed to enable labeling through the Al^{18}F -labeling method, pioneered by McBride et al. [30]. Several comprehensive reviews of this radiolabeling method have been published [31, 32].

Preliminary reports regarding the synthesis of [^{18}F]AlF-FAPI-74 have started emerging. The first manual synthesis of [^{18}F]AlF-FAPI-74 was reported by Giesel et al. [33], though it lacked in-depth description of additional quality control procedures associated with cGMP (current Good Manufacturing Practice) implementation. A more optimized manual radiosynthesis was later reported by Wang et al. [34], including a radio-HPLC method using an acidic solvent system, as well as preclinical evaluation and clinical translation.

Two publications report the fully automated synthesis of [^{18}F]AlF-FAPI-74, one utilizing the CFN-MPS200 cassette-type multi-purpose synthesizer [35] and another comparing the GE TracerLab Fx2N platform with the Trasis AllInOne system [36]. However, the automated radiosynthesis of [^{18}F]AlF-FAPI-74 still faces challenges, with radiochemical yields remaining low (around 20%) and batch activities (11 GBq) insufficient for centralized production and shipment. Additionally, the radio-HPLC analytical methods reported rely on an acidic gradient solvent system, which is not ideal for routine clinical applications. Our group has previously shown that acidic solvents negatively affect the recovery of free [^{18}F]F $^-$ on silica-based columns [37].

In this manuscript, we describe an optimized fully-automated cGMP-compliant production method for [^{18}F]AlF-FAPI-74 on the Trasis AllinOne $^{\circ}$ synthesis module, demonstrating its robustness and reproducibility with high batch activities and, consequently,

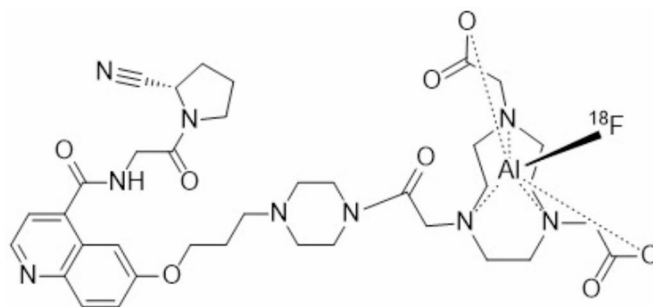


Fig. 1 Chemical structure of [^{18}F]AlF-FAPI-74

high apparent molar activity. We also introduce a more suitable radio-HPLC method for quality control that eliminates the use of an acidic mobile phase and employs an isocratic approach. This method enables optimal impurity separation, including radiolysis products, while maximizing the recovery of free $[^{18}\text{F}]\text{F}^-$.

Recent literature has highlighted the need for a more rational approach to molar dosing of FAPI radiopharmaceuticals. In preclinical animal models, increasing the molar dose from low to medium levels was associated with reduced off-target uptake in healthy tissues, whereas high molar doses raised concerns about tumor saturation [38–40]. As part of our validation process, we conducted both in vitro and in vivo preclinical studies, to assess the impact of molar dose on biodistribution. With the clinical FAPIDO trial (NCT06782412) now underway at University Hospital Leuven, we also present a representative PET/CT image from a human subject to illustrate biodistribution and contribute to the discussion on the molar dose effect in patients.

Methods

Validation of analytical procedures

Validation of the radio-HPLC method

Radio-HPLC analysis was performed using a Shimadzu LC-20 A HPLC System (Shimadzu, Kyoto, Japan), equipped with a diode array detector (DAD-UV) set to 220 nm. For detection of the radioactive signal, a shielded 3-inch NaI(Tl) scintillation detector coupled to a single-channel analyzer (Gabi box, Elysia-Raytest, Straubenhardt, Germany) was placed in series with the UV detector. Chromatographic separation was performed using an XBridge® C18 column (3.5 μm , 3.0 $\times$ 100 mm; Waters, Milford, Massachusetts, USA). Elution was performed isocratically over 20 min at a flow rate of 0.8 mL/min, with a mobile phase consisting of: (A) 0.05 M ammonium acetate buffer (pH 5.5) and (B) acetonitrile (91:9; v/v). To assess the presence of lipophilic impurities, a subsequent 40-minute gradient elution was applied during validation runs, transitioning from 91:9 to 10:90 (A: B). Consequent absence of lipophilic impurities during validation runs resulted in the removal of this gradient in clinical routine.

The radio-HPLC method was validated through a system suitability test following the approach of Tsibangu et al. (2020), assessing specificity, precision, linearity, limits of quantification and detection (LOQ/LOD), and recovery of $[^{18}\text{F}]\text{F}^-$, $[^{18}\text{F}]\text{AIF}$ and $[^{18}\text{F}]\text{AIF}$ -FAPI-74 recovery. A concentration range of 0.32–31.81 $\mu\text{g/mL}$ of AIF-FAPI-74 (ABX, Radeberg, Germany) was analyzed following an initial blank run to correct for non-specific signals. Chromatographic parameters evaluated included main peak resolution (R_s), peak width, and relative standard deviation (RSD) of retention time (R_t) and area under the curve (AUC) of the main peak. Linearity was assessed via a calibration curve for AIF-FAPI-74, and LOQ/LOD values were calculated based on signal-to-noise ratios from peak and background signals.

To demonstrate the superiority of the validated method over previously published approaches, a comparative recovery study was conducted using an HPLC method described in literature employing an acidic solvent system [35]. This method used an XBridge® C18 column (3.5 μm , 3.0 $\times$ 100 mm, Waters) and a 15-minute gradient run at 0.8 mL/min. Mobile phase A consisted of 0.1% trifluoroacetic acid in water, while mobile phase B was acetonitrile. The gradient was programmed as follows: 0–3 min: 95% A; 3–15 min: linear gradient from 95% to 50% A [34].

Validation of the GC method

The analysis of residual solvents was performed using a Shimadzu GC-20i0 (Shimadzu), containing a Shimadzu AOC-20i auto-injector and a Shimadzu AOC-20s autosampler. The system was equipped with a Supelco Equity™ (30 m length, 0.32 mm internal diameter, 5 µm film thickness). The analysis was conducted under isothermal conditions at a column temperature of 40 °C, with a linear carrier gas velocity of 40 cm/s. The gas chromatography method was validated for specificity, precision, linearity, and LOQ/LOD, as previously described [41]. Specificity testing was evaluated for acetonitrile and ethanol.

Radiosynthesis

The GMP-grade NOTA-FAPI-74 precursor was manufactured by ABX (Radeberg, Germany) and supplied by SOFIE Biosciences (Dulles, VA, USA). GMP-compliant single-use synthesis cassettes and reagent kits were obtained from Trasis (Ans, Belgium) for use with the AllInOne® synthesis module (Trasis). Each cassette included a Sep-Pak light QMA anion exchange cartridge (Waters), and a Sep-Pak light tC18 cartridge (Waters), which were placed on the synthesizer without manual pre-conditioning. An additional Alumina N cartridge (Waters) was added on the transfer line connection. The reagent kits comprised acetonitrile (≥99.5%), QMA eluent solution (saline in acetonitrile), sodium ascorbate, L-ascorbic acid, and buffered aluminum(III) solution. Additional reagents not included in the kit but used in the synthesis included water for injection (WFI, Ph. Eur; ABX). Sterile filtration of the final product was performed using a combination of a 0.22 µm sterile vent filter (Cathivex-GV, 25 mm, Merck KGaA, Darmstadt, Germany) and a 0.22 µm sterile filter (Pall Acrodisc, Merck KGaA). The final product was collected in a sterile 25 mL vial (Huaiyi Isotopes Co., Changshu, China).

[¹⁸F]F[−] was obtained using an ¹⁸O(p, n)¹⁸F reaction by irradiation of 2 mL of 97% enriched [¹⁸O]H₂O (CortecNet, France) in a Niobium target with 18-MeV protons from a Cyclone 18/9 cyclotron (Ion Beam Applications, Louvain-la-Neuve, Belgium).

Before transfer of [¹⁸F]F[−], a cassette preconditioning sequence (±16 min.) was executed by the module. This included dissolving L-ascorbic acid and sodium ascorbate in WFI, and preparing the precursor and Al(III) solutions with acetonitrile and radioprotectant. Finally, 6 mL of the sodium ascorbate solution (50 mM) was sterile-filtered into the final product vial within a Class A dispensing cell.

Following the production of [¹⁸F]F[−], the activity (89.7 ± 1.8 GBq) was transferred to the synthesis module and the automated sequence was initiated. Al(III) (108 nmol, 0.3 mL) solution was first transferred to the reactor. [¹⁸F]F[−] was trapped on the QMA cartridge, separated from the [¹⁸O]H₂O and eluted using the QMA eluent solution (0.4 mL) into the reactor containing Al(III) to form [¹⁸F]AlF. The radiolabeling was initiated by adding the precursor solution (98 nmol, 0.35 mL of metal-free H₂O/acetonitrile solution) to the reactor, which was then heated to 70 °C for 10 min. The reaction was quenched with 6 mL sodium ascorbate buffer and allowed to cool. The diluted reaction mixture was loaded onto a preconditioned tC18 Sep-Pak cartridge (conditioned with 5 mL ethanol and 10 mL WFI). After rinsing with 20 mL of diluted sodium ascorbate buffer, [¹⁸F]AlF-FAPI-74 was eluted with 1.8 mL of pure ethanol, passed through an Alumina N cartridge (Waters), and filtered into the final product vial and further diluted with 14.2 mL of sodium ascorbate buffer. The final product consisted of [¹⁸F]AlF-FAPI-74 in ~22 mL WFI/ethanol (92:8 v/v), buffered with 135 mM sodium ascorbate. A complete schematic

of the synthesis cassette layout and a table detailing the position of all reagents and consumables are available in Figure S1 and Table S1.

Quality control

Table 1 summarizes the quality control methods along with the corresponding acceptance criteria (specifications) for cGMP produced [^{18}F]AIF-FAPI-74. Prior to batch release, the following tests were performed: visual inspection (appearance), identification, radiochemical purity, chemical purity, pH measurement, sterile membrane filter integrity, residual solvent analysis, and total activity measurement. Retrospective quality control tests included radionuclide identity verification (based on physical half-life and gamma-spectrum analysis), sterility testing, and bacterial endotoxins determination.

The identity of [^{18}F]AIF-FAPI-74 was confirmed using the validated radio-HPLC QC method by comparing the retention time of the principal radioactive peak with that of the authentic reference material (AIF-FAPI-74), as detected by UV/VIS. Chemical purity was assessed based on the UV/VIS chromatogram. To further confirm the identity of [^{18}F]AIF-FAPI-74, radio-LC-HRMS analysis was performed using a Waters Alliance HPLC system coupled to a Waters Acquity QDa Detector (Milford, MA, USA), a single quadrupole mass spectrometer with electron spray ionization. A gradient elution method was developed for this purpose, transitioning from 0% to 10% mobile phase B over 20 min. The mobile phases were: (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. UV, radio, and mass spectra were recorded for the precursor,

Table 1 Acceptance criteria and test methods applied for quality control of [^{18}F]AIF-FAPI-74

Test parameters	Acceptance Criteria	Test Methods
Identification	Difference between retention time of [^{18}F]AIF-FAPI-74 and retention time of the reference $\leq 10\%$.	HPLC with Radiodetection and UV/VIS detection
Radiochemical purity 1. [^{18}F]AIF-FAPI-74	1. $\geq 93\%$ of total radioactivity	Radio-HPLC
Chemical purity 1. FAPI-74 ($\mu\text{g/ml}$) 2. Amount of unidentified chemical impurities ($\mu\text{g/ml}$)	1. $\leq 2.0 \mu\text{g/ml}$ 2. $\leq 3.0 \mu\text{g/ml}$	HPLC with UV/VIS detection
pH of the finished product	pH = 4.5–8.5	pH strip
Integrity of the sterile filter membrane	Bubble point ≥ 3.2 bar	Membrane integrity testing with the bubble point test
Appearance	Clear and colorless solution with no visible particles	Visual inspection
Residual solvent detection 1. Acetonitrile 2. Ethanol	1. $\leq 4.1 \text{ mg/day}$ (Ph. Eur) 2. $\leq 10\% \text{ v/v}$ (Ph. Eur.)	Gas Chromatography
Total radioactivity	No criteria defined	Dose calibrator
Radionuclide identity - approximate half-life	$T_{1/2} = 105\text{--}115 \text{ min}$	Two-time point radioactivity measurement in dose calibrator
Radionuclide identity - gamma spectrometry	Gamma energy is 501–521 keV	Gamma spectrum on NaI (TI) spectrometer
Sterility	No growth after 14 days of incubation at 37°C	Current Ph. Eur. <2.6.1>
Bacterial endotoxins	< 175 IU per injected volume	LAL-Test Current Ph. Eur. <2.6.14>

reference compound, and the final [^{18}F]AIF-FAPI-74 product. Residual solvent levels in the final drug product were determined using the validated GC method.

Radioactivity measurements were performed using a calibrated dose calibrator (VIK-202, Comecer, Italy). Radionuclide Identity was confirmed through half-life determination and gamma spectrometry using a shielded 3 × 3 inch NaI(Tl) detector (Elysia-Raytest, Mucha, GinaStar6 software). pH values were measured with a pH strip (MQuant® pH 0–14, Merck KGaA Darmstadt, Germany).

Environmental bioburden during final formulation was monitored by continuous particle counting and active air sampling within the Class A dispensing cell using the single-use microbial impactor particle measuring system (BioCapt, Boulder, Colorado, USA). Sterile filter integrity was determined by the bubble point test. All production batches of [^{18}F]AIF-FAPI-74 were subjected to sterility testing via direct inoculation in fluid thioglycolate medium and soya-casein digest medium, in accordance with Ph. Eur. 2.6.1. Endotoxins levels were measured using the Limulus Amebocyte Lysate (LAL) chromogenic assay (Endosafe PTS, Charles River, Wilmington, USA), in compliance with Ph. Eur. 2.6.14.

The stability (chemical and radiochemical purity) of [^{18}F]AIF-FAPI-74 was assessed using the validated radio-HPLC method. Stability testing was conducted at room temperature over a period of up to 10 h post-synthesis, across a range of radioactivity concentrations (1242–2019 MBq/ml).

In vitro assays

DMEM-High Glucose and Fetal Bovine Serum were obtained from Gibco (Waltham, MA, USA), and the prescribed selection antibiotic Puromycin Dihydrochloride was purchased from Sigma-Aldrich. HEK293T cells and HEK293T.HumanFAP cells were purchased from Creative Biogene Biotechnology (NY, USA). Cell passage numbers for this study were <5 and cells were regularly tested for mycoplasma contamination (MycoStrip®, InvivoGen, San Diego, CA, USA). Other cell culture reagents and consumables, including TrypLE™, PBS, culture flasks, and counting cartridges (incl. tryptan blue) were obtained from Gibco, Greiner Bio-One (Kremsmünster, Austria), and Nano-Entek (Seoul, Korea) respectively. For LigandTracer® experiments, Nunc Petri Dishes (Thermo-Fisher Scientific, USA) and CO₂-independent media (Gibco) were used. The following consumables were used during the internalization assays: 24-well plates (Corning, NY, USA), PBS (Gibco), Glycine-HCl (Sigma Aldrich), Reagent A100, Reagent B, and NucleoCassettes™ (ChemoMetec, Allerød, Denmark). The blocking agent UAMC-1110 was obtained from AmBeed (Arlington Heights, IL, USA). For *in vivo* experiments, SCID Beige mice (C B17.Cg-Prkdc scid Lystbg-J/ CrJ) were procured from Charles River Laboratories (Sulzfeld, Germany) and inoculated cells were prepared in Cultrex (1:1; Cultrex Basement Membrane Extract, R&D systems, Minneapolis, MN).

A cellular uptake and internalization assay of [^{18}F]AIF-FAPI-74 was performed following methods similar to Ahenkorah et al. [42]. In brief, 1.5×10^5 HEK293T-HumanFAP cells were cultured per well, in a 24-well plate. The following day, adherent cells were incubated in fresh media (same composition) containing [^{18}F]AIF-FAPI-74 (500 µL, ± 16.5 nM, ± 180 kBq/well) ($n = 3$) at 37 °C and 5% CO₂ for 15 min, 30 min, 1 h, and 2 h. As a control, wells with blocking conditions (100 µM vector molecule UAMC-1110), or containing the negative HEK293T cell line, were allowed to incubate for 1 h. After

incubation the supernatant was removed, and the cells were washed three times with ice-cold PBS. Thereafter, 500 μ L of Glycine.HCl (50 mM, pH 2.8) was added to the wells, incubated for 5 minutes, and transferred to a second tube, including three PBS washes. Lastly, the cells were lysed by adding 500 μ L Reagent A100 (Chemometec, Allerød, Denmark). Reagent A100 (Chemometec), containing the remaining [18 F]AIF-FAPI-74, was transferred to a third tube, along with a 500 μ L wash of Reagent B (Chemometec). All tubes were counted in a Wallac Wizard gamma counter (Perkin-Elmer, Waltham, MA, USA), directly after the samples were processed, and the lysate in tube 3 was counted on a NucleoCounter NC-100 (Chemometec). The percentage of membrane-bound (acid-released) and internalized (acid-resistant) radioactivity was calculated relative to the total activity added per well (%Added Dose; %AD) and normalized to 1.5×10^5 cells, expressed as %AD per 1.5×10^5 cells.

The kinetic binding parameters of [18 F]AIF-FAPI-74 were evaluated using the LigandTracer[®] Yellow system (Ridgeview Instruments AB, Uppsala, Sweden). This system, which includes an uptilted dish holder, rotates the cell sample between media containing [18 F]AIF-FAPI-74 and a radiodetector (See Figure S2). This setup enables real-time monitoring of [18 F]AIF-FAPI-74 uptake, in contrast to conventional endpoint binding assays, and additionally allows testing of multiple concentrations within a single run. The resulting data can be analyzed using various kinetic models in the associated software (TraceDrawer, version 1.9.2; Ridgeview Instruments AB) to determine kinetic parameters related to association, dissociation and affinity.

HEK293T.HumanFAP cells (0.5×10^6) were seeded on a Petri Dish in a 0.5 mL spot of DMEM supplemented with 10% FBS and incubated overnight at 37 °C and 5% CO₂. The culture media was replaced with 3 mL CO₂-independent medium, and a 15-minute background measurement was recorded. Subsequently, binding kinetics of [18 F]AIF-FAPI-74 were assessed at concentrations of 1, 3, and 9 nM in sequential order. After the final addition, dissociation was monitored overnight. All obtained Time (h)/Detected Activity (CPS) curves were decay-corrected and analyzed using TraceDrawer software. Kinetic parameters, including the dissociation constant (K_d), were calculated by fitting the data to a one-to-one binding model. This experiment was performed in triplicate.

In vivo imaging

All animal procedures were approved by the KU Leuven ethical review board (ethical approval reference P054/2021) and were carried out following the Directive 2010/63/EU and reported according to the ARRIVE guidelines [43]. Animals were kept in individually ventilated cages in a temperature-controlled (approximately 22 °C) and humidity-controlled facility with a 12 h/12 h light-dark cycle with *ad libitum* access to food and water. The subcutaneous tumor xenograft model was prepared using a published procedure [42]. Briefly, female SCID Beige mice (C B17.Cg-Prkdc scid Lystbg-J/ Crl), procured from Charles River Laboratories (Sulzfeld, Germany), were xenografted with two different tumor cell lines, namely HEK293T (1×10^6 in 100 μ L) as negative control and HEK293T.HumanFAP (1×10^6 in 100 μ L) as positive control. Cells in growth media (DMEM) were mixed with Cultrex (1:1) and were implanted subcutaneously into the right (negative control) and left (positive control) shoulders of female 6- to 8-week-old SCID mice, respectively. After an average of four weeks, the tumor mice were used in biodistribution and PET/computed tomography (CT) imaging studies. Tumor volumes

ranged from 70 to 500 mm³ (measured by caliper, $h \times l \times w$), while the tumor mass-to-body weight ratio was 0.16–2.44%. All animals included in the study were randomly selected among the in-house bred mice of the correct age. There were no exclusion criteria, and all subsequent studies and analyses were unblinded.

Whole-body PET imaging was performed after i.v. administration of [¹⁸F]AIF-FAPI-74 (1.5–3.0 MBq/mouse; 2.5–100 nmol/kg) via the tail vein in xenografted mice, either in presence or absence of the FAP inhibitor UAMC-1110 (co-injected, 7.3 µmol/kg). To allow for bladder clearance, mice were briefly awake following injection of the radiopharmaceutical (30 min) before undergoing static PET imaging (10 min) at 90 min post-injection (p.i.). Scans were acquired using a Beta-Cube PET scanner (Molecubes, Ghent, Belgium). Mice were kept under gas anesthesia during the entire procedure (2.5% isoflurane in O₂ at 1 L/min flow rate), with temperature and respiration monitored throughout. Prior to PET acquisition, a CT image was acquired with an X-cube CT scanner (Molecubes, Gent, Belgium) for anatomic co-registration, using the following settings: 50 kVp, 480 exposures, 85 ms/projection, 100 µA tube current, and a 60-second rotation time.

PET data were reconstructed into a 192 × 192 image matrix with 0.4 mm voxels using 30 iterations, using a vendor-supplied MLEM algorithm, with corrections for randoms, scatter, attenuation, and decay. CT data were reconstructed using a regularized statistical image reconstruction algorithm using non-negative least squares, using an isotropic 200 µm voxel size and scaled to Hounsfield Units (HUs) after calibration against a standard air/water phantom. Images were analysed using the fusion module in PMOD v4.0 (PFUS) for optimal volume delineations (PMOD Technologies GmbH, Zürich, Switzerland). Regions of interest were manually drawn for the positive and negative tumor sites, the lungs, and the femur. Spheres of 2 mm diameter were sampled for liver and muscle, to act as references.

Radio-metabolite analysis

Three female SCID Beige mice received 2–2.5 MBq of [¹⁸F]AIF-FAPI-74 and were sacrificed 10 min post-injection. Urine and blood were collected from each animal. Urine was analyzed without sample preparation, whilst plasma samples were extracted from blood. Samples were processed as described before [41] and analyzed by the radio-HPLC method as described, with the exception that the eluate was collected in 1-minute fractions and measured on a gamma counter (the Wallac Wizard, Perkin-Elmer) due to low signal levels.

Clinical PET/CT scan

The clinical PET/CT scan was acquired using a Discovery MI4 PET/CT system (GE Healthcare) and included a low-dose CT for attenuation correction and anatomical localization. In accordance with the study protocol (NCT06782412), the patient received an intravenous bolus injection of [¹⁸F]AIF-FAPI-74 at a dose of 3.5 MBq/kg, corresponding to a total activity of 242.0 MBq (molar amount: 440 pmoles). PET/CT image acquisition was performed 60 min post-injection. During the 60 min waiting time, oral contrast (three cups of 4 ml Telebrix (30 g/100 ml), dissolved in 300 ml water) was administered. The patient was monitored for adverse events for 30 min following the examination. PET images were reconstructed according to EARL-2 standards.

Statistical analysis

Quantitative data are expressed as mean $\pm$ standard deviation (SD) unless stated otherwise. Statistical analyses were performed using GraphPad Prism (v10, GraphPad Software, USA). Data are presented as mean $\pm$ standard deviation (SD), and all tests were two-tailed with a significance threshold of $p < 0.05$. For comparisons involving more than two independent groups (e.g. tumour-to-organ ratios across injected molar amounts of [^{18}F]FAPI-74), data distribution was evaluated using the Shapiro–Wilk test and homogeneity of variance by Levene’s test. Where normality and equal variance assumptions were satisfied, differences were assessed using one-way ANOVA followed by Tukey’s multiple-comparisons test. When these assumptions were not met or when sample sizes were small, a Kruskal–Wallis test with Dunn’s post-hoc correction (Holm adjustment) was applied.

Results

Validation of analytical procedures

The HPLC method developed for the identification, quantification and purity analysis of [^{18}F]AIF-FAPI-74 was validated for key analytical parameters, as summarized in Table 2. System suitability was assessed by injecting the non-radioactive reference standard (AIF-NOTA-FAPI-74) prior to batch analysis. Each analysis began with a 20 μL injection of

Table 2 Method validation summary for HPLC and GC method

Validation parameters	Results	
<i>HPLC method</i>		
Specificity	UV/VIS signal The method is specific for AIF-FAPI-74 and [¹⁸ F]AIF-FAPI-74.	Radiometric signal The method is specific for [¹⁸ F]AIF-FAPI-74. Resolution with radiochemical impurity > 1.5
Linearity	The method is linear in the tested range: Range 0.32–31.81 µg/ml, R ² > 0.9999 for AIF-FAPI-74	The method is linear in the tested range.
Method precision	6 repeated injections of 20 µl of a 3.19 µg/ml AIF-FAPI-74 solution yield a % _{RSD} of the AUC < 0.3%. The retention time has a % _{RSD} < 0.2%.	At least 6 repeated injections of 20 µl of a [¹⁸ F]AIF-FAPI-74 solution yield a % _{RSD} of the AUC < 3%. The retention time has a % _{RSD} < 2%.
Quantification of detection limits	LOD AIF-FAPI-74 = 34 ng/ml LOQ AIF-FAPI-74 = 114 ng/ml	LOD [¹⁸ F]AIF-FAPI-74 = 2.6 kBq LOQ [¹⁸ F]AIF-FAPI-74 = 8.5 kBq
Recovery	N/A	[¹⁸ F]F [−] : 96 ± 3% [¹⁸ F]AIF: 97.8 ± 0.3% [¹⁸ F]FAPI-74: 97.9 ± 1.3%
Relative peak width	The relative peak width of AIF-FAPI-74 is < 0.04 (peak FWHM/R _f)	N/A
<i>GC method</i>		
Specificity	The method is specific for CH ₃ CN and EtOH.	
Linearity	R ² > 0.99 for all solvents, range: 0.005% to 5% for CH ₃ CN and 0.005% to 10% for EtOH.	
Method precision	The precision of the method was determined by repeat injections (N = 10) of methanol (1%), acetone (1%), tetrahydrofuran (1%) dimethylformamide (1%), dimethyl sulfoxide (1%), t-amylOH (1%), EtOH (1%) and CH ₃ CN (1%). The % _{RSD} of the AUC was < 5% and the % _{RSD} of the retention time was < 1% for all tested solvents.	
Quantification of detection limits	LOD EtOH: 0.006%; LOQ EtOH: 0.0020% LOD CH ₃ CN: 0.007%; LOQ CH ₃ CN: 0.0022%	

a 5 µg/mL reference solution (in batch vehicle), followed by a blank run to confirm the absence of carryover.

The method demonstrated high specificity for AIF-FAPI-74 and [^{18}F]AIF-FAPI-74, with baseline separation from its nearest impurity, the FAPI-74 precursor (retention time: 9.2 min), achieving a resolution > 1.5. The main product peak consistently eluted at 12.5 min. Signal linearity was confirmed over the full tested concentration range (0.32–31.81 µg/mL), with a correlation coefficient (R^2) > 0.9995.

Repeat injections ($n = 6$) of a 3.19 µg/mL FAPI-74 solution confirmed excellent precision. Sensitivity was high, with a LOD and LOQ of 34 ng/mL and 114 ng/mL, respectively. The method also yielded sharp peak profiles, with a relative peak width < 0.04. Equivalent validation results were obtained for the radiometric signal, with an LOD of 2.6 kBq and LOQ of 8.5 kBq, demonstrating specificity, linearity, and reproducibility for detection of [^{18}F]AIF-FAPI-74.

Importantly, by using the new HPLC method, an excellent recovery (> 95%) of [^{18}F]F $^-$, [^{18}F]AIF and [^{18}F]AIF-FAPI-74 was observed. In contrast, while the recovery of [^{18}F]AIF and [^{18}F]AIF-FAPI-74 was above 95% for the acidic radio-HPLC system, only a recovery of $59 \pm 20\%$ was observed for [^{18}F]F $^-$, which might result in an overestimation of the radiochemical purity.

Figure 2 shows representative UV- and radio-chromatograms of the validated HPLC method. The identity of [^{18}F]AIF-FAPI-74 was confirmed by matching retention times with the reference standard (UV/VIS, 220 nm). Additionally, the UV chromatogram from a representative production batch illustrates the chemical purity of the final formulation.

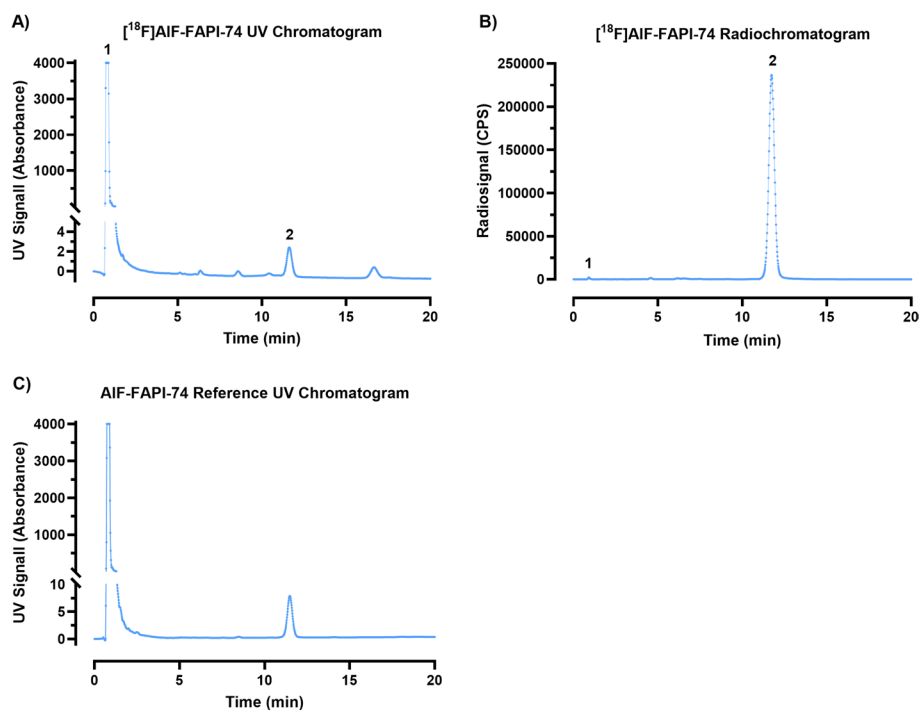


Fig. 2 **A** Representative chromatogram at 220 nm and **B** corresponding radio-chromatogram of [^{18}F]AIF-FAPI-74 (EOS). In panel A, peak 1 (0.8 min) corresponds to ascorbic acid, peak 2 (11.6 min) corresponds to AIF-FAPI-74, and additional peaks represent unidentified chemical impurities. In panel B, peak 1 (0.8 min) corresponds to free [^{18}F]F $^-$ /[^{18}F]AIF, while peak 2 (11.7 min) corresponds to [^{18}F]AIF-FAPI-74; other peaks indicate unidentified radiochemical impurities. **C** UV chromatogram of the non-radioactive reference solution, with AIF-FAPI-74 eluting at 11.5 min

GC validation confirmed specificity for residual solvents (ethanol and acetonitrile), with excellent linearity observed over the tested ranges (0.005–5% for acetonitrile; 0.005–10% for ethanol). The method also demonstrated adequate precision for all solvents. Additional details are presented in Table 2.

Radiosynthesis and quality control

Three validation runs of [^{18}F]AIF-FAPI-74 were performed using the fully automated synthesis protocol with a starting activity of 90 ± 2 GBq [^{18}F]F $^-$. The average produced activity of [^{18}F]AIF-FAPI-74 was 36 ± 7 GBq (up to 42.5 GBq), corresponding to a decay-corrected radiochemical yield of $50.0 \pm 10.3\%$, calculated relative to the starting activity in the target. All three batches demonstrated high radiochemical purity, with an average of $97.1 \pm 0.2\%$ at the end of synthesis (EOS). The total synthesis time was 30 min, measured from receipt of [^{18}F]F $^-$ to collection of the sterile, purified final product.

Quality control results for the three validation batches (A–C) are summarized in Table 3. The apparent molar activity, calculated based on the combined content of NOTA-FAPI-74, AIF-NOTA-FAPI-74, and other metal complexes of NOTA-FAPI-74, was 1125 ± 311 GBq/ μmol . The final product consistently exhibited a pH of 5. The ethanol content was $7.7 \pm 1.0\%$, and acetonitrile levels were below the LOD ($<0.0007\%$). Radionuclidic identity and purity were confirmed for all batches in accordance with established protocols. Sterile filter integrity tests met the predefined acceptance criteria in all cases. Additionally, all batches complied with the European Pharmacopoeia (Ph. Eur.) requirements for sterility and bacterial endotoxins.

Table 3 [^{18}F]AIF-FAPI-74 validation runs batch analysis

Test Parameters	Acceptance Criteria	Batch A	Batch B	Batch C
Total radioactivity (MBq)	Not defined	28 090	42 510	38 440
Molar activity concentration (EOS; GBq/ μmol)	Not defined	826	1446	1102
Identification	Difference between retention time of [^{18}F]FAPI74 and retention time of the reference $\leq 10\%$.	1.7%	1.3%	1.6%
Radiochemical purity	1. $\geq 93\%$ of total radioactivity	97.0%	97.0%	97.3%
1. [^{18}F]AIF-FAPI-74	2. $\leq 5\%$	0.3%	0.4%	0.3%
2. [^{18}F]F $^-$ or [^{18}F]AIF				
Chemical purity	1. ≤ 2.0 $\mu\text{g}/\text{ml}$	1.2 $\mu\text{g}/\text{ml}$	1.1 $\mu\text{g}/\text{ml}$	1.3 $\mu\text{g}/\text{ml}$
1. FAPI-74	2. ≤ 3.0 $\mu\text{g}/\text{ml}$	0.8 $\mu\text{g}/\text{ml}$	1.2 $\mu\text{g}/\text{ml}$	1.0 $\mu\text{g}/\text{ml}$
2. Amount of unidentified chemical impurities				
pH of the finished product	pH = 4.5–8.5	5	5	5
Integrity of the sterile filter membrane	Bubble point ≥ 3.2 bar	3.4 bar	3.3 bar	3.3 bar
Appearance	Clear, colorless solution with no visible particles	Conforms	Conforms	Conforms
Residual solvent detection	1. ≤ 4.1 mg/day (Ph. Eur.)	Conform	Conform	Conform
1. Acetonitrile	2. $\leq 10\%$ v/v (Ph. Eur.)	(< LOD)	(< LOD)	(< LOD)
2. Ethanol		6.3% v/v	8.5% v/v	8.2% v/v
Radionuclide identity - approximate half-life	$T_{1/2} = 105\text{--}115$ min	107.3 min	108.5 min	109.5 min
Radionuclide identity - gamma spectrometry	Gamma energy is 501–521 keV	514 keV	520 keV	518 keV
Sterility	No growth after 14 days of incubation at 37°C	Conforms	Conforms	Conforms
Bacterial endotoxins	< 175 IU per injection	Conforms	Conforms	Conforms

Radio-LC/MS analysis confirmed the identity of [^{18}F]AIF-FAPI-74. The co-eluting UV- and radiometric main peak was identified in the mass spectrum as $m/z = 390.85$ ($[\text{M} + 2\text{H}]^{2+}$). The theoretical monoisotopic mass of the reference AIF-FAPI-74 ($\text{C}_{36}\text{H}_{47}\text{O}_8\text{N}_9\text{AlF}$) is 390.67 ($[\text{M} + 2\text{H}]^{2+}$) (**Figure S3-S6**).

The stability of [^{18}F]AIF-FAPI-74 was assessed using the validated radio-HPLC method. At the highest tested batch concentration of 2019 MBq/mL, the radiochemical purity remained >95% for at least 10 h post-synthesis, indicating excellent formulation stability. Detailed quantitative stability data are provided in Table 4, and representative radio-HPLC chromatograms are available in **Figure S7**.

In vitro assays

[^{18}F]AIF-FAPI-74 demonstrated rapid and specific binding to FAP-expressing cells, with significant uptake observed as early as 15 min post-incubation. A plateau phase was reached between 30 min and 4 h, indicating fast binding kinetics. Blocking experiments using the FAP-specific inhibitor UAMC-1110 resulted in near-complete inhibition of [^{18}F]AIF-FAPI-74 uptake, confirming target-specific binding. No uptake was observed in the FAP-negative control cell line, further validating the high specificity. Additionally, a substantial fraction of the bound activity was internalized over time, reflecting efficient cellular uptake and internalization of [^{18}F]AIF-FAPI-74. These binding and internalization data are shown in Fig. 3A.

LigandTracer® data for [^{18}F]AIF-FAPI-74 were analyzed using a OneToOne interaction model, which provided an excellent fit to the experimental curves and yielded reliable estimates of the kinetic parameters. A representative binding curve, along with the fitted model, is shown in Fig. 3B. The experiments yielded a mean dissociation constant (K_d) of 1.9 ± 0.8 nM, demonstrating high binding affinity of [^{18}F]AIF-FAPI-74 for FAP.

In vivo imaging and radio-metabolite analysis

Whole-body small animal PET imaging was performed across all dosing groups and is shown in Fig. 4A–E. Across all administered molar amounts, [^{18}F]AIF-FAPI-74 exhibited fast renal clearance with limited retention in the kidneys (SUV at 90 min p.i.: 2.5 nmol/kg = 1.64 ± 2.47 ; 100 nmol/kg = 0.15 ± 0.09) and low liver uptake (SUV at 90 min p.i.: 2.5 nmol/kg = 0.26 ± 0.15 ; 100 nmol/kg = 0.04 ± 0.02), indicating favorable pharmacokinetics. Additionally, low bone uptake was observed across all groups, suggesting minimal in vivo defluorination of the radiopharmaceutical.

To assess specificity of the radiopharmaceutical, positive-to-negative tumor standardized uptake value (SUV) ratios were calculated under non-blocked conditions (Fig. 4F). SUV_{mean} values were derived from PET images acquired 90 min post-injection. No statistically significant differences were found in SUV_{mean} or SUV_{max} values of FAP-positive tumors across the different molar doses (SUV_{mean} 2.5 nmol/kg = 1.47 ± 0.39 ; 30 nmol/kg = 1.86 ± 0.54 ; 100 nmol/kg = 1.82 ± 0.74 / SUV_{max} 2.5 nmol/kg = 4.604 ± 1.715 ; 30nmol/

Table 4 Stability of [^{18}F]AIF-FAPI-74 in formulation buffer

Batch	Radioactivity concentration (EOS)	Radio-chemical purity (EOS)	Radio-chemical purity after incubation
*Batch A	1242 MBq/ml	97.0%	>95%; 2, 4, 6, 8 and 10 h after EOS
*Batch B	2019 MBq/ml	97.0%	>95%; 2, 4, 6, 8 and 10 h after EOS
*Batch C	1784 MBq/ml	97.3%	>95%; 2, 4, 6, 8 and 10 h after EOS

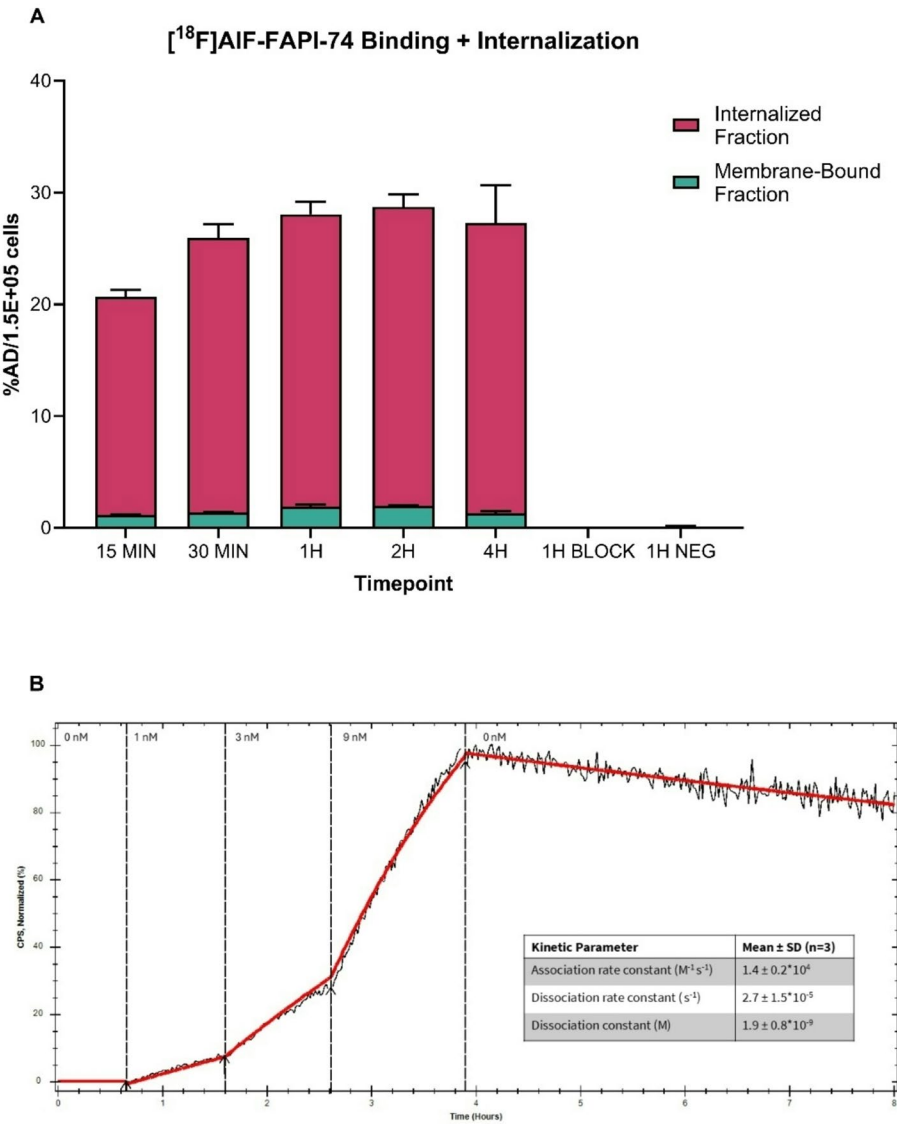


Fig. 3 In vitro characterization of [¹⁸F]AIF-FAPI-74 kinetic characteristics. **(A)** Results of a 4-hour binding assay showing membrane-bound (green) and internalized (purple) fractions of [¹⁸F]AIF-FAPI-74. Two negative controls, a FAP-negative cell line and UAMC-1110 block, were included to confirm specificity of uptake. Data points represent mean ± SD (n=3). **(B)** Representative binding curve, with arrows indicating increase/decrease of [¹⁸F]AIF-FAPI-74 concentration. The curve includes background measurement, three consecutive association phases with increasing [¹⁸F]AIF-FAPI-74 concentrations (indicated above the plot), and a dissociation phase, separated by dotted vertical lines. Raw signal (black line) and fitted model (red line) are plotted over an 8-hour period. Calculated kinetic parameters are presented as mean ± SD (n=3)

kg = 5.238 ± 1.911 ; 100nmol/kg = 4.913 ± 1.875. However, the 2.5 nmol/kg group showed a markedly reduced selectivity between FAP-positive and FAP-negative tumors, with a positive-to-negative tumor ratio of 4.87 ± 4.4 (vs. 39.0 ± 9.6 and 44.0 ± 10.3, for 30, 100 nmol/kg resp.), indicating lower specificity at suboptimal molar doses.

Tumor-to-organ SUV_{mean} ratios were also analyzed to evaluate differential accumulation of [¹⁸F]AIF-FAPI-74 and image contrast across dosing groups. Statistically significant differences were observed between the ratio of the 2.5 nmol/kg group and both the ratios of the 30 and 100 nmol/kg groups for all evaluated ratios (tumor-to-muscle, tumor-to-lung, and tumor-to-bone). No significant differences were observed between

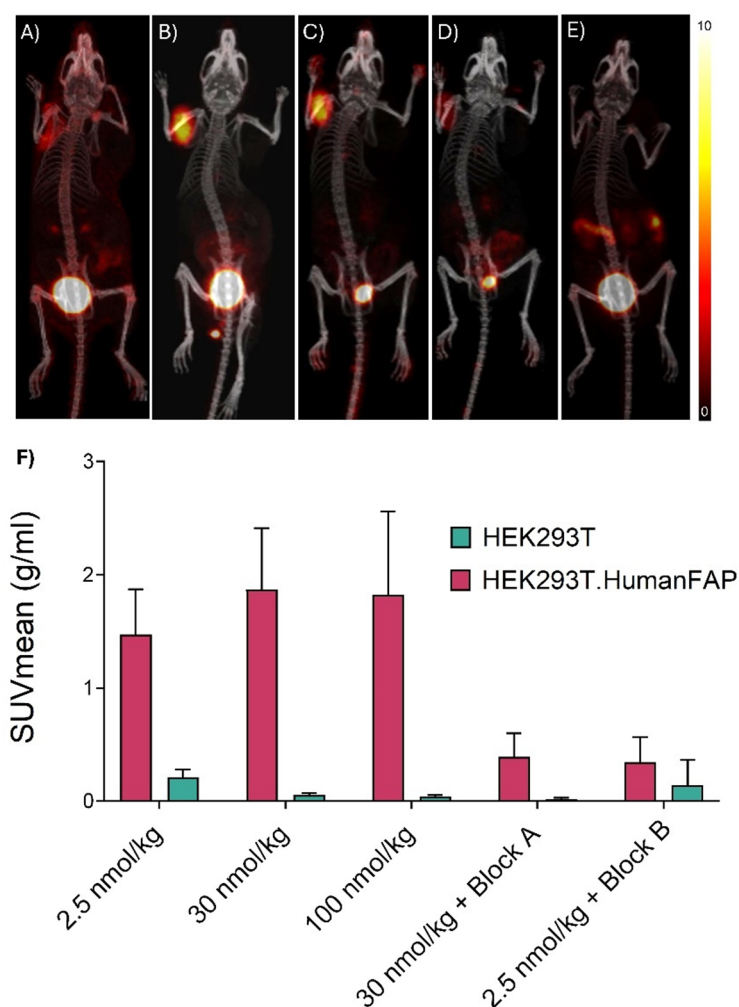


Fig. 4 Co-registered PET/CT maximum intensity projection images acquired 90 min post-injection of [^{18}F]AIF-FAPI-74 in SCID mice bearing subcutaneous HEK293T.hFAP (left shoulder, FAP-positive) and HEK293T (right shoulder, FAP-negative) tumors. [^{18}F]AIF-FAPI-74 was administered at the following molar doses: **A** 2.5 nmol/kg, **B** 30 nmol/kg, **C** 100 nmol/kg, **D** 30 nmol/kg + 7.3 $\mu\text{mol/kg}$ UAMC-1110, **E** 2.5 nmol/kg + 14.6 $\mu\text{mol/kg}$ UAMC-1110. (F) Quantification of tracer uptake ($\text{SUV}_{\text{mean}} \pm \text{SD}$) in positive (green) and negative (red) tumors. Positive-to-negative tumor SUV_{mean} ratios were 4.9 ± 4.4 for the 2.2 nmol/kg group, 39.0 ± 9.6 for the 30 nmol/kg group and 44.0 ± 10.3 for the 100 nmol/kg group; across all treatment groups selectivity differed significantly ($p=0.007$)

the 30 and 100 nmol/kg groups, indicating a plateau in imaging performance at higher molar doses. The 2.5 nmol/kg group consistently showed higher background activity, as evident in the $\mu\text{PET/CT}$ images (Fig. 4A–E) and reduced tumor-to-background contrast (Fig. 5).

Radio-metabolite analysis confirmed the in vivo stability of [^{18}F]AIF-FAPI-74, with more than 95% of intact radiopharmaceutical in urine and >85% intact in plasma at 10 min p.i. Representative radio-chromatograms are provided in **Figure S8–S9**.

Clinical PET/CT scan

Figure 6 presents the [^{18}F]AIF-FAPI-74 PET/CT scan of a 34-year-old female patient diagnosed with a well to moderately differentiated, hormone receptor-positive (ER8, PR8, HER2-negative) non-specific breast carcinoma located in the right breast. Ultrasound-mammography revealed a multifocal lesion, predominantly in the upper-outer

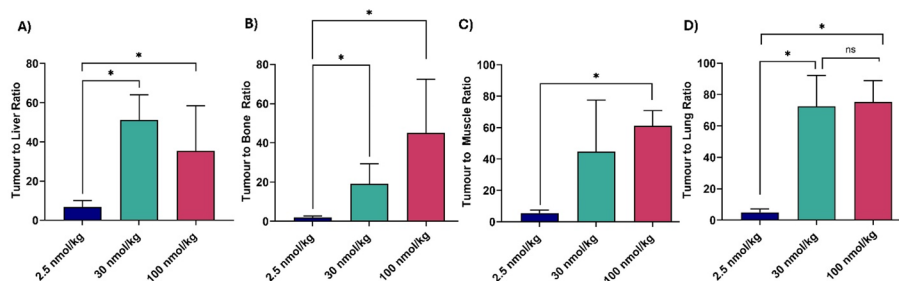


Fig. 5 **A** Tumour-to-liver, **B** tumour-to-bone, **C** tumour-to-muscle, and **D** tumour-to-lung ratios in mice injected with 2.5, 30, or 100 nmol/kg of [^{18}F]FAPI-74. Data are presented as mean $\pm$ SD ($n=4$ per group). Overall group differences were significant for all organs ($p < 0.05$), driven primarily by increased tumour selectivity at higher molar amounts. $p < 0.05$ was considered statistically significant

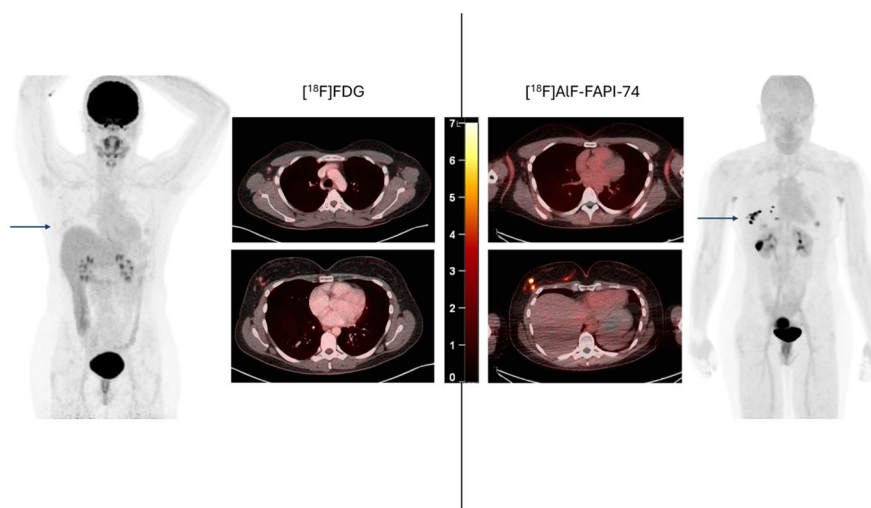


Fig. 6 A case report of a 34-year-old female patient with well to moderately differentiated breast carcinoma of the right breast (ER8 PR8 HER2 negative) with discordant [^{18}F]FDG PET/CT (left) and [^{18}F]AIF-FAPI-74 PET/CT (right) studies (blue arrow). All images were scaled according to the same scale (center; SUV: 0–7). Multifocal and multicentric disease is shown with high contrast by the [^{18}F]AIF-FAPI-74 FAPI PET (SUV_{max}: 12; tumor-to-contralateral breast ratio (TBR): 24), but not by [^{18}F]FDG (SUV_{max}: 2; TBR: 4). This demonstrates that the current molar activity (549 GBq/ μmol EOS) allows high contrast tumor depiction that can outperform [^{18}F]FDG

quadrant, with additional foci in the upper-inner quadrant. A nodular cortical thickening of a right axillary lymph node prompted further evaluation via fine-needle aspiration cytology (FNAC).

[^{18}F]FDG PET/CT demonstrated only faint uptake in a portion of the primary breast lesion and mild hypermetabolism in the axillary lymph node. In contrast, [^{18}F]AIF-FAPI-74 PET/CT, using high apparent molar activity [^{18}F]AIF-FAPI-74 (700 GBq/ μmol EOS), revealed intense and well-delineated uptake of [^{18}F]AIF-FAPI-74 corresponding to all identified tumor foci, with the highest signal in the upper-outer quadrant and additional uptake in the upper-inner quadrant. No abnormal uptake was observed in axillary lymph nodes or distant sites, and FNAC of the suspicious lymph node was negative, consistent with the [^{18}F]AIF-FAPI-74 findings.

[^{18}F]AIF-FAPI-74 demonstrated fast renal clearance with low retention in the kidneys and no detectable bone uptake, indicating high in vivo stability and minimal defluorination. Additionally, background signal in non-target tissues was low, resulting in excellent tumor-to-background contrast.

Discussion

[¹⁸F]AIF-FAPI-74 presents several advantages over ⁶⁸Ga-labeled FAPI derivatives, including its longer half-life, higher resolution, and cost-effectiveness. The longer half-life of fluorine-18 (approximately 110 min) compared to gallium-68 (68 minutes) offers more flexibility for imaging protocols, including delayed imaging, higher patient throughput, and centralized production. The cyclotron-based production of fluorine-18 ensures a more reliable and scalable supply than the generator-based gallium-68, which can be constrained by production limitations and decreasing yield over time. Although gallium-68 can also be produced in cyclotrons, this method is not yet routinely implemented in the clinical setting. While ⁶⁸Ga-based FAPI radiopharmaceuticals are still valuable for their rapid imaging and generator-based production, [¹⁸F]AIF-FAPI-74 is a superior choice for large-scale diagnostic applications due to its logistical advantages [44, 45].

The GMP-compliant, automated production method for [¹⁸F]AIF-FAPI-74 integrates the advantages of fluorine-18, the pseudo-metal approach, and automated strategies in clinical radiopharmacy. Previously, the highest reported batch activity was 11.3 ± 1.1 GBq (starting activity 32–40 GBq) with a radiochemical yield of $37 \pm 4\%$ [35]. In contrast, our optimized synthesis method consistently produced batches ($N = 3$) of 36.3 ± 7.4 GBq and a decay-corrected radiochemical yield of $50.0 \pm 10.1\%$. The maximum batch activity reached 42.5 GBq, with no production limitations encountered. This improved method offers the potential for even larger batch sizes, although expanded stability testing would be required for these larger quantities.

The average molar activity per synthesis was 1124 ± 254 GBq/ μ mol. Final purification was efficiently achieved using a tC18 SepPak cartridge and a Sep-Pak Alumina N Plus Light Cartridge. Compared to the method reported by Naka et al. [35], key differences include the type of cartridge for purification (HLB vs. tC18/Alumina N) and the absence of DMSO in our radiolabeling mixture. Both methods are one-pot, one-step radiolabeling procedures. In our method, we use 98 nmol of precursor, whereas Naka et al. [35] used 80 nmol. Our method involves radiolabeling at 70 °C for 10 min, while Naka et al. [35] performed the process at room temperature for 5 min followed by 15 min at 95 °C. Overall, total synthesis times were similar.

Radiolabeling at lower temperatures, combined with the inclusion of radioprotectant during the radiosynthesis, effectively mitigated radiolysis and degradation. We demonstrated both radiochemical and chemical stability for up to 10 h post-EOS. At a maximum concentration of 2019 MBq/ml (42,510 MBq/21.1 ml), only minimal radiolysis was observed, and the radiochemical purity remained >95% throughout the 10-hour period. All three batches maintained adequate radiochemical purity (>95%) for the entire duration. The levels of total unidentified chemical impurities (including FAPI-74 precursor) remained consistent with the initial analysis. As a result, the shelf-life of the compound was determined to be 10 h post-EOS at room temperature.

The quality control methods were validated following ICH Q2(R1) 2005 guidelines [46]. Previous studies [37] have indicated that fluoride recovery improves with higher pH levels of the mobile phase when using silica-based C18 columns for radiochemical purity assessment by radio-HPLC. To address this, we validated an alternative radio-HPLC method, differing from the standard acidic approach commonly used in the literature [33–36]. Our method utilizes an isocratic program with a mobile phase of 0.05

M NH_4OAc (pH = 5.5) and acetonitrile (91:9; v/v) for the first 20 min, followed by a 40-minute gradient up to 90% acetonitrile. This method provides optimal separation of radiolytic impurities, allows for detection of high-lipophilicity impurities, and ensures efficient recovery of fluorine-18. We strongly recommend this method for its superior performance in both impurity separation and fluorine-18 recovery.

The specifications for radiochemical purity, endotoxins, and sterility were established in accordance with the guidelines for other PET radiopharmaceuticals outlined in the Ph. Eur. Stability requirements (e.g. max. required shelf-life) were determined based on the properties of fluorine-18. For FAPI-74 content, a maximum concentration of 2.0 $\mu\text{g}/\text{ml}$ was set, derived from toxicological testing based on the microdosing concept. The sum of unidentified chemical impurities is limited to 3.0 $\mu\text{g}/\text{ml}$. No maximum limit was set for the precursor, as it is quantified as an unidentified chemical impurity. For the residual solvent CH_3CN , a Class 2 solvent, a daily limit of 4.1 mg conforms to ICH Q3C acceptance criteria. To minimize irritation during intravenous administration and comply to Ph. Eur. standards, the concentration of ethanol (EtOH) is limited to 10% of the total formulation.

In vitro experiments were conducted to investigate the kinetic properties of ^{18}F AIF-FAPI-74. The results from both assays confirmed the rapid kinetics previously mentioned for quinoline-based FAPI-radiopharmaceuticals [5]. High and fast uptake was observed in the internalization assay, with stabilization occurring within 60 min. Within this timeframe, almost all bound ^{18}F AIF-FAPI-74 (>90%) was internalized. Similar outcomes were obtained in the LigandTracer[®] experiment. The OneToOne model was applied to fit the data, as it makes minimal assumptions regarding ligand-target interactions. The model provided a good fit, as indicated by the fitting parameters (Chi-Squared, U-value) and visual inspection. Overall, the association rates were in line with expectations, while dissociation rates were lower than anticipated. Typically, association and dissociation rates for small molecules are $>10^4$ and $>10^{-4}$, respectively. The lower dissociation rate observed in this study may be attributed to the high fraction of internalized radiopharmaceutical, as demonstrated in the cellular binding/internalization assay. Further comparisons with other investigational FAPI radiopharmaceuticals could offer deeper insights into the relative affinity of these radiopharmaceuticals.

Radio-HPLC analysis demonstrated that ^{18}F AIF-FAPI-74 remained >95% intact in urine and >85% in serum, 10 min post-injection, indicating high in vivo stability in mice at early timepoints. Recently, Adhikari et al. reported a more comprehensive in vivo stability study, which revealed late-point instability of the ^{18}F AIF-FAPI-74 at 120 min p.i [29]. While such extensive stability evaluations are valuable for characterizing FAPI radiopharmaceuticals, the high stability observed at 60 min. p.i. suggests that ^{18}F AIF-FAPI-74 possesses sufficient in vivo stability given its rapid pharmacokinetics. Although the in vivo evaluation of ^{18}F AIF-FAPI-74 in animal models has been previously reported [47], we opted to perform additional in vivo validation to further assess the quality of the high molar activity ^{18}F AIF-FAPI-74 produced at our hospital's GMP facility (University Hospital Leuven) prior to its clinical application. In this study, we utilized transfected HEK-293.hFAP cells, which express high levels of human FAP [47]. While we acknowledge that transfection models may not entirely reflect natural expression patterns, the selection of appropriate animal models for evaluating FAP-targeting radiopharmaceuticals remains a subject of ongoing debate in the literature [48]. The

current system serves as a “best-case scenario” positive control for validation of FAPI radiopharmaceuticals. It is important to note that interactions between the FAP-targeting radiopharmaceutical and transfected HEK cells may differ substantially from those in cancer-associated fibroblasts (CAFs) expressing FAP [47]. Additionally, as pointed out in the literature [48], these animal models do not fully replicate clinical conditions.

Initial small-animal imaging studies were performed using an injected molar amount of approximately 2.5 nmol/kg (1.5–3.0 MBq/mouse), which resulted in suboptimal image quality and low tumor-to-background contrast. Around that time, emerging pre-clinical literature began to highlight the so-called “molar amount effect” in FAPI-based radiopharmaceuticals [37–39]. Based on these findings, we expanded our evaluation to include higher molar doses of 30 nmol/kg and 100 nmol/kg. A previously published study reported an even lower molar dose of 0.65 nmol/kg with 3.7 MBq injected activity but did not specifically address the molar amount effect. In that study, [^{18}F]AIF-FAPI-74 showed inferior tumor-to-background ratios compared to a novel FAP-targeting radiopharmaceutical [47].

Given the high expression of FAP in the HEK293.HumanFAP model, complete blocking was not achieved under either of the tested blocking conditions (30 nmol/kg FAPI-74 + 7.3 $\mu\text{mol/kg}$ UAMC-1110 or 2.5 nmol/kg FAPI-74 + 14.6 $\mu\text{mol/kg}$ UAMC-1110). Nevertheless, tumor uptake was sufficiently reduced to become undetectable on $\mu\text{PET/CT}$, confirming the specificity of the radiopharmaceutical.

Tumor-to-organ ratios were calculated to facilitate the comparison of relative uptake of [^{18}F]AIF-FAPI-74 across tissues and treatment groups, enabling a clearer assessment of biodistribution and selectivity. Ratios such as tumor-to-muscle, tumor-to-lung, tumor-to-liver, and tumor-to-bone provide valuable insight into the pharmacokinetic profile of [^{18}F]AIF-FAPI-74. Statistical analysis of these ratios allowed for a robust evaluation of dose-dependent effects and blocking efficiency.

Among the tested dosing groups, the 2.5 nmol/kg group consistently demonstrated elevated background uptake and reduced tumor-to-background ratios across all organs analyzed. In this group, [^{18}F]AIF-FAPI-74 accumulation in the HEK293.HumanFAP tumors was not substantially higher than in background tissues, likely due to non-specific uptake in reference organs. By contrast, the 100 nmol/kg group yielded significantly improved tumor-to-background ratios, highlighting the importance of optimizing molar dosing for accurate imaging contrast and specificity in preclinical setting.

While the preclinical data strongly support the existence of a molar amount effect in rodent models, this phenomenon did not appear to translate directly to the clinical setting. In patient scans performed at our institution as part of the prospective FAPIDO trial (NCT06782412), [^{18}F]AIF-FAPI-74, administered with an apparent molar activity of approximately 549 GBq/ μmol , demonstrated high tumor-to-background contrast. The clinical case highlighted in this study illustrates that [^{18}F]AIF-FAPI-74 provided superior delineation of the local tumor extent compared to [^{18}F]FDG. Following mastectomy and sentinel lymph node dissection, pathology confirmed the presence multicentric and multifocal disease, with a single micrometastasis (1.9 mm), consistent with the imaging findings. This demonstrates that high molar activity [^{18}F]AIF-FAPI-74 allows high contrast tumor depiction, capable of outperforming [^{18}F]FDG in certain tumors.

These results suggest that the molar amount effect observed in preclinical models may not have the same clinical relevance, possibly due to differences in pharmacokinetics,

tumor microenvironment, or FAP expression between species. As this is a single case-study, no definitive conclusions can be drawn. Further clinical studies are warranted to clarify the impact of molar activity on image quality and diagnostic performance in human subjects.

Conclusions

This study presents the successful development, optimization, and validation of a high-yield, fully automated, and GMP-compliant production method for [^{18}F]AIF-FAPI-74, establishing a reliable platform suitable for centralized and large-scale radiopharmaceutical manufacturing. All analytical methods were successfully validated. Importantly, by using the new QC HPLC method, an excellent recovery of [^{18}F]F $^-$, [^{18}F]AIF and [^{18}F]AIF-FAPI-74 was observed. In contrast, while the recovery of [^{18}F]AIF and [^{18}F]AIF-FAPI-74 was above 95% for the acidic radio-HPLC system, only a recovery of $59 \pm 20\%$ was observed for [^{18}F]F $^-$, which might result in an overestimation of the radiochemical purity. We recommend to use a mobile phase with $\text{pH} > 5$ for silica-based C18 columns for quality control of ^{18}F -labelled radiopharmaceuticals.

In vitro and in vivo evaluations confirmed the compound's high specificity for FAP. Preclinical imaging in a rodent xenograft model revealed improved image quality at increasing injected molar amounts. Low injected masses resulted in insufficient tumor-to-background contrast due to high background uptake.

Importantly, this molar amount effect was not observed in patient scans conducted at our institution, where [^{18}F]AIF-FAPI-74 consistently produced high diagnostic performance even at high apparent molar activity. These findings highlight the importance of cautious interpretation of preclinical data when projecting to clinical use. Further studies are warranted to elucidate the clinical relevance and underlying mechanisms of the observed molar amount effect in preclinical models.

Abbreviations

AUC	Area Under the Curve
CAF	Cancer Associated Fibroblast
(c)GMP	(current) Good Manufacturing Practices
DAD-UV	Diode Array Detector-UV
DMEM	Dulbecco's Modified Eagle Medium
DPP	DiPeptidylPeptidase
EOS	End Of Synthesis
ESI-MS	Electron Spray Ionization Mass Spectrometry
FAP(I)	Fibroblast Activation Protein (Inhibitor)
FDG	FluoroDeoxyGlucose
FNAC	Fine Needle Aspiration Cytology
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IU	International Units
LC/(HR)MS	Liquid Chromatography/(High Resolution) Mass Spectrometry
LOD/LOQ	Limit Of Detection/Quantification
PBS	Phosphate Buffered Saline
PET/CT	Positron Emission Tomography/Computed Tomography
Ph. Eur.	European Pharmacopoeia
QMA	Quaternary Methyl Ammonium
RCP/RCY	RadioChemical Purity/Yield
WFI	Water For Injection

Supplementary Information

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Supplementary Material 1

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Authors' contributions

All authors contributed to the study conception and design. Experimental design, execution and data analysis was performed by W.S., S.C., J.K. and J.M. Writing – original draft preparation was performed by W.S., J.K., J.M. and F.C. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the KU Leuven ethical review board (ethical approval reference P054/2021). The clinical study (FAPIDO trial) is performed at University Hospital Leuven after approval by the Ethics Committee. (ClinicalTrials.gov identifier, NCT06782412).

Consent for publication

Subject gave written informed consent.

Competing interests

L.T., C.W., and R.G. are employed by Trasis.

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References

1. Litt HK, Kwon DH, Velazquez AI. FDG PET scans in cancer care. *JAMA Oncol.* 2023;9(9):1304. <https://doi.org/10.1001/jamaoncol.2023.0265>.
2. Fletcher JW, Djulbegovic B, Soares HP, Siegel BA, Lowe V, et al. Recommendations on the use of 18F-FDG PET in oncology. *J Nucl Med.* 2008;49(3):480–508.
3. Xin L, Gao J, Zheng Z, Chen Y, Lv S, et al. Fibroblast activation protein- α as a target in the bench-to-bedside diagnosis and treatment of tumors: a narrative review. *Front Oncol.* 2021;11:648187. <https://doi.org/10.3389/fonc.2021.648187>.
4. Mori Y et al. Clinical applications of fibroblast activation protein inhibitor positron emission tomography (FAPi-PET). *NPIJ Imaging* 2024 2:1 2, 1–21 (2024).
5. Altmann A, Haberkorn U, Siveke J. The latest developments in imaging of fibroblast activation protein. *J Nucl Med.* 2021;62(2):160–7. <https://doi.org/10.2967/jnumed.120.244806>.
6. Hope TA, Calais J, Goenka AH, Haberkorn U, Konijnenberg M, et al. SNMMI procedure Standard/EANM practice guideline for fibroblast activation protein (FAP) PET. *J Nucl Med.* 2025;66(1):26–33. <https://doi.org/10.2967/jnumed.124.269002>.
7. Ruan D, et al. Evaluation of FAPi PET imaging in gastric cancer: a systematic review and meta-analysis. *Theranostics.* 2023;13:4694–710.
8. van Veldhuijzen SEM et al. FAPi PET versus FDG PET, CT or MRI for staging Pancreatic-, Gastric- and cholangiocarcinoma: systematic review and Head-to-Head comparisons of diagnostic performances. *Diagnostics (Basel)* 12, (2022).
9. Röhrich M, et al. Impact of 68 Ga-FAPi PET/CT imaging on the therapeutic management of primary and recurrent pancreatic ductal adenocarcinomas. *J Nucl Med.* 2021;62:779–86.
10. Ding J et al. Prognostic value of preoperative [68 Ga]Ga-FAPi-04 PET/CT in patients with resectable pancreatic ductal adenocarcinoma in correlation with Immunohistological characteristics. <https://doi.org/10.1007/s00259-022-06100-4>
11. Karbhari A et al. Phase 2, Multicenter, single Arm, open label Non-Randomized study of [68Ga]FAPi-46 PET in patients with resectable or borderline resectable pancreatic ductal carcinoma. *J Nucl Med* 64, (2023).
12. Kessler L, et al. 68 Ga-Labeled fibroblast activation protein inhibitor (68 Ga-FAPi) PET for pancreatic adenocarcinoma: data from the 68 Ga-FAPi PET observational trial. *J Nucl Med.* 2023;64:1910–7.
13. Jiao M, Mingsong T, Hao J, Chunyin Z. Diagnostic value of 18F-FDG PET/CT and 68Ga-FAPi PET/CT in primary liver cancer: A systematic review and meta-analysis. *Hell J Nucl Med.* 2023;26:123–31.
14. Zhuang Z, Zhang Y, Yang X, Deng X, Wang Z. Head-to-head comparison of the diagnostic performance between 68Ga-FAPi-04 PET/CT and 18F-FDG PET/CT in colorectal cancer: a systematic review and meta-analysis. *Abdom Radiol (NY).* 2024. <https://doi.org/10.1007/S00261-024-04266-Z>.
15. Evangelista L, Filippi L, Schillaci O. What radiolabeled FAPi pet can add in breast cancer? A systematic review from literature. *Ann Nucl Med.* 2023;37:442–50.
16. Rizzo A et al. Diagnostic yield of FAP-guided positron emission tomography in thyroid cancer: a systematic review. *Front Med (Lausanne)* 11, (2024).

17. Yang Q, et al. Performance of [18F]FDG PET/CT versus FAPI PET/CT for lung cancer assessment: a systematic review and meta-analysis. *Eur Radiol.* 2024;34:1077–85.
18. Gu B, et al. Imaging of tumor stroma using 68 Ga-FAPI PET/CT to improve diagnostic accuracy of primary tumors in head and neck cancer of unknown primary: A comparative imaging trial. *J Nucl Med.* 2024;65:365–71.
19. Shu Q, et al. The additional role of [68Ga]Ga-FAPI-04 PET/CT in patients with unknown primary lesions with a negative or equivocal [18F]FDG. *Eur J Nucl Med Mol Imaging.* 2023;50:1442–52.
20. Serfling S, et al. Improved cancer detection in waldeyer's tonsillar ring by 68 Ga-FAPI PET/CT imaging. *Eur J Nucl Med Mol Imaging.* 2021;48:1178–87.
21. Dendl K, et al. FAP imaging in rare cancer entities—first clinical experience in a broad spectrum of malignancies. *Eur J Nucl Med Mol Imaging.* 2022;49:7.
22. Mosessian S, Jensen JD, Enke AS. Current state of clinical trials and regulatory approvals with fibroblast activation protein targeting interventions. *PET Clin.* 2023;18:429–39.
23. Zhong K, et al. Comparison of [18F]FAPI-42 and [18F]FDG PET/CT in the evaluation of systemic vasculitis. *Eur J Nucl Med Mol Imaging.* 2025;52:1083–94.
24. Mori Y et al. FAPI PET: fibroblast activation protein inhibitor use in oncologic and nononcologic disease. *Radiology* 306, (2023).
25. Bentestuen M, Al-Obaydi N, Zacho HD. FAPI-avid nonmalignant PET/CT findings: an expedited systematic review. *Semin Nucl Med.* 2023;53:694–705.
26. Leupe H, Ahenkorah S, Dekervel J, Unterrainer M, Van Cutsem E, Verslype C, Cleeren F, Deroose CM. ¹⁸F-Labeled somatostatin analogs as PET tracers for the somatostatin receptor: ready for clinical use. *J Nucl Med.* 2023;64(6):835–41. <https://doi.org/10.2967/jnumed.123.265622>.
27. Conti M, Eriksson L. Physics of pure and non-pure positron emitters for PET: a review and a discussion. *EJNMMI Phys.* 2016;3(1):8. <https://doi.org/10.1186/s40658-016-0144-5>.
28. Hoffmann C, Gröner B, Bahutski V, Endepols H, Lindemeyer J, Saniternik S, Drewes B, Timmer M, Gokhadze O, Brugger M, Neumaier F, Neumaier B, Zlatopolskiy BD. Comparative evaluation of three ¹⁸F-fluorinated FAP ligands in rodent tumor models. *Eur J Med Chem.* 2025;299:118103. <https://doi.org/10.1016/j.ejmech.2025.118103>.
29. Adhikari K, Grintsevich S, de Groot A, Verhulst E, van Rymenant Y, Vangestel C, Lauwerys L, Miranda A, De Meester I, Van der Veken P, Elvas F. Synthesis and preclinical evaluation of druglike ¹⁸F-labeled fibroblast activation protein (FAP) inhibitors with enhanced tumor retention. *Bioconjug Chem.* 2025;36(8):1887–906. <https://doi.org/10.1021/acs.bioconjchem.5c00366>.
30. McBride WJ, Sharkey RM, Karacay H, D'Souza CA, Rossi EA, Laverman P, et al. Novel method of ¹⁸F radiolabelling for PET/CT for imaging neuroendocrine neoplasms. *Clin Nucl Med.* 2019;44:452–8.
31. Archibald SJ, Allot L. The aluminum-[18F]fluoride revolution: simple radiochemistry with a big impact for radiolabelled biomolecules. *EJNMMI Radiopharm Chem.* 2021;6:30. <https://doi.org/10.1186/s41181-021-00141-0>.
32. Fersing C, Bohlhel A, Cantelli C, Garrigue P, Lisowski V, Guillet B. A comprehensive review of non-covalent radiofluorination approaches using aluminum [¹⁸F]fluoride: will [¹⁸F]AlF replace 68Ga for metal chelate labeling? *Molecules.* 2019;24:16. <https://doi.org/10.3390/molecules24162866>.
33. Giesel FL, Adeberg S, Syed M, Lindner T, Jimenez-franco LD, Mavriopoulou E et al. FAPI-74 PET/CT using either ¹⁸F-AIF or cold-kit ⁶⁸Ga-labelling: biodistribution, radiation dosimetry and tumor delineation in lung cancer patients. *J Nucl Med* 62(2): 201–7. <https://doi.org/10.2967/jnumed.120.245084>.
34. Wang S, Zhou X, Xu C, Ding J, Liu S, Hou X et al. Clinical translation evaluation of Al18F-NOTA-FAPI for fibroblast activation protein-targeted tumour imaging. *Eur J Nucl Med Mol Imaging.* 48(13): 4259–71. <https://doi.org/10.1007/s00259-021-05470-5>.
35. Naka S, Watabe T, Lindner T, Cardinale J, Kurimoto K, Moore M, et al. One-pot and one-step automated radio-synthesis of [18F]AlF-FAPI-74 using a multi-purpose synthesizer: a proof-of-concept experiment. *EJNMMI Radiopharm Chem.* 2021;6:28. <https://doi.org/10.1186/s41181-021-00142-z>.
36. Dahl K, Kissing E, Bylund L, Moein MM, Samen E, Tran T. Fully automated production of the fibroblast activation protein Radiotracer [18F]FAPI-74. *J Label Comp Radiopharm.* 64(8): 346–52. <https://doi.org/10.1002/jlcr.3926>.
37. Ory D, Van den Branden J, De Groot T, Serdons K, Bex M, Declercq L, et al. Retention of [18F] fluoride on reversed-phase HPLC columns. *J Pharm Biomed Anal.* 2015;111:209–14.
38. Zhao L, Niu B, Fang J, Pang Y, Li S, Xie C, Sun L, et al. Synthesis, preclinical evaluation, and a pilot clinical PET imaging study of 68Ga-labeled FAPI dimer. *J Nucl Med.* 2022;63(6):862–8. <https://doi.org/10.2967/jnumed.121.263016>.
39. Galbiati A, Bocci M, Neri D, Cazzamalli S. Effect of molar dose on the in vivo tissue biodistribution profile of FAP-targeted radioligand therapeutics. *EJNMMI.* 2025;52:1339–405. <https://doi.org/10.1007/s00259-024-06969-3>.
40. Liu L, Shi Y, He S, Yang J, Song S, Wang D, et al. The molar dose of FAPI administered impacts on the FAP-targeted PET imaging and therapy in mouse synergistic tumor models. *EJNMMI.* 2025. <https://doi.org/10.1007/s00259-025-07071-y>.
41. Tshibangu T, Cawthorne C, Serdons K, Pauwels E, Gsell W, Bormans G, Deroose CM, Cleeren F. Automated GMP compliant production of [18F]AlF-NOTA-octreotide. *EJNMMI Radiopharm Chem.* 2020;5(1):4. <https://doi.org/10.1186/s41181-019-0084-1>.
42. Ahenkorah S, Cawthorne C, Murce E, Deroose CM, Cardinaels T, Seimille, et al. *Nucl Med Biol.* 2023;118–9. <https://doi.org/10.1016/j.nucmedbio.2023.108338>. Direct comparison of [18F]AlF-NOTA-JR11 and [18F]AlF-NOTA-octreotide for PET imaging of neuroendocrine tumors: antagonist versus agonist.
43. Du Sert NP, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *BMC Vet Res.* 2020;16:242. <https://doi.org/10.1186/s12917-020-02451-y>.
44. Krasikova RN. Fluorine-18 or gallium-68: the perspective of the PET radiochemist. *Radiochemistry.* 2023;65(2):158–76. <https://doi.org/10.1134/s1066362223020030>.
45. Kesch c, Kratochwil C, Mier W, Kopka K, Giesel FL. 68Ga or 18F for prostate cancer imaging. *J Nucl Med.* 58(5): 687–8. <https://doi.org/10.2967/jnumed.117.190157>.
46. International Council for Harmonisation guideline on analytical validation (ICH Q2 (R1)). 2019: https://database.ich.org/sites/default/files/Q2_R1_Guideline.pdf

47. Bian L, Liu x, Wang X, Sun Y, Du X, et al. Preclinical and first-in-human imaging of novel [18F]F-FAPI-FUSCC-07 tracer: comparative prospective study with [18F]F-FAPI-42 and [18F]F-FAPI-74. *Mol Pharm*. 2025;22(3):16264–1632. <https://doi.org/10.1021/acs.molpharmaceut.4c01360>.
48. Van der Heide CD, Campeiro JD, Ruigrok EAM, Van den Brink L, Ponnala S, Hillier SM, Dalm SU. In vitro and ex vivo evaluation of preclinical models for FAP-targeted theranostics: differences and relevance for Radiotracer evaluation. *EJNMMI Res*. 2024;14:125. <https://doi.org/10.1186/s13550-024-01191-6>.

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