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Marlène Wiart, Clément Tavakoli, Violaine Hubert, Inès Hristovska, Chloé Dumot, et al.. Use of metal-based contrast agents for in vivo MR and CT imaging of phagocytic cells in neurological pathologies. *Journal of Neuroscience Methods*, 2023, 383, pp.109729. 10.1016/j.jneumeth.2022.109729 . hal-03865234

HAL Id: hal-03865234

<https://hal.science/hal-03865234>

Submitted on 22 Nov 2022

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Use of metal-based contrast agents for *in vivo* MR and CT imaging of phagocytic cells in neurological pathologies

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ARTICLE INFO

Keywords:

Phagocytic cells
Contrast agents
Metal-based nanoparticles
Magnetic resonance imaging
Computed tomography
Neurological diseases

ABSTRACT

The activation of phagocytic cells is a hallmark of many neurological diseases. Imaging them in their 3-dimensional cerebral environment over time is crucial to better understand their role in disease pathogenesis and to monitor their potential therapeutic effects. Phagocytic cells have the ability to internalize metal-based contrast agents both *in vitro* and *in vivo* and can thus be tracked by magnetic resonance imaging (MRI) or computed tomography (CT). In this review article, we summarize the different labelling strategies, contrast agents, and *in vivo* imaging modalities that can be used to monitor cells with phagocytic activity in the central nervous system using MRI and CT, with a focus on clinical applications. Metal-based nanoparticle contrast agents such as gadolinium, gold and iron are ideal candidates for these applications as they have favourable magnetic and/or radiopaque properties and can be fine-tuned for optimal uptake by phagocytic cells. However, they also come with downsides due to their potential toxicity, especially in the brain where they might accumulate. We therefore conclude our review by discussing the pitfalls, safety and potential for clinical translation of these metal-based neuroimaging techniques. Early results in patients with neuropathologies such as multiple sclerosis, stroke, trauma, cerebral aneurysm and glioblastoma are promising. If the challenges represented by safety issues are overcome, phagocytic cells imaging will be a very valuable tool for studying and understanding the inflammatory response and evaluating treatments that aim at mitigating this response in patients with neurological diseases.

1. Introduction

Phagocytic cells play a critical role in the development of the central nervous system (CNS) and brain homeostasis maintenance (Galloway et al., 2019). Resident macrophages (microglia, as well as perivascular, meningeal and choroid plexus macrophages) represent the main professional phagocytic cells in the CNS. In case of injury, they are joined by infiltrating immune cells: monocyte-derived macrophages and neutrophils. Non-professional phagocytic cells such as endothelial cells,

dendritic cells and astrocytes complement the CNS phagocytosis arsenal (Ge et al., 2022). Under pathological circumstances, phagocytic cells clean the brain of toxic substances, dying cells and debris, and are thus thought to exert beneficial effects. Microglial phagocytosis of apoptotic cells increases neurogenesis and may thus promote regeneration in neurodegenerative disease (Diaz-Aparicio et al., 2020). In turn, microglia can selectively eliminate synapses, a process that is beneficial in development but deleterious in many conditions: excessive phagocytosis of synapses is thought to contribute to neurodegeneration in diseases

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<https://doi.org/10.1016/j.jneumeth.2022.109729>

Received 10 June 2022; Received in revised form 27 September 2022; Accepted 17 October 2022

Available online 19 October 2022

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such as Alzheimer's disease and multiple sclerosis, while reduced phagocytosis of synapses contributes to neurodevelopment diseases such as Autism Spectrum Disorder (Taheri et al., 2022; Wang et al., 2021). Decreased microglial phagocytic activity also plays a role in the impaired clearance of beta-amyloid aggregates and thus in the progression of Alzheimer's disease (Hong et al., 2016). In acute injuries, inadequate phagocytosis (whether excessive or insufficient) induces secondary brain damages and compromises repair (Galloway et al., 2019). For instance, it has been shown that stressed but viable neurons may be eaten alive by activated microglia at the acute stage of ischemic stroke (a process coined 'phagoptosis') (Brown and Neher, 2012), and that therapeutic intervention aiming at mitigating phagocytosis was able to salvage viable neurons from premature death in this context (Zhang et al., 2020; Yang et al., 2021). Targeting and/or manipulating phagocytic cells therefore appears as a novel and promising therapeutic strategy that may have applications in a range of neurological diseases, such as multiple sclerosis (Jansen et al., 2022), Alzheimer's disease (Machhi et al., 2022), glioblastoma (Zhai et al., 2021), brain trauma (Krukowski et al., 2021) and ischemic stroke (Yang et al., 2021).

Imaging phagocytic cells in their 3-dimensional environment over time is crucial to better understand their role in disease pathogenesis and to monitor their potential therapeutic effects. Due to their phagocytic properties, these cells are easy to label with a contrast agent in order to be tracked by *in vivo* imaging. In this review article, we will summarize the different labelling strategies, contrast agents, and *in vivo* imaging modalities that can be used to monitor cells with phagocytic activity in the CNS and introduce quantification tools for image analysis, with a focus on clinical applications. In a translational perspective, we will consider radiological imaging modalities only, *i.e.*, magnetic resonance imaging (MRI) and computed tomography (CT) techniques. We also introduce the novel imaging modalities that are promising for clinical cell tracking: magnetic particles imaging (MPI) and spectral

photon-counting CT (SPCCT). Nanoparticle contrast agents based on metals such as gadolinium, gold and iron are ideal candidates for these applications as they possess favourable magnetic and/or radiopaque properties and can be fine-tuned for optimal uptake by phagocytic cells. However, they also have drawbacks due to their potential toxicity, especially in the brain where they can accumulate. We will therefore conclude our review by discussing the pitfalls, safety and clinical translation potential of these metal-based neuroimaging techniques. Of note, non-metal-based MRI or CT contrast agents such as fluorine-19 and iodine are beyond the scope of this review. We also excluded nuclear medicine tracers such as Technetium-99 that are used for cell labelling (Dudhia et al., 2015) because they do not allow long term (>week) cell tracking.

2. Methods for labelling phagocytic cells

There are two main strategies to label phagocytic cells: the first one is to inject the contrast agent intravenously to label endogenous cells *in vivo* and the second one is to label exogenous phagocytic cells *ex vivo* and re-inject them *in vivo* (Fig. 1).

2.1. *In vivo* labelling of endogenous phagocytic cells

In vivo labelling is based on the intravenous injection of a contrast agent that will be taken up by phagocytic cells *in vivo*. Cells that have engulfed the contrast agent become visible with the imaging technique of interest and may thus be tracked over time. This approach is usually used as an *in vivo* biomarker of (neuro)inflammation. Table 1 summarizes clinical applications of this technique in the neurological field. For information about the contrast agents used in these studies, please see Table 2. In addition, there are currently two on-going clinical trials that make use of iron oxide nanoparticles for evaluating phagocytic cell

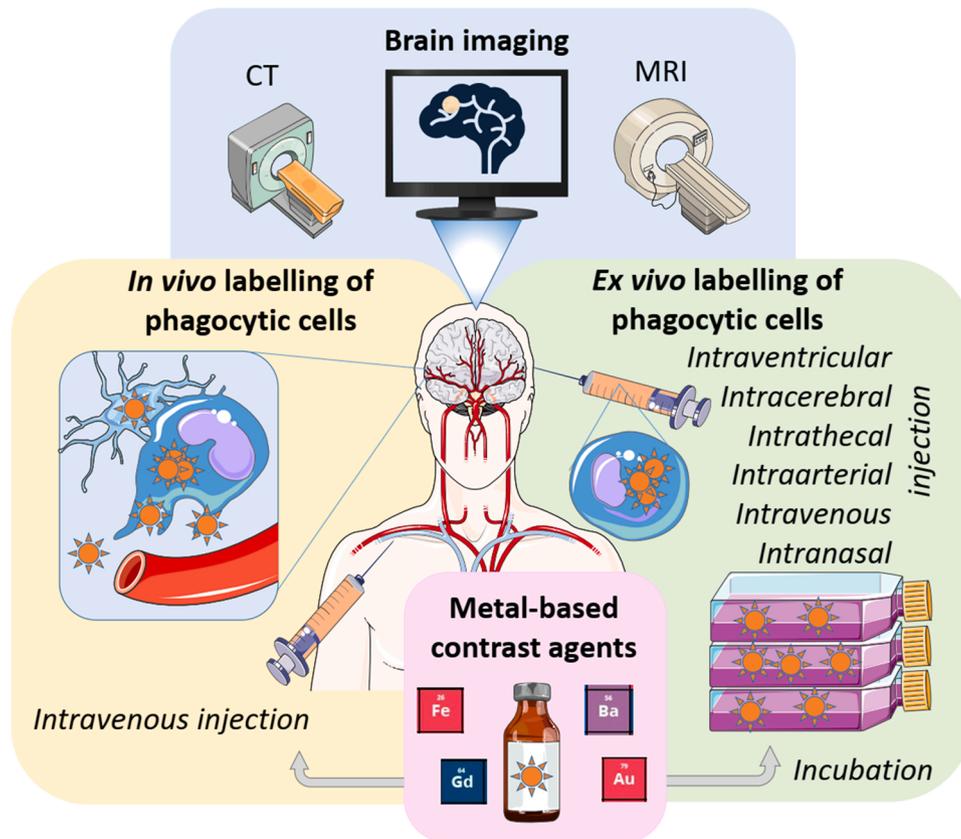


Fig. 1. Principle of MR and CT imaging of phagocytic cell labelled with metal-based contrast agents. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>).

Table 1
In vivo labelling of endogenous phagocytic cells: clinical applications in CNS.

Neurological disease	Modality	Metal-based contrast agent	Administration route	Imaging time points	Number of patients	References
Multiple sclerosis	MRI	Ferumoxtran-10	Intravenous	Before administration and 24–48 h after	24, 19, 10	(Tourdias et al., 2012; Vellinga et al., 2008; Dousset et al., 2006)
CNS inflammatory diseases & CNS lymphoma	MRI	Ferumoxtran-10 Ferumoxytol	Intravenous	Before administration and 24 h after	20	(Farrell et al., 2013)
Stroke	MRI	Ferumoxtran-10	Intravenous	Before administration and 24–72 h after, up to 11 days after in [23]	10, 5, 10, 12	(Nighoghossian et al., 2007; Cho et al., 2007; Saleh et al., 2004, 2007)
Cerebral aneurysm	MRI	Ferumoxytol	Intravenous	Before and immediately after administration, 24–72 h after	22, 5, 11	(Hasan et al., 2012a, 2013a, 2013b)
Arteriovenous malformation	MRI	Ferumoxytol	Intravenous	Before and either 1 or 5 days after	4	(Hasan et al., 2012b)
Migraine	MRI	Ferumoxytol	Intravenous	Before and 27 h after	28	(Khan et al., 2019)
Brain tumours	MRI	Ferumoxtran-10 Ferumoxytol	Intravenous	Before and 27 h after, up to 12 days after in [30]	10, 26, 7	(Iv et al., 2019; Dosa et al., 2011; Neuwelt et al., 2004)

Table 2

Physicochemical properties of metal-based contrast agents that have been used in patients for imaging phagocytic cells in the brain. SPIO: superparamagnetic particles of iron oxide. USPIO: ultrasmall superparamagnetic particles of iron oxide.

Metal-based contrast agent	Modality	Contrast agent type	Hydrodynamic diameter (nm)	Coating	Zeta potential (in water at pH7, in mV)	Remarks
Ferumoxide (Endorem, Guerbet or Feridex, Advanced Magnetics)	MRI	SPIO	80–150	Dextran	– 15 ± 0 (Berger et al., 2006)	Removed from the market
Ferumoxtran-10 (Sinerem, Guerbet or Combidex, Advanced Magnetics)	MRI	USPIO	20–40	Dextran	– 19 ± 1 (Berger et al., 2006)	Removed from the market, recently re-introduced as Ferrotran® by SPL Medical
Ferumoxytol (AMAG pharmaceuticals)	MRI	USPIO	27–33	Polyglucose sorbitol carboxymethylether	– 24 ± 9 (Bryant et al., 2017)	Approved for anemia therapy, off-label use in radiology

activation with MRI (<https://clinicaltrials.gov/>: NCT05357833 and NCT04925557). These two trials are being conducted in patients with multiple sclerosis. Fig. 2 shows examples of brain MR imaging after intravenous administration of iron oxides in patients with multiple sclerosis (Dousset et al., 2006), ischemic stroke (Nighoghossian et al., 2007) and glioblastoma (Iv et al., 2019).

Initially, it was thought that circulating monocytes and neutrophils were primary labelled and that this imaging approach specifically imaged infiltrating immune cells (Dousset et al., 1999; Rausch et al., 2001). That idea is still widespread, although it has been challenged by several teams, who have expressed concerns, for example, that some agents will be taken up in already resident cells, therefore the observed signal is not specific for infiltrating cells (Farr et al., 2011; Brisset et al., 2017; Desestret et al., 2009). We have recently performed a comprehensive study to address this issue using MRI back-to-back with two-photon microscopy in CX3CR1/GFP+ mice subjected to permanent ischemic stroke (Hubert et al., 2021). NanoGd, a hybrid multimodal imaging probe specifically designed to be internalized by phagocytic cells *in vivo*, was administered intravenously 24 h after ischemic stroke. Immediately and 24 h after injection, two-photon microscopy revealed that both circulating and resident phagocytic cells were labelled by NanoGd. The proportion of labelled cells depended on the brain regions considered, with an ascending gradient from extralesional to lesional areas. In the core of the lesion, where phagocytic activity is the highest in this model (Zarruk et al., 2018), more than 80 % of parenchymal CX3CR1-GFP/+ cells and around 60 % of circulating CX3CR1-GFP/+ cells had engulfed NanoGd at these early stages (the chemokine receptor CX3CR1 is expressed both in microglia and peripheral myeloid cells). Highly mobile NanoGd-laden CX3CR1-GFP/- cells were also observed, most probably corresponding to neutrophils. The same ascending gradient was seen for the presence of free NanoGd in the parenchyma. This suggested that at least some of the contrast agent had been transported through the blood brain barrier (BBB) where it had been taken up by resident activated microglia (as identified by

their typical morphophenotype). Therefore, this approach provides information about phagocytic cells activation, regardless of their type or origin. Of note, only those regions with intense phagocytic activity (*i.e.* lesion core) were visualized on MRI. We will further discuss this question in the section ‘Imaging of phagocytic cells *in vivo*’.

2.2. *Ex vivo* labelling of exogenous cells with phagocytic properties

To overcome the problem of contrast agent delivery to target regions, cells may be labelled *ex vivo* prior to their *in vivo* administration. This approach is usually used for two distinct aims: (i) to study the role of a given cell type in the physiopathology of the disease of interest and (ii) to monitor malignant cells (tumor cells) or therapeutic cells (stem cells). Table 3 summarizes clinical applications of this technique in the neurological field. For information about the contrast agents used in these studies, please see Table 2. Fig. 3 shows examples of brain MR imaging after intracerebral and intrathecal administration of stem cells labelled with iron oxides in patients with trauma (Zhu et al., 2006) and global cerebral ischemia (Janowski et al., 2014).

Regarding the type of cells that could be labelled, the advantage of professional phagocytes such as macrophages is the fact that they can easily incorporate high payloads of contrast agents by simple incubation with the contrast agent. But a number of cells, including progenitor and stem cells, also exhibit phagocytic properties and are thus amenable to labelling this way. Practical aspects of *ex vivo* cell labelling have been described elsewhere and are not detailed here (Kolonsjaj-Tabi et al., 2013). The main difficulty with this approach is to determine the optimal administration route for labelled cells to reach their target in large enough quantities to be visualized. Indeed, the intravenous route results in an important loss of cells at first pass through the lungs, liver and spleen. Although this might not be a problem for them to act as therapeutic cells (because they can have peripheral effects), this represents a major limitation for imaging purposes, as MRI and especially CT have relatively low sensitivities and thus require high accumulation of

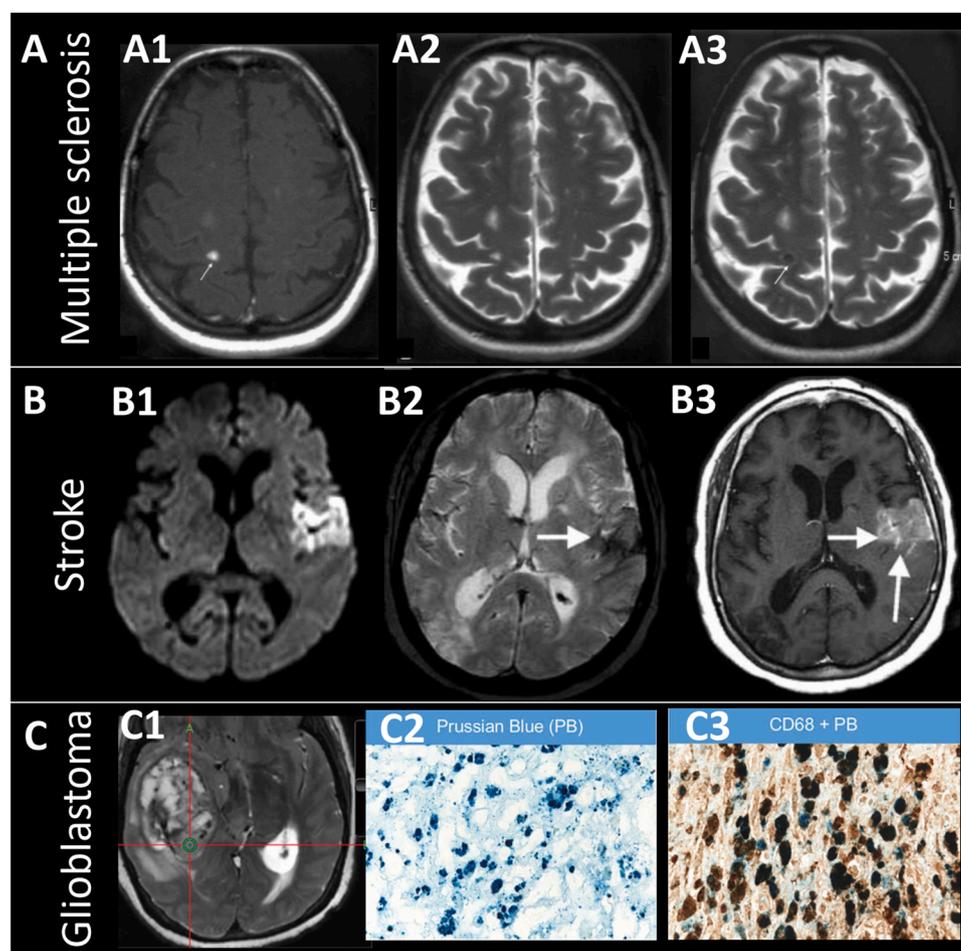


Fig. 2. Clinical applications of in vivo labeling of endogenous phagocytic cells in neurological diseases. (A) MRIs of a patient with MS: (A1) T1-weighted MRI shows a lesion enhancement after small gadolinium chelate administration (arrow); (A2) T2-weighted MRI without contrast agent; (A3) T2-weighted MRI, 24h after administration of Ferumoxytol, shows a markedly hypointense area (arrow); (B) MRIs of a patient with ischemic stroke: (B1) Diffusion-weighted imaging without contrast shows the ischemic lesion; (B2) T2*-weighted MRI 72h after administration of Ferumoxtran-10 shows a markedly hypointense area (arrow); (B3) Corresponding T1-weighted imaging shows a T1 effect of USPIOs; (C) MRIs and histology of a patient with a glioblastoma: (C1) T2-weighted MRI 24h after administration of Ferumoxytol shows hypointense signals within the tumor (green circle) that was sampled at surgery; (C2) Staining of biopsy using Prussian Blue (PB) shows iron particles (blue particles); (C3) Dual staining with PB and phagocytic marker CD68 show colocalization of the iron particles (blue particles) and CD68-positive macrophages (brown cells). (A) Adapted from (Doussset et al., 2006). (B) Adapted from (Nighoghossian et al., 2007). (C) Adapted from (Iv et al., 2019).

Table 3
Ex vivo labelling of endogenous phagocytic cells: clinical applications in CNS.

Neurological disease	Modality	Metal-based contrast agent	Cell type	Administration route	Imaging time points	Number of patients	References
Traumatic brain injury	MRI	Feridex	Neural stem cells	Intracerebral	Before transplantation, 1 day after, then every 7 days for 10 weeks	2 (1 with labelled cells and one with unlabelled cells)	(Zhu et al., 2006)
Global cerebral ischemia	MRI	Ferumoxtran-10	Umbilical stem cells	Intrathecal	Before transplantation, 1 week, 1 month, 2 months and 4 months after	1	(Janowski et al., 2014)
Multiple sclerosis (MS) & Amyotrophic lateral sclerosis (ALS)	MRI	Feridex	Mesenchymal stem cells	Intracerebral, intrathecal & intravenous	4–48 h post-administration then 1 month and 3–6 months	15 MS 19 ALS Incl. 9 with labelled cells	(Karussis et al., 2010)

contrast agents for detection. The intraarterial route is an alternative option to the intravenous one, but it is not suitable for every pathology (Grudzenski et al., 2017). In acute ischemic stroke for instance, we have shown that bone-marrow derived macrophages labelled with iron oxides and delivered through the ipsilateral carotid artery induced brain lesions, probably due to their embolization in the activated (sticky) microvasculature (Riou et al., 2013). Despite their invasiveness, the intracerebral and intrathecal/intraventricular routes are now emerging as viable options for stem cells delivery in clinical trials (Chiu et al., 2021; Muir et al., 2020; Steinberg et al., 2016; Portnow et al., 2017). These administration routes are particularly well suited for imaging as they maximize the number of labelled cells in the targeted area. On the other hand, pilot clinical trials using intranasal administration have shown to be safe and potentially efficient to deliver drugs to the brain in

patients with early Alzheimer's disease (Craft et al., 2012; Tuszyński et al., 2015). This administration route has been successfully tested in animal models of neurological diseases for delivering both nanocarriers and stem cells (Crowe and Hsu, 2022) and thus represents a promising option for the delivery and imaging of phagocytic cells (Perets et al., 2019).

3. Metal-based contrast agents that can be used to label phagocytic cells

The choice of the contrast agent obviously depends on the imaging technique of interest and on the strategy chosen to label phagocytic cells. Physicochemical properties of the contrast agent are paramount for both internalization efficiency and *in vivo* pharmacokinetics.

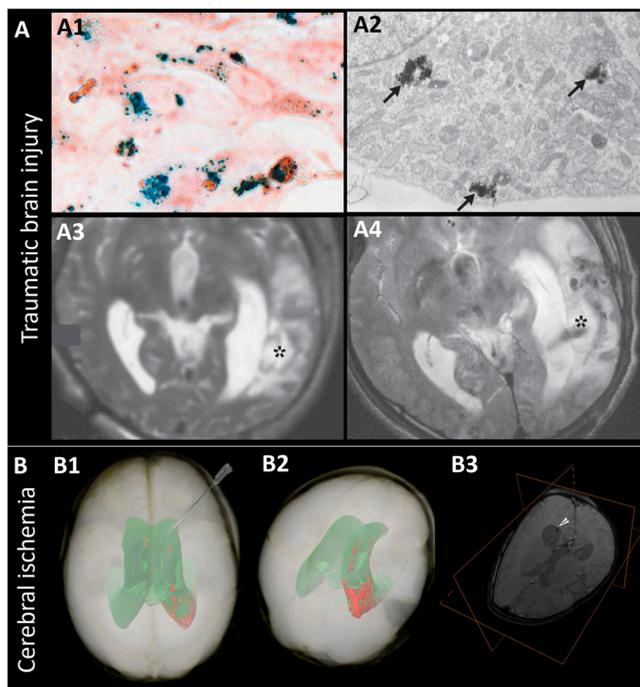


Fig. 3. Clinical applications of *ex vivo* labelling of phagocytic cells in neurological diseases. (A) Data of a patient with traumatic brain injury: (A1) Prussian Blue staining of neural cells labelled with iron oxide nanoparticles (blue particles); (A2) Corresponding Transmission Electron Microscopy (TEM) shows the presence of iron oxides as black inclusions (arrow); (A3) T2-weighted MRI without contrast shows the brain lesion resulting from trauma (asterisk); (A4) T2-weighted MRI 24 h after cell transplantation shows hypointense signals at the injection site. (B) MRIs of a patient with global cerebral ischemia: (B1) Volume rendering of MRI data of the patient's head obtained 24 h post-transplantation showing the ventricular system (green) and the distribution of the iron oxides signal from the transplanted cells (red); (A2) Postero-superior view of the patient's head; (A3) T2*-weighted MRI with an orthogonal view centered on the cellular signal (white arrowhead)
(a) Adapted from (Zhu et al., 2006). (b) Adapted from (Janowski et al., 2014).

Nanoparticles are particularly suited for the purpose of cell labelling as they can be fine-tuned to optimize physicochemical features (Hsu et al., 2020). Additionally, contrast agents should be biocompatible in order not to affect the viability or functionality of cells (Betzer et al., 2015).

3.1. Specifications for contrast agents to get efficiently engulfed by cells

The efficacy through which contrast agents are taken up by phagocytic cells mainly depends on their size, shape, surface charge (zeta potential), and coating material (Hsu et al., 2020). The role that these factors play depends on the cell type in question and complex relationships have been observed between the size and coating of nanoparticles and their uptake by phagocytic cells (Chhour et al., 2017). Some coatings such as dextran or poly(ethylene) glycol (PEG) render contrast agents stealthier upon their intravenous administration. Table 2 summarizes the physicochemical properties of metal-based contrast agents that have been used in clinical studies aiming at imaging phagocytic cells in the neurological field.

In case of intravenous administration, there are several issues to consider. First, the fact that the nanoparticle will get opsonized and that its ability to be phagocytosed efficiently will likely be modified compared to *in vitro* conditions. Second, phagocytic cells from the reticuloendothelium system (RES) will be the first ones to take up the nanoparticles, resulting in their rapid and long-term sequestration in organs such as the liver, spleen, lungs, lymph nodes and bone marrow (with a biodistribution that depends on the contrast agent

physicochemical characteristics). Large doses of contrast agents are usually employed to prolong their blood half-life, as a longer vascular remanence increases the chances of the compound to get internalized by circulating monocytes and/or to accumulate in the target tissue, here the brain. For neuroimaging, transport of the contrast agent through intact BBB represents another hurdle to consider. A number of strategies have been proposed to deliver nanomedicine through the BBB (Shilo et al., 2014; Ayub and Wettig, 2022). For example, the physicochemical properties of metal nanoparticles themselves can be tuned to pass the BBB (Sintov et al., 2016), just as they can be tuned to enhance internalization by phagocytic cells, for example by designing small lipophilic nanoparticles. Furthermore, nanoparticles may be functionalized with ligands specific to receptors that are expressed in the BBB such as transferrin receptors (TfR) (Ramalho et al., 2022; Karatas et al., 2009) or large neutral amino acid transporter (LAT)-1 receptors (Gonzalez-Carter et al., 2019), to enhance transport across the BBB through receptor-mediated transcytosis. Exosomes and extravesicles loaded with cells and/or gold or iron oxides nanoparticles have also been shown to cross the BBB following their intranasal administration in animal models of neurological diseases such as stroke, autism, Parkinson's and Alzheimer's disease (Perets et al., 2019; Kutchy et al., 2022). Additionally, ultrasound coupled to the administration of microbubbles may be used to enhance permeability of the BBB (Barbato et al., 2022; Gandhi et al., 2022), a strategy that is already evaluated in clinical trials to optimize chemotherapy delivery (Carpentier et al., 2016). But it should also be noted that most brain pathologies, including the ones just mentioned, induce some kind of BBB dysfunction and endothelium activation that facilitates intra-parenchymal delivery of nanoparticles.

3.2. Available metal-based MRI contrast agents suited for phagocytic cell labelling

Conventional MR contrast agents based on chelates of gadolinium are not well suited for labelling phagocytic cells as they are not efficiently internalized by cells (Liu and Frank, 2009). Iron oxide particles represent the most widely used metal-based contrast agents for cell labelling (Chauveau et al., 2011). They can be classified into 3 categories: (i) superparamagnetic particles of iron oxides (SPIO) with a size > 50 nm; (ii) ultrasmall SPIO (USPIO) with a size < 50 nm and (iii) micron-sized particles of iron oxides (MPIO) with a size > 0.9- μ m. The SPIO family (Endorem®, Guerbet; Feridex®, Berlex; Resovist®, Shering) were initially used for the diagnosis of hepatic tumors. They are clinically approved but have been withdrawn from the market due to the improvement in MRI techniques that made them no longer needed by radiologists for liver imaging. The USPIO family comprises compounds that were initially dedicated to the imaging of lymph nodes (Ferumoxtran-10: Sinerem®, Guerbet; Combidex®, AMAG Pharmaceutical) or angiography (SHU555C, Bayer). Their commercial development was however interrupted years ago, despite potential benefit and acceptable safety profile, because of the difficulty to prove their diagnostics value in patients with cancer. One member of the USPIO family, Ferumoxtyl® (AMAG Pharmaceutical), became FDA-approved not as a contrast agent but as a therapeutic drug prescribed to treat anaemia in patients with kidney failure. Nowadays, Ferumoxtyl® is the only approved (U)SPIO that may be used as a superparamagnetic contrast agent, although 'off-label' (Toth et al., 2017; Huang et al., 2022). Finally, MPIOs are clusters of USPIOs dispersed in a polymer matrix. This has the advantage to enhance the MR signal. To date, no MPIO is yet biodegradable so they are not clinically approved and used for research applications only. However, the development of biocompatible MPIOs is an active field of research (Gauberti et al., 2018). The main application of MPIOs in the CNS is the assessment of endothelial activation thanks to functionalization targeted at adhesion molecules (Belliere et al., 2015), but they are also efficient for labelling cells *in vivo* (Foley et al., 2009) and *ex vivo* (Dallet et al., 2021).

Beside (U)SPIO, other types of contrast agents are noteworthy to

mention for their use as cell labelling compounds. First, Gadofluorine M (invivoContrast GmbH) has been used to label phagocytic cells in the CNS (Hubert et al., 2021; Wuerfel et al., 2010; Giesel et al., 2006). Gadofluorine M is a preclinical small molecular weight gadolinium-based contrast agent with high protein binding affinity that forms micelles in aqueous solution. Like USPIOs, Gadofluorine M is characterized by a prolonged blood half-life and is up taken by the RES, which, as we have seen, are favourable characteristics for the *in vivo* labelling of phagocytic cells. Manganese-based contrast agents are another class of compounds that may be used in pre-clinical studies, preferentially to label cells *ex vivo* (Gilad et al., 2008; Haedicke et al., 2019).

3.3. Available CT metal-based contrast agents suited for phagocytic cell labelling

Barium sulfate suspensions are clinically approved contrast agents that are used to improve visualization of the gastrointestinal tract on CT. These metal-based contrast agents appear suited for labelling cells (mouse macrophages and human epithelial cells) *ex vivo* (Krenkel et al., 2015; Shepherd et al., 2018), but, to the best of our knowledge, no neurological application has been reported to date.

A few nanoparticles have been developed for cell tracking with CT in the preclinical field. Barium is one of the metals of interest for designing CT nanoparticles for cell tracking (Arifin et al., 2012; Barnett et al., 2006), but gold nanoparticles appear as the most promising candidates for this application (Meir and Popovtzer, 2018; Meir et al., 2014). Indeed, gold is an inert metal with good biocompatibility profile and gold nanoparticles are easy to synthesize and customize. Moreover, gold nanoparticles are extremely dense, which represents an advantage for achieving the high payloads needed due to the low sensitivity of CT. On the other hand, gadolinium nanoparticles such as NanoGd, which is based on a gadolinium fluoride (GdF₃) core are metal-based contrast agents that may be used either with MRI or CT (Cuccione et al., 2020). Fig. 4 shows an example of brain MR imaging after intravenous NanoGd administration in a murine model of ischemic stroke and Fig. 5 shows an example of CT imaging after administration of macrophages labelled with 11-MUDA gold nanoparticles (Cuccione et al., 2020) embedded in a NanoGd-labelled scaffold in a rat model of focal cerebral injury (author's own unpublished data).

CT contrast agent development is an active field of research, due in part to the recent advent of photon counting CTs that now allow to image multiple elements simultaneously (see 'Imaging phagocytic cells labelled with metal-based contrast agents *in vivo*' section below). In addition, metal-based nanoparticles are currently being developed to enhance radiotherapy and phototherapy for tumour treatment. Some of them are already on the path to clinical approval like the gadolinium-based nanoparticle AguiX® (Muir et al., 2020) and the gold-based nanoparticle AuroShell® (Naha et al., 2015). As a consequence, a number of novel contrast agents based on different metals (gadolinium, gold, silver, ytterbium, tantalum etc) are emerging (Kim et al., 2018) and may prove useful for clinical cell tracking in the coming years.

4. Imaging phagocytic cells labelled with metal-based contrast agents *in vivo*

The choice of MRI or CT is mainly determined by the availability of the imaging modality and by their intrinsic characteristics: MRI is more sensitive than CT (0.01 mM vs 10 mM (Bernstein et al., 2016)), however, acquisition is faster and quantification is easier with CT. Because the exquisite soft tissue contrast and functional information provided by MRI is locally lost in the presence of an MR contrast agent, multi-parametric MRI evaluation of tissue may gain to be coupled with CT tracking of cells (using a contrast agent not seen on MRI), to combine the best of two worlds (Fig. 5).

One limitation of conventional MRI and CT, however, is the fact that

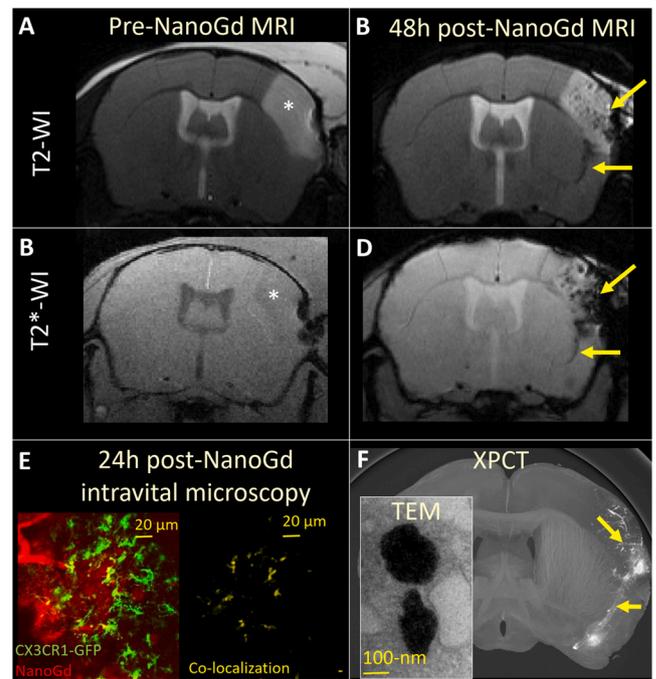


Fig. 4. Pre-clinical application of phagocytic cells imaging with MRI: original example in a mouse model of ischemic stroke. (A) T2-weighted MRI without contrast 24 h after permanent occlusion of the middle cerebral artery (pMCAO) shows the ischemic lesion (asterisk); (B) T2-weighted MRI, 48 h after administration of NanoGd, shows markedly hypointense areas in the lesion (arrows); (C) Corresponding T2* -weighted MRI without contrast; (D) Corresponding T2* -weighted MRI 48 h after administration of NanoGd shows markedly hypointense areas in the lesion with blooming effects compared to T2-weighted MRI (arrows); (E) Intravital microscopy in a CX3CR1/GFP mouse with pMCAO shows NanoGd (red) and CX3CR1-GFP+ cells (green) in the lesion, and their co-localization (yellow) demonstrates internalization of NanoGd by CX3CR1/GFP+ cells; (F) Post-mortem X-ray phase contrast computed tomography (XPCT) of the same mouse as in A-D shows NanoGd-induced hyperintense signals in the lesion (arrows) and Transmission Electron Microscopy (TEM) confirms NanoGd internalization in the lysosomes of brain macrophages (insert). Authors' own unpublished data. Experimental procedures involving animals and their care were carried out in accordance with the European regulations for animal use (EEC Council Directive 2010/63/EU, OJ L 276, Oct. 20, 2010) and the study was approved by the local review board ("Comité d'éthique pour l'Expérimentation Animale Neurosciences Lyon:" CELYNE; CNREEA no. 42; APAFIS agreement no. #4688).

the signal produced by cells labelled with a given metal-based contrast agent is not specific: it is thus impossible to distinguish the signal of labelled cells from similar endogenous (for example haemorrhages, skull or brain calcifications) or exogenous signals induced by another distinct metal-based contrast agent (for example small gadolinium chelates or iodine). A baseline scan is therefore needed to eliminate confounding signals. MPI and SPCCT represent novel imaging modalities that are emerging for cell tracking with the advantage of allowing a quantitative and specific detection of metal-based contrast agents, without the need for a baseline scan.

4.1. MRI and MPI

For MRI, depending on the contrast agent and strength of the magnetic field used, T1-weighted or T2/T2* -weighted imaging will be used to track labelled cells in the CNS (Fig. 2, Fig. 4). T2* -weighted imaging and related methods such as susceptibility-weighted imaging (SWI) (Janowski et al., 2014) and positive contrast techniques (Brisset et al., 2011) are the most sensitive ways to detect cells labelled with a super-paramagnetic agent, but at the cost of quantification and exact

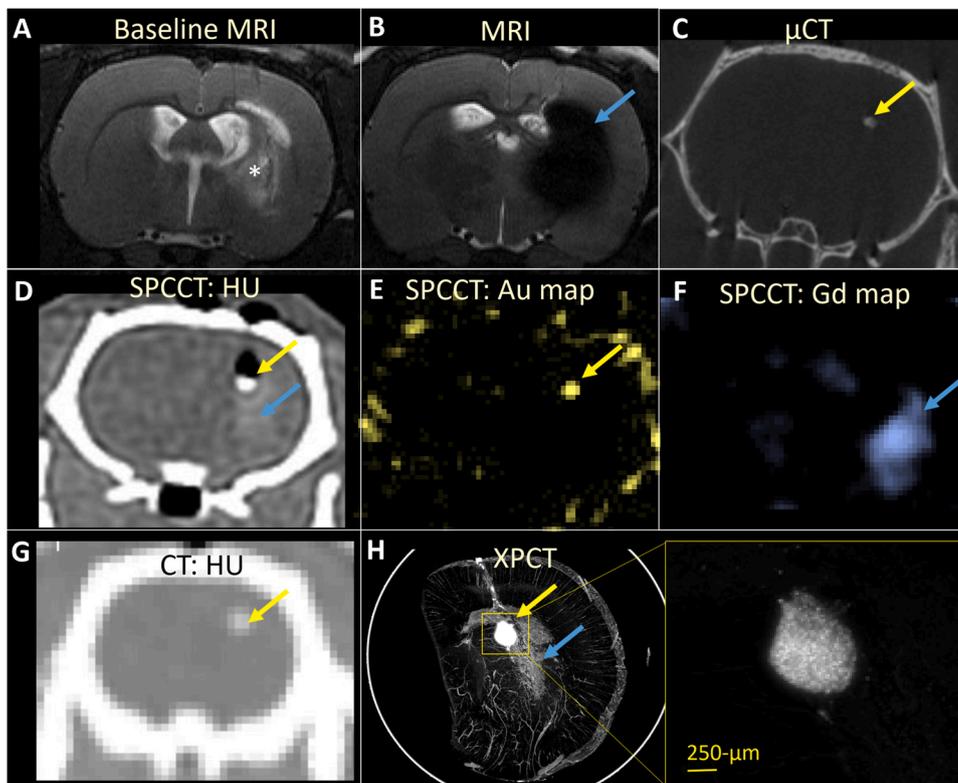


Fig. 5. Pre-clinical application of phagocytic cells imaging with CT: original example in a rat model of focal cerebral injury. (A) T2-weighted MRI without contrast shows the focal cerebral lesion (asterisk); (B) T2-weighted MRI immediately after the administration of gold (11-MUDA nanoparticles)-labelled macrophages embedded in gadolinium (GdF3)-labelled hydrogel shows the hypointense signal induced by gadolinium (arrow); (C) μ CT of the same rat shows hyperdensities, probably induced by gold-labelled cells (arrow); (D) SPCCT of the same rat. Conventional HU image shows hyperintense signals: a hot spot (yellow arrow) and more diffuse signal (blue arrow); Material decomposition images of SPCCT acquisition show that: (E) the hot spot corresponds to gold-labelled cells; and (F) the more diffuse signal corresponds to NanoGd-labelled hydrogel that have filled in the lesion; (G) Clinical CT of the same rat brain shows the detection of gold-labelled cells only (arrow); (H) Post-mortem XPCT of the ipsilateral hemisphere shows hyperintense signals corresponding to both gold and gadolinium (arrows), with a magnification on the hot spot. Authors' own unpublished data. Experimental procedures involving animals and their care were carried out in accordance with the European regulations for animal use (EEC Council Directive 2010/63/EU, OJ L 276, Oct. 20, 2010) and the study was approved by the local review board ("Comité d'éthique pour l'Expérimentation Animale Neurosciences Lyon:" CELYNE; CNREEA no. 42; APAFIS agreement no. #4688).

localization (due to the "blooming effect"), which is more difficult than with T1.

One of the limitations of the *in vivo* labelling approach is the fact that the signal may arise both from contrast agent accumulation in the brain parenchyma and from phagocytic cells. Different imaging strategies have been proposed to decipher those contributions, for instance by using MRI sequences that are more sensitive to the internalization of iron oxides into cells than to free iron oxide in the parenchyma (Yang et al., 2014; Brisset et al., 2009). Numerous studies, including clinical ones, have shown that brain areas showing enhancement with gadolinium chelates did not totally overlap with areas that show MR signals induced by the cell labelling contrast agent (Nighoghossian et al., 2007; Tourdias et al., 2012; Dosa et al., 2011; Dousset et al., 1999; Desestret et al., 2009; Wiart et al., 2007). This suggests that another phenomenon than BBB leakage is captured, at least 24 h post-injection and is therefore in favour of phagocytic cell imaging. Internalization of contrast agents into lysosomes results in a high payload of metals per cell. Once compartmentalized and clustered in cells, metal-based contrast agents are more likely to dominate the MR signal than the ones dispersed into the parenchyma, especially if MR sequences sensitive to susceptibility effects are used. We have confirmed this finding in the NanoGd study mentioned above and further showed that areas with strong signal voids on T2- and T2* -weighted imaging did correspond to regions with high phagocytic activities (Fig. 4) (Hubert et al., 2021).

The quantification of iron with T2* -weighted MRI is a difficult task because the relationship between MR signal and iron quantity is not linear (Ghassaban et al., 2018). For this reason, authors have used different strategies to produce quantitative results when tracking cells. One of them consists in segmenting regions of signal voids produced by superparamagnetic contrast agents on T2* -weighted images using various criteria (Riou et al., 2013). Another one is to quantify R2 relaxivity rather than R2* (Rad et al., 2007). We have shown that

segmentation of regions with $R2 > 35 \text{ ms}^{-1}$ correlated well with the quantity of iron (Marinescu et al., 2013a). Finally, a system of score based on both the intensity and extent of signal void represents a robust way to analyse images (Hubert et al., 2019; Di Cataldo et al., 2016), very much like what is done in clinical practice and thus probably easier to translate in clinical trials.

One way to address the issue of quantification, blooming effects and lack of specificity of MRI is to switch to MPI. MPI is a (relatively) new imaging modality that allows the quantitative and specific detection of iron oxides, with $0.1 \mu\text{M}$ sensitivity (Harvell-Smith et al., 2022). The first MPI cell tracking studies monitored stem cells labelled with SPIO transplanted in the brain of healthy animals (Bulte et al., 2015; Zheng et al., 2015). Since then, it has been applied in rodent models of brain pathologies such as cancer (Melo et al., 2022; Rivera-Rodriguez et al., 2021). Because MPI does not provide information about anatomy, it is usually combined with MRI and CT, with hybrid scanners becoming available for pre-clinical research. Human-sized MPI scanners are also emerging, with the monitoring of immunotherapy as a primary clinical application (Graeser et al., 2019). Interestingly, MPI can take advantage of iron oxides with different harmonics response behaviour to perform multicolour imaging and can discriminate free from compartmentalized iron oxides (Harvell-Smith et al., 2022). Therefore, MPI appears as a highly promising technology for future clinical applications of cell tracking in the CNS (Sehl et al., 2020).

4.2. CT and SPCCT

Two parameters may be tuned for image acquisition with clinical CT and bench μ CT: the current and the X-ray tube voltage. The current value has minimum impact on contrast agent attenuation while the tube voltage, by modifying the X-ray spectrum, has a significant effect on the attenuation induced by a given contrast agent, depending on the atomic

number of the element and its K-edge (Bernstein et al., 2016). For instance, gold nanoparticles are better seen with tube voltage > 80 kVp (Bernstein et al., 2016). Alternatively, synchrotron X-rays have been used for the μ CT tracking of cells labelled with gold nanoparticles (Astolfo et al., 2013). Detectability of metal-based contrast agents (and thus labelled cells) may be improved by optimizing reconstruction methods (Bernstein et al., 2016). Quantification is straightforward, as the signal is directly proportional to metal quantity.

The next CT generation, namely dual-energy CT and spectral photon counting CT (SPCCT), have the potential to generate concentration maps of given elements. Just like MPI, cell tracking is emerging as an application of these techniques, which have the advantage of being both specific and quantitative (Cuccione et al., 2020). Furthermore, SPCCT allows to discriminate several distinct elements, thus enabling to perform multicolour imaging for the first time with CT. This has been used to simultaneously track M2-polarized macrophages labelled with gold nanoparticles and their embedding scaffold labelled with iodine nanoparticles in a rat model of focal brain injury (Cuccione et al., 2020). Fig. 5 show an example of brain SPCCT imaging in this context. *In vivo* labelling of phagocytic cells by intravenous administration of gold nanoparticles was recently performed in a rabbit model of atherosclerotic plaque: imaging with SPCCT allowed to discriminate gold-labelled macrophages, calcifications and iodine-enhanced vessel lumen (Si-Mohamed et al., 2021). This paves the way for future works in the neurological field. Quantification is here based on materials decomposition and hence necessitates post-processing of the data.

Validation of these approaches is difficult because they are performed *in vivo* in 3-dimensions while methods of reference such as ICP-OES and immunohistology are performed *ex vivo* and are either destructive or necessitate to slice and stain the brain tissue. To address this issue, we have recently proposed to use *in vivo* high resolution synchrotron K-edge subtraction CT (SKES-CT) as a method of reference (Tavakoli et al., 2021). In addition, in-line phase contrast CT with synchrotron x-ray (XPCT) may complement *in vivo* analysis as a 3-dimensional virtual histology tool that allows to visualize cells labelled with metal-based contrast agents (Albers et al., 2018; Chourout et al., 2022; Marinescu et al., 2013b). Fig. 4 F and 5 H show examples of XPCT in rodent models of brain injury in the context of cell tracking (author's own unpublished data).

Altogether, these innovative imaging tools based on X-rays seem very promising for the tracking of phagocytic cells and should expand in the future with new neurological applications.

5. Pitfalls, safety and clinical translation

Imaging phagocytic cells is a powerful tool to get information about biological processes *in vivo*. However, there are a number of limitations that end-users of these approaches should be aware of in order to avoid interpretation errors. Regarding safety, two issues should be considered: the safety of the contrast agent to cells (whether they induce mortality, activation and hamper or modify functionality) and the safety of the contrast agent to patients (whether they induce side effects or do not get eliminated) (Helfer et al., 2021).

5.1. Which cells are being imaged? Are they alive? Are they altered by labelling?

In case of brain injury, macrophages represent the most abundant activated phagocytic cells and therefore the methods that relies on *in vivo* labelling are mostly referred to as the imaging of macrophages. In rodent models of neurological diseases (ischemic stroke, multiple sclerosis, Alzheimer's disease), the different kind of resident macrophages (microglia, perivascular and plexus choroid macrophages), as well intravascular monocytes, have all been shown to contribute to image contrast (Desestret et al., 2009; Hubert et al., 2021; Klohs et al., 2016). However, other cell types may also contribute, for example neutrophils

(Hubert et al., 2021; Kirschbaum et al., 2016), endothelial cells (Halama KENZAOUI et al., 2013; Machtoub, 2012) and astrocytes (Neuwelt et al., 2004; Kutchy et al., 2022).

One of the limitations of these imaging approaches is the fact that they do not provide any information about cell viability. When cells are labelled *ex vivo*, they may die and get ingested by endogenous macrophages, which also raises the question of which cell type is imaged. Another concern is the potential cytotoxicity of metal-based contrast agents themselves. Several mechanisms have been described *in vitro* in relation to the cytotoxicity of iron oxides such as the generation of reactive oxygen species (ROS), mitochondrial dysfunction and induction of apoptosis and ferroptosis (Singh et al., 2010; Kim et al., 2016). Coating may be responsible in part for this toxicity as shown in some gold nanoparticles (Naha et al., 2015). Another effect that may be induced by metal-based contrast agent is the alteration of inflammatory response. Some authors reported that iron oxides shifted macrophages towards an anti-inflammatory phenotype *in vitro* (Siglienti et al., 2006) while others have shown that they favoured a pro-inflammatory shift (Zanganeh et al., 2016). This discrepancy may be explained by the differences in nanoparticle types and dosage (Wei et al., 2021). For a given neurological application in the pre-clinical setting, thorough cytotoxicity studies should be conducted *in vitro*. In addition, MRI and CT approaches should be coupled with other imaging modalities such as bioluminescence imaging to get an idea about how long labelled cells stay alive in the brain microenvironment and immunohistochemistry to determine which cells represent the signal substrate.

5.2. Is clinical translation feasible?

Clinical translation has already been achieved using MRI coupled to USPIOs intravenous injection in a variety of neurological diseases (Table 1). In addition, 3 clinical studies have already reported the transplantation of stem cells labelled *ex vivo* with (U)SPIO into the CNS (Table 3). This demonstrates the translation potential of both approaches in the neurological field.

There are some constraints to the use of these techniques in the clinical setting. For *in vivo* labelling, contrast agent delivery to the brain parenchyma and cell labelling is favoured by a long vascular remanence, therefore a delay between injection and imaging is recommended to minimize the signal in the vessels. This delay is usually comprised between 24 h and 72 h post-administration and represents one of the hurdles for widespread clinical use, as it complicates care organization, especially for outpatients. This is also one of the reasons why MPI or spectral CT techniques that do not necessitate a baseline scan are attractive options: beside reducing costs both X-ray dose (for SPCCT), they also improve care flow. The next generation of dual energy CT scanners and SPCCT are performed at much lower dose than conventional CT so they are more adapted to longitudinal imaging if cell tracking is needed on the long term. Other obstacles are represented by the 'off-label' use of clinically-approved iron oxide nanoparticles, the difficulty of signal interpretation, and the toxic challenge represented by metal accumulation into the brain (Rasschaert et al., 2020).

5.3. What about metal accumulation in the brain?

In clinical routine, about 50 % of CT scans and 40 % MRI scans use iodine- or gadolinium-based contrast agents, respectively, to improve image contrast and thus diagnostics, and this figure raises to 60 % for neurological exams (Wahsner et al., 2019). These contrast agents benefit from the experience and knowledge gained during > 30 years of clinical use. Clinical evidence shows they are safe in most patients, although the safety record of gadolinium-based contrast agents has been challenged with the finding of nephrogenic systemic fibrosis (NSF) in patients with reduced kidney function and, more recently, concerns about brain deposition (Rasschaert et al., 2020). There is an active on-going debate about the clinical and biological significance of retained gadolinium in

the brain, if any (Bruno and Veale, 2021). In turn, iron oxide contrast agents are attractive alternative to gadolinium chelates because they are not associated with a risk of NSF and because humans have endogenous iron recycling machinery. Preclinical data indicate that intravenous Ferumoxytol® doses of 5–10 mg Fe/kg (corresponding to typical clinical doses) do not lead to iron deposition in the brain of pigs (Theeruvath et al., 2020). However, patients that received multiple iron administrations may exhibit signal voids in the choroid plexus lasting up to several weeks (Daldrup-Link, 2017). In addition, Ferumoxytol® has been associated with ferroptosis-related cell death in tumours and thus proposed as a theranostics agent (Kim et al., 2016; Modo et al., 2013). These side effects should thus be thoroughly investigated prior to use in neurological diseases.

In summary, metal accumulation is a sensitive issue in the field of brain imaging regardless of the metal considered. Further studies are obviously warranted to clarify these issues. It is noteworthy that the elimination pathways of nanoparticles may differ from that of small molecular weight contrast agents: for instance, it has been shown that macrophages were able to scavenge gold nanoparticles and eliminate them through exosomes (Logozzi et al., 2019). Long-term studies of metal-based contrast agent fate in the CNS should inform us about the safety profile of these neuroimaging techniques.

6. Conclusions and future perspectives

The activation of phagocytic cells is a hallmark of many neurological diseases, such as Alzheimer's disease, multiple sclerosis, acute ischemic stroke or glioblastoma. These cells can internalize metal-based contrast agents and may thus be tracked by MRI or CT. In addition, cells that are currently being evaluated for regenerative medicine therapy in many neurological pathologies, such as M2-polarized macrophages (Chernykh et al., 2016) or adipose-derived stem cells (Chiu et al., 2021), have phagocytic properties and may serve as theranostics tools once labelled with a metal-based contrast agent.

Numerous different preclinical applications have been explored with these imaging techniques and clinical applications are still being developed in neurology. One of the limitations of these approaches is the fact that they do not allow to define which cell type is being imaged. In the future, targeting of specific cells could be achieved through the development of functionalized contrast agents. For example, the integrin CD11b is involved in the phagocytosis of apoptotic cells and CD11b-targeted SPIOs have been designed with the aim of improving macrophage detection in atherosclerotic plaques (von zur Muhlen et al., 2010). However, this strategy was not very successful because non-targeted SPIO were also internalized (von zur Muhlen et al., 2010). Hence there is still a need for the development of smart metal-based contrast agents that would help decipher the contributions of the different phagocytic cells in pre-clinical studies. Another limitation is the fact that the term “imaging of phagocytic cells” may be misleading, for two main reasons. First, the process by which metal-based contrast agents are internalized may be different from that of actual phagocytosis (*i.e.* pinocytosis or endocytosis). Second, phagocytosis is a function that is not restrained to engulfment but includes ‘find-me’ and ‘digest-me’ steps, which are not captured by the imaging methods here described. As a consequence, the value of these imaging techniques as biomarkers of phagocytosis remains to be demonstrated. They should rather be seen as the *in vivo* equivalent of *in vitro* ‘phagocytosis assays’ based on the engulfment of fluorescent beads for example. In the future, the development of imaging methods that can provide insight into the mechanistic process of efferocytosis (*i.e.* the phagocytosis of apoptotic cells) would be very valuable. Finally, the main difficulty for widespread clinical translation is the fact that to date, no metal-based contrast agent is clinically approved for cell tracking *per se*. However, if the challenges represented by the issues mentioned above are taken up, safety issues in particular, these cellular imaging approaches will be a most valuable tool to start studying and understanding the phagocytic response to

many neurological diseases and to evaluate treatments that aim at mitigating this response in patients.

CRedit authorship contribution statement

Marlène Wiart: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Clément Tavakoli:** Data curation, Investigation, Resources, Software, Visualization, Writing – review & editing. **Violaine Hubert:** Data curation, Investigation, Resources, Visualization, Writing – review & editing. **Inès Hristovska:** Data curation, Investigation, Resources, Writing – review & editing. **Chloé Dumot:** Data curation, Investigation, Resources, Writing – review & editing. **Stéphane Parola:** Investigation, Writing – review & editing. **Frédéric Lerouge:** Investigation, Writing – review & editing. **Fabien Chauveau:** Investigation, Resources, Validation, Writing – review & editing. **Emmanuelle Canet-Soulas:** Funding acquisition, Validation, Writing – review & editing. **Olivier Pascual:** Data curation, Funding acquisition, Investigation, Validation, Writing – review & editing. **David P. Cormode:** Investigation, Writing – review & editing. **Emmanuel Brun:** Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing. **Hélène Elleaume:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflict of interests to declare.

Data Availability

No data was used for the research described in the article.

Acknowledgements

This review was written in the framework of the French National Research Agency (ANR) grants NanoBrain (ANR-15-CE18-0026-01) and Breakthru (ANR18-CE19-0003) of the EU's H2020 Research and Innovation Program under the grant agreement no. 633937. The authors thank ESRF for allocation of beamtime (MD1094).

We thank Radu Bolbos and Jean-Baptiste Langlois at Lyon's multi-modal imaging platform Cernep (MRI and SPCCT), Marc Vandamme and Emmanuel Chereul at VOXCAN (μ CT and CT) and Herwig Requardt (XPCT) for acquisition of the original data sets presented in Fig. 4 and Fig. 5. The authors also thank Amanda Sierra from Achucarro Basque Center for Neuroscience for fruitful discussions about the topic of this review.

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