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Review

In vivo imaging biomarkers of neuroinflammation in the development and assessment of stroke therapies – towards clinical translation

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Abstract

Modulation of the inflammatory microenvironment after stroke opens a new avenue for the development of novel neurorestorative therapies in stroke. Understanding the spatio-temporal profile of (neuro-)inflammatory imaging biomarkers in detail thereby represents a crucial factor in the development and application of immunomodulatory therapies. The early integration of quantitative molecular imaging biomarkers in stroke drug development may provide key information about (i) early diagnosis and follow-up, (ii) spatio-temporal drug-target engagement (pharmacodynamic biomarker), (iii) differentiation of responders and non-responders in the patient cohort (inclusion/exclusion criteria; predictive biomarkers), and (iv) the mechanism of action. The use of targeted imaging biomarkers for may thus allow clinicians to decipher the profile of patient-specific inflammatory activity and the development of patient-tailored strategies for immunomodulatory and neuro-restorative therapies in stroke. Here, we highlight the recent developments in preclinical and clinical molecular imaging biomarkers of neuroinflammation (endothelial markers, microglia, MMPs, cell labeling, future developments) in stroke and outline how imaging biomarkers can be used in overcoming current translational roadblocks and attrition in order to advance new immunomodulatory compounds within the clinical pipeline.

Key words: Neuroinflammation; Stroke; Imaging; Microglia; TSPO; Matrix Metalloproteinases; Brain-gut axis

Introduction

Cerebral ischemia is one of the major causes of death and disability world-wide due to an overall increase in stroke burden. Ischemic stroke represents the most prevalent form of stroke [1, 2]. Currently available treatment options are limited to tissue plasminogen activator (tPA) and endovascular treatment after stroke solely aiming at recanalization

of an occluded vessel [3–5]. Over a thousand neuroprotective candidates, targeting different parameters of the pathophysiological cascade after stroke, have been suggested by preclinical research [3]. However, there is a remarkable gap between the huge number of candidates and their clinically effective translation to stroke patients. The failure of

most clinical trials is partially due to the complexity of molecular alterations and their kinetics with regards to the variety of cell types and molecules and their respective functions at different stages after cerebral ischemia [6, 7]. Together with the enormous average costs of clinical development programs of up to 1.4 billion dollars [8], there is a need for a refinement of preclinical and clinical stroke research [9]. Apart from improving quality of research, data acquisition and collaborations, novel non-invasive molecular imaging biomarkers and techniques may help to overcome the translational roadblock in stroke research and to facilitate drug development processes [10].

As current recanalization treatment options are limited to the first couple of hours after stroke, there is an urgent need for molecular imaging techniques employing other markers besides those targeting cerebral blood flow, oxygen and glucose consumption. Therefore, a rapid imaging workup is necessary, and processes like the inflammatory response after stroke remain to be investigated as these represent a variety of attractive novel targets for neurorestorative stroke therapies and extension of the therapeutic window. The multi-factorial and multi-phasic nature of cerebral ischemia, with an initial pro-inflammatory and a delayed repair and regeneration phase, emphasizes the intriguing possibility to shift the inflammatory response towards a pro-resolving, anti-inflammatory phenotype [11, 12]. The detailed understanding of the dynamics of cerebral and peripheral inflammation markers is a key step towards the development of next generation immunomodulatory therapies for ischemic stroke.

Molecular imaging combining positron emission tomography (PET) and single photon computed emission tomography (SPECT) together with functional and anatomical imaging by magnetic resonance imaging (MRI) in hybrid preclinical and clinical scanners represents an important approach to decipher these molecular alterations after stroke in a spatio-temporal manner. Multi-modality imaging bears the advantage of being non-invasive, allowing intra-individual follow-up of multiple disease processes at a time, while at the same time reducing experimental attrition and animal numbers as well as increasing statistical power and translatability [13]. The superb sensitivity of PET (pico-nanomolar range) and SPECT [14, 15] in combination with the fast acquisition time, clinical availability and excellent soft tissue contrast obtained by MRI, allows for the parallel intra-individual determination of various parameters after stroke [16].

In particular, nuclear imaging techniques like PET have been widely used for drug development purposes: (i) direct radiolabeling of a new drug

candidate for distribution (microdosing; $< \mu\text{g}$), (ii) mathematical modeling approaches to decipher drug-receptor interactions and subsequent assessment of pharmacodynamic responses, (iii) lead validation and optimization, (iv) following the (patho-)physiological response to a new drug over time, (v) allowing early diagnosis, and (vi) following longitudinally an imaging biomarker for patient monitoring [13, 17–19]. Ideally these imaging parameters can be used to enhance our understanding of disease pathogenesis and serve as predictive biomarkers for patient selection and stratification to improve clinical study protocols.

This review will highlight innovative preclinical and clinical imaging biomarkers targeting various aspects of neuroinflammation beyond monitoring and re-establishing cerebral blood flow (CBF). We will highlight their potential use in disease modulation and drug development processes towards personalized stroke therapies.

Imaging biomarkers of neuroinflammation

The lack of blood supply to certain brain regions after stroke leads to an orchestrated inflammatory response [12]. Resident and peripheral immune cells become rapidly activated and peripheral immune cells can infiltrate the brain through three major routes including (i) direct crossing of the BBB of (meningeal) blood vessels, (ii) direct crossing over the subarachnoid space into the brain parenchyma and (iii) infiltration via the blood-CSF barrier at the choroid plexus (Figure 1) [20]. The inflammatory response can roughly be divided into an initial damaging period with expression and release of pro-inflammatory mediators and into a late neuroprotective phase of inflammation that is hallmarked by release and expression of anti-inflammatory mediators [12, 21]. However, Hu and colleagues described an increase of anti-inflammatory biomarkers a few days after stroke followed by a progressive increase of pro-inflammatory markers in a mouse stroke model [22]. These contradictory results underline the complex overall dynamics, heterogeneity and multi-phasic nature of the immunological response after stroke making the development and application of immunomodulatory therapies challenging [12, 21]. This heterogeneity is an interesting possibility to extend the therapeutic window after stroke [23]. Given the heterogeneous location and size of infarctions in the clinical setting, the non-invasive assessment of the time course of inflammatory parameters by molecular imaging (Table 1) may help to investigate the pathophysiological response to immunomodulatory

therapies in a temporal manner (Table 2). Another possible application of molecular imaging is therapy selection and patient stratification for immunomodulatory treatment, thereby increasing the power of a clinical study.

The following paragraphs will highlight recent advances in imaging of several aspects of the inflammatory cascade and their use in the investigation of treatment responses in preclinical and clinical settings.

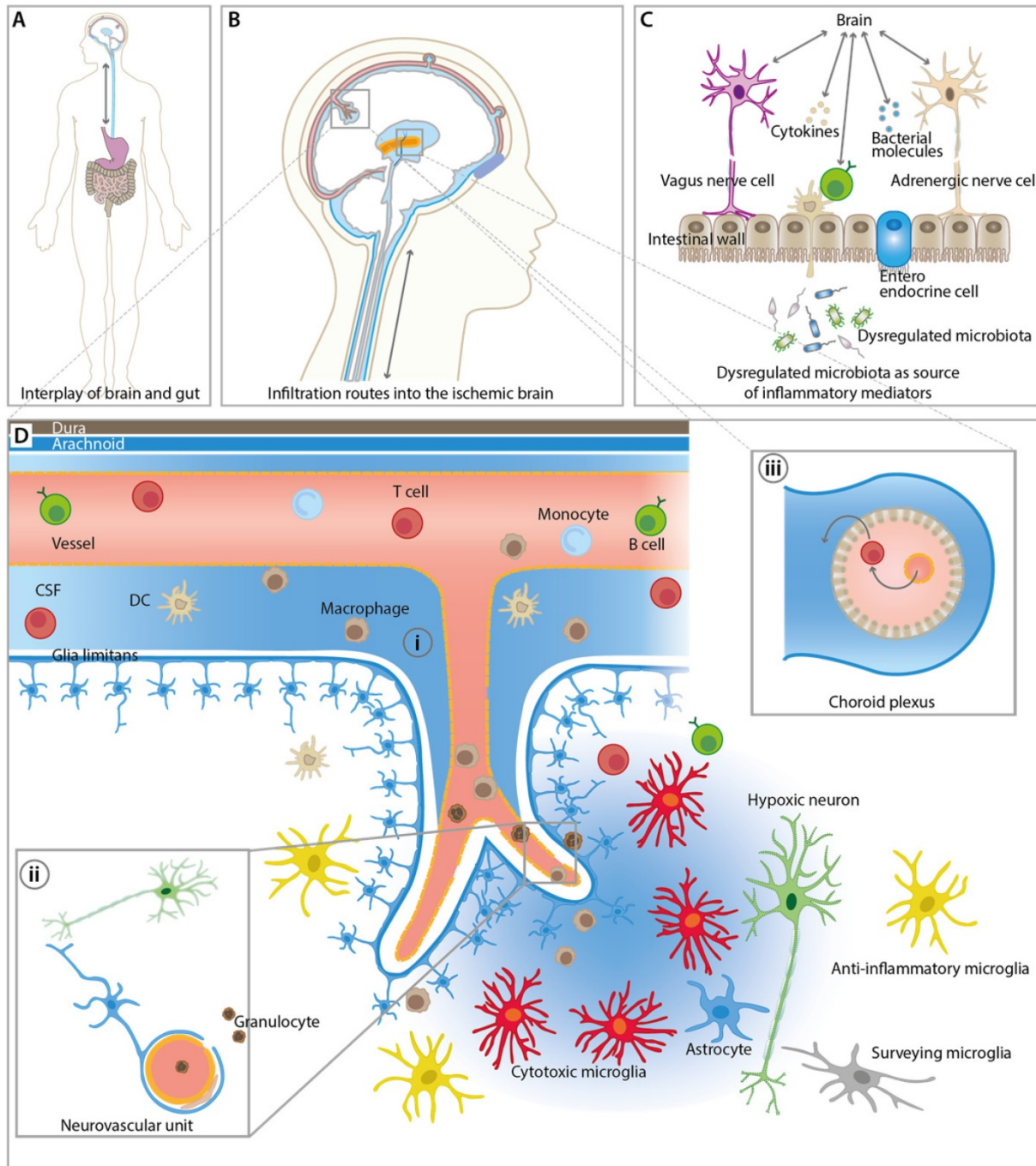


Figure 1. Overview of inflammatory pathways and imaging targets in the CNS and periphery. (A) Interplay between gut and the brain as an important route for inflammatory signals from and to the brain influencing stroke outcome. (B) Overview of key infiltration routes of peripheral innate and adaptive immune cells. Peripheral immune cells can enter into the ischemic brain from the (i) subarachnoid space (SAS), (ii) direct crossing of the blood brain barrier, and (iii) the choroid plexus across the blood–cerebrospinal fluid (CSF) barrier. (C) Dysregulated microbiota and resident intestinal immune cells send inflammatory mediators including cytokines via the hypothalamus–pituitary–adrenal glands axis (HPA), the autonomic nervous system, and the enteric nervous system (ENS). (D) Detailed view of the inflammatory events after ischemic stroke at the neurovascular unit (NVU) and SAS. CNS-resident microglia and astrocytes become activated and, depending on their polarization state, promote disease progression and repair. Resident and infiltrating immune cells exert their bi-modal functions via cell contact-dependent mechanisms and through the action of soluble inflammatory mediators. The NVU represents the target structure for functional tissue repair by neuroinflammatory processes. The NVU components may serve as imaging targets for the investigation of novel image-guided therapies targeting the rescue of NVU function.

Table 1. Selected molecular imaging studies highlighting the dynamics of imaging biomarkers after stroke.

Marker/Target	Imaging Modality	Compound/Tracer	Species	Occlusion time	Peak signal after stroke	Reference
VCAM-1	MR	MPIOs- α VCAM-1	C57BL/6 mice	pMCAo and tMCAo (various)	24 hours – 5 days	[34]
P-Selectin	MR	MPIOs- α P-selectin	Swiss mice	MCAo and TIA (15 min)	48 hours	[40]
TSPO	PET	[11 C]PK11195	Sprague-Dawley rat	tMCAo (1h)	7 days	[52]
TSPO	PET	[18 F]DPA-714	Sprague-Dawley rats	tMCAo (2h)	11 days	[53]
TSPO	PET	[18 F]DPA-714	Sprague-Dawley rats	tMCAo (1.5 h)	7 days	[72]
TSPO	PET	[18 F]DPA-714	C57BL/6 mice	tMCAo (30 min)	14 days	[60]
TSPO	PET	[18 F]DPA-714	Balb/c mice	tMCAo (60 min)	10 days	[70]
TSPO	PET	[11 C]PBR28	Sprague-Dawley rats	M2Cao (1.5 h)	4-14 days	[71]
TSPO	PET	[18 F]DPA-714	human	n.a.	n.a.	[64]
cystine-glutamate antiporter (system xc-)	PET	[18 F]FSPG	Sprague-Dawley rats	tMCAo (1.5h)	3-7 days	[72]
α 2 Nicotinic Acetylcholine Receptor	PET	2-[18 F]-fluoro-A85380	Sprague-Dawley rats	tMCAo (2h)	7 days	[77]
Matrix metallo-proteinases	PET	[18 F]BR-351	C57BL/6 mice	30 min	7 days	[60]

Table 2. Selected studies of anti-inflammatory treatments in stroke and their respective nuclear imaging readouts.

Drug	Target	Imaging readout	Effect	Reference
Multi-nutrient diet	Broad spectrum	[18 F]DPA-714, DTL, rsfMRI	Inflammation \downarrow , DTL, rsfMRI \uparrow , functional outcome \uparrow	[54]
Minocycline	Broad spectrum	[18 F]DPA-714 and [18 F]PBR06	Inflammation \downarrow , functional outcome \uparrow	[69, 76]
sulfasalazine and S-4-CPG	inhibition of system xc	[18 F]DPA-714, [18 F]FSPG	Inflammation \downarrow	[72]
Dh β E	α 2 antagonist	[11 C]PK11195 and 2[18 F]-fluoro-A85380	Inflammation \uparrow	[77]
AMD3100	CXCR4 antagonist	[18 F]DPA-714	Inflammation \downarrow	[70]

Infiltration of immune cells

Leukocytes are among the first inflammatory cells to enter the ischemic brain within hours after ischemia and, depending on the animal model, infiltration peaks about three days after stroke [6, 7, 24]. The infiltration of leukocytes depends on adhesion markers that are expressed on leukocytes and endothelial cells, including P/E-Selectin, ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1). These adhesion markers represent an early and essential step in the recruitment of leukocytes into the ischemic brain and, therefore, provide an important therapeutic target for modulation of leukocyte infiltration [25].

Beneficial effects of blocking or genetic deletion of these adhesion molecules on functional and molecular parameters in different animal models of ischemic stroke have been studied extensively (reviewed by Yilmaz and Granger [25]).

Several imaging approaches targeting intracellular and cell surface markers have been used to investigate their temporal expression profile after cerebral ischemia (for review: [16, 26, 27]). Among the imaging methods for tracking neutrophil recruitment to the infarction site, intravital imaging is increasingly being used. The applicability of intravital microscopy, however, is limited by the invasiveness of the disease models, as well as the relatively low temporal resolution and translatability into clinical practice [28]. Imaging approaches with PET, SPECT, and MRI represent imaging techniques with a higher

translational value due to their non-invasive character.

Most of the imaging paradigms targeting cell adhesion markers are based on molecular MRI. Molecular MRI utilizes contrastophores (either gadolinium- or iron oxide-based) that are linked to a protein of interest using a targeting moiety (pharmacophore: protein, peptide, antibody or enzymatic substrate). Over the last years, micro-sized particles of iron oxide (MPIOs) have been shown to possess superior characteristics in terms of sensitivity and specificity compared to classical ultra-small particles of iron oxide (USPIOs) [16, 29]. However, data on the application of imaging biomarkers for the assessment of treatment efficacy in stroke is still limited.

VCAM-1

Several approaches have been undertaken to image VCAM-1 expression in infarcted areas [30–32]. More recent studies focus on the use of MPIOs targeted to VCAM-1. VCAM-1 activation was visualized within hours after stroke by MRI and the VCAM MPIOs-mediated signal was compared with diffusion weighted imaging (DWI). Interestingly, the spatial extent of VCAM-1 expression was found to be considerably larger than the detectable lesion delineated by DWI. The authors concluded that the VCAM MPIOs might therefore delineate penumbral and core regions of the stroke. Ischemic preconditioning was found to significantly reduce VCAM MPIOs levels and improved functional outcome after stroke [33]. In line with these findings,

Gauberti *et al.* demonstrated spatio-temporal kinetics of VCAM-1 surface expression on endothelial cells after stroke by using MPIOs conjugated to monoclonal VCAM-1 antibodies (MPIOs- α VCAM-1). VCAM-1 expression was found perilesional and peaked after 24 hours for up to 5 days, depending on the severity of the stroke model. The activation of VCAM-1 in the periphery suggests that noninvasive imaging of this inflammatory reaction could be useful to identify the penumbra.

Interestingly, MPIOs- α VCAM-1 may be used to demonstrate the efficacy of immunomodulatory therapies with celecoxib and atorvastatin. Both compounds reduced VCAM-1 expression in the contralateral cortex, but not in the ischemic cortex [34]. Results in other disease models including Alzheimer's disease encourage the use of MPIOs- α VCAM-1 as an imaging biomarker for VCAM-1 expression [35]. To overcome current limitations of contrastophores, another study used liposomes loaded with the neuroprotectant Citicoline. Citicoline has the advantage of inherent chemical exchange saturation transfer (CEST) signal, which can be detected by MRI. In a targeted theranostic approach, Citicoline-loaded liposomes were specifically targeted against VCAM-1, leading to improved imaging-guided drug delivery to the ischemic area in a label-free fashion [36, 37]. However, it should be noted that this signal was detected in areas of BBB damage, potentially indicating the presence of unspecific leakage of liposomes into damaged tissue [36].

Selectins

E- and P-selectin expression, as an important marker of the tether-roll phase of leukocytes, was visualized *in vivo* by T2-type glyconanoparticle reagent GNP-sLex [38]. In this study, E- and P-selectin could be visualized by cross-linked iron oxide (CLIO) nanoparticles conjugated to anti-human E-selectin (CLIO-F(ab')(2)). Interestingly, this study described a hypointensity in the T2* area only 3 hours after permanent stroke; whereas, this hypointense area could only be detected 24 hours after transient ischemia. These results underline the sensitivity issues related to these techniques. To address this issue, paramagnetic gadolinium diethylenetriamine-pentaacetic acid (Gd-DTPA) conjugated to Sialyl Lewis X (Slex) was designed. The authors reported improved targeting of the tracer to E- and P-selectin mediated by Slex, as it mimics P-selectin glycoprotein ligand-1 tetrasaccharide Slex, which mediates the binding of leukocytes and platelets to activated endothelium [39].

Strikingly, antibody-based microparticles of iron oxide targeting P-selectin (MPIOs- α P-selectin), similar

to the previously addressed MPIOs- α VCAM-1 [34, 35], could be found in mouse brains 24 hours after stroke induction. In contrast to MPIOs- α VCAM-1, MPIOs- α P-selectin was able to identify and delineate transient ischemic attacks (TIA) 24 hours after induction. Using these microparticles, the authors could discriminate TIAs from epilepsy and migraine-mimicking TIAs in the clinical setting [40], suggesting sensitivity and specificity of the compound for TIAs.

Despite the high translational value of MRI-based assessment of adhesion marker profiles, and first successful theranostic approaches, these techniques still suffer from various drawbacks, including (i) low sensitivity, (ii) low specificity, (iii) quantification issues, and (iv) problems with possible *in vivo* toxicity of the molecular imaging marker [34, 37, 41]. Drug-loaded micelles/liposomes or activatable probes based on enzymatic substrates [16, 42] in combination with paramagnetic particles may further allow the combination of a therapeutic approach with improved diagnosis (theranostics) [36, 37, 41].

Microglia

Microglia have been implicated as important mediators in a variety of diseases. Under physiological conditions, microglia constantly screen the brain parenchyma for pathophysiological alterations [12, 43]. Under pathological conditions, microglia fulfill a dual role. In the acute phase (days) of ischemic stroke, microglia produce pro-inflammatory mediators such as TNF- α , IL-1 β and reactive oxygen species (ROS), i.e., proinflammatory M1-like microglia. Thereafter, under chronic (days-weeks) conditions, microglia obtain an anti-inflammatory M2-like phenotype, i.e., anti-inflammatory M2-like microglia. The neuroprotective function of these M2-like microglia is mediated by the release of IL-10 and TGF- β and other growth factors. However, it should be noted that the concept of M1 and M2 microglia is rather simplified and only represents two aspects of microglia polarization, at the same time neglecting a variety of different sub-phenotypes [44]. However, it has to be pointed out that in chronic post-stroke phases inflammation may spread from focal to remote areas following Wallerian degeneration and may thus not solely be beneficial [45]. Contrarily, it may worsen the outcome and lead to cognitive impairment, again highlighting the transient function of the inflammatory response. (for review: [46]).

One important marker of general microglial activation (i.e., not function of microglia and infiltration of macrophages), is the 18 kDa translocator protein (TSPO) located on the outer mitochondrial membrane. TSPO is part of

mitochondrial permeability transition pore (MPTP) and is involved in shuttling cholesterol across the mitochondrial membrane and in steroid synthesis [47–49]. In the past 30 years, TSPO has become an attractive imaging target in neuroinflammation since basal expression levels in the brain cells (microglia, astrocytes, endothelial and smooth muscle cells, subpial glia, intravascular monocytes and ependymal cells) are relatively low under physiological conditions, while it becomes highly upregulated upon neuroinflammatory stimuli in glial cells (microglia and astrocytes) and macrophages in different models of neurological disease [48–55]. Imaging studies in ischemic stroke revealed TSPO to be highly expressed by microglia/macrophages in the infarct core, peri-infarct areas, as well as in remote areas [45, 56–60].

TSPO tracers can be subdivided into first and second generation tracers. The most prominent first generation TSPO ligand is [^{11}C]PK11195. However, it suffers from a poor signal-to-noise ratio, and short half-life of the ^{11}C -label leading to the development of second generation tracers with improved bio-availability, higher specificity, and favorable signal-to-noise ratios. Second generation TSPO tracers like [^{18}F]PBR06, [^{11}C]PBR-28, [^{125}I]CLINDE, [^{18}F]GE-180, [^{18}F]DPA-714 were used in target validation studies in preclinical stroke (Figure 2) [53, 60–63] and clinical stroke [64, 65], allowing an improved understanding of the spatio-temporal profile of TSPO as an imaging biomarker.

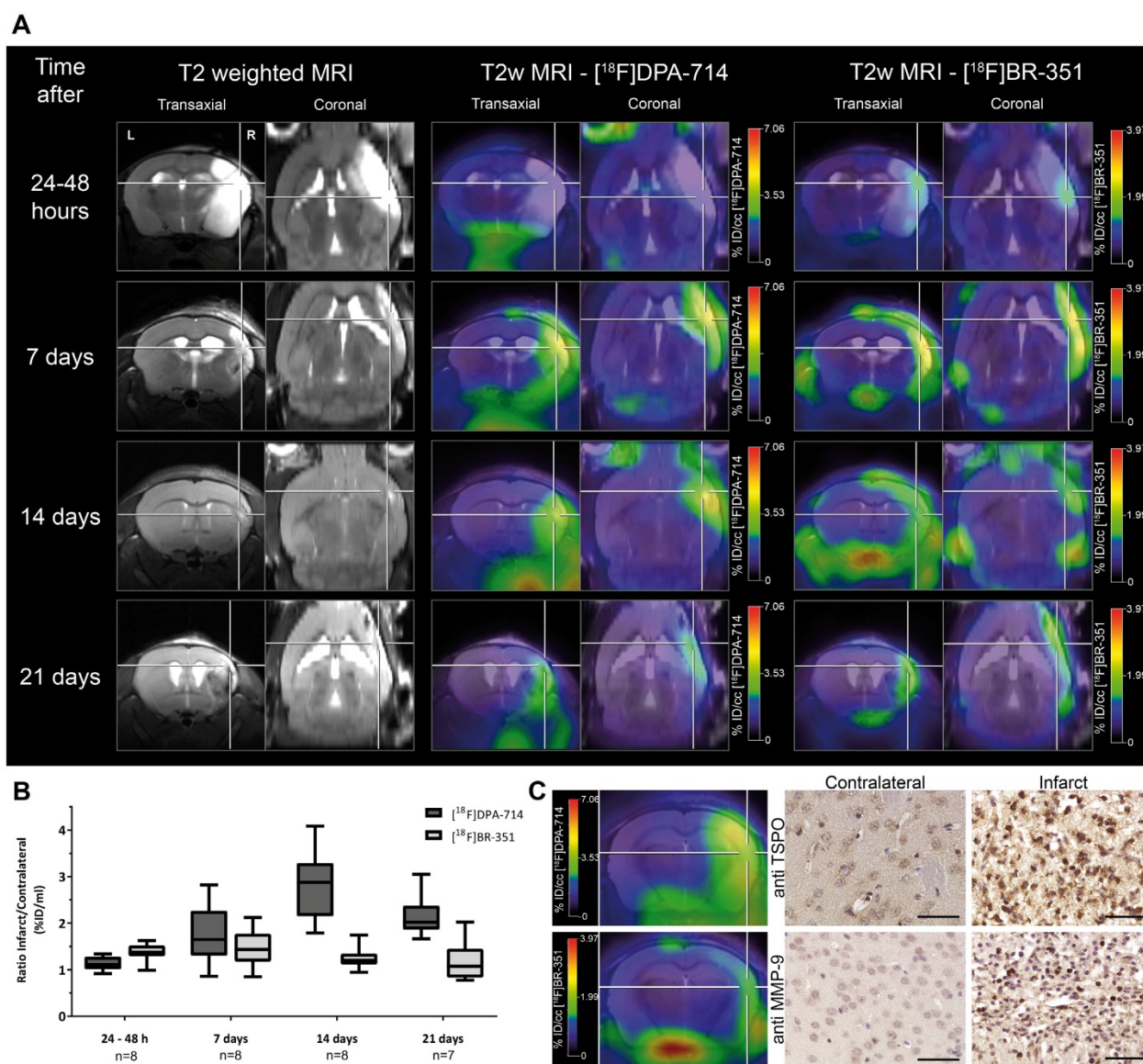


Figure 2. Investigation of the temporal dynamics of [^{18}F]DPA-714 (TSPO) and [^{18}F]BR-351 (MMPs) uptake after transient middle cerebral artery occlusion (tMCAo). (A) The inter-individual comparison highlights differential time-dependent radiotracer uptake patterns. (B) [^{18}F]DPA-714 uptake was significantly increased from day 7, peaked at day 14, and was still significantly elevated 21 days after tMCAo. [^{18}F]BR-351 was significantly increased after 24 to 48 hours after tMCAo and 7 days. Data are represented as ratio \pm SD. (C) Comparison of *in vivo* PET data with immunohistochemistry for TSPO and MMP-9. Both markers are increased in the infarct zone. Scale bar: 50 μm . (Adapted with permission from [60], copyright 2015 SAGE Publishing Group).

Recently, a third generation of TSPO tracers was described. Although a general definition of the requirements for a third generation tracer is lacking, [¹¹C]JER176 and [¹⁸F]GE180 were recently described as third generation tracers being less susceptible for the rs6971 polymorphism [66–68]. However the experimental data supporting this is relatively sparse [68] or do not fulfill the expectations [67].

Application of second generation tracers in stroke allowed detection of increased inflammation in the infarct region from subacute 3 days post-stroke, while the peak of microglia/macrophage response was found around 7–14 days after stroke for up to 7 months post-stroke in remote areas, depending on the stroke model in mice and rats (Table 1) [53, 60, 69–73]. Especially at late time points after stroke, TSPO-positive astrocytes contribute to the amount of TSPO-positive cells [53, 71, 74]. Thus, TSPO is a useful biomarker of inflammation from sub-acute to chronic phases after stroke.

The knowledge about the spatiotemporal profile of TSPO expression was further used to monitor therapy effects in experimental stroke for subsequent translation (Table 2). Minocycline, a well described antibiotic with anti-inflammatory function [75], was applied in a rat model of cerebral ischemia. TSPO levels, indicated by [¹⁸F]DPA-714 PET, were reduced in response to minocycline treatment. However, no effect on stroke size was observed and functional parameters were not obtained [76]. This study underlines the still open question of when and how to therapeutically interfere with the post-ischemic neuroinflammatory cascade to serve a beneficial tissue and functional outcome. Another study using the PET ligand [¹⁸F]PBR06 showed a correlation between [¹⁸F]PBR06 radiotracer uptake and impaired motor function [69]. In contrast to the previous study by Martin *et al.*, this group demonstrated treatment effects of minocycline through improved motor function 22 days after stroke, along with decreased [¹⁸F]PBR06 uptake after minocycline treatment. However, this study involved longer observation times and more sophisticated treatment readouts, i.e., motor function by rot-a-rod [69]. Domercq *et al.* have further shown that the inhibition of Cystine-glutamate antiporter (system xc⁻) resulted in reduced [¹⁸F]DPA-714 uptake and microglial M1 proinflammatory markers 7 days after treatment onset (CCL2, TNF and iNOS) [72]. In line with these results, treatment with an α4β2 antagonist reduced neuroinflammation seven days post stroke in rats, as measured with the first generation TSPO tracer [¹¹C]PK11195 [77]. The anti-inflammatory effect of

AMD3100, a CXCR4 antagonist, was evaluated by [¹⁸F]DPA-714 in a stroke model for up to 16 days after stroke induction. In line with previous studies, the peak of [¹⁸F]DPA-714 uptake was found around 10 days after stroke. A reduction of [¹⁸F]DPA-714 after AMD3100 was observed three days after stroke, but was not detectable after seven days. No effects on functional parameters were investigated [70].

Recent studies highlight the potential of novel diet-based interventions in a variety of disease models [54, 78–83]. In a multicenter study, the potential therapeutic use of a multi-component diet enriched with, e.g., omega-3 long-chain polyunsaturated fatty acids (n3-LCPUFAs), membrane precursors, vitamins, and anti-oxidants on several parameters including microglia were investigated. The same diet is already being applied in the management of patients with early Alzheimer's disease [84, 85]. In order to assess the potential treatment effects of this multi-component diet, a bi-modal translational neuroimaging approach was employed to assess treatment effects on perfusion (arterial spin labeling, ASL), brain structure (diffusion tensor imaging, DTI) and function (resting state functional MRI, rsfMRI), inflammation/microglia ([¹⁸F]DPA-714 PET) and functional parameters (behavioral testing). Interestingly, it could be shown that the multi-component diet was able to improve the imaging parameters, as well as functional outcome after stroke (Figure 3) [54]. The beneficial effects of dietary interventions enriched with n3-LCPUFAs may be attributed to different metabolites of n3-LCPUFAs, so called pro-resolving factors (resolvins). These are able to increase phagocytosis and exert anti-inflammatory functions on glial cells via peroxisome proliferator-activated receptor-γ (PPARγ) modulation, ω-3 FA incorporation into the cell membrane, and inhibition of ion currents [for review: 62]. Moreover, in preclinical studies, docosahexaenoic acid (DHA), an n3-LCPUFA, and its oxygenated derivatives, docosanoids, have been shown to regulate gene expression of inflammatory mediators like PPARγ; for further review see [87].

These results underline the striking potential of dietary interventions in modulation of stroke pathogenesis and should be considered for clinical translation in the management of stroke patients. Notably, microglia/macrophage imaging in combination with sophisticated MR neuroimaging techniques and *ex vivo* tissue biomarkers (e.g., polarization markers) has proven a powerful tool for detection of treatment effects and may serve as a blueprint for clinical translation of other neuroprotective compounds.

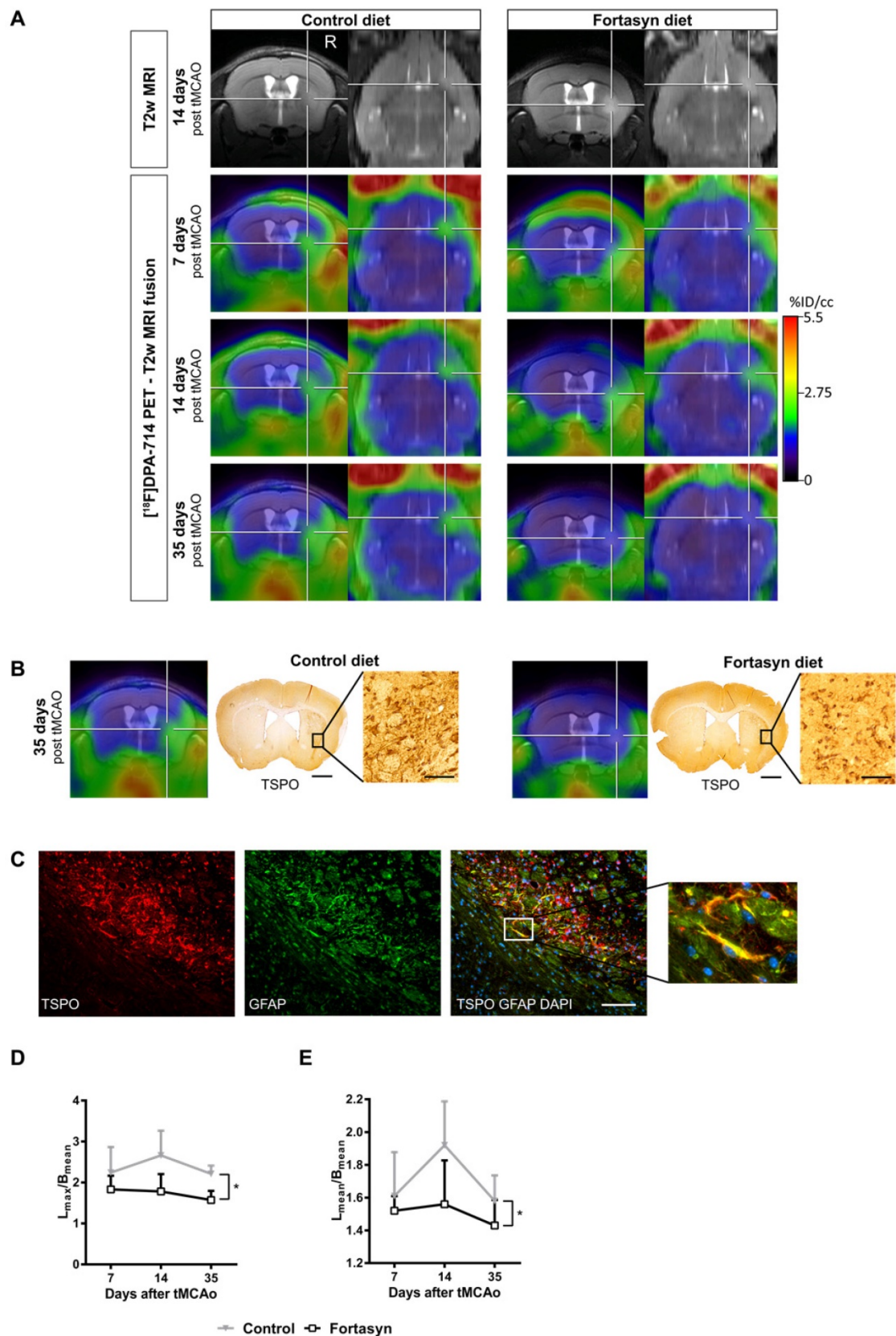


Figure 3. [¹⁸F]DPA-714 PET in the investigation of diet-mediated immunomodulatory effects on microglial activation. (A) Intra-individual follow up with [¹⁸F]DPA-714 PET/MRI of mice fed with a control diet (left) versus mice fed with the Fortasyn diet (right). Fortasyn-fed mice showed a reduced uptake of [¹⁸F]DPA-714 in the infarction after 35 days. (B) Comparison of [¹⁸F]DPA-714 with TSPO immunohistochemistry. TSPO levels were reduced in Fortasyn-fed animals (scale bars: 1000 μm and 50 μm). (C) TSPO (red) was also expressed by astrocytes (green) 35 days after tMCAO (scale bar: 100 μm). (D) Values represent mean ± SD. [¹⁸F]DPA-714 mean uptake ratios were significantly reduced from 7 to 35 days after tMCAO in the Fortasyn group when compared to control animals (p<0.029). (E) The maximum [¹⁸F]DPA-714 radio uptake ratios show reduced [¹⁸F]DPA-714 uptake in the Fortasyn diet group from 7 to 35 days (p<0.016). (Adapted with permission from [54], copyright 2017 Ivyspring International Publisher).

Despite the advances in preclinical microglia imaging, clinical data on TSPO in ischemic stroke is still relatively sparse. Imaging with the first generation TSPO ligand [¹¹C]PK11195 revealed early (72 hours post stroke) changes as well as chronic changes (up to months) in remote areas, indicative of Wallerian degeneration [45, 57, 58, 88, 89]. Besides [¹¹C]PK11195, second generation TSPO ligands have been used in initial clinical studies. Gulyás and colleagues investigated the TSPO signal evolution within the ischemic region and peri-infarct regions using the second generation TSPO tracer [¹¹C]vinpocetine. The authors reported the highest radiotracer uptake in the peri-infarct regions independent of the time after the insult. However, the authors also reported very moderate BP_{ND} values for [¹¹C]vinpocetine [90]. In another study, the same group reported no significant improvements of [¹¹C]vinpocetine in comparison to the first generation tracer [¹¹C]PK11195 [91]. In a single case study, Kreisl *et al.* found increased binding of [¹¹C]PBR28 after subacute infarction [92], but this tracer has not yet been used in further stroke research. The fluorine 18 label of [¹⁸F]DPA-714 has the advantage of allowing longer scan times and broader clinical availability, as evaluated in nine stroke patients. Increased [¹⁸F]DPA-714 uptake was found within and around the infarct, exceeding the blood brain barrier damage, as indicated by Gd-enhanced T1-weighted MRI [64]. Nevertheless, future studies will have to investigate the relative contribution of the BBB damage for radiotracer uptake in stroke.

Using the SPECT TSPO ligand [¹²⁵I]CLINDE, Feng *et al.* investigated the spatial and temporal change of TSPO levels 28 days and 8 months after middle cerebral artery stroke. Increased distribution volume (V_T , in mL cm⁻³) values were observed mostly in peri-infarct areas after 28 days and decreased to baseline values after 8 months. Interestingly, accumulation of [¹²⁵I]CLINDE was found in the insula of the contralesional hemisphere at this late time point [65], indicating the involvement of the entire brain in ischemia-induced neuroinflammation. Next to TSPO ligands, novel targets for microglia imaging may include the 18F-FSPG /system xc- and 2-18F-fluoro-A85380 ($\alpha4\beta2$). Both system xc- and $\alpha4\beta2$ have been reported to be overexpressed in microglia after stroke [72, 77].

One of the limitations of second generation TSPO ligands exacerbating clinical translational hurdles is the existence of an rs6971 polymorphism within the gene encoding for TSPO that affects the binding affinity of TSPO ligands [93–97]. This polymorphism results in a non-conservative single amino acid substitution from alanine to threonine at

amino acid residue 147 (Ala147Thr) of the TSPO gene causing an inter- and intra-individual variability in binding affinity, hampering quantification of imaging data [98], while at the same time no effect of Ala147Thr on inflammatory and cerebrovascular biomarkers was observed [99].

Based on the expression of high and low affinity sites of TSPO, patients can be subdivided into low-, mixed-, and high-affinity binders, whereas different ligands display a different sensitivity for the rs6971 polymorphism [94]. This implicates that the choice of the TSPO ligand, as well as the TSPO status of the individuals included in a study, may severely influence the outcome of the clinical trial. Thus, statistical methods accounting for the TSPO status and matching of patients should be performed [98].

Newly developed third generation TSPO ligands, being unsusceptible to the rs6971 polymorphism, and new non-TSPO molecular microglial targets are currently under investigation within the EU FP7 program Imaging Inflammation in Neurodegenerative diseases (INMiND consortium; <http://www.uni-muenster.de/InMind/>) [100, 101].

Nevertheless, current second generation TSPO ligands are under intense clinical investigation in a variety of diseases including Alzheimer's disease, mild cognitive impairment, multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, rheumatoid arthritis, brain tumors and alcoholism.

In conclusion, TSPO imaging provides a valuable tool to study the neuroinflammatory response in stroke and allows for assessment of immunomodulatory treatments targeting inflammation in pre-clinical and clinical settings.

Matrix Metalloproteinases (MMPs)

MMPs belong to the family of secreted or membrane-bound zinc- and calcium-dependent endopeptidases [102]. Most of the MMPs are secreted in their inactive (zymogen) form. After secretion into the extracellular space they become rapidly activated through proteolytic processing of the cysteine switch by plasmin, trypsin, kallikrein, mast cell tryptase and other MMPs, as well as ROS and hypochlorous acid from inflammatory processes [102–105]. MMPs have been implicated in a variety of physiological events such as neoangiogenesis, neural plasticity, wound healing, tissue remodeling, embryogenesis and apoptosis [104, 106]. Besides the physiological functions of MMPs, different pathologies have been strongly linked to MMP function, i.e., rheumatoid arthritis, osteoarthritis, cardiovascular disease, fibrosis, tumorigenesis, tumor neovascularization, invasion, metastasis, blood brain barrier damage, atherosclerosis, multiple sclerosis, meningitis,

Alzheimer's disease, stroke and many more [102, 104, 107–111]. MMPs contribute to the acute (neuro-) inflammatory reaction by modulating the expression, activity, and integrity of chemokines, inflammatory cytokines, growth factors, and matrix components and by affecting receptor turnover and leukocyte transmigration (diapedesis) [104, 106, 112, 113].

In ischemic stroke, MMPs are known to play a key role during disease progression. They are associated with BBB damage, hemorrhagic transformation, and vasogenic edema formation [110, 114–116]. It should be pointed out that over-simplification of MMPs as purely extracellular matrix (ECM)-degrading enzymes should be avoided since *in vivo* evidence that MMPs degrade ECM is lacking [117]. Several MMPs have been described to modulate the inflammatory response in cerebral ischemia via a network of complex functions and sources, including glial cells and neutrophils as important cellular sources. In particular, the gelatinases MMP-2 and MMP-9 are elevated after cerebral ischemia and have been correlated with BBB opening and hemorrhagic transformation [118, 119]. Interestingly, early inhibition of MMP-9 activity in experimental stroke, as well as genetic knockout, has beneficial effects on stroke outcome [120–124]. In contrast to acute MMP inhibition, it was shown that late inhibition can have detrimental effects, leading to increased brain damage characterized by reductions in numbers of neurons and newly formed blood vessels, supporting the hypothesis of dynamic multi-phasic effects of MMPs [119, 125–127].

Moreover, it has been reported that MMP inhibition leads to reduced migration of doublecortin (microtubule-associated protein expressed by neuronal precursor cells)-positive cells from the subventricular zone to infarcted areas after stroke [128]. Problems with the study design, definition of endpoints, the design of MMP inhibitors and heterogeneous nature of MMPs might so far explain the lack of efficacy and adverse effects of MMP inhibitors in clinical studies (for review: [129]). The multitude of available data on beneficial effects of MMP modulation in different disease settings makes MMPs attractive as biomarkers and therapeutic targets. Thus, there is a strong need for MMP inhibitors with increased specificity and selectivity for MMP subtypes, as well as sophisticated methods to detect MMPs *in vivo* in real time.

Most of the currently available preclinical imaging probes for tracking MMP activity in stroke rely on fluorescently labeled broad spectrum inhibitors or activatable (smart) probes. Smart probes are designed to remain in a quenched state until the quencher is degraded by activated MMPs under

pathophysiological conditions, resulting in emission of near-infrared fluorescence (NIRF) [130].

Klohs and colleagues were the first to describe the use of an activatable, near-infrared fluorescent probe to image general MMP activity after stroke 24 hours after injection of the tracer. Treatment with the MMP inhibitor GM6001 resulted in significantly lower tracer signal and lesion volume, suggesting successful imaging of *in vivo* MMP activity [131]. Another study made use of MMPsense 750 FAST with improved characteristics including a shorter optical imaging time for early (within hours) visualization of MMP activity. Increased changes in fluorescence intensities were found in early ischemic reperfusion and at 24 hours post ischemia, but not in sham controls. The results correlated with MMP-9 protein levels, 5 and 24 hours after ischemia reperfusion. Overall MMP activity could be attenuated by hypothermia [132]. In contrast to these planar imaging approaches, Martín and colleagues further applied tomographic optical imaging describing an increase of MMP activity as early as 3 to 6 hours correlating with the volume of the ischemic damage [133]. Next to smart probes, a recent study suggested gelatinase-activatable cell-penetrating peptides (ACPP) with a cleavable l-amino acid linker linked to Cy5 or gadolinium for imaging MMP activity and therapy response in ischemic tissue in the femtomolar range [134]. These studies emphasize the potential of *in vivo* assessment of MMP activity after stroke and investigation of therapy response. The drawback of both probes is, however, the relatively long circulation required before imaging and/or the relatively low signal intensities due to physical limitations due to tissue scattering and limited penetration depth of light, requiring invasive measurements [131, 132]. To address this, the probe MMP-P12 with improved characteristics including optimized stability, blood half-life and enzyme susceptibility was developed. The probe allowed observation of significant signal enhancement from as early as 15 minutes to 24 hours after injection, with a peak 1 hour after injection. The spatiotemporal signal was followed for up to 14 days after stroke. Treatment with the MMP inhibitor GM6001 reduced the MMP-P12 fluorescence signal, suggesting MMP substrate specificity, though no complete blocking of the signal was observed. The feasibility of using this probe for drug development purposes was further tested by treating mice with RWJ67657, an inhibitor of the p38 mitogen-activated protein kinase (MAPK) signaling pathway, which resulted in modulation of MMP activity after stroke accompanied by improved functional outcome. Moreover, p38 MAPK inhibition rescued MMP-P12 NIRF intensity [130]. A direct

comparison of NIRF probes is difficult due to the differences in stroke models being used and the different imaging time points. Despite the various advantages of NIRF imaging for real-time visualization of MMP activity in preclinical drug discovery including high sensitivity, low cost, non-ionizing radiation and vast applicability [135], there is a lack of clinical translatability due to the physical limitations of light only allowing for intra-operative applications.

Another important step towards clinical translation is the use of radioactively labeled compounds for nuclear imaging applications. The first radiofluorinated hydroxamate-based MMP inhibitor, the I-2-(*N*-benzyl-4-(2-[¹⁸F]fluoroethoxy)phenylsulphonamido)-*N*-hydroxy-3-methylbutanamide compound CGS 27023A 1 ([¹⁸F]BR-351), was developed by Wagner and colleagues. Due to the underlying radiochemistry, [¹⁸F]BR-351 can only bind the activated forms of MMP-2 (IC₅₀ = 4 nM), -8 (IC₅₀ = 2 nM), -9 (IC₅₀ = 50 nM), and -13 (IC₅₀ = 11 nM) [136].

In a multi-tracer study [¹⁸F]BR-351 was used in combination with the TSPO ligand [¹⁸F]DPA-714 for spatio-temporal assessment of MMP activity in a murine model of ischemic stroke (Figure 2) [60]. Interestingly, MMP expression, as measured by [¹⁸F]BR-351, appeared already 24 hours after tMCAo, peaked at 7 days post stroke, and was only seen in a subset of mice at later time points (14-21 days post stroke) [60]. Although this study did not focus on targeted MMP therapy, the results raise the intriguing possibility that [¹⁸F]BR-351 might be useful in selecting individuals who may benefit from MMP inhibitory therapy. Future studies should focus on how radioligands like [¹⁸F]BR-351 can be used to select and assess MMP-based therapies in stroke. Encouraging results come from a recent clinical study in patients with multiple sclerosis where [¹⁸F]BR-351 was successfully applied to monitor the early signs of multiple sclerosis and for monitoring anti-inflammatory treatment, thus opening new avenues for personalized therapies [137].

The combination of longitudinal MMP imaging in conjunction with improved inhibitors, which selectively modulate MMPs, is needed to rejuvenate the idea of using MMP modulation as a personalized therapy approach after stroke.

Cell-based therapies and *in vivo* cell tracking

Molecular imaging has been applied to track different cell populations after stroke. This is important to improve our understanding of the spatiotemporal immune cell infiltration and activation

after cerebral ischemia. One example is the early clinical study by Price *et al.* [138], in which migration of [¹¹¹In]troponin-labeled neutrophils in stroke patients was successfully tracked by gamma scintigraphy. Additionally, molecular imaging provides a powerful tool to evaluate the success of (stem) cell-based therapies, aiming to stimulate tissue repair after stroke. Investigated cell types showing promising therapeutic success in preclinical studies include, but are not limited to, neural stem cells (NSCs) [139], mesenchymal stem cells (MSCs) [140] and bone marrow mononuclear cells (BMMNCs) [141]. Interestingly, the reported success of those therapies is attributed to paracrine effects rather than to the replacement of destroyed neurological tissue. Observed effects include increased neurological plasticity [142] and stimulation of angiogenesis and, therefore, revascularization of the ischemic tissue [143-147]. Another important mode of action is immunomodulation induced by the transplanted cells. Brenneman *et al.* showed a downregulation of inflammatory cytokines in the brains of rats treated with BMMNCs as well as upregulation of IL-10 in acute stroke [148]. Yoo *et al.* reported immune suppression via TGF- β produced by transplanted MSCs themselves [149]. Interestingly, the immunomodulatory effects were systemic rather than restricted to the brain, possibly explaining why intravenous application of stem cells has therapeutic effects, even though only very few cells reach the brain [140, 150]. Next to MSCs and BMMNCs, specialized regulatory B- and T-lymphocytes may also importantly contribute to therapeutic capacity in ischemic stroke and should be pursued as imaging and therapeutic targets (for review: [151]).

Non-invasive imaging is ideally suited to investigate the migration and engraftment of transplanted cells. For all, direct or indirect labeling of the transplanted cells is necessary. Long term tracking and engraftment of transplanted MSCs or BMMNCs has been successfully conducted in animal models and first human stroke patients using the SPECT tracers [^{99m}Tc]HMPAO and [¹¹¹In]Oxine [152, 153] as well as small iron oxide-based agents such as SPIO-based particles for MRI [154, 155]. The severe limitation of direct labeling approaches for both nuclear imaging as well as MRI is the low retention rate and accompanied tracer leakage from the transplanted cells [156, 157]. In the case of SPIOs, leaked particles can be taken up by phagocytic cells in the ischemic area and might result in positive signals in the absence of transplanted cells [156]. Alternatively, cells can be labeled indirectly, e.g., by using reporter genes that are only expressed by transplanted cells [158]. Bioluminescence imaging

(BLI) has been applied to visualize and monitor endogenous stem cell vitality and response post stroke [159] and to image the fate of *fluc*-transduced MSCs [157, 160], NSCs [159] and monocytes/macrophages [161]. BLI is limited by the low penetration depth of light and, while visualization of cells through the skull is possible, imaging remains restricted to surface structures. Genetic reporters have also been investigated for tracking of cells using nuclear imaging. The best characterized reporter is based on the herpes simplex virus type 1 thymidine kinase (HSV-1-*tk*) that can phosphorylate isotope-labeled pyrimidine analogs like [¹²⁴I]FIAU or [¹⁸F]FHBG and thereby trap them in transplanted cells [162]. Severe disadvantages of the HSV-1-*tk*-system are possible immune reactions against the viral protein and the relatively low blood brain barrier penetration of the applied imaging probes [163]. A promising alternative is the human sodium iodide symporter (hNIS) that can transport all radioactive forms of I⁻. While hNIS so far has not been applied after stroke, it has been successfully used to track MSCs, for example, in animal models of breast cancer [164], glioma [165] and myocardial infarction [166]. Similarly, indirect labeling of cells, e.g., via reporters based on CEST, has been investigated for tracking cells by MRI [167]. However, to date this approach has not been applied to track cells after cerebral ischemia.

Novel imaging approaches will focus not only on tracking the fate of transplanted cells, but at the same time monitoring therapy responses in the form of immunomodulation, changes in the microenvironment and activation of endogenous neural stem cells. In a promising approach Daadi *et al.* applied longitudinal multimodal imaging to track NSCs labeled both directly (SPIOs for tracking with MRI) and indirectly (HSV-1-*tk* reporter for PET imaging with [¹⁸F]FHBG) [168]. They elegantly combined tracking of grafted cells with analysis of infarct size by MRI and neurological metabolic activity by [¹⁸F]FDG-PET over the course of several months. In the future, multimodal- and multitracer imaging assessing microglia and tissue remodeling, in combination with cell tracking will help to monitor immunomodulation and changes in brain microenvironment [60]. In addition, monitoring of endogenous neural stem cells, for example by 3'-deoxy-3'-[¹⁸F]fluoro-L-thymidin ([¹⁸F]FLT) -PET, could elucidate reparative processes induced by stem cell therapies. Rueger *et al.* applied [¹⁸F]FLT to monitor endogenous NSC proliferation in the subventricular zone following minocycline treatment in rats with cerebral ischemia [169]. In this multimodal- and multitracer imaging study, [¹⁸F]FLT imaging was combined with [¹¹C]PK11195-PET to

assess neuroinflammation and MRI to monitor infarct size.

Future perspectives

Imaging the brain-gut axis

Investigation of the role of intestinal microbiota in neurological diseases and recovery has drawn a lot of attention for microbiota as possible disease-modulating entities in stroke (for review: [170, 171]). Immune cells, hormones and neurotransmitters originating from the gut may reach the brain via a recently discovered lymphatic subsystem, which allows for direct or indirect transport of inflammatory mediators from the gut to the brain (Figure 1). This specialized lymphatic system drains from the cerebrospinal fluid in the adjacent subarachnoid space and the interstitial fluid to the deep cervical lymph nodes and may be visualized by molecular imaging [172–174].

Another aspect of intestinal microbiota is their role in regulating the permeability of the BBB. Germ-free animals have been shown to exhibit reduced expression levels of tight junction proteins, which results in increased permeability of the BBB for molecules [174–176]. In line with these observations, complete depletion of gut microbiota with a broad-spectrum antibiotic cocktail led to increased mortality rates and development of severe colitis in a mouse model of stroke [177].

In response to stroke stages, various reports exemplify significant changes in the composition of gut microbiota in preclinical and clinical stroke that partially correlate with disease severity [178–181]. This raises the intriguing possibility of selective modulation of the gut microbiome to treat stroke. In line with this notion, beneficial effects on stroke severity were observed by therapeutic transplantation of fecal microbiota partially restoring bacterial species diversity and improving stroke outcome likely via a lymphocyte-dependent mechanism [179]. Further evidence for a link between microbiota and the lymphocytic response after stroke was deduced by studies of Benakis and colleagues. They induced microbiota dysbiosis by antibiotic treatment, which led to an increase in regulatory T cells, suppression of IL-17-positive $\gamma\delta$ T cells and thus reduced trafficking of effector T cells from the gut to the leptomeninges after stroke, resulting in smaller infarcts and improved behavioral outcome [182]. Little is known about the *in vivo* dynamics of specialized gut microbiota and trafficking cells (i.e., lymphocytes) after stroke. Therefore, the *in vivo* follow-up by molecular imaging of bacterial dysbiosis and trafficking cells may have strong indications in patient

stratification and selection for therapy. Novel imaging agents are needed to study gut microbiota non-invasively over time.

Imaging astrocytes:

Astrocytes are the key cells within the neurovascular unit mediating exchange of signals between the vascular system and neurons and are an important mediator of damage and repair after stroke [183–185]. As for many other immune cells, astrocytes also have detrimental functions through hyper-reactivity and glial scar formation, as well as beneficial functions through neuroprotective, angiogenic, immunomodulatory, neurogenic, and antioxidant functions (for review: [186]). Therefore, astrocytes also represent an important therapeutic and thus imaging target. Despite encouraging data on monoamine oxidase B (MAO-B) binding PET ligands like [¹¹C]deuterium-L-deprenyl (DED) in Alzheimer's disease [187, 188], *in vivo* imaging data on astrocyte distribution and activity after stroke still represents an underexplored aspect of the disease. Besides DED, TSPO and $\alpha 4\beta 2$ nicotinic acetylcholine receptor-targeted PET ligands are able to visualize astrocytic activation, depending on the imaging time point after stroke [54, 77, 189]. Nevertheless, there is still a need for more specific astrocytic imaging biomarkers with longer half-life isotopes for monitoring disease progression and therapy responses.

Discussion

Multi-modality and multi-tracer imaging have the potential to support drug development processes by answering key questions about location, extent, temporal profile, and therapy effect on an imaging biomarker after stroke, thereby facilitating our understanding of disease progression. By simultaneously providing profiles of different markers after stroke, multi-modality and multi-tracer imaging can assist in development, selection, application and follow-up of targeted therapies for stroke patients at specific time-points (personalized medicine). These types of technologies are important, especially in diseases such as stroke with its highly dynamic disease process and with heterogenous and variable molecular signatures within different parts of the diseased brain.

Current imaging paradigms aim at identifying the location and extent of the stroke lesion, estimation of the penumbra and patient selection for recanalization therapy. CT and several MRI techniques are used to obtain physiological data and to exclude hemorrhagic transformation [190, 191]. However, next to physiological and anatomical

information, molecular parameters are important for targeted and personalized therapies. This review was not dedicated to pathophysiological and molecular markers such as cerebral blood flow, oxygen and glucose consumption, but on imaging biomarkers that may allow the assessment of further consequences after stroke such as neuroinflammatory and tissue repair processes. Current tracers fall short in discriminating between different inflammatory cell types and/or polarization states. Thus, further imaging biomarkers and novel techniques, e.g., optogenetics, specifically targeting these differences in cell activation and function need to be developed and are currently under evaluation [72, 77, 100]. Ideally, these novel markers should be longitudinally studied (intra-individual imaging) and combined with, e.g., other imaging and molecular techniques including “-omics” and liquid biopsies to obtain a patient-specific detailed characterization of disease. This will allow stratification of patients for treatment and drug development paradigms. As target availability and resulting patient stratification is a key determinant of sample sizes in clinical trials, molecular imaging will provide important information to determine sample sizes and to define inclusion and exclusion information earlier than standard clinical assessments and at the same time increase the sensitivity and predictivity of clinical studies [192].

In parallel with defining new imaging targets, innovative disease-resolving and neurorestorative therapies need to be developed. These may include immunomodulatory therapies, dietary interventions and related alterations of the microbiota-gut-brain axis [82, 193].

Despite the multitude of advances in imaging-guided therapies, there is still a strong need to further improve the quality of research to obtain predictive and clinically translatable data. To this end, research activities should involve collaborative community efforts, including multi-center studies as suggested [10, 194] and applied recently [54, 195–198]. The concept of reverse-translating quality standards from clinical studies into preclinical work has shown promise and overall acceptance to further enhance translatability of research [199].

Next to general quality measures in stroke research, imaging research must overcome additional issues specific to the field. Obtaining meaningful, quantifiable data remains an important challenge in preclinical and clinical research. Nuclear imaging techniques provide the essential pharmacodynamic data of radiolabeled compounds, but they also need to be harmonized [200–202]. In particular, quantification in small animals remains challenging, since

pharmacokinetic/pharmacodynamic modeling usually requires determination of an arterial input function by repeated blood sampling. The choice of the right mathematical model, either model- or data-based, applied for determination of transfer rate constants, volumes of distribution or binding potentials may also vary between different ligands and targets [201, 202]. This problem can be exemplified by means of TSPO tracer studies, which suffer from a lack of a suitable reference region in the brain. Multiple approaches have been undertaken to overcome these limitations to create parametric images including population-based approaches, Logan analysis and spectral analysis [203–205].

In summary, over the last couple of years, the molecular imaging field has rapidly evolved and many novel approaches to dissect molecular processes over time and during the course of a disease process in various tissue compartments *in vivo* are under way. Molecular imaging will therefore represent an important asset to the drug development process by providing essential information about patient-specific disease progression and therapy response, while allowing for patient selection, stratification and personalized therapy.

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Competing Interests

The authors have declared that no competing interest exists.

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