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„Quantitative imaging of energy-dependent processes –  
opportunities and limitations of 2-[<sup>18</sup>F]FDG“

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***„Wir wissen mitunter nicht, wonach wir suchen, bis wir es schließlich gefunden haben.“***

Ludwig Wittgenstein

*Dedicated to my family.*





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## ABSTRACT

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The glucose analogue 2-[ $^{18}\text{F}$ ]fluoro-2-deoxy-D-glucose (2-[ $^{18}\text{F}$ ]FDG) is the most applied radiotracer in positron emission tomography and builds the center of this dissertation.

In one of the presented publications, 2-[ $^{18}\text{F}$ ]FDG was used to determine the effect of unconjugated bilirubin (UCB) on the metabolism of *in vitro* cultivated cell lines. Literature suggests that mild hyperbilirubinemia (Gilbert's syndrome) protects against diabetes mellitus type 2 and improves the lipid profile, but mechanistic studies are lacking. In the course of this dissertation, it was shown that low UCB concentration reduces the accumulation of lipids in skeletal muscle and hepatoblastoma cells *in vitro* and increases the consumption of glucose visualized by 2-[ $^{18}\text{F}$ ]FDG.

Furthermore, new approaches to absolutely quantify glucose demand with the help of 2-[ $^{18}\text{F}$ ]FDG are constantly developed. Because manual determination of the arterial input function (AIF; from arterial blood) is invasive and laborious, there are increasing attempts to derive the IF directly from the images. In this dissertation, an advanced computational approach for an image-derived input function was developed for the absolute quantification of glucose demand in the brain. The method used a combined PET/magnetic resonance imaging system and showed good comparability with the AIF.

Moreover, the exact behavior of 2-[ $^{18}\text{F}$ ]FDG in tissues is still unclear despite the long-standing use of the radiotracer. In principle, the radiotracer is taken up *via* glucose transporters. It then gets phosphorylated to 2-[ $^{18}\text{F}$ ]FDG-6-phosphate (2-[ $^{18}\text{F}$ ]FDG-6-P) and is said to be mainly trapped as such. However, recent studies indicate that 2-[ $^{18}\text{F}$ ]FDG-6-P accumulates in the endoplasmic reticulum, where it can be processed by hexose-6-phosphate dehydrogenase (H6PD), an enzyme of the oxidative pentose phosphate pathway (oxPPP). According to literature, this could be crucial for 2-[ $^{18}\text{F}$ ]FDG accumulation beyond phosphorylation. However, whether 2-[ $^{18}\text{F}$ ]FDG can also be metabolized by the related enzyme glucose-6-phosphate dehydrogenase (G6PD) is ill-defined. Furthermore, the metabolism of 2-[ $^{18}\text{F}$ ]FDG is poorly understood in general. Most importantly, it is unclear whether metabolism beyond 2-[ $^{18}\text{F}$ ]FDG-6-P alters uptake kinetics or overall tissue accumulation of the radiotracer, which could affect imaging. Therefore, in this doctoral thesis, 2-[ $^{18}\text{F}$ ]FDG metabolism was first investigated in different human cancer cell lines *in vitro* and several radiometabolites were identified. Metabolism beyond 2-[ $^{18}\text{F}$ ]FDG-6-P

depended substantially on glucose availability and the tumor entity (origin of the cell lines). In a second step, a map of 2-[<sup>18</sup>F]FDG metabolism in interaction with accumulation over time was defined for various murine organs, two xenograft tumors and corresponding cell lines *in vitro* for the first time. The extent of accumulation and metabolism of 2-[<sup>18</sup>F]FDG was significantly different among the tissues studied, and a different metabolic pattern was observed. Most interestingly, levels of radiometabolites within glycogen metabolism or the oxPPP correlated inversely with total tracer accumulation. This suggests that metabolism beyond 2-[<sup>18</sup>F]FDG-6-P or the activity of specific glucose metabolic pathways could be relevant for imaging. Mechanistic *in vitro* studies concerning the role of the oxPPP suggested that while both G6PD and H6PD could be involved in 2-[<sup>18</sup>F]FDG metabolism, only H6PD determines 2-[<sup>18</sup>F]FDG accumulation.

Finally, while 2-[<sup>18</sup>F]FDG is a highly versatile tracer, its nonspecific mode of action is also a drawback. For example, 2-[<sup>18</sup>F]FDG is not satisfactory for the imaging of brown adipose tissue (BAT), although it is currently considered the gold standard. Following preclinical imaging with our melanin-concentrating hormone receptor 1 tracer (MCHR1) [<sup>11</sup>C]SNAP-7941 that revealed BAT uptake, the MCHR1 was described in rodent and human BAT for the first time in the course of this doctoral thesis. This finding could promote research on organ-crosstalk in diabetes and obesity, as both the MCHR1 and BAT regulate energy homeostasis. Furthermore, [<sup>11</sup>C]SNAP-7941 was shown to be a potentially suitable alternative radiotracer for BAT imaging.

## ZUSAMMENFASSUNG

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Das Glucose-Analogon 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucose (2-[<sup>18</sup>F]FDG) ist der am häufigsten verwendete Radiotracer in der Positronen-Emissions-Tomographie und steht im Zentrum dieser Dissertation.

In einer der Studien wurde 2-[<sup>18</sup>F]FDG verwendet, um den Einfluss von unkonjugiertem Bilirubin (UCB) auf den Stoffwechsel von *in vitro* kultivierten Zelllinien zu bestimmen. Die Literatur legt nahe, dass eine milde Hyperbilirubinämie (Gilbert-Syndrom) vor Diabetes mellitus Typ 2 schützt und das Lipidprofil verbessert, jedoch fehlen mechanistische Studien. Im Zuge dieser Dissertation wurde gezeigt, dass eine niedrige UCB-Konzentration die Anreicherung von Lipiden in Skelettmuskel- und Hepatoblastomzellen *in vitro* reduziert und die Aufnahme von Glucose – visualisiert durch 2-[<sup>18</sup>F]FDG – erhöht.

Trotz der langjährigen Anwendung des Radiotracers werden laufend neue Ansätze zur absoluten Quantifizierung des Glukosebedarfs mit Hilfe von 2-[<sup>18</sup>F]FDG entwickelt. Da die manuelle Bestimmung der arteriellen Inputfunktion (AIF; aus arteriellem Blut) zu diesem Zwecke invasiv und aufwendig ist, versucht man zunehmend, die IF direkt aus den generierten Bildern abzuleiten. Im Rahmen dieser Doktorarbeit wurde mithilfe eines kombinierten PET-Magnetresonanztomographen ein neuartiger Ansatz für eine aus dem Bild abgeleitete IF zur absoluten Quantifizierung des Glukosebedarfs im Gehirn entwickelt, wobei sich eine gute Vergleichbarkeit mit der AIF zeigte.

Darüber hinaus ist das genaue Verhalten von 2-[<sup>18</sup>F]FDG im Gewebe trotz der langjährigen Anwendung immer noch unklar. Prinzipiell wird der Radiotracer über Glukosetransporter aufgenommen, anschließend zu 2-[<sup>18</sup>F]FDG-6-Phosphat (2-[<sup>18</sup>F]FDG-6-P) phosphoryliert und laut aktuellem Stand der Wissenschaft hauptsächlich als solches in der Zelle gespeichert. Neuere Studien deuten jedoch darauf hin, dass 2-[<sup>18</sup>F]FDG-6-P im endoplasmatischen Retikulum akkumuliert, wo es von der Hexose-6-phosphat-Dehydrogenase (H6PD), einem Enzym des oxidativen Pentosephosphatwegs (oxPPP), metabolisiert werden kann. Dies könnte laut Studien über die Phosphorylierung hinaus für die Anreicherung des Radiotracers im Gewebe entscheidend sein. Die Literatur darüber, ob 2-[<sup>18</sup>F]FDG auch durch das verwandte Enzym Glucose-6-phosphat-Dehydrogenase (G6PD) metabolisiert werden kann, ist uneinheitlich. Ferner ist der Metabolismus von 2-[<sup>18</sup>F]FDG im Allgemeinen unzureichend erforscht. Vor allem ist unklar, ob der Metabolismus über 2-

[<sup>18</sup>F]FDG-6-P hinaus die Aufnahmekinetik oder Gesamtanreicherung des Radiotracers im Gewebe beeinflusst, was die Bildgebung beeinträchtigen könnte. Daher wurde im Zuge dieser Doktorarbeit zunächst der 2-[<sup>18</sup>F]FDG-Metabolismus in verschiedenen menschlichen Krebszelllinien *in vitro* untersucht, wobei mehrere Radiometaboliten identifiziert wurden. Es zeigte sich, dass der Metabolismus von 2-[<sup>18</sup>F]FDG wesentlich von der Verfügbarkeit von Glucose und der ursprünglichen Tumorentität abhängt. In einem zweiten Schritt wurde erstmals eine Übersicht über den 2-[<sup>18</sup>F]FDG-Metabolismus im Zusammenspiel mit der Akkumulation über die Zeit in verschiedenen Mausorganen, humanen Xenotransplantations-Tumoren und den entsprechenden Zelllinien *in vitro* erarbeitet. Das Ausmaß der Akkumulation und des Metabolismus von 2-[<sup>18</sup>F]FDG war signifikant unterschiedlich zwischen den untersuchten Geweben und es wurde ein unterschiedliches Stoffwechsellmuster beobachtet. Interessanterweise korrelierten Radiometaboliten über 2-[<sup>18</sup>F]FDG-6-P hinaus, die den Glykogenstoffwechsels oder den oxPPP widerspiegeln, invers mit der Gesamtakkumulation des Radiotracers. Dies deutet darauf hin, dass der Metabolismus über 2-[<sup>18</sup>F]FDG-6-P hinaus, bzw. die Aktivität bestimmter Glucose-Stoffwechselwege für die Bildgebung relevant sein könnte. Mechanistische *in vitro* Versuche bezüglich der Rolle des oxPPP zeigten, dass sowohl G6PD als auch H6PD am Metabolismus von 2-[<sup>18</sup>F]FDG beteiligt sein könnten, aber möglicherweise nur H6PD die Anreicherung von 2-[<sup>18</sup>F]FDG bestimmt.

Zu guter Letzt ist 2-[<sup>18</sup>F]FDG zwar ein vielseitig anwendbarer Radiotracer, doch seine unspezifische Aufnahme ist auch von Nachteil. Beispielsweise ist 2-[<sup>18</sup>F]FDG für die Darstellung des braunen Fettgewebes (BAT) nicht zufriedenstellend, obwohl es derzeit als Goldstandard gilt. Angestoßen durch die präklinische Bildgebung mit unserem Radiotracer für den Melanin-konzentrierenden Hormonrezeptor-1 (MCHR1), [<sup>11</sup>C]SNAP-7941, die eine Aufnahme des Radiotracers im BAT aufzeigte, konnte der MCHR1 im Zuge dieser Doktorarbeit erstmals im BAT von Nagetieren und Menschen nachgewiesen werden. Dieses Wissen könnte die Erforschung der Interaktion von Organen und Signalwegen bei Diabetes und Adipositas fördern, da sowohl der MCHR1 als auch BAT die Energiehomöostase regulieren. Darüber hinaus konnte gezeigt werden, dass [<sup>11</sup>C]SNAP-7941 ein geeigneter alternativer Radiotracer für die BAT-Bildgebung sein könnte.

# 1. INTRODUCTION

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## 1.1 Positron emission tomography and the indicator method

Positron emission tomography (PET) is a molecular imaging technique that allows for a non-invasive visualization and/or quantification of metabolic processes in a living organism (*functional imaging*). It requires the injection of a radiopharmaceutical, a so-called PET-tracer. PET-tracers are radioactively labelled, mostly organic molecules. Many of them aim at a defined target of interest, such as a receptor, while some do not bind to a specific target structure but are non-specifically taken up by tissues due to altered metabolic processes (e.g., enhanced lipid or glucose metabolism). As indicated by the name, PET-tracers are applied in trace amounts (pico- to nanomolar range) to not provoke unwanted pharmacological or systemic effects (1,2).

In the most elegant case, the fate of a molecule can be monitored by using a PET-tracer consisting of the very same, but radioactive, molecule. For example, at a defined position of a substance, the non-radioactive isotope carbon-12 might be exchanged to the radioisotope carbon-11. As isotopes of an element bear the same biochemical properties, this approach enables the unadulterated imaging of a metabolic process or pathway. This *indicator (tracer) method* was described for the first time by George Charles de Hevesy, who was honored with the Nobel Prize for this discovery in 1943 (3,4).

The radionuclide of a PET-tracer decays by emitting a positron. As it travels, the positron annihilates with an electron, which results in the simultaneous emission of two 511 keV annihilation-quanta in a relative 180° angle. These quanta are then coincidentally detected by a detector pair of the PET-scanner's detector ring. As the annihilation must have occurred on a line between the two detected quanta (*line of response*), the source of annihilation can be reconstructed with the help of numerous coincidences. In the end, the obtained PET-image displays the spatial distribution of the injected radioactivity over time and provides current metabolic information about a disease. Most often, PET is combined with Computed tomography (CT) to additionally acquire anatomical information (*structural imaging*). PET can further be combined with Magnetic resonance imaging (MRI), which is favorable for visualizing soft tissue, such as ligaments or the brain (5,6).

Total-body PET (TBP) devices, the new generation of PET-scanners, promise simultaneous imaging of the entire human body with higher resolution in much less time, but also a

significant dose reduction (7). Importantly, simultaneous dynamic imaging of the whole body could enable real-time visualization and quantification of inter-organ communication, revolutionizing the role of nuclear medicine in disease characterization.

## 1.2 From past to present: The history of 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucose

As the first tomographic devices could only image the brain, PET-tracer development was initially oriented towards understanding regional cerebral function (8).

In the late 1960s, the teams around Louis Sokoloff and Martin Reivich from the National Institutes of Health (NIH) and the University of Pennsylvania (UP) proved that the <sup>14</sup>C-labelled glucose analogue 2-deoxy-D-glucose (2-[<sup>14</sup>C]DG) could be used to map regional brain metabolism. Its success was based on the so-called “metabolic trapping” of 2-[<sup>14</sup>C]DG. Sokoloff and Reivich described that, in contrast to the rapid degradation of glucose, 2-[<sup>14</sup>C]DG remained intact in the tissue after its phosphorylation to 2-[<sup>14</sup>C]DG-6-phosphate, as it cannot be processed by enzymes except for glucose-6-phosphatase (G6Pase) (8,9). Due to these favorable properties, the beta-emitter was soon extensively used in *ex vivo* autoradiographic studies of animal brains analyzing physiologic or pathologic metabolic alterations (10–13). However, to conduct human studies using early PET technology, 2-DG needed to be labelled with a suitable positron-emitting nuclide. Fluorine-18 with its half-life of 109.8 min was more suitable than carbon-11 (half-life 20 min), both in terms of patient application and distribution to other facilities. Led by the chemists Alfred Wolf, Tatsuo Ido and Joanna Fowler, 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucose (2-[<sup>18</sup>F]FDG) was developed in Brookhaven National Laboratory (BNL) in collaboration with the UP and the NIH in the mid-1970s (8,14). However, the initial synthesis mode *via* electrophilic addition, using the precursor 3,4,6-tri-*O*-acetyl-D-glucal and [<sup>18</sup>F]F<sub>2</sub> gas, only yielded round 8 % (14). In parallel with the radiochemical advances at BNL, the team at the UP equipped its scanner with high-energy collimators for upcoming human studies. In August 1976, two healthy volunteers finally received the first doses of 2-[<sup>18</sup>F]FDG at the UP, revealing strong uptake in the human brain. In the following years, scanner technology was continuously improved and 2-[<sup>18</sup>F]FDG imaging was expanded to other centers and research groups (8). An important reason for the success of 2-[<sup>18</sup>F]FDG was the fact that cancer cells switch their metabolism to aerobic glycolysis instead of using oxidative phosphorylation in the mitochondria to generate adenosine triphosphate (ATP), as observed by Otto Warburg in the 1930s



(*Warburg effect*). This metabolic rewiring causes tumors to dramatically increase their glucose uptake compared to the surrounding tissue (15). Building on this, 2-[<sup>18</sup>F]FDG imaging was shown in the 1980s to predict tumor grade (16–18) as well as patient outcomes in brain tumors (18) and to perform better than CT (19,20) or MRI (20) in distinguishing recurrent tumors from radiation necrosis. Since then, the efficacy of 2-[<sup>18</sup>F]FDG for the detection and treatment monitoring of various malignancies has been demonstrated (8).

Today, 2-[<sup>18</sup>F]FDG remains the most widely used and versatile PET-tracer. In the prevailing synthesis procedure developed by Hamacher *et al.* (21), it is prepared by nucleophilic substitution, with [<sup>18</sup>F]fluoride replacing the triflate leaving group at position two of the precursor, which gives a much higher yield compared to the original synthesis route. In general, 2-[<sup>18</sup>F]FDG synthesis is highly automated and industrialized, reflecting the consistently high worldwide importance of this PET-tracer.

### 1.3 Imaging possibilities with 2-[<sup>18</sup>F]FDG

The unbroken importance of 2-[<sup>18</sup>F]FDG can largely be attributed to its versatile application. As it is taken up nonspecifically *via* glucose transporters (GLUTs), it can theoretically visualize any disease or condition resulting in aberrant glucose uptake.

2-[<sup>18</sup>F]FDG is primarily used in the detection and management of various malignancies (22,23). Common indications for tumor imaging are diverse and include, for example, discriminating between benign and malign lesions, searching for the primary tumor when metastases are detected first, staging, monitoring of treatment effects, differentiating between post-treatment tumor residuals and fibrosis or necrosis, tumor recurrence, and guiding biopsies or radiation treatment (23–28). 2-[<sup>18</sup>F]FDG uptake was shown to be of predictive significance for the prognosis of numerous different tumor types, such as hepatocellular carcinoma (29,30), lymphoma (30), prostate cancer (30), lung adenocarcinoma (31), non-small cell lung cancer (32,33), nasopharyngeal carcinoma (34), gastric cancer (35), or colorectal carcinoma (36).

Apart from tumor imaging, 2-[<sup>18</sup>F]FDG is used to assess tissue viability, such as in myocardial ischemia, where 2-[<sup>18</sup>F]FDG aids in delineating healthy from scarred tissue, or surgical and medical decision making (37). As for the brain, the tracer can measure neuronal activity and is therefore used to visualize neurodegeneration and its progression before

macrostructural changes occur (38). In this regard, 2-[<sup>18</sup>F]FDG-PET is recommended to aid in the early diagnosis of Lewy body dementia (38–40), Alzheimer's disease and frontotemporal lobar degeneration (38,39). It is also used to classify dementia subtypes (38,41). Furthermore, 2-[<sup>18</sup>F]FDG can help to discriminate Parkinson's disease from atypical parkinsonian syndromes (APS) and to classify the APS subtypes (38,42–44). As for other diseases of the brain, 2-[<sup>18</sup>F]FDG is used in the evaluation of epilepsy in adults and children. For this indication, 2-[<sup>18</sup>F]FDG is most commonly applied between seizures to identify the epileptogenic zone of focal pharmaco-resistant epilepsy pre-surgery (38,45–48).

Lastly, 2-[<sup>18</sup>F]FDG PET/CT imaging has also gained importance in the detection of inflammation and infection (49,50). Inflammation at an early stage leads to hyperemia and increased vascular permeability, resulting in enhanced perfusion and thus 2-[<sup>18</sup>F]FDG accumulation (50). At a later stage, characterized by cell migration, recruitment and proliferation, inflammatory cells also exhibit the Warburg effect (50). According to the current guideline of the European Association of Nuclear Medicine and the Society of Nuclear Medicine and Molecular Imaging, major evidence-based indications include sarcoidosis, fever of unknown origin, peripheral bone osteomyelitis, suspected spinal infection, or vasculitis (51). Other frequent applications are inflammatory bowel disease (22,52) and tuberculosis (22,53).

Conventional static 2-[<sup>18</sup>F]FDG imaging is recommended 60 min after radiotracer application, which is considered suitable for pharmacokinetic distribution (23). However, dual-time-point imaging (DTPI) and delayed-time-point imaging, where PET imaging is repeated after a certain amount of time or performed later than usual, respectively, have been introduced. The goal of these approaches is to reduce false-positive findings and enhance the tumor-to-background ratio. Several studies have shown that in general 2-[<sup>18</sup>F]FDG uptake tends to increase over time in tumor lesions, while wash-out or stable values are observed in healthy organs (54–56) and sites of inflammation (54,55,57,58). In literature, this phenomenon has been on the one hand attributed to the tumors' higher proliferation rate (56,59) as well as their higher GLUT1 and hexokinase (HK) II expression (60), simply resulting in higher "glucose craving" compared to healthy tissue throughout the observed time-period. On the other hand, G6Pase levels tend to be higher in healthy tissue (56,61) and in inflammatory cells (62), when compared to tumors, leading to higher tracer efflux over time. Importantly, also the background activity is significantly reduced at

later time points due to the advanced clearance from the blood pool and urinary tract (63). Overall, DTPI or delayed-time-point imaging can be a powerful tool to increase sensitivity and specificity of 2-[<sup>18</sup>F]FDG imaging (62,63). At the same time, some studies applying DTPI have revealed overlapping 2-[<sup>18</sup>F]FDG uptake patterns for benign and malign tissue (64,65), contributing to the debate on the appropriate use of DTPI.

#### 1.4 Evaluation and quantification of 2-[<sup>18</sup>F]FDG imaging

2-[<sup>18</sup>F]FDG PET images can be analyzed qualitatively by visual inspection, semi-quantitatively and quantitatively (66). The most common approach in clinical routine is to determine the standardized uptake value (SUV) (66,67). The SUV is a semi-quantitative parameter, usually defined by the formula *tissue activity concentration/(injected dose/body mass)*, while definitions are slightly varying (66–68). For determining *tissue activity concentration*, a two- or three-dimensional region of interest (ROI), in the latter case also called volume of interest, is drawn around the respective area of interest (e.g., the tumor) at a single time point (*static imaging*) (66,67). According to the formula, the radioactivity concentration in the ROI is normalized to the total concentration in the body. While the SUV is easy to apply, it is a highly simplified parameter that is influenced by a variety of biological, technical, and physical factors, e.g., the time between tracer application and observation, the uptake by other tissues and the location or dimensions of the ROI (68,69). Furthermore, this semi-quantitative approach cannot distinguish between non-metabolized and metabolized tracer and does not consider plasma dynamics (70).

Most commonly, the SUV is reported as SUV<sub>max</sub> or SUV<sub>mean</sub> (67). The SUV<sub>max</sub> represents the highest voxel value of the respective ROI and is therefore independent from ROI size (given that the highest voxel value is still included in the ROI) (67). However, it is sensitive to noise (71) and might overestimate the metabolic activity in a region (70). In contrast, the SUV<sub>mean</sub>, which is less sensitive to noise, averages information from multiple voxels and therefore depends on which voxels are included in the analysis (67,72).

2-[<sup>18</sup>F]FDG PET can further be absolutely quantified. However, this more elaborate approach requires dynamic scanning, the assessment of the plasma time-activity curve (to define the *input function*, (IF)) and pharmacokinetic modeling (70).

For generating the IF, arterial blood or arterialized venous blood sampling is performed at several time points to evaluate the plasma-to-blood radioactivity ratio over time (*arterial input function*, AIF) (73). However, blood sampling is invasive and time-consuming and thus inapplicable for clinical routine. Among other approaches, it has been proposed to derive the IF directly from PET images, creating the so-called *image-derived input function* (IDIF). In humans, this has been successfully applied by defining ROIs in bigger blood pools like the femoral arteries (74), the aorta (75), or the heart (76). The carotid arteries (CA) are most often used for brain studies, as the other structures are not in the field of view. However, these vessels are comparatively small, and one must correct for spill-in and spill-out effects (activity from the surroundings spills into the vessels vs. activity within the CA spills over into the surroundings) (77). For extracting the IDIF from CA, studies have either used data from PET (78–80), PET and MRI (both standalone) (81–83), or combined PET/MRI (84–86) devices.

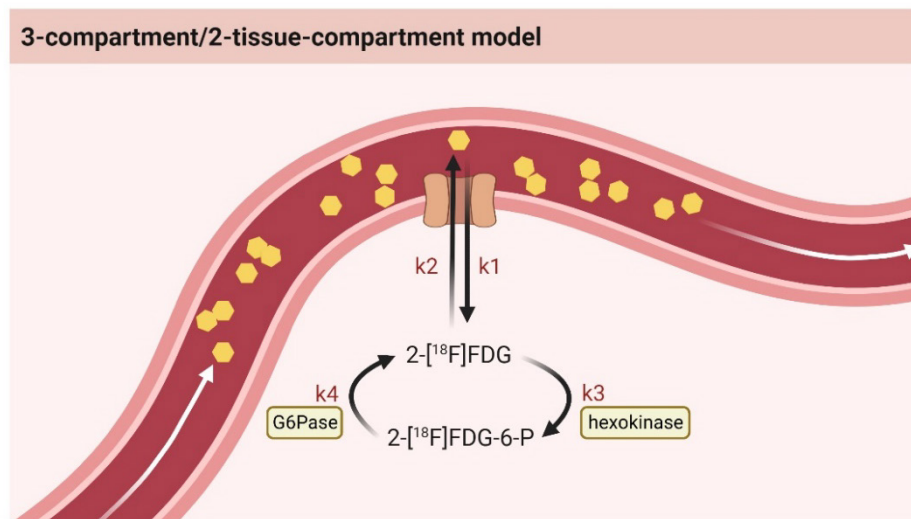
Regarding kinetic modeling, tracer kinetics can be derived from ROIs or, more elaborately, from voxels of dynamic PET data. In the first approach, a kinetic model is fitted to the time-activity curve of a pre-defined ROI, whereas in the second approach, a voxel-wise calculation is performed (*parametric imaging*), allowing to also assess spatial heterogeneity (70,87,88). Mathematical models are used to describe tracer distribution within different so-called *compartments* of the body (e.g., blood pool, specific tissues), as well as different metabolic states of the tracer. The number of interconnected compartments depends on the chemical, physiological, and biological properties of the tracer (89–91). Rate constants (abbreviated with  $k$ ) describe the exchange of the tracer between compartments and the action of enzymes involved in tracer metabolism (68).

Kinetic modeling of radiotracers is in general based on numerous conditions (68):

- 1.) The concentration of the tracer is negligible and cannot influence physiologic processes.
- 2.) The rate constants are independent of the concentration. Therefore, transport of the tracer follows first order kinetics.
- 3.) The tracer is readily distributed homogeneously within each compartment.
- 4.) Metabolic processes or conditions are in a steady state during the measurement.

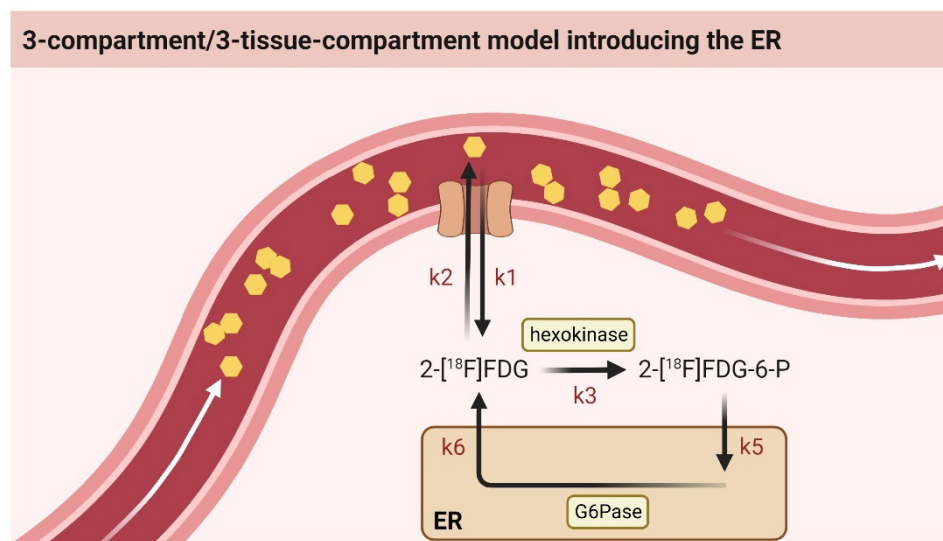
- 5.) The arterial tracer concentration is also an acceptable approximation for capillary concentration and tracer bound in blood is disregarded.

In the case of 2-[<sup>18</sup>F]FDG, the oldest model is an irreversible 3-compartment/2-tissue-compartment model with three rate constants, originally developed by Sokoloff *et al.* for rat brain studies using 2-[<sup>14</sup>C]DG (9). While there are only two physically separated compartments, namely the blood pool and tissue, the intracellular, phosphorylated pool is sometimes referred to as third compartment (92). The original model was later modified for human studies by Phelps *et al.* (92) and Reivich *et al.* (93). According to the currently valid model, unmodified 2-[<sup>18</sup>F]FDG from the blood pool enters and leaves the tissue *via* GLUTs ( $k_1$  in,  $k_2$  out). Inside the tissue, 2-[<sup>18</sup>F]FDG gets phosphorylated to 2-[<sup>18</sup>F]FDG-6-phosphate (2-[<sup>18</sup>F]FDG-6-P) by HK ( $k_3$ ) and can be dephosphorylated again by G6Pase ( $k_4$ ). In the original rat brain model by Sokoloff,  $k_4$  is set to zero, describing irreversible trapping of 2-[<sup>18</sup>F]FDG-6-P, while  $k_4$  is calculated in Phelps' model for human brain (92) (Figure 1). To date,  $k_4$  is considered slow and is usually set zero (94), especially if the acquisition time is below 40 (92) or 60 min (95). Still, significant dephosphorylation has been described for the liver, an organ with particularly high G6Pase expression (94,96,97), as well as for other organs and tumors (98).



**Figure 1:** 3-compartment/2-tissue-compartment model by Phelps *et al.* (92) based on Sokoloff *et al.* (9). Adapted from “Tumor Cell Metastasis”, by BioRender.com (2023). Retrieved from <https://app.biorender.com/biorender-templates>. G6Pase = glucose-6-phosphatase.

Recently, a 3-compartment/3-tissue-compartment model has been suggested, including the endoplasmic reticulum (ER) as physically separated, intracellular compartment (99,100). As G6Pase is an enzyme of the ER (101,102), dephosphorylation happens only after 2-[ $^{18}\text{F}$ ]FDG-6-P is transported into the ER, which is also suggested to be the main site of tracer accumulation (100,103,104). Hence, this model adds the rate constants  $k_5$  and  $k_6$ , which describe the influx of 2-[ $^{18}\text{F}$ ]FDG-6-P into the ER and the outflow of dephosphorylated 2-[ $^{18}\text{F}$ ]FDG into the cytosol, while  $k_4$  is omitted (Figure 2) (99).



**Figure 2:** Proposed 3-compartment/3-tissue-compartment model based on (99,100).

Adapted from “Tumor Cell Metastasis”, by BioRender.com (2023). Retrieved from <https://app.biorender.com/biorender-templates>. ER = endoplasmic reticulum, G6Pase = glucose-6-phosphatase.

However, while it provides underlying physiological information, compartmental analysis is computationally intensive (105). Thus, based on the irreversible model, the most used, simplified method to quantify irreversible 2-[ $^{18}\text{F}$ ]FDG uptake is a multiple-time graphical analysis, the so-called *Patlak plot* (106) (or *Gjedde-Patlak plot* (107)). In graphical analyses, compartmental equations are linearized, which makes the kinetic analysis faster and less sensitive to noise (105). In short, the concentration curves of the arterial plasma and the region of interest are combined to one curve that reaches linearity when there is an equilibrium between plasma and tissue. Fitting a line to the plotted data in the graph allows for the determination of the slope, which reflects the net influx rate of the radiotracer ( $K_i$ ) (106).

Furthermore, modifications of the standard Patlak plot have been suggested (98,108). For example, to account for the fact that the irreversible model is not always applicable, a Patlak plot extended by the net efflux rate constant  $k_{loss}$  has been developed (98).

In general, 2-[<sup>18</sup>F]FDG is transported and phosphorylated analogously to glucose, but at a different rate. Thus, to determine the glucose metabolic rate ( $MR_{gluc}$ ), the 2-[<sup>18</sup>F]FDG metabolic rate ( $MR_{FDG}$ ) needs to be corrected. This correction is done using the so-called lumped constant (LC), consisting of 6 constants itself (109,110). The LC originally developed by Sokoloff *et al.* that allows quantification of glucose demand using 2-[<sup>14</sup>C]DG is defined as (9,109):

$$LC = \frac{\lambda * K_m * V_{max}'}{\varphi * V_{max} * K_m'}$$

$\lambda$ ...Ratio of the distribution volume between 2-[<sup>14</sup>C]DG and glucose

$\varphi$ ...Fraction of glucose introduced into glycolysis

$K_m$ ...Michaelis-Menten constant for glucose phosphorylation ( $K_m' =$  for 2-[<sup>14</sup>C]DG)

$V_{max}$ ...Maximum velocity of glucose phosphorylation ( $V_{max}' =$  for 2-[<sup>14</sup>C]DG)

To obtain  $MR_{gluc}$ , the calculated  $MR_{FDG}$  is then divided by the LC (109). While the LC can be determined from dynamic PET data, one can also use LC values that have been reported over the years for different organs under various conditions. For example, the recommended brain LC is 0.65 applying the irreversible model with three rate constants or Patlak plot without  $k_{loss}$ , and 0.81 using four rate constants or Patlak plot with  $k_{loss}$  (110,111). However, there also exist other formulas that are independent of the used model. Importantly, since the LC approach is based on kinetics of competitive substrates, it can be used as a correction factor for all tracers competing with natural metabolic substrates (110).

## 1.5 New aspects and limitations of 2-[<sup>18</sup>F]FDG imaging

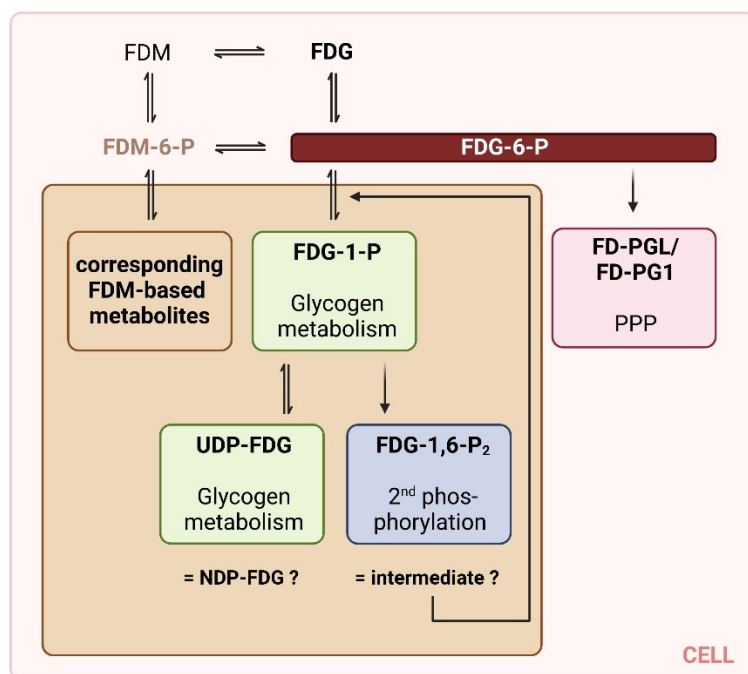
### 1.5.1 Metabolism of 2-[<sup>18</sup>F]FDG

Regarding tracer metabolism, the above described kinetic models for quantifying 2-[<sup>18</sup>F]FDG PET only consider phosphorylation of 2-[<sup>18</sup>F]FDG to 2-[<sup>18</sup>F]FDG-6-P. In contrast to

glucose, 2-[ $^{18}\text{F}$ ]FDG-6-P cannot be introduced into glycolysis, because the fluoride at position 2 prevents the conversion to fructose-6-phosphate by phosphoglucose isomerase (PGI) (9,112). However, glucose metabolism after glucose-6-phosphate (G6P) formation is not unidirectional and restricted to glycolysis. G6P is a key intermediate that is also introduced into the pentose phosphate pathway (PPP) or glycogen synthesis to enable various cellular reactions or glucose storage, respectively. Over the years, some publications have shown that while escaping glycolysis, 2-[ $^{18}\text{F}$ ]FDG can also be metabolized beyond 2-[ $^{18}\text{F}$ ]FDG-6-P. A lot of research groups have applied non-radioactive 2-FDG and  $^{19}\text{F}$  nuclear magnetic resonance spectroscopy ( $^{19}\text{F}$  NMR) to follow its metabolism. Besides studies in e.g., plants and yeast (113,114), which will not be discussed in detail here, its intracellular fate has been investigated in tissues and tumors of mice and rats (115–125).  $^{19}\text{F}$  NMR allows an evaluation at later time points, which is not feasible with 2-[ $^{18}\text{F}$ ]FDG. However, 100-400 mg/kg 2-FDG were used in the latter  $^{19}\text{F}$  NMR studies, which is approximately 100.000 times more than what is usually applied in 2-[ $^{18}\text{F}$ ]FDG PET studies (126). As 2-DG-6-phosphate can inhibit glycolysis (112,127,128) such high amounts of 2-FDG could itself influence metabolism and these studies might not be transposable to 2-[ $^{18}\text{F}$ ]FDG. Still,  $^{19}\text{F}$  NMR has revealed the formation of several metabolites other than 2-FDG-6-P. For example, fructose-6-phosphate cannot be formed from 2-FDG-6-P, but it was shown that PGI can produce 2-fluoro-2-deoxy-D-mannose-6-phosphate (2-FDM-6-P) (117,125,129). Although never confirmed, it was then suggested that measuring 2-FDM-6-P might be used to determine the glycolytic activity of tissues (117). Unphosphorylated 2-FDG can also be metabolized to 2-FDM (125), which in turn is also a substrate for HK (130). Main additional metabolites detected include, e.g., 2-fluoro-2-deoxy-D-glucose-1-phosphate (2-FDG-1-P) and 2-fluoro-2-deoxy-D-glucose-1,6-bisphosphate (2-FDG-1,6-P<sub>2</sub>) (115,116,119), as well as 2-FDG bound to uridine diphosphate (UDP-2-FDG) (116,117). Instead of specific UDP-2-FDG, nucleoside-bound 2-FDG (NDP-2-FDG) has sometimes been described (115,116,118). Corresponding metabolites beyond FDM-6-P have also been detected (115,116,118,119). Furthermore, the PPP metabolite 2-fluoro-2-deoxy-6-phosphogluconolactone (2-FD-PGL) and its hydrolyzation product 2-fluoro-2-deoxy-6-phosphogluconate (2-FD-PG1) have been described (120,121). Besides these frequently detected metabolites, Nakada *et al.* also reported the formation of the catabolite 2-fluoro-2-deoxy-glycerol in rat brain (123).



A lot of studies have also looked into the metabolism of the radiotracer 2- $^{18}\text{F}$ FDG in various animals and tumor models, or specific cells, confirming the main metabolites observed in  $^{19}\text{F}$  NMR studies (126,131–141). The radiometabolites 2- $^{18}\text{F}$ FDG-1-P and 2- $^{18}\text{F}$ FDG-1,6-P<sub>2</sub> were successfully produced through the addition of phosphoglucomutase (PGM) (132,135), a key enzyme of glycogen metabolism (142). However, it is unclear whether 2- $^{18}\text{F}$ FDG-1,6-P<sub>2</sub> is formed as an intermediate during the reaction with PGM or results from a second phosphorylation by HK after 2- $^{18}\text{F}$ FDG-1-P formation (132,143). UDP-glucose pyrophosphorylase, which is also involved in glycogen metabolism (142), was shown to be the enzyme forming UDP-2- $^{18}\text{F}$ FDG (144). Additionally, Fedders *et al.* proved the formation of the radiometabolites 2-fluoro-2-deoxy-D-[U- $^{14}\text{C}$ ]galactose and 2-fluoro-2-deoxy-D-[U- $^{14}\text{C}$ ]glucuronic acid *via* the enzymes UDP-glucose-4-epimerase and UDP-glucose dehydrogenase, respectively (135), and reported the incorporation of 2-fluoro-2-deoxy-D-[U- $^{14}\text{C}$ ]glucose into proteoglycans of human chondrocytes (139). Lastly, 2- $^{18}\text{F}$ FD-PGL and/or 2- $^{18}\text{F}$ FD-PG1 were successfully synthesized using the PPP enzyme glucose-6-phosphate dehydrogenase (G6PD) (132,140). Figure 3 gives an overview of the most often described (radio)metabolites using either 2-FDG or 2- $^{18}\text{F}$ FDG.



**Figure 3:** Schematic representation of the most commonly detected (radio)metabolites of 2-FDG or 2- $^{18}\text{F}$ FDG to date. The correct nomenclature has been omitted. Created with BioRender.com. FDM = 2-fluoro-2-deoxy-D-mannose, FDM-6-P = 2-fluoro-2-deoxy-D-mannose-6-phosphate, FDG-1-P = 2-fluoro-2-deoxy-D-glucose-1-phosphate, FDG-1,6-P<sub>2</sub> =

2-fluoro-2-deoxy-D-glucose-1,6-bisphosphate, FD-PGL = 2-fluoro-2-deoxy-6-phosphogluconolactone, FD-PG1 = 2-fluoro-2-deoxy-6-phosphogluconate, NDP-FDG = nucleoside-bound 2-FDG, UDP-FDG = 2-FDG bound to uridine diphosphate, PPP = pentose phosphate pathway.

Except for the studies by Fedders *et al.* (135,139), the mentioned publications specifically using the radiotracer reported the formation of single or few radiometabolites. Suolinna *et al.* detected up to 4 % of “nucleotide-derived 2-[<sup>18</sup>F]FDG” as only radiometabolite beyond 2-[<sup>18</sup>F]FDG-6-P in several organs and tumors of anesthetized rats after 45 min (140). Presumably the same radiometabolite, named UDP-2-[<sup>18</sup>F]FDG in this case, was detected by Haaparanta *et al.* in various organs and tumors of another rat tumor model (136). In the recent publication by Rokka *et al.*, a different radiometabolite, namely 2-[<sup>18</sup>F]FD-PGL, was detected in the liver of conscious mice and rats (132).

To date, little is known about the relevance of 2-FDG or 2-[<sup>18</sup>F]FDG metabolism for the tracer's uptake and accumulation, or how its metabolism can be used as readout for metabolic response to, e.g., treatment. In the above-mentioned study by Haaparanta *et al.*, an attempt was made to correlate tumor growth and treatment effects with the uptake and metabolism of 2-[<sup>18</sup>F]FDG (136). However, 2-[<sup>18</sup>F]FDG-6-P and UDP-2-[<sup>18</sup>F]FDG were summed up to one metabolized compartment, therefore the effects of metabolism beyond 2-[<sup>18</sup>F]FDG-6-P were not evaluated. In a <sup>19</sup>F NMR study with mice, the conversion to 2-FDM and 2-FDM-based phosphorylated metabolites was predictive of the treatment response to 5-fluorouracil (119). Furthermore, the conversion between the 2-FDG and 2-FDM group was proposed as a readout for brain activity (117). Another study with pigs showed substantial formation of 2-[<sup>18</sup>F]FD-PGL/PG1 in the liver, especially from 90 min on, suggesting to extend the pharmacokinetic model in prolonged studies (126).

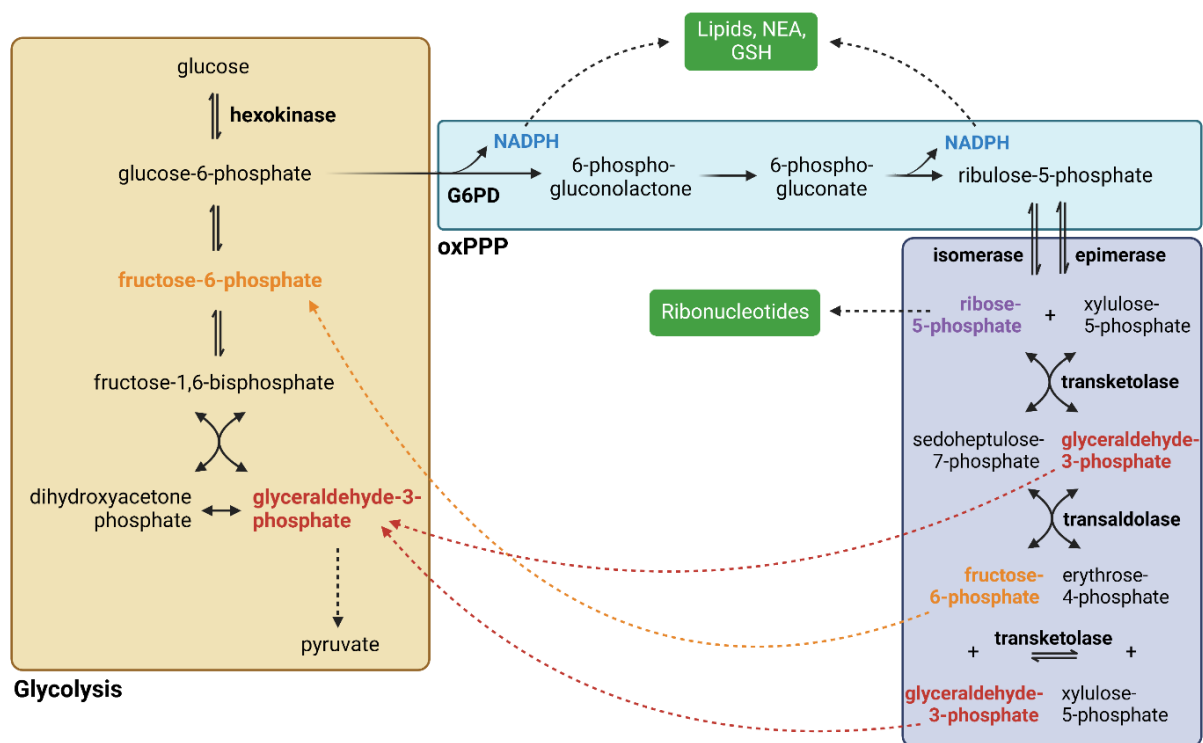
Importantly it was recently discovered that 2-[<sup>18</sup>F]FDG accumulation could be a function of hexose-6-phosphate dehydrogenase (H6PD), an enzyme of the PPP located in the ER (99,100,145,146). This indicates that 2-[<sup>18</sup>F]FDG metabolism beyond 2-[<sup>18</sup>F]FDG-6-P or at least specific parts of it could indeed affect 2-[<sup>18</sup>F]FDG imaging.

#### 1.5.1.1 The pentose phosphate pathway

The PPP is a carbohydrate metabolic pathway that is tightly connected to glycolysis. It involves an oxidative, irreversible branch (*oxPPP*) and a non-oxidative branch consisting of reversible enzymatic reactions (*non-oxPPP*) (147,148).

Like the glycolytic pathway, the *oxPPP* uses glucose-6-phosphate (G6P) as a starting substrate and thus provides an alternate metabolic glucose pathway generating reduced nicotinamide-adenine dinucleotide phosphate (NADPH) instead of ATP. The first and rate-limiting step is the conversion of G6P to 6-phosphogluconolactone by the enzyme G6PD (147). It is generally believed that G6PD does only recognize G6P (146,149), although it was also reported that this enzyme could process 2-[<sup>18</sup>F]FDG-6-P *in vitro* (140). The metabolite 6-phosphogluconolactone is then hydrolyzed to 6-phosphogluconate by 6-phosphogluconolactonase (6PGL) and subsequently converted to ribulose-5-phosphate by the enzyme 6-phosphogluconate dehydrogenase. Ribulose-5-phosphate is lastly converted to ribose-5-phosphate, which serves as a precursor for ribonucleotide synthesis (147,148). Furthermore, the catalyzed reactions produce NADPH, which is required for other vital processes like the synthesis of fatty acids, non-essential amino acids, and reduced glutathione (147), which plays a key role in reducing reactive oxygen species (150).

In contrast to the *oxPPP*, the *non-oxPPP* involves a series of reversible reactions producing several carbohydrate intermediates including the glycolytic molecules fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P) (147,148). Due to this reversible branch, the PPP can easily adapt to the present metabolic demands of the cell. For example, if NADPH is needed to maintain redox homeostasis, the *non-oxPPP* can be directed towards F6P and G3P synthesis, thus enabling the re-formation of G6P *via* the glycolytic pathway, which in turn serves the formation of NADPH by the *oxPPP* (147). On the other hand, if cell growth is prioritized, both the *oxPPP* and the *non-oxPPP* can be directed towards pentose phosphate formation (147,151,152). A scheme of the PPP and its interconnection with glycolysis is given in Figure 4.



**Figure 4:** Scheme of the PPP and its metabolite exchange with glycolysis. Created with BioRender.com. G6PD = glucose-6-phosphate dehydrogenase, GSH = glutathione, NEA = non-essential amino acids, non-oxPPP/oxPPP = non-oxidative/oxidative pentose phosphate pathway.

It has been reported that in general, the flux through PPP in healthy organs is highest in those carrying out lipid- and steroid-synthesis, such as the liver, adrenal glands, gonads, or adipose tissue. Still, basal PPP activity greatly varies between cells and tissues, as many conditions interfere with the  $\text{NADP}^+/\text{NADPH}$  ratio (153).

From the multiple and central functions of PPP described above, it is evident that this pathway is also highly regulated in tumors and various other diseases, such as neurodegenerative diseases or diabetes (147). For example, G6PD overexpression is associated with poor prognosis in hepatocellular carcinoma (154), breast (155,156) and colorectal cancer (157). Furthermore, numerous tumor suppressor proteins and oncoproteins such as the tumor suppressor p53, the TP53-induced glycolysis and apoptosis regulator, oncogenic Ras, or the mammalian target of rapamycin complex 1 (mTOR) interfere with the PPP (147).

While the described PPP enzymes are located in the cytosol of the cell, there exists another, less considered PPP within the ER, represented by H6PD. In contrary to the well-studied,

sex-linked G6PD, H6PD is autosomal and capable of oxidizing other hexoses in addition to glucose (149,158). Furthermore, H6PD itself also has 6PGL function, thus accounting for both step one and two of the oxPPP (159). In contrast to what has been postulated in earlier years (160), recent *in vitro* data suggest that H6PD significantly contributes to the overall cellular oxPPP rate. Both G6PD and H6PD silencing led to a comparable decrease in the cellular pool of NADPH, PPP intermediates and D-ribose (146). Analogous to G6PD, H6PD also contributes to cellular proliferation and migration (146,161). Due to its location within the ER, knockdown of H6PD has additional implications compared to an interference with G6PD expression. For example, H6PD silencing affects the protein-folding machinery in the ER by attenuating the unfolded-protein response pathway and increases intraluminal calcium levels (161). In a recent study, the ER-PPP rather than cytosolic G6PD was shown to be the major determinant of cancer aggressiveness in a triple-negative breast cancer cell line (104). Even though the PPP has become increasingly interesting for tumor metabolism research, the interplay of G6PD and H6PD regarding tumor development and progression has not yet been fully elucidated. Also, open access human proteome data suggest that G6PD and H6PD cannot be strictly assigned to the cytoplasm and the ER, respectively, but that both enzymes could be expressed in either compartment (162,163).

Naturally, the PPP represents an attractive target for tumor therapy. While many different inhibitors interfering with the individual enzymes of the PPP have been developed, no efficient PPP inhibitor has made it to the clinic yet (164). As for the oxPPP, the steroid dehydroepiandrosterone (DHEA) is by far the most used and prominent inhibitor, reducing cellular NADPH and increasing oxidative stress (165,166). Importantly, DHEA is a precursor of androgen and estrogen derivatives and also acts as a hormone itself (167,168), raising the question of whether its inhibitory effect on G6PD is paramount. Furthermore, DHEA is heavily metabolized in the liver (169,170) and the metabolite DHEA sulfate is a much less potent G6PD inhibitor (171), thus high oral doses would be required.

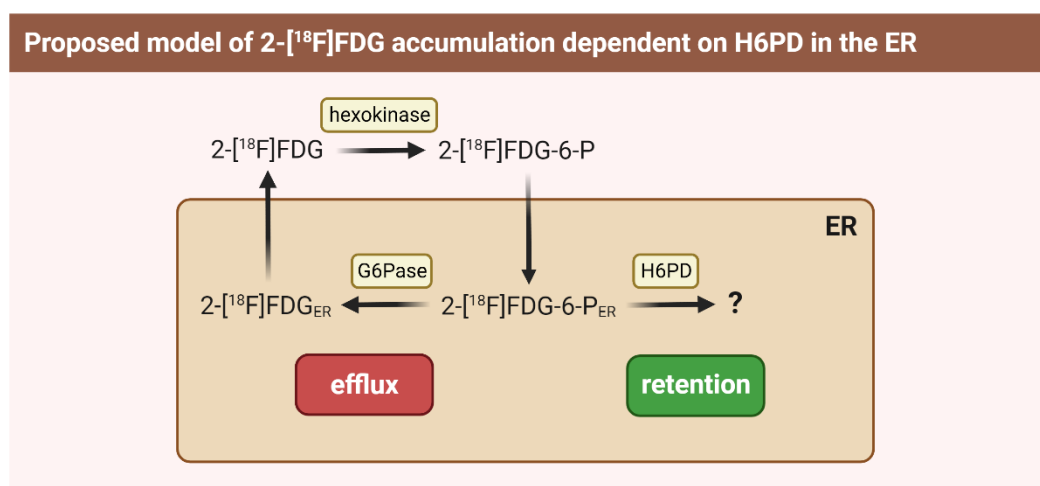
Even though DHEA has been extensively used over the years, the direct inhibitory effect of DHEA on G6PD has recently been questioned. Ghergurovich *et al.* have shown that DHEA inhibits the purified enzyme but fails to inhibit G6PD when applied in cell assays, suggesting that oxidative stress is induced independent from G6PD engagement. Therefore, they have introduced a new, non-steroidal G6PD inhibitor, G6PDi-1 (166).

Carbenoxolone has been suggested as indirect inhibitor of H6PD and thus ER-PPP activity (172), as it inhibits 11 $\beta$ -hydroxysteroid dehydrogenase type 1 in the ER and thereby limits NADP<sup>+</sup> supply (173,174).

### 1.5.2 Molecular mechanisms of 2-[<sup>18</sup>F]FDG accumulation: What do we visualize?

2-[<sup>18</sup>F]FDG uptake was and is considered a marker for glucose demand. Many publications over the years have shown 2-[<sup>18</sup>F]FDG accumulation to highly correlate with, e.g., the expression of GLUT1 (175–178) and HK II (176,179,180). However, recent findings have supplemented or challenged these accepted requirements for 2-[<sup>18</sup>F]FDG accumulation, as well as its use as a marker for glucose demand.

For example, it has been described that 2-[<sup>18</sup>F]FDG accumulation does not (only) depend on GLUT transport and phosphorylation by HK, but on the catalytic function of H6PD in the ER (100,145,172). Cossu *et al.* have proposed that H6PD competes with G6Pase for 2-[<sup>18</sup>F]FDG-6-P, hence, higher H6PD expression or activity should increase intracellular 2-[<sup>18</sup>F]FDG retention (Figure 5) (100). Metformin treatment that impairs H6PD function has been shown to reduce 2-[<sup>18</sup>F]FDG uptake while increasing glucose uptake, raising the question of whether 2-[<sup>18</sup>F]FDG uptake mimics glucose demand under all circumstances (172).



**Figure 5:** Schematic illustration of the proposed relationship between 2-[<sup>18</sup>F]FDG accumulation and H6PD activity. Based on Cossu *et al.* (100) and created with BioRender.com. ER = endoplasmic reticulum, G6Pase = glucose-6-phosphatase, H6PD = hexose-6-phosphate dehydrogenase.

Others have raised the question of whether 2-[<sup>18</sup>F]FDG actually visualizes the Warburg effect. In general, it is widely accepted that an acidic environment as present in the tumor core promotes lactate utilization and oxidative phosphorylation, while proliferating cancer cells on the outskirts are reprogrammed towards aerobic glycolysis and thus preferably use glucose (181). However, in a study by Kernstine *et al.* with non-small cell lung cancer patients undergoing an 2-[<sup>18</sup>F]FDG scan and a <sup>13</sup>C-glucose infusion, the SUV<sub>max</sub> of the lesion did not correlate with <sup>13</sup>C-enrichment in glycolytic metabolites (182). The authors suggested that since SUV<sub>max</sub> correlated with the proliferation index instead, 2-[<sup>18</sup>F]FDG imaging might depict the use of glucose in other pathways than glycolysis. This lack of correlation between evident glycolytic metabolism and 2-[<sup>18</sup>F]FDG accumulation was also described elsewhere (183). In another publication it was suggested that 2-[<sup>18</sup>F]FDG accumulation is rather a marker for anaerobic glycolysis (*Pasteur effect*) than for aerobic glycolysis (184).

In summary, even though 2-[<sup>18</sup>F]FDG has been the most used PET-tracer worldwide for the past decades, the molecular requirements or cellular metabolic states responsible for its uptake and intracellular accumulation are not yet fully understood.

### 1.5.3 General limitations of 2-[<sup>18</sup>F]FDG (tumor) imaging

While there are many more parameters affecting 2-[<sup>18</sup>F]FDG imaging, such as limited spatial resolution, or image artefacts (e.g., due to motion), we focus on those directly related to the physicochemical characteristics of the tracer. For example, due to its renal excretion, the value of 2-[<sup>18</sup>F]FDG PET in urological oncology, like bladder, adrenal gland, prostate or kidney cancer is limited, except for specific indications (185). The non-specific uptake mode of 2-[<sup>18</sup>F]FDG, while enabling various applications, is also its greatest limitation. Glucose uptake is ubiquitous and influenced by a plethora of parameters, potentially limiting 2-[<sup>18</sup>F]FDG PET-scan performance. To reduce physiologic uptake into the skeletal muscles, which depict high glucose uptake upon insulin release or physical activity (186), the guidelines for 2-[<sup>18</sup>F]FDG PET specify fasting for at least 4 h before the scan (for non-diabetic patients), as well as avoiding strenuous exercise for at least 6 h before the scan (23). During the uptake period, patients should not move and be kept warm so as not to provoke uptake into muscle or brown adipose tissue (BAT) (23), the latter being activated by cold temperatures (187). Importantly, glucose is the main energy substrate of the brain.

Therefore, healthy brain in general shows high physiologic baseline 2-[<sup>18</sup>F]FDG uptake (188–190). In gliomas, the most common brain tumors, 2-[<sup>18</sup>F]FDG PET is therefore largely outperformed by radiolabeled amino acid tracers (190–193). However, glucose loading could enhance the efficacy of 2-[<sup>18</sup>F]FDG PET/CT for glioma characterization and tumor delineation (194).

False-positive 2-[<sup>18</sup>F]FDG PET findings may be due to benign tumors, infection, or inflammation. In this context, interventions like chemo- or radiotherapy, or surgery can also non-specifically alter 2-[<sup>18</sup>F]FDG uptake (195). Even though the differentiation between inflammatory and malignant lesions by DTPI is at least partially possible as described above, this approach is not included in daily clinical practice. Furthermore, the question of how the tumor microenvironment contributes to the overall uptake of 2-[<sup>18</sup>F]FDG has been debated for decades. Some publications have reported that 2-[<sup>18</sup>F]FDG rather accumulates in macrophages or granulation tissue than in malignant tissue itself (133,142). Also, cancer associated fibroblasts seem to greatly contribute to the amount of 2-[<sup>18</sup>F]FDG uptake (143).

As stated before, 2-[<sup>18</sup>F]FDG uptake is said to largely correlate with tumor aggressiveness, hence the tracer can fail to detect slow-growing, well-differentiated malignancies (196–200). Naturally, lesions with high G6Pase expression, like some hepatocellular carcinomas (201), can also be less 2-[<sup>18</sup>F]FDG-avid.

#### 1.5.4 Brown adipose tissue: Characteristics and imaging

Since it led to the discovery of active BAT in adult humans in the early 2000s, 2-[<sup>18</sup>F]FDG PET/CT imaging has been the most widely used technique to assess the activity of BAT in humans and rodents (202–204). BAT is a major site for non-shivering thermogenesis (NST), a form of adaptive thermogenesis. This ability to generate heat in response to external stimuli like cold represents an important evolutionary development (187,205). In contrast to white adipose tissue (WAT), BAT dissipates energy in the form of heat instead of storing it (187,206). Especially in rodents, classical brown adipocytes display a higher mitochondrial density, a high expression of uncoupling protein 1 (UCP1) and a multilocular phenotype when compared to WAT (Figure 6) (207,208).

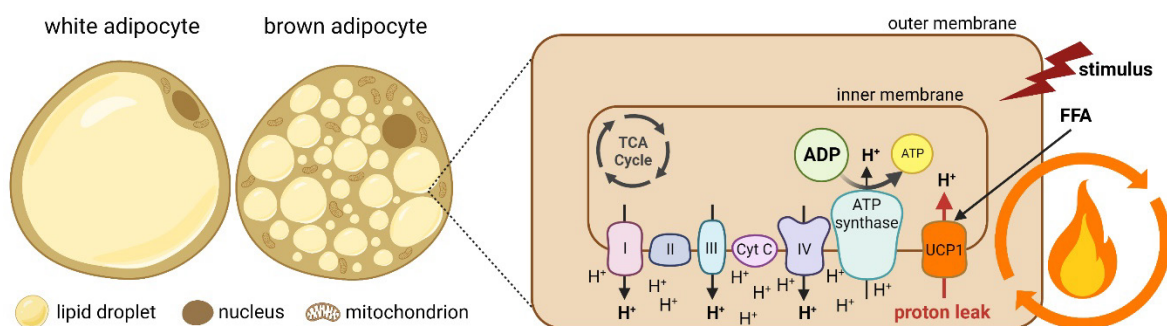
In addition to white and brown adipocytes, BAT-like cells, termed *beige* or *brite adipocytes*, have been described in WAT depots (206–209). In rodents, the molecular differences



between beige and brown adipocytes are distinct, as beige adipocytes derive from white adipocytes whereas brown adipocytes originate from dermomyotomal precursors. They display a very low baseline UCP1 expression but acquire a BAT-like phenotype with thermogenic potential upon activation, a process termed *browning* (209). Although human BAT is morphologically more similar to rodent beige adipocytes, it still appears to be most comparable to classical rodent BAT at the molecular level, as demonstrated with “physiologically humanized” mice (208).

In rodents, “classical” BAT is located in the interscapular region, but other BAT sites (e.g., perirenal, axillary, supraclavicular) have also been described (210). In humans, the expression of BAT was long thought to be restricted to infants before studies finally revealed the existence of BAT in adults, preferably in the neck and supraclavicular area (211–213).

In detail, BAT is activated *via* norepinephrine binding to adrenergic receptors triggered by cold exposure or other stimuli such as thyroid hormones. Activation *via*  $\beta$ 3-adrenoceptors (ADRB3) represents the most important pathway in rodents. This adrenergic stimulation induces lipolysis, and free fatty acids (FFA) in turn activate UCP1 located in the inner mitochondrial membrane (187). The respiratory chain in the mitochondria oxidizes substrates and thereby pumps protons into the intermembrane space. The ensuing electrochemical gradient is usually used to synthesize ATP. When UCP1 is activated, protons can be transported back passively across the inner mitochondrial membrane, creating a proton leak. Thus, active UCP1 uncouples fatty acid oxidation from ATP synthesis, resulting in the generation of heat (Figure 6) (187,214,215). This process secondarily leads to an increased uptake of circulating substrates like FFA or glucose, laying the basis for a visualization with 2- $^{18}\text{F}$ FDG (187,216).



**Figure 6:** Morphological differences between (rodent) white and brown adipocytes and molecular mechanisms of thermogenesis. Adapted from “Electron transport chain (in mitochondria)”, by BioRender.com (2023). Retrieved from <https://app.biorender.com/biorender-templates>. FFA = free fatty acids, UCP1 = uncoupling protein 1.

In 2016, the *Brown Adipose Reporting Criteria in Imaging Studies* (BARCIST 1.0) have been published to standardize inclusion and exclusion criteria, imaging and cooling protocols and documentation for studies using 2-[<sup>18</sup>F]FDG for BAT imaging (202). Nevertheless, BAT imaging with 2-[<sup>18</sup>F]FDG does have several limitations. Firstly, based on the mechanism described above, only active BAT can be detected with 2-[<sup>18</sup>F]FDG. In addition, the primary substrate for thermogenesis is thought to be endogenous fatty acids first and circulating FFAs and triglycerides second, which is why 2-[<sup>18</sup>F]FDG imaging may underestimate active BAT (203). Secondly, 2-[<sup>18</sup>F]FDG uptake may not be a reliable indicator for thermogenic activity (203,217,218). It has been shown that glucose uptake into BAT not only occurs during active thermogenesis but also, e.g., after stimulation by insulin (187). Moreover, BAT uses glucose predominantly for lipid *de novo* synthesis (187), hence glucose uptake might be largely determined by intracellular triglyceride content (219). Glucose serves as a fuel for a plethora of physiologic processes that are not linked to thermogenesis, which is why increased 2-[<sup>18</sup>F]FDG uptake in BAT is highly unspecific in general (203). Consistent with this, a recent study indicates that UCP1 expression is not a prerequisite for 2-[<sup>18</sup>F]FDG uptake upon  $\beta$ -adrenergic stimulation. Instead, acute tracer uptake seems to be mediated by mTOR, representing a process independent of thermogenesis (218).

In addition to 2-[<sup>18</sup>F]FDG, several other PET-tracers have been proposed for BAT imaging. Among them are tracers representing other metabolic substrates, like the fatty acid tracer 14(*R,S*)-[<sup>18</sup>F]Fluoro-6-thia-heptadecanoic acid ([<sup>18</sup>F]FTHA) or [<sup>11</sup>C]acetate (measuring the oxidative activity) (220). Also, (*S,S*)-[<sup>11</sup>C]O-methylreboxetine (221) and [<sup>11</sup>C]-(-)-*m*-hydroxyephedrine (222,223), both ligands for the norepinephrine transporter, have been evaluated. Lastly, PET-tracers targeting the mitochondria, like 4-[<sup>18</sup>F]Fluorobenzyl-triphenylphosphonium (224) and PET-tracers binding to the translocator protein (225,226), can be used to image BAT. However, unlike 2-[<sup>18</sup>F]FDG, which is commercially available, the

above mentioned tracers are not applied on a large scale. Additionally, [<sup>18</sup>F]FTHA reportedly provides only little contrast between BAT and WAT (210,217).

As active BAT, dissipating calories in the form of heat, could positively influence metabolic diseases, research on BAT has been intensified during the last decades regarding obesity, diabetes, and fatty liver disease. While the full potential of BAT in the treatment of obesity remains unclear, BAT volume/activity has been shown to inversely correlate with visceral fat mass in obese individuals (227). Also, among obese patients, those with active BAT display a healthier metabolic phenotype characterized, e.g., by lower systemic inflammation and lower insulin resistance (228). Pharmacotherapy (with, e.g., sympathomimetics or thyroid hormones), browning agents and cooling protocols have been studied with the goal to increase BAT mass/activity (229). So far, cold exposure (lower room temperature or cooling-vests (220,227,228,230,231)) has been the most common approach. For example, repeated cooling for six weeks not only led to increased BAT activity, but also to decreased body fat mass (231).

After decades of research, BAT is not only considered a thermogenic fat compartment, but an organ with endocrine and paracrine function. Its so-called *BATokines* enable BAT-tissue-crosstalk and thereby influence systemic metabolism. Accordingly, BAT function is of interest to better characterize obesity and find new treatment options (232).

## 1.6 The MCH/MCHR1 system

The melanin-concentrating hormone (MCH), a cyclic polypeptide, is expressed in all groups of vertebrates and structurally identical among mammals (233). The hormone was first discovered in fish where it was found to regulate the lighting of skin color in response to their surrounding environment (234). In mammals, it acts as a neuropeptide and is primarily expressed in the lateral hypothalamus and zona incerta (235), but also in the peripheral tissues like the pancreas (236).

MCH has been shown to be a key modulator of energy homeostasis, regulating e.g., metabolic rate, food intake, and body weight (237,238). Evidently, the hormonal balance of leptin and MCH is also tightly connected. Leptin-deficient mice are not only obese and hyperphagic, but also show significantly increased MCH levels (239). Transgenic MCH-deficient mice are significantly leaner compared to wildtype mice (240), whereas MCH-overexpressing mice are hyperphagic and obese when placed on a high-fat diet (241). The

latter effect can also be observed under chronic MCH infusion of non-transgenic mice on normal (242) or moderately high-fat diet (238,243). Chronic infusion further leads to increased WAT and liver mass and reduces the mRNA expression of UCP1, acyl-CoA-oxidase, and carnitine palmitoyltransferase I, which are crucial molecules for BAT thermogenesis (238).

MCH binds to the G protein-coupled receptors MCHR1 and MCHR2 (244,245). Analogous to MCH itself, the MCHR1 shows high structural homology across species (246,247). On a molecular level, its activation leads to the stimulation of extracellular-signal-regulated kinase and phospholipase C. The latter lastly results in an enhanced intracellular accumulation of calcium. In contrast, adenylate cyclase is inhibited, reducing the production of cyclic adenosine monophosphate (248). These alterations in signaling cascades are known to stimulate changes in gene transcription and ion channel activity (249–251).

In comparison to MCHR1, little is known about the physiological role of the MCHR2, which shares only 38 % amino acid sequence identity with MCHR1 (252). The MCHR2 is a pseudogene in rodents, hence the lack of suitable animal models (253). However, one study with a transgenic MCHR1R2 mouse model has revealed that MCHR2 might partly antagonize the physiological actions of MCHR1 (253).

Given the involvement of MCH/MCHR1 in obesity and diabetes, many antagonists for the MCHR1 have been developed over the years. Among them, SNAP-7941 is a non-peptidic antagonist with excellent binding affinity ( $K_d = 0.18 \text{ nM}$ ) and a more than 1000-fold selectivity for the MCHR1 (254). [ $^{11}\text{C}$ ]SNAP-7941, a carbon-11-labelled version of this antagonist, was the first described PET-tracer targeting the MCHR1 (255–257). Although originally developed for brain imaging, [ $^{11}\text{C}$ ]SNAP-7941 seems to be a substrate of the efflux transporter p-glycoprotein (P-gp) (255,258), limiting its use to the peripherally located receptor, at least in rodents where the P-gp density at the blood brain barrier is higher (259,260).

## 2. RATIONALE FOR THE SELECTION OF MANUSCRIPTS

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Each of the five presented manuscripts deals with the possibilities, challenges and limitations of using the radiotracer 2-[<sup>18</sup>F]FDG as outlined in the introduction.

The two manuscripts “Inhibition of lipid accumulation in skeletal muscle and liver cells: A protective mechanism of bilirubin against diabetes mellitus type 2” (**manuscript 1**) and “Towards quantitative [<sup>18</sup>F]FDG-PET/MRI of the brain: Automated MR-driven calculation of an image-derived input function for the non-invasive determination of cerebral glucose metabolic rates” (**manuscript 2**) both describe new aspects in the use of 2-[<sup>18</sup>F]FDG. In **manuscript 1**, 2-[<sup>18</sup>F]FDG was utilized to study the metabolic behavior of skeletal muscle and hepatoblastoma cells in the presence of unconjugated bilirubin mimicking mild hyperbilirubinemia (Gilbert's syndrome). In **manuscript 2**, an advanced approach to extract an IDIF for absolute quantification of glucose utilization in brain was developed and compared with the AIF (gold standard).

The two manuscripts “If it works, don't touch it? A cell-based approach to studying 2-[<sup>18</sup>F]FDG metabolism” (**manuscript 3**) and “A fingerprint of 2-[<sup>18</sup>F]FDG radiometabolites - how tissue-specific metabolism beyond 2-[<sup>18</sup>F]FDG-6-phosphate could affect tracer accumulation” (**manuscript 4**) deal with 2-[<sup>18</sup>F]FDG metabolism beyond 2-[<sup>18</sup>F]FDG-6-P. **Manuscript 3** describes the metabolism and accumulation of 2-[<sup>18</sup>F]FDG in different human cancer cell lines under various conditions. **Manuscript 4** follows up on the previous work by describing 2-[<sup>18</sup>F]FDG metabolism over time in different murine organs, as well as in a translational context (cell culture versus xenograft tumors). Furthermore, effects of metabolism beyond 2-[<sup>18</sup>F]FDG-6-P on tracer accumulation in murine organs and xenograft tumors are explored. The relationship between 2-[<sup>18</sup>F]FDG metabolism and accumulation, as well as the role of a specific carbohydrate metabolic pathway, namely the PPP, are studied in detail.

The manuscript “Discovery of melanin-concentrating hormone receptor 1 in brown adipose tissue” (**manuscript 5**) deals with the limitations of 2-[<sup>18</sup>F]FDG, as it presents the MCHR1 antagonist [<sup>11</sup>C]SNAP-7941 as an alternative tracer for BAT imaging. Furthermore, in analogy to the detection of BAT in humans through 2-[<sup>18</sup>F]FDG PET/CT, it describes the discovery of the MCHR1 in BAT through  $\mu$ PET/CT imaging with [<sup>11</sup>C]SNAP-7941.

### 3. SCIENTIFIC PART

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#### 3.1 Author contributions

I herewith declare to have significantly contributed to all peer-reviewed articles included in this thesis. The following section lists the tasks I performed for each publication:

*Regarding **manuscript 1**, I designed and established the radioactive experiments, which I supervised and partially performed (all together with Theresa Balber). Furthermore, I helped with the interpretation of the results and the writing of the manuscript:*

Published: Hana CA, **Klebermass EM**, Balber T, Mitterhauser M, Quint R, Hirtl Y, Klimpke A, Somloi S, Hutz J, Sperr E, Eder P, Jašprová J, Valášková P, Vítek L, Heiss E, Wagner KH. Inhibition of Lipid Accumulation in Skeletal Muscle and Liver Cells: A Protective Mechanism of Bilirubin Against Diabetes Mellitus Type 2. Front Pharmacol. 2021 Jan 25;11:636533.

*For **manuscript 2**, I was responsible for generating the AIF data as the gold standard and revised the manuscript:*

Published: Sundar LK, Muzik O, Rischka L, Hahn A, Rausch I, Lanzenberger R, Hienert M, **Klebermass EM**, Füchsel FG, Hacker M, Pilz M, Patariaia E, Traub-Weidinger T, Beyer T. Towards quantitative [18F]FDG-PET/MRI of the brain: Automated MR-driven calculation of an image-derived input function for the non-invasive determination of cerebral glucose metabolic rates. J Cereb Blood Flow Metab. 2019 Aug;39(8):1516-1530.

*In the case of **manuscript 3**, I designed the study, partly produced the data, analyzed, and interpreted the data, and wrote the manuscript:*

Published: **Klebermass EM**, Mahmudi M, Geist BK, Pichler V, Vranka C, Balber T, Miller A, Haschemi A, Viernstein H, Rohr-Udilova N, Hacker M, Mitterhauser M. If It Works, Don't Touch It? A Cell-Based Approach to Studying 2-[18F]FDG Metabolism. Pharmaceuticals (Basel). 2021 Sep 9;14(9):910.

*For **manuscript 4**, I designed the study, partly produced, and analyzed the data, interpreted the data, and wrote the manuscript:*

Under Revision in iScience (ISCIENCE-D-23-02552): **Patronas EM**, Balber T, Miller A, Geist BK, Michligk A, Vranka C, Krisch M, Rohr-Udilova N, Haschemi A, Viernstein H, Hacker M, Mitterhauser M. A fingerprint of 2-<sup>[18F]</sup>FDG radiometabolites - how tissue-specific metabolism beyond 2-<sup>[18F]</sup>FDG-6-phosphate could affect tracer accumulation.

*In the case of **manuscript 5**, I was involved in the design of the study, partly produced, analyzed, and interpreted the data, and drafted the manuscript (together with Cécile Philippe):*

Published: Philippe C<sup>a</sup>, **Klebermass EM<sup>a</sup>**, Balber T, Kulterer OC, Zeilinger M, Egger G, Dumanic M, Herz CT, Kiefer FW, Scheuba C, Scherer T, Fürnsinn C, Vranka C, Pallitsch K, Spreitzer H, Wadsak W, Viernstein H, Hacker M, Mitterhauser M. Discovery of melanin-concentrating hormone receptor 1 in brown adipose tissue. Ann N Y Acad Sci. 2021 Jun;1494(1):70-86.

<sup>a</sup>**These authors contributed equally to the study.**

### 3.1.1 Manuscript I

Hana CA, **Klebermass EM**, Balber T, Mitterhauser M, Quint R, Hirtl Y, Klimpke A, Somloi S, Hutz J, Sperr E, Eder P, Jašprová J, Valášková P, Vitek L, Heiss E, Wagner KH. Inhibition of Lipid Accumulation in Skeletal Muscle and Liver Cells: A Protective Mechanism of Bilirubin Against Diabetes Mellitus Type 2. *Front Pharmacol.* 2021 Jan 25;11:636533.





# Inhibition of Lipid Accumulation in Skeletal Muscle and Liver Cells: A Protective Mechanism of Bilirubin Against Diabetes Mellitus Type 2

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Ectopic lipid accumulation in skeletal muscle and liver drives the pathogenesis of diabetes mellitus type 2 (DMT2). Mild hyperbilirubinaemia has been repeatedly suggested to play a role in the prevention of DMT2 and is known for its capacity to shape an improved lipid phenotype in humans and in animals. To date, the effect of bilirubin on lipid accumulation in tissues that are prone to ectopic lipid deposition is unclear. Therefore, we analyzed the effect of bilirubin on lipid accumulation in skeletal muscle and liver cell lines. C2C12 skeletal muscle and HepG2 human liver cells were treated with physiological concentrations of free fatty acids (FFA) (0.5 mM and 1 mM) and unconjugated bilirubin (UCB) (17.1 and 55  $\mu$ M). The intracellular presence of UCB upon exogenous UCB administration was confirmed by HPLC and the lipid accumulation was assessed by using Nile red. Exposure of both cell lines to UCB significantly reduced lipid accumulation by up to 23% ( $p \leq 0.001$ ) in HepG2 and by up to 17% ( $p \leq 0.01$ ) in C2C12 cells at 0.5 and 5 h under hypoglycaemic conditions. Simultaneously, UCB slightly increased FFA uptake in HepG2 cells after 0.5 and 5 h and in C2C12 cells after 12 h as confirmed by gas chromatographic analyses of the remaining FFA content in the incubation media. The effects of UCB on lipid accumulation and uptake were abolished in the presence of higher glucose concentrations. Monitoring the uptake of a radiolabeled glucose analogue [<sup>18</sup>F]FDG: (2-deoxy-2-[<sup>18</sup>F]fluoro-D-glucose) into both cell types further indicated higher glucose consumption in the presence of UCB. In conclusion, our findings show that UCB considerably decreases lipid accumulation in skeletal muscle and liver cells within a short incubation time of max. 5 h which suggests that mildly elevated bilirubin levels could lower ectopic lipid deposition, a major key element in the pathogenesis of DMT2.

**Keywords:** bilirubin, mild hyperbilirubinaemia, ectopic lipid accumulation, C2C12 skeletal muscle cells, HepG2 cells, [<sup>18</sup>F]FDG uptake, insulin resistance, lipid accumulation

**Abbreviations:** DMT2, diabetes mellitus type 2; [<sup>18</sup>F]FDG, 2-deoxy-2-[<sup>18</sup>F]fluoro-D-glucose; FFA, free fatty acids; GS, Gilbert's syndrome; LA, linoleic acid; NR, Nile red; OA, oleic acid; PA, palmitic acid; UCB, unconjugated bilirubin.

## INTRODUCTION

Globally, the increase in diabetes mellitus type 2 (DMT2) is emerging with 463 million cases worldwide, a prevalence that tripled over the last 20 years and is projected to rise to 700 million cases in 2045 (International Diabetes Federation, 2019). Obesity and an increased fat mass are major contributors to the development of insulin resistance and DMT2. Many studies have shown that the distribution of body fat and particularly the deposition of lipids within non-adipose tissues drive insulin resistance. Indeed, ectopic lipid accumulation in tissues such as skeletal muscle and liver that are responsible for the majority of insulin stimulated glucose disposal drive the pathogenesis and progression of DMT2 (Shulman 2000; Moore et al., 2003; Goodpaster and Wolf 2004).

Prospective and retrospective human studies repeatedly found a protective role of mildly elevated blood bilirubin levels in DMT2 and a Mendelian Randomization study observed a causal risk reduction for DMT2 by mild hyperbilirubinaemia (Vitek 2012; Abbasi et al., 2015; Nano et al., 2016; Yang et al., 2019). Further, subjects with Gilbert's syndrome (GS), an existing human model with constant mildly increased unconjugated blood bilirubin (UCB) levels have repeatedly been linked to a reduced fat mass, BMI and blood lipids (Wallner et al., 2013) and also exhibited a health beneficial glucose metabolic phenotype including a reduction in fasting glucose, insulin, C-peptide and insulin resistance (Mölzer et al., 2016; Khoei et al., 2018). This might suggest that mild hyperbilirubinaemia reduces the risk of DMT2 by protecting from disadvantageous, diabetes related changes in lipid metabolism. To date, data about bilirubin and its relation to ectopic lipid accumulation is scarce and limited to the liver, showing a decreased hepatic lipid content in mildly hyperbilirubinaemic mice (Hinds et al., 2017). However, experimental studies on lipid accumulation in skeletal muscle cells and hepatocytes, tissues that are prone to ectopic lipid deposition and simultaneously responsible for the majority of the insulin-stimulated glucose disposal are lacking. Therefore, this study aims for the first time to investigate the effect of UCB on intracellular lipid accumulation in C2C12 skeletal mouse muscle cells and HepG2 hepatoblastoma-derived cells.

## MATERIALS AND METHODS

### Cell Culture and Treatment

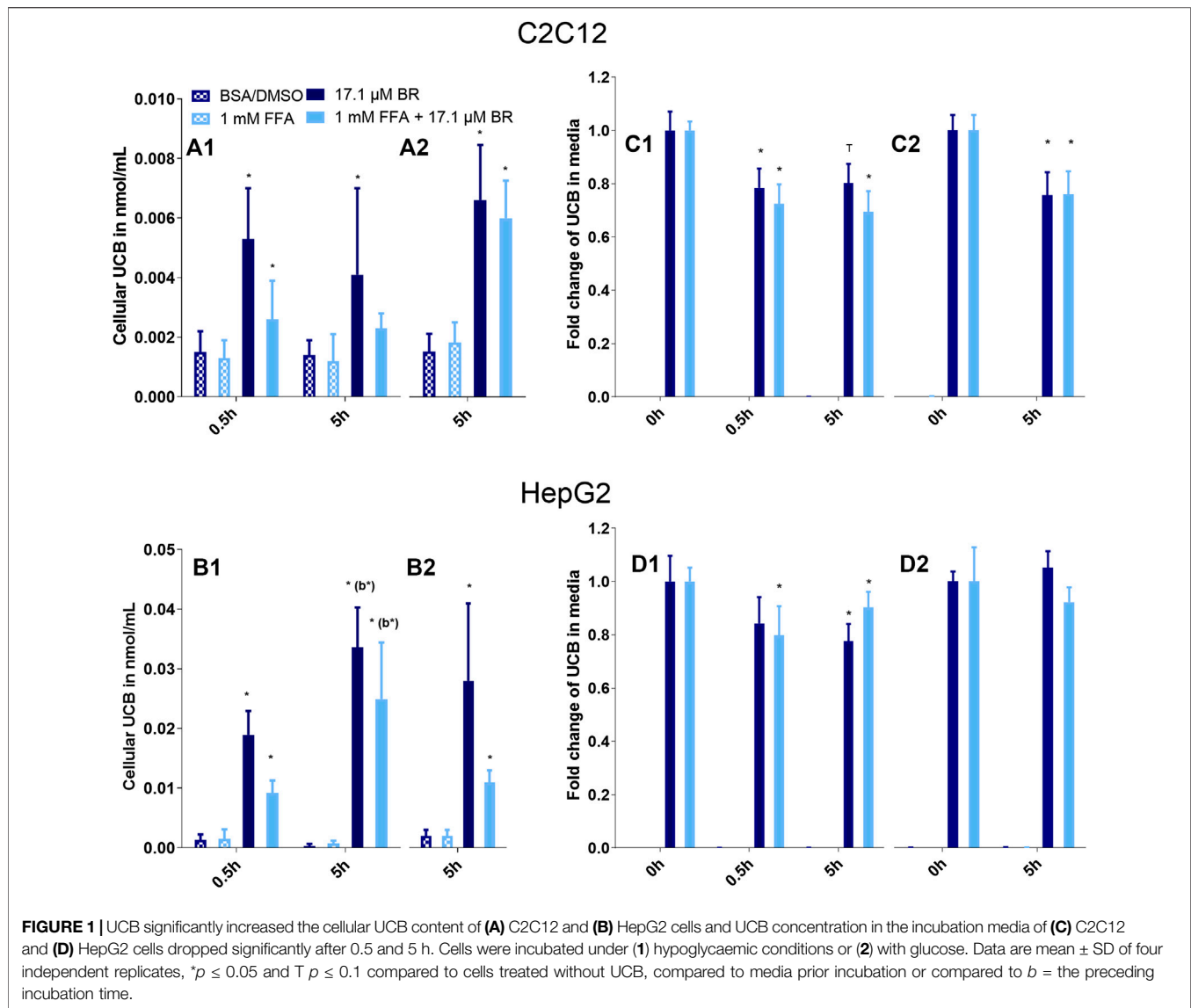
C2C12, murine myoblast (ATCC Cat# CRL-1772, RRID: CVCL\_0188) and HepG2, human hepatoblastoma cells (ATCC Cat# HB-8065, RRID:CVCL\_0027) purchased from LGC Standards GmbH were routinely cultured in high glucose (25 mM) containing DMEM (D1145, Sigma-Aldrich, US) with 10% FBS (10270-098, Gibco, Thermo Fisher, US), 4 mM L-glutamine (G7513, Sigma-Aldrich, US) and 1% penicillin/streptomycin (P0781, Sigma-Aldrich, US) at 37°C, 5% CO<sub>2</sub> under subconfluent conditions. Prior to experiments, HepG2 cells were grown to confluence for 2 days and confluent C2C12 cells were supplemented for another 6 days with 2% horse serum (10500-056,

Gibco, Thermo Fisher, US) to allow differentiation into multinucleated myotubes. Cells were then treated with 0.5 mM or 1 mM BSA-coupled free fatty acids (FFA) (Svedberg et al., 1990) using palmitic acid (PA, P9767, Sigma-Aldrich, US), oleic acid (OA, O7501, Sigma-Aldrich, US) and linoleic acid (LA, L8134, Sigma-Aldrich, US) at a ratio of 2:1:2 which is consistent with the physiological FFA concentration and composition in human blood (Hodson et al., 2008). Simultaneously cells were exposed to 17.1 μM UCB (B412, Sigma-Aldrich, US), the threshold of the UCB blood concentration that characterizes GS (Wagner et al., 2018). In addition, 55 μM UCB was used for the principal lipid-based experiments to reflect the higher UCB concentrations in the liver compared to other tissues such as muscle (Zelenka et al., 2008) using an upper UCB concentration seen in sera of GS subjects. UCB was dissolved in DMSO (0.275% final DMSO concentration in all treatments) and then added to the BSA-containing media by shaking. Prior to the experiments, the solubility of UCB was routinely checked for possible UCB microprecipitate formation under the microscope. All experiments were performed in a darkened room. The control and reference for all treatments consisted of DMEM supplemented with 4 mM L-glutamine, 0.45 mM BSA and 0.275% DMSO. Cytotoxicity of the treatments was tested by MTT assay with  $1 \times 10^4$  C2C12 and  $0.5 \times 10^5$  HepG2 in 96 well plates. Formazan crystals were formed with 1 mg/ml MTT (M2128-1G, Sigma-Aldrich, US) in DMEM (37°C, 5% CO<sub>2</sub>, 4 h), dissolved in 200 μL DMSO (37°C, 5% CO<sub>2</sub>, 1 h) and measured at 485 nm using a plate reader (Fluorostar Optima, BMG Labtech, Germany).

### Cellular Unconjugated Bilirubin Uptake

The cellular UCB uptake and the remaining UCB concentration in the incubation media were analyzed by HPLC. Briefly,  $4 \times 10^5$  C2C12 and  $4.5 \times 10^6$  HepG2 cells were seeded into 100 × 20 mm cell culture dishes for treatment (10 ml). After exposure to UCB for 0.5 and 5 h, the supernatant was immediately frozen at -80°C. The cells were put on ice, washed with 10 ml PBS (4°C), collected using a cell scraper (5 ml PBS 4°C) and centrifuged at 179 g, 4°C for 5 min. The cell pellet was stored in 1 ml PBS (4°C) at -80°C after adding N<sub>2</sub>.

The sample solutions for HPLC analysis consisted of 500 μL of UCB-non-treated media, 50 μL of UCB-treated media or 450 μL of cellular homogenate, disintegrated by ultrasound on ice as well as 50 μL of internal standard (5 mM mesobilirubin, Frontier Scientific, US) and the trace of BHT (antioxidant) (Sigma-Aldrich, US). UCB was extracted into 6 ml of methanol/chloroform/hexane (10/5/1 v/v/v) and concentrated into a small droplet of carbonate buffer (100 mM, pH 10) which was injected (50 μL) onto HPLC Agilent 1200 (CA, US) equipped with a diode-array detector. Bilirubin was then separated on the Luna C8 column (4.6 mm × 150 mm, 3 mM/100A, Phenomenex, CA, US). The mobile phase for the analysis contained methanol (450 g), water (300 g) and tetrabutylammonium hydroxide (7.5 ml) and its pH was adjusted by phosphoric acid (9–9.3). The signal was detected at 440 nm with 550 nm as the reference wavelength (Zelenka et al., 2008). The final amount of bilirubin in



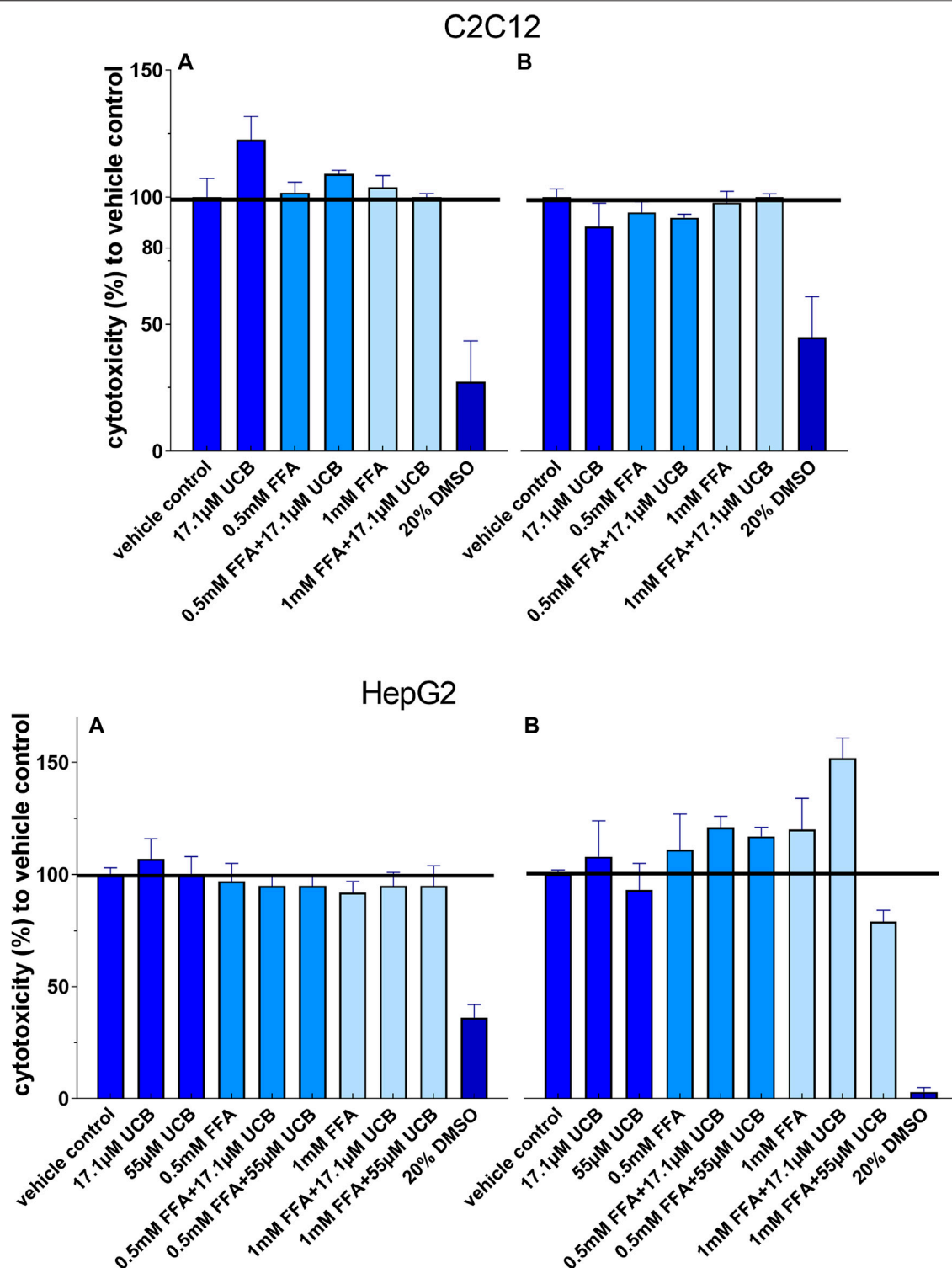
the medium was calculated as nmol of UCB per mL of sample. The amount of protein in the cellular homogenate was measured by Bio-Rad Protein Assay (Bio-Rad, CA, US) using the microplate reader (Sunrise, Tecan, Austria). Concentration of bilirubin in cells was calculated as pmol of UCB per mg of protein.

## Lipid Accumulation and Uptake

Lipid accumulation was measured by the Nile red (NR) assay that selectively stains intracellular lipids. Therefore,  $1 \times 10^4$  C2C12 and  $0.5 \times 10^5$  HepG2 cells were seeded into a black 96 well microplate (Greiner, Austria) for treatment (final volume 200 μL). Then cells were fixed with 3% paraformaldehyde (F1635, Sigma-Aldrich, US) (24°C, 20min), stained with NR (3.3 μg/mL in PBS, 37°C, 5% CO<sub>2</sub>, 2 h) and rinsed with 100 μL PBS. NR fluorescence was measured from the bottom of the plate at ex: 485 nm and em: 590 nm, the background fluorescence was

subtracted prior staining and NR fluorescence was adjusted by Bradford protein content (B6916, Sigma-Aldrich, US). Images of intracellular lipids were obtained with the confocal microscope Leica TCS SPE including the inverse microscope DMi8 and analyzed using the LAS X 2.0.014332 software from Leica.

Lipid accumulation at a single cell level was measured using flow cytometry after NR staining. C2C12 ( $0.2 \times 10^6$ ) and HepG2 ( $0.6 \times 10^6$ ) cells were seeded into a six well plate (Greiner, Austria) with 2 mL treatment solution. Cells were harvested with accutase (A6964, Sigma-Aldrich, US), collected in a 5 mL Polystyrene Round-Bottom Tube (352058, Corning, US), centrifuged (179 g, 5 min), washed with 1 mL PBS (37°C) and incubated in the dark for 15 min with 1 mL 0.75 μg/mL NR in PBS. Cells were centrifuged and analyzed in 400 μL NR solution. 50,000 cells were measured in FL2 excluding cell debris using a four-channel FACS Calibur™ flow cytometer (BD, Europe).

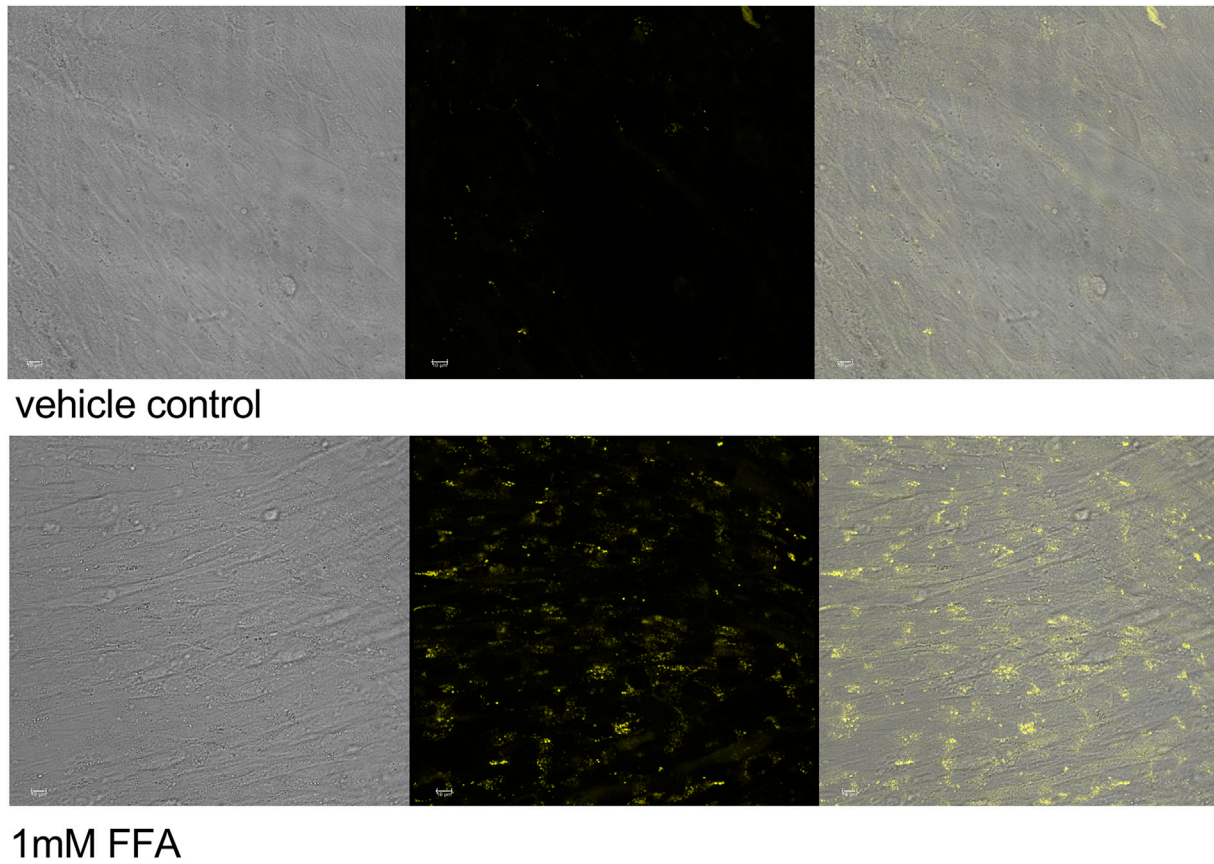


**FIGURE 2 |** None of the treatments was cytotoxic to C2C12 and HepG2 cells: Cells were incubated (A) at hypoglycaemic conditions for 12 h (C2C12) and 5 h (HepG2) or (B) with glucose for 24 h. Absorbance 485 nm, data are mean  $\pm$  SD of at least  $n = 2$  (each in sextuplicate).

The cellular lipid uptake was analyzed indirectly by measuring the remaining FFA concentrations from the incubation media by gas chromatography. The lipid extraction was performed according to

modified protocols from Folch and Metcalfe (Folch et al., 1957; Metcalfe et al., 1966). First, the sample solution consisting of 2 ml incubation media and 100  $\mu$ L of internal standard (2 mM stearic acid



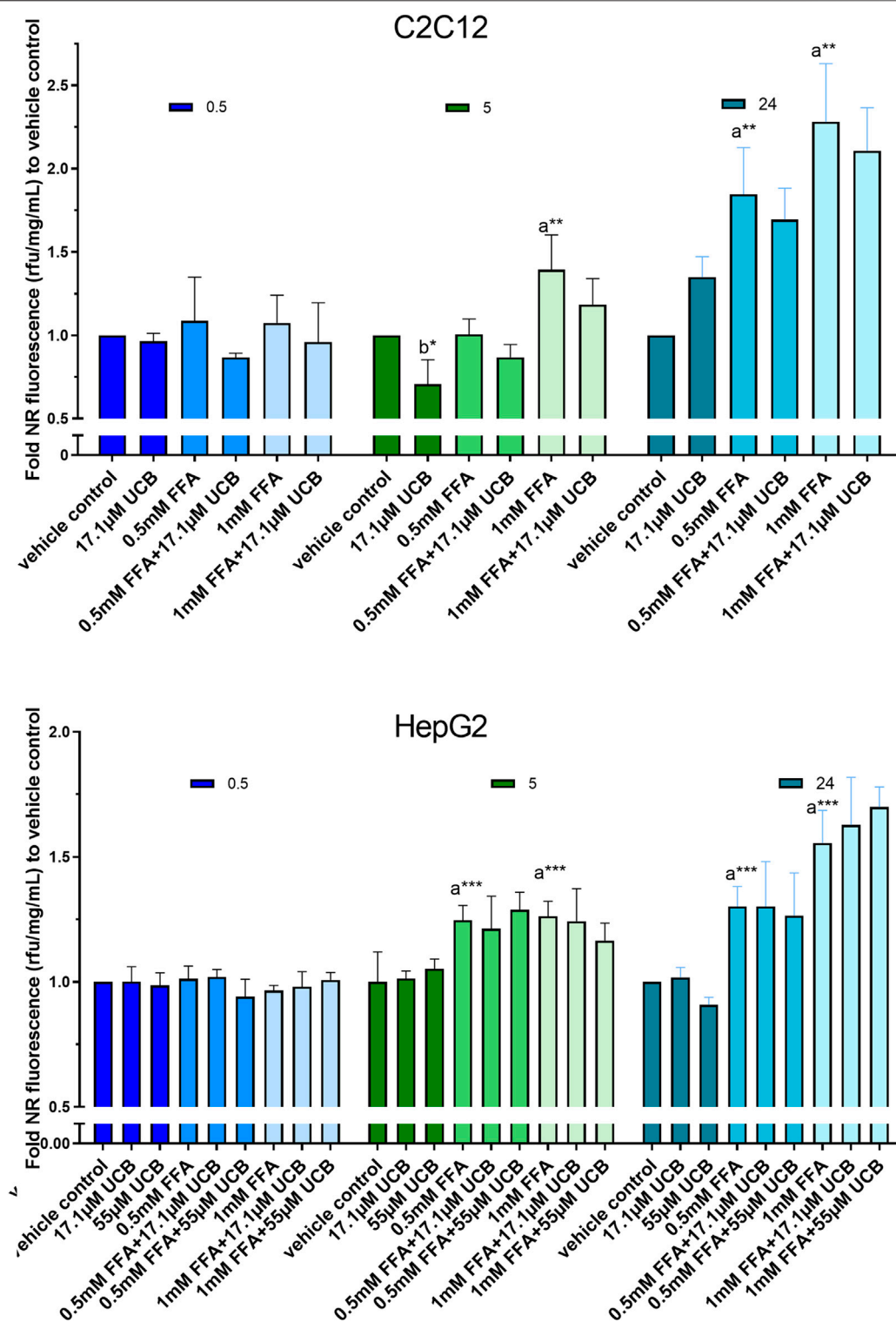


**FIGURE 3 |** Lipid accumulation and lipid droplet formation in C2C12 myotubes. Cells were incubated for 5 h, stained with NR and viewed by fluorescence confocal microscopy. Transmission (**left**), fluorescence (**center**) images and overlay (**right**), ex: 488 nm, em: 509–560 nm, no adjustment of gain.

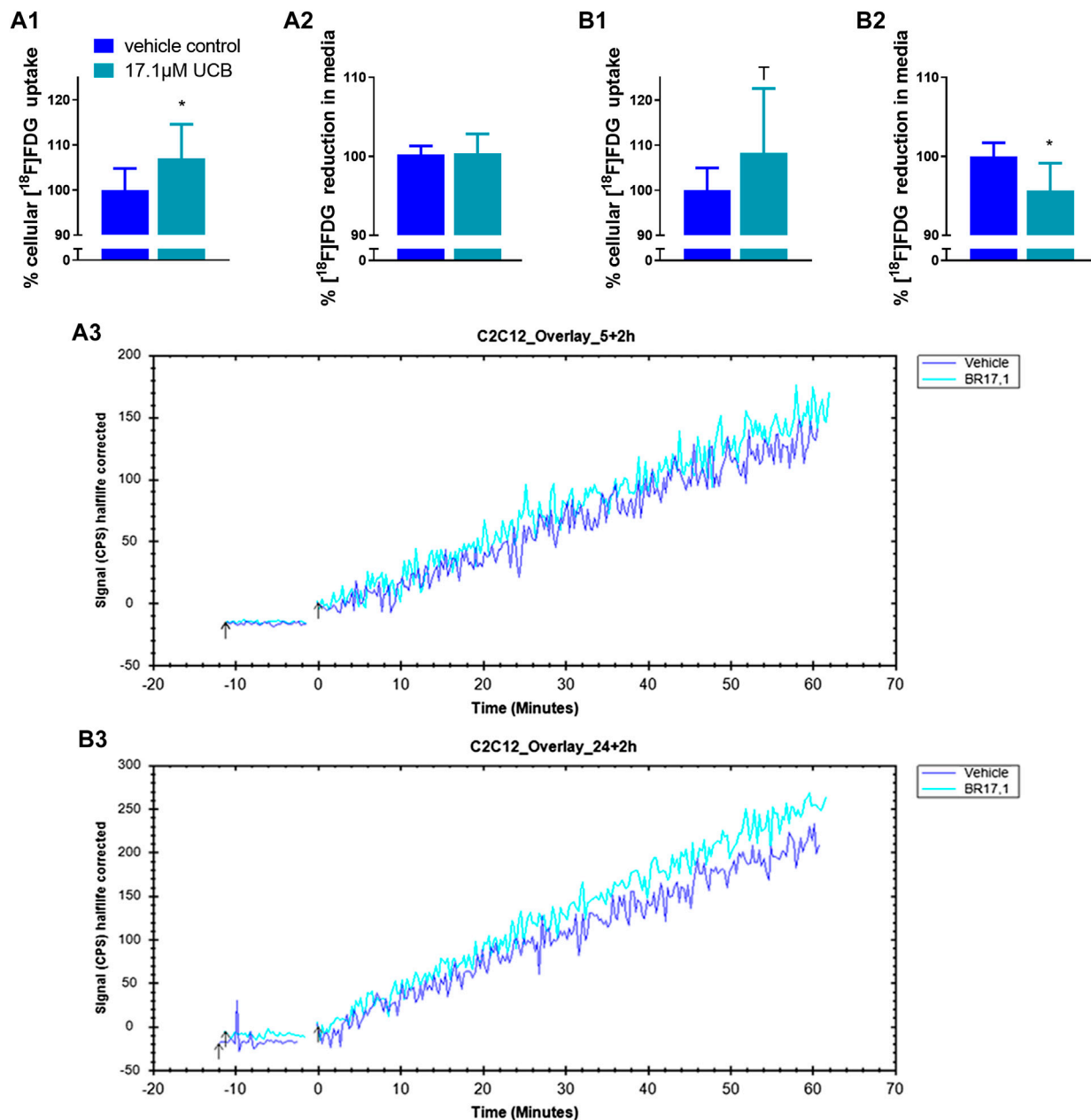
in isopropanol) was extracted twice using 10 ml/5 ml chloroform/methanol (2:1) for 20 min on ice while vortexing twice for 0.5 min. The lipophilic phases were collected (2,000 g for 10 min), pooled and evaporated with  $N_2$  (37°C). Then, saponification and methylation were performed using 1 ml methanolic NaOH (0.5 M NaOH and 0.27 mM BHT) and 1 ml BF<sub>3</sub> (14% in methanol) respectively at 100°C for 5 min. The samples were immediately cooled on ice for 10 min and fatty acid methyl esters (FAMES) were collected twice in 2 and 1.5 ml hexane by shaking for 20 min. The hexane phases were pooled, evaporated with  $N_2$  (37°C) and dissolved in 200 ml hexane. Samples were analyzed with an auto system gas chromatograph (Perkin Elmer Clarus 500, US), a Rtx-2330 30 m  $\times$  0.25 mm i.d. silica column, a flame ionization detector (270°C) and helium as a carrier gas. FAMES, 1  $\mu$ l per sample were injected at an initial injector temperature of 250°C. The initial oven temperature was set to 90°C and was increased 3 times. First to 150°C by 13.0°C/min, then to 180°C by 2.9°C/min with an isothermal period of 5 min and finally to 230°C by 4.0°C/min and an isothermal period of 7 min. FFAs were quantified based on a six point calibration curve (200  $\mu$ g/ml–800  $\mu$ g/ml) using a five component FAMES Mix Standard ME28 (8691.1, Carl Roth, Germany). The concentration of the FFAs was adjusted according to recovery of the internal standard.

### Glucose Uptake Using [<sup>18</sup>F]FDG

We analyzed the cellular uptake of 2-deoxy-2-[<sup>18</sup>F]fluoro-D-glucose ([<sup>18</sup>F]FDG), a radiolabeled glucose analogue, which allows to mimic glucose uptake. [<sup>18</sup>F]FDG is GLUT-dependently transported into cells mainly via GLUT1 (Wiebe 2001) which is predominantly expressed in C2C12 (Calderhead et al., 1990) and HepG2 cells (Takanaga et al., 2008) and allows to monitor basal glucose uptake. [<sup>18</sup>F]FDG uptake was investigated at a single time point and by real-time kinetic measurements over 60 min using LigandTracer<sup>®</sup> Yellow (Ridgeview Instruments, Sweden) (Balber et al., 2019). Briefly, C2C12 ( $0.16 \times 10^6$ ) and HepG2 ( $1.2 \times 10^6$ ) cells were seeded into a six well plate and incubated with 4 ml UCB treatment solution for 5 and 24 h followed by 2 h glucose starving in the presence of UCB. 150 kBq of [<sup>18</sup>F]FDG were added to the cells for 1 h at 37°C, subsequently, supernatant and cells (accutase) were collected and measured with a gamma counter (Wizard<sup>2</sup>™ 3", PerkinElmer, USA) for 30 s. Results were expressed relative to a reference adding [<sup>18</sup>F]FDG to PBS instead of treatment and cells. For the real-time kinetic [<sup>18</sup>F]FDG measurements,  $0.8 \times 10^6$  C2C12 and HepG2 cells were seeded onto one half of a 100  $\times$  20 mm cell culture dish, the opposite side was used as a reference. Cells were treated analogue to the six well assay and cell associated radioactivity



**FIGURE 4** | UCB had no significant effect on lipid accumulation in C2C12 and HepG2 cells in the presence of glucose. Cells were exposed to treatments for 0.5, 5 and 24 h. NR fluorescence was adjusted by protein content (rfu/mg/mL) and compared to vehicle control. Data are mean  $\pm$  SD of  $n = 3$  (each in triplicate). Significant differences according to Bonferroni ( $***p \leq 0.001$ ,  $**p \leq 0.01$ ,  $*p \leq 0.05$ ) of a = compared to vehicle control and b = UCB treated vs. untreated cells.



**FIGURE 5** | UCB changed [ $^{18}\text{F}$ ]FDG uptake in C2C12 cells. **(1)** Cellular uptake (%) of [ $^{18}\text{F}$ ]FDG and **(2)** reduction (%) of [ $^{18}\text{F}$ ]FDG in the supernatant determined by gamma counting after UCB incubation compared to vehicle control (100%) at **(A)** 5 h and **(B)** 24 h. Data are mean  $\pm$  SD of  $n = 3$  (each in triplicate). \* $p \leq 0.05$ , T  $p \leq 0.1$ . **(3)** Representative [ $^{18}\text{F}$ ]FDG kinetics (0–60min) after UCB/vehicle treatment.

was measured starting directly after addition of [ $^{18}\text{F}$ ]FDG for 60 min.

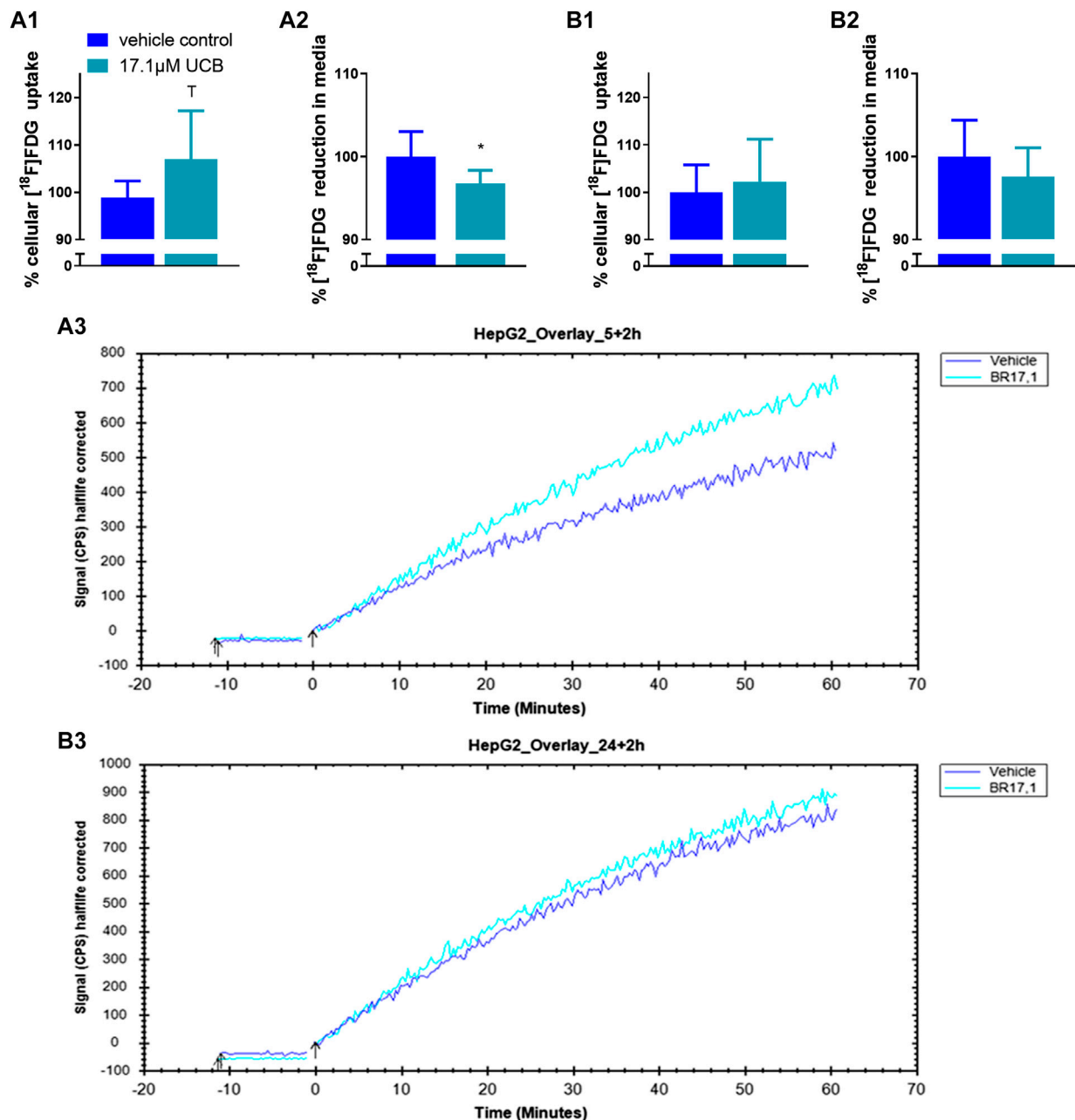
### Statistical Analysis

Statistical analysis and calculations were completed using IBM SPSS 24 (IBM SPSS Statistics, RRID: SCR\_019096). Data distribution was checked based on histograms and box plots. For parametric data One-Way ANOVA and for non-parametric data Kruskal-Wallis test were performed. Alpha was set at  $p \leq 0.05$  according to Bonferroni.

## RESULTS

### Exogenous Administration of Unconjugated Bilirubin Increased the Intracellular Unconjugated Bilirubin Content and Was Not Cytotoxic

The exposure of C2C12 and HepG2 cells to 17.1  $\mu\text{M}$  UCB significantly increased the intracellular UCB concentration after 0.5 and 5 h (**Figures 1A, B**). Consistently, the UCB concentration in the



**FIGURE 6 |** UCB changed [ $^{18}\text{F}$ ]FDG uptake in HepG2 cells. (1) Cellular uptake (%) of [ $^{18}\text{F}$ ]FDG and (2) reduction (%) of [ $^{18}\text{F}$ ]FDG in the supernatant determined by gamma counting after UCB incubation compared to vehicle control (100%) at (A) 5 h and (B) 24 h. Data are mean  $\pm$  SD of  $n = 3$  (each in triplicate). \* $p \leq 0.05$ , T  $p \leq 0.1$ . (3) Representative [ $^{18}\text{F}$ ]FDG kinetics (0–60 min) after UCB/vehicle treatment.

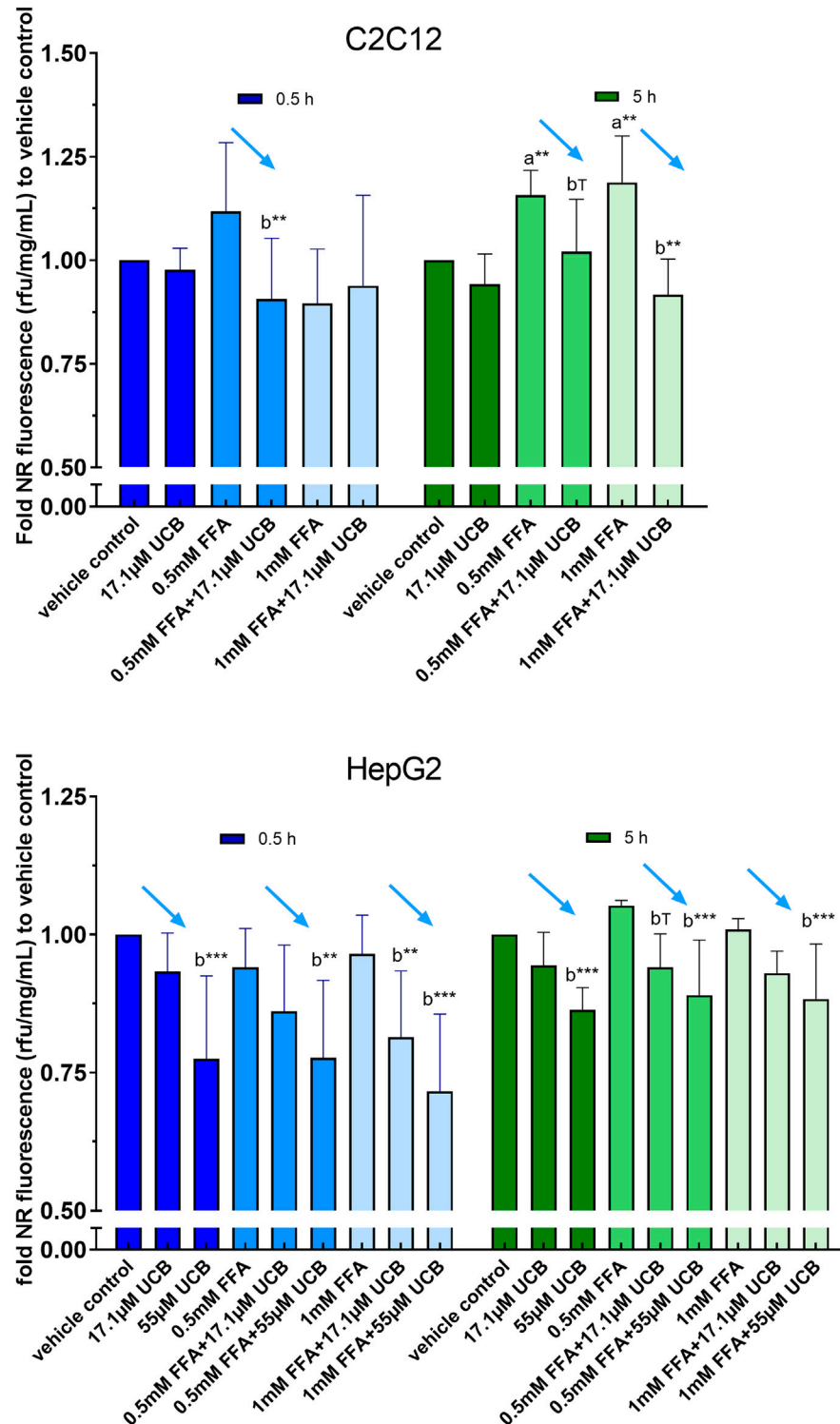
incubation media of both cell lines decreased significantly after 0.5 and 5 h (Figures 1C, D). None of the treatments was cytotoxic to C2C12 or HepG2 cells compared to the vehicle control (Figure 2).

### Unconjugated Bilirubin did Not Change Lipid Accumulation in C2C12 and HepG2 Cells in the Presence of Glucose

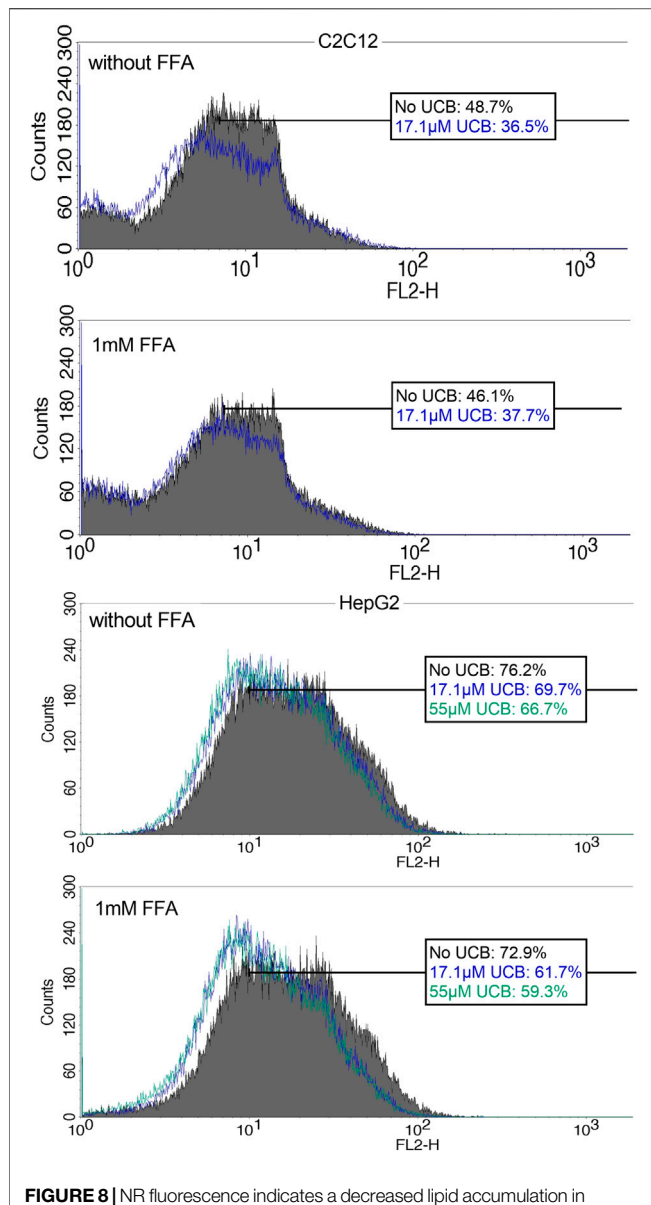
First, intracellular lipids and lipid droplet formation upon FFA incubation for 5 h were confirmed via confocal microscopy

(Figure 3). In a second step, lipid accumulation was quantified via NR assay. C2C12 and HepG2 cells were exposed to 0.5mM and 1 mM FFAs which significantly increased the content of intracellular lipids in both cell lines compared to vehicle control after 5 and 24 h of incubation. However, the treatment with UCB did not significantly change the basal or the FFA-driven lipid accumulation in C2C12 and HepG2 cells. We only observed a reproducible but non-significant slight reduction in lipid accumulation in all FFA treatments in C2C12 cells (Figure 4). This was somewhat unexpected, however, the treatments were





**FIGURE 7** | UCB significantly decreased lipid accumulation in C2C12 and HepG2 cells at hypoglycaemic conditions. Cells were exposed to treatments for 0.5 and 5 h. NR fluorescence was adjusted by protein content (rfu/mg/mL) and compared to vehicle control. Data are mean  $\pm$  SD of  $n = 3$  (each in quadruplicate). \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ ,  $T p \leq 0.1$  of  $a =$  compared to vehicle control and  $b =$  compared to UCB treated vs. untreated cells according to Bonferroni.



**FIGURE 8 |** NR fluorescence indicates a decreased lipid accumulation in C2C12 and HepG2 cells upon UCB exposure. Cells were exposed to the respective treatments for 5 h at hypoglycaemic conditions. Cell associated fluorescence was analyzed by flow cytometry (FL2). The blue (17.1 μM UCB) and green (55 μM UCB) histograms show a shift to the left toward decreased NR fluorescence intensity in comparison to the gray histogram (without UCB). The boxes show the % of cells with a higher NR fluorescence intensity after incubation without UCB, with 17.1 μM or 55 μM UCB. Representative histogram of **Supplementary Tables S2** of 50,000 cells.

performed in presence of glucose. It is known that skeletal muscle and liver preferentially rely on glucose as energy source upon sufficient glucose availability but switch from glucose toward lipids at hypoglycaemic conditions. Further, people with GS beside having health beneficial lipid blood and anthropometric biomarkers (Wallner et al., 2013; Wagner et al., 2015), also exhibited beneficial changes in glucose metabolism (decreased fasting glucose, insulin and C-peptide) in a more recent study (Mölzer et al., 2016). This could suggest that UCB has effects on the glycaemic

response of our cell model masking or overriding changes in lipid metabolism. Therefore, we assessed in a next step basal glucose uptake into C2C12 and HepG2 cells upon UCB treatment by using the radiolabeled glucose analogue [ $^{18}\text{F}$ ]FDG.

## Unconjugated Bilirubin Mildly Increased Cellular [ $^{18}\text{F}$ ]FDG Uptake in C2C12 and HepG2 Cells

In C2C12 cells, UCB significantly increased the intracellular [ $^{18}\text{F}$ ]FDG content by 7% after 5 h and by 8% after 24 h (**Figures 5A1,B1**). Simultaneously, UCB lowered the [ $^{18}\text{F}$ ]FDG concentration in the incubation media significantly by 4% after 24 h (**Figure 5B2**). Consistent with these observations, cell associated radioactivity derived from [ $^{18}\text{F}$ ]FDG decay was slightly higher in real-time kinetic measurements after exposure of C2C12 cells to UCB for 5 and 24 h (**Figure 5A3,B3**). In HepG2 cells, UCB increased the intracellular [ $^{18}\text{F}$ ]FDG content by trend by 8% and significantly reduced the [ $^{18}\text{F}$ ]FDG concentration in the media by 7% only after 5 h of incubation (**Figure 6A1,A2**). In real-time kinetic measurements, cell associated radioactivity derived from [ $^{18}\text{F}$ ]FDG decay was again higher after exposure to UCB for 5 and 24 h (**Figure 6A3,B3**).

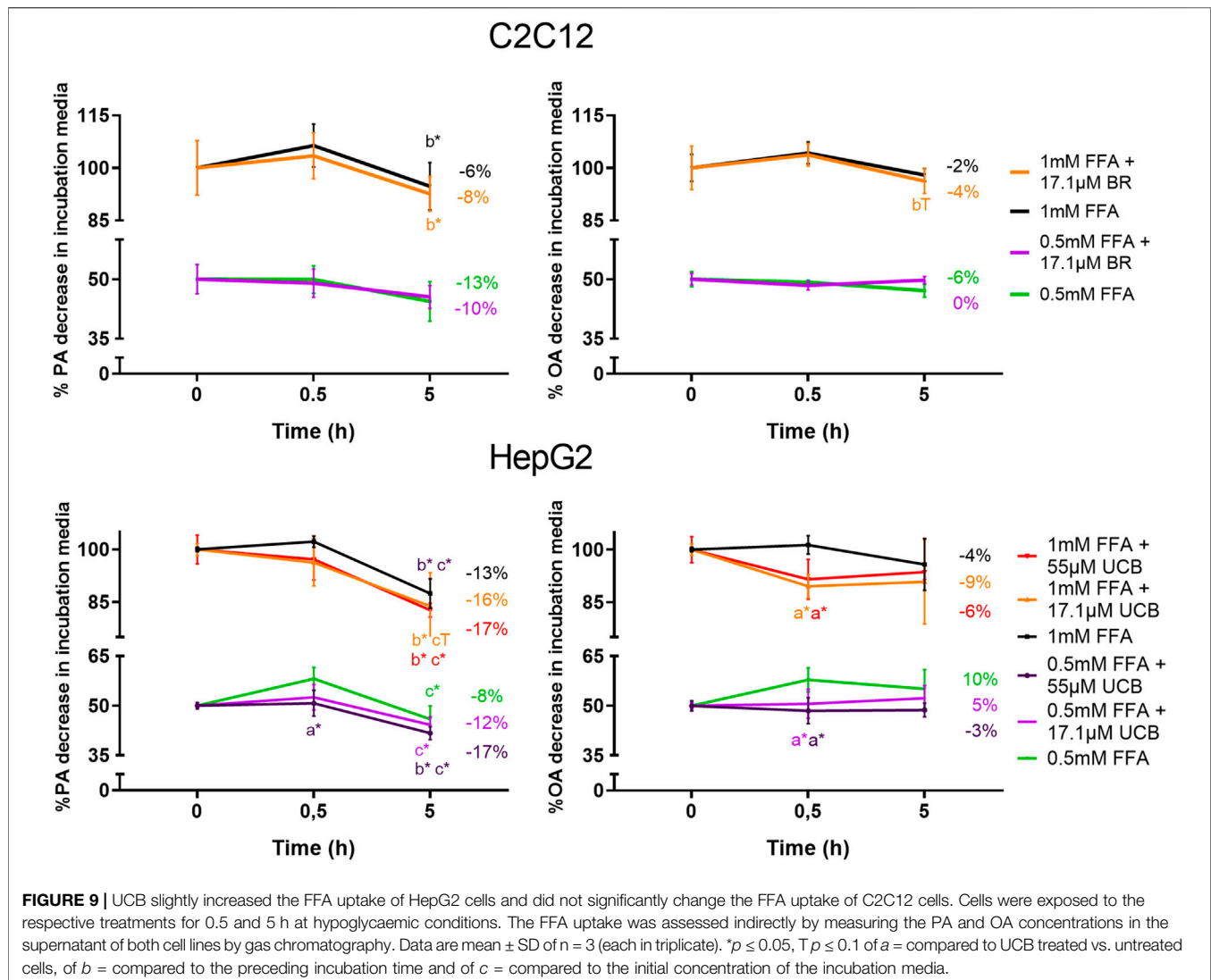
## Unconjugated Bilirubin Decreases Lipid Accumulation in C2C12 and HepG2 Cells at Hypoglycaemic Conditions

In a second batch of experiments the effect of UCB on lipid accumulation was investigated in the absence of glucose with incubation times up to 5 h. We decided to remove glucose 1) due to the effects of UCB on [ $^{18}\text{F}$ ]FDG uptake in our cell model and 2) to challenge lipid catabolism since glucose deprived conditions stimulate the oxidative metabolism as shown in HepG2 cells (Weber et al., 2002). Prior to the lipid accumulation experiment, we confirmed that the UCB uptake was largely unaltered in the absence of glucose compared to glucose conditions and that the treatments were not cytotoxic to the cells (**Figures 1A1,B1,C1,D1,2A**).

C2C12 cells exposed to 17.1 μM UCB significantly decreased the intracellular lipid accumulation by up to 17% ( $p \leq 0.01$ ) compared to vehicle controls at 0.5 and 5 h. In HepG2 cells, UCB significantly decreased lipid accumulation in all treatments after 0.5 and 5 h by up to 23% ( $p \leq 0.001$ ), whereas 55 μM UCB further decreased the lipid accumulation compared to 17.1 μM UCB, indicating a dose dependency (**Figure 7**). Lipid accumulation was also decreased after UCB treatment in C2C12 and HepG2 cells using flow cytometry at hypoglycaemic conditions, in contrast to treatments with glucose (**Figures 7, 8, Supplementary Tables S1, S2**).

## Unconjugated Bilirubin Slightly Increased Cellular Lipid Uptake

To exclude a decreased lipid uptake as a reason for the decreased lipid accumulation upon UCB treatment, we analyzed the remaining FFA concentrations in the incubation media by gas chromatography. UCB did not change the concentration of PA and OA in the treatments of C2C12 cells, which indicates that UCB did not change the cellular FFA uptake after 0.5 and 5 h under



hypoglycaemic conditions (Figure 9). Further investigations of FFA uptake up to 12 h showed that UCB slightly reduced the FFA concentrations by approximately 5% in the incubation media of C2C12 cells at hypoglycaemic conditions in contrast to treatments with glucose (Supplementary Figures S1, S2). In detail, PA dropped by 22% and OA by 10% significantly in the presence of UCB and 1 mM FFA, whereas the drop of PA by 17% and OA by 6% was not significant in the absence of UCB (Supplementary Figure S1). In HepG2 cells, PA and OA concentrations were consistently lower ( $\bar{x} \approx 8\%$ ) in the presence of UCB over all treatments and incubation times which was significant at 0.5 h. Thus, this data not only excludes that UCB decreases lipid uptake, it also indicates that UCB slightly increases FFA uptake in both cell lines.

## DISCUSSION

Over the last decade, accumulating evidence has been suggesting a role of mildly elevated systemic bilirubin concentrations in the

prevention of metabolic diseases and particularly in DMT2. Epidemiologic studies repeatedly showed a protection against DMT2 at mildly increased blood bilirubin levels (Abbasi et al., 2015; Nano et al., 2016). In case control studies, GS individuals with constant mild hyperbilirubinaemia revealed a reduced fat mass, BMI and blood lipids next to improved insulin resistance, fasting glucose, insulin and C-peptide levels (Wallner et al., 2013; Mölzer et al., 2016). Mice with diet induced obesity displayed an improved insulin sensitivity and reduced body weight and fat mass when treated with bilirubin (Dong et al., 2014; Liu et al., 2015; Gordon et al., 2020). Genetically modified mice with mild hyperbilirubinaemia are resistant to high fat diet induced hepatic steatosis, obesity, hypertriglyceridaemia, glucose intolerance, hyperglycaemia and hyperinsulinaemia due to an increased PPAR $\alpha$  activity (Hinds et al., 2017). This evidence indicates that mildly elevated bilirubin levels have a strong DMT2-preventive potential via shaping an improved lipid phenotype. However, the mechanisms of this lipid based diabetes-protective capacity of UCB remain unclear.

The pathogenesis of DMT2 involves ectopic lipid accumulation in tissues that account for most of the insulin-dependent glucose disposal. Studies about the effects of UCB on ectopic lipids are rare, only focused on the liver and show a reduced diet induced hepatic lipid content in the presence of mild hyperbilirubinaemia in mice (Hinds et al., 2017). However, to date there are no experimental studies about the effect of UCB on ectopic lipid deposition in skeletal muscle cells and hepatocytes, cell types that are responsible for the majority of the whole-body glucose uptake. Thus, this study focused on the UCB driven changes of lipid accumulation in C2C12 skeletal muscle cells and HepG2 hepatocytes.

Mild hyperbilirubinaemic conditions (17.1  $\mu\text{M}$  UCB) significantly decreased the intracellular lipid accumulation by up to 17% ( $p \leq 0.01$ ) in C2C12 skeletal muscle cells after 0.5 and 5 h. In HepG2 cells, exposure to mildly elevated UCB concentrations in the medium (17.1 and 55  $\mu\text{M}$ ) significantly reduced the lipid accumulation by up to 23% ( $p \leq 0.001$ ) dose dependently and consistently in all FFA treatments at 0.5 and 5 h of incubation (Figure 7). Simultaneously, a slightly decreased PA and OA concentration in the UCB containing incubation media indicated that UCB slightly increased FFA uptake in both cell lines (Figure 9, Supplementary Figure S1). This confirms that the lipid content in the presence of UCB is reduced not based on a decrease in lipid uptake but rather due to a reduction of intracellular lipids. This strong UCB-induced reduction in lipid accumulation by up to 23% within a short incubation period of max. 5 h suggests that mild hyperbilirubinaemia might considerably lower ectopic lipid accumulation.

In contrast to these experiments that were performed with hypoglycaemic media, UCB had no effect on lipid accumulation or uptake in both cell lines when using glucose at a concentration recommended for both cell lines (Figure 4, Supplementary Figure S2, Table S1). Under hypoglycaemic fasting conditions PPAR $\alpha$  and AMPK are activated to upregulate the cellular lipid oxidative metabolism and lipid uptake (Kersten et al., 1999; Towler and Hardie 2007). Thus while in the presence of glucose, our cell model might preferentially metabolize glucose for energy production, the hypoglycaemia activates PPAR $\alpha$  and AMPK and forces the cells into the oxidative lipid metabolism. We and other groups showed that the beneficial metabolic profile in mild hyperbilirubinaemia might be PPAR $\alpha$  and AMPK dependent (Mölzer et al., 2016; Gordon et al., 2020) and UCB was shown to be an activator of PPAR $\alpha$  (Stec et al., 2016). This might explain the differences of lipid accumulation and lipid uptake at varying glucose concentrations (Figures 4, 7, 9, Supplementary Figures S1, S2) and suggests an increased lipid oxidation as a reason for the UCB-induced reduction of lipid accumulation in muscle and liver cells.

Mild hyperbilirubinaemia has been associated with beneficial changes in the blood glucose profile in humans and in mice (Liu et al., 2015; Mölzer et al., 2016; Hinds et al., 2017). Hence, it is possible that UCB increased the glycaemic response of our cell

model in the presence of glucose masking or overriding changes in lipid metabolism. UCB had indeed effects on the [ $^{18}\text{F}$ ]FDG uptake in both cell lines which was one of the main reasons why we continued our experiments under hypoglycaemic conditions. These experiments showed a slightly increased [ $^{18}\text{F}$ ]FDG uptake in C2C12 and HepG2 cells, indicating a rise of mainly GLUT1-dependent glucose uptake (Figures 5, 6). To our knowledge there is no other data to date about the effects of UCB on glucose uptake. However, there is one study that observed a significantly increased GLUT4 expression in the muscle cell membrane of bilirubin-treated mice (Dong et al., 2014).

This is the first study that reports a UCB driven decrease in lipid accumulation in skeletal muscle and liver cells, types of tissues that are most affected by ectopic lipid deposition. Lipid accumulation was investigated at varying physiological FFA concentrations considering the level of glucose and was simultaneously compared to the lipid uptake. Another strength of this study was that the uptake and the intracellular presence of UCB was confirmed by HPLC (Figure 1). For future experiments it is necessary to explore the effect of UCB on ectopic lipid deposition in animals or humans to further support our results.

## CONCLUSION

Our findings show that UCB considerably decreases lipid accumulation in skeletal muscle and liver cells by up to 23% within a short incubation time of max. 5 h. This suggests that mild hyperbilirubinaemia could lower ectopic lipid deposition, a major key element in the pathogenesis of DMT2. Thus, the reduced ectopic lipid accumulation in skeletal muscle and liver cells could indeed, at least partly explain the reduced insulin resistance in GS and the protective role of mild hyperbilirubinaemia in DMT2.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

CAH designed the project and the experiments, performed experiments and statistical analysis and wrote the manuscript. K-HW. conceived the original idea, designed and supervised the project and revised the manuscript. E-MK and TB designed and performed glucose uptake experiments and revised manuscript. MM contributed his radiopharmaceutical expertise, supervised radioactive uptake experiments and revised the manuscript. RQ, YH, AK, JH, SS, PE, ES, JJ, and PV performed experiments. LV contributed the UCB uptake method and revised the manuscript. EH provided support with cell culture expertise and revised the manuscript. All authors provided final approval of the manuscript.



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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2020.636533/full#supplementary-material>.

Zelenka, J., Leníček, M., Muchová, L., Jirsa, M., Kudla, M., Balaž, P., et al. (2008). Highly sensitive method for quantitative determination of bilirubin in biological fluids and tissues. *J. Chromatogr. B.* 867 (1), 37–42. doi:10.1016/j.jchromb.2008.03.005

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary Material

### C2C12

#### Lipid accumulation by flow cytometry

| Treatments             | 0.5h             | 5h                | 24h               |
|------------------------|------------------|-------------------|-------------------|
| BSA                    | 1.00 ± 0.03      | 1.00 ± 0.02       | 1.00 ± 0.05       |
| 17.1µM UCB             | 0.97 ± 0.08 n.s. | 1.03 ± 0.05 n.s.  | 1.01 ± 0.08 n.s.  |
| 0.5mM FFA              | 1.00 ± 0.06 n.s. | 1.15 ± 0.07 0.005 | 1.19 ± 0.09 0.000 |
| 0.5mM FFA + 17.1µM UCB | 0.94 ± 0.07 n.s. | 1.20 ± 0.08 n.s.  | 1.19 ± 0.07 n.s.  |
| 1mM FFA                | 1.02 ± 0.08 n.s. | 1.27 ± 0.08 0.000 | 1.39 ± 0.07 0.000 |
| 1mM FFA + 17.1µM UCB   | 1.00 ± 0.09 n.s. | 1.22 ± 0.12 n.s.  | 1.35 ± 0.09 n.s.  |

### HepG2

#### Lipid accumulation by flow cytometry

| Treatments             | 0.5h             | 5h               | 24h               |
|------------------------|------------------|------------------|-------------------|
| BSA                    | 1.00 ± 0.10      | 1.00 ± 0.08      | 1.00 ± 0.10       |
| 17.1µM UCB             | 0.99 ± 0.09 n.s. | 0.94 ± 0.15 n.s. | 0.96 ± 0.11 n.s.  |
| 55µM UCB               | 0.95 ± 0.09 n.s. | 0.99 ± 0.14 n.s. | 0.92 ± 0.08 n.s.  |
| 0.5mM FFA              | 0.90 ± 0.06 n.s. | 1.03 ± 0.14 n.s. | 1.17 ± 0.18 n.s.  |
| 0.5mM FFA + 17.1µM UCB | 0.84 ± 0.12 n.s. | 0.93 ± 0.06 n.s. | 1.05 ± 0.16 n.s.  |
| 0.5mM FFA + 55µM UCB   | 0.99 ± 0.31 n.s. | 0.88 ± 0.05 n.s. | 1.08 ± 0.24 n.s.  |
| 1mM FFA                | 0.94 ± 0.11 n.s. | 1.14 ± 0.07 n.s. | 1.44 ± 0.16 0.000 |
| 1mM FFA + 17.1µM UCB   | 0.93 ± 0.16 n.s. | 0.94 ± 0.18 n.s. | 1.43 ± 0.21 n.s.  |
| 1mM FFA + 55µM UCB     | 0.88 ± 0.15 n.s. | 0.95 ± 0.18 n.s. | 1.39 ± 0.23 n.s.  |

**Table S1:** Lipid accumulation in C2C12 and HepG2 single cells in the presence of glucose. Cells were analysed using flow cytometry. The geometric mean fluorescence (FL2) of 50,000 NR-stained cells per treatment was compared to vehicle control. Data are mean ±SD of n=3 (each in duplicate). n.s.=non significant.

## C2C12

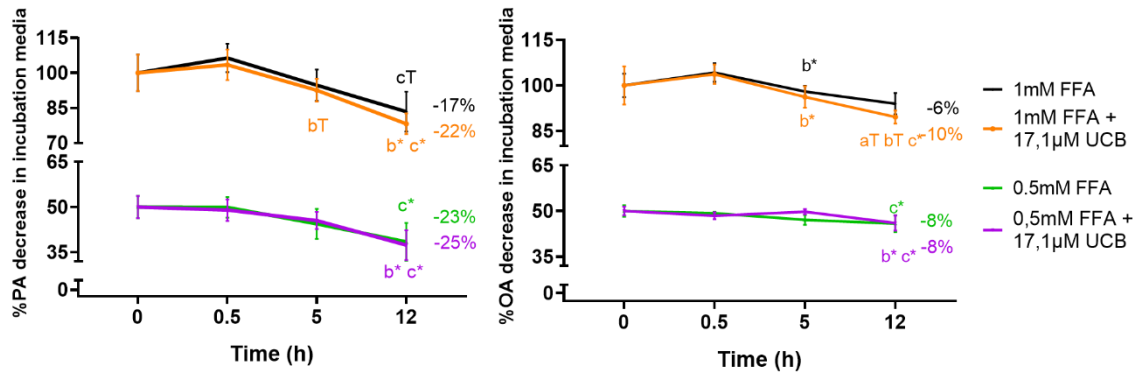
| Lipid accumulation by flow cytometry |                    |                            |
|--------------------------------------|--------------------|----------------------------|
| Treatments                           | 0.5h               | 5h                         |
| BSA                                  | 1.00 ± 0.18        | 1.00 ± 0.03                |
| 17.1µM UCB                           | 1.04 ± 0.13 n.s.   | ↓ 0.88 ± 0.06 n.s.         |
| 0.5mM FFA                            | ↓ 0.93 ± 0.14 n.s. | ↓ 0.87 ± 0.07 n.s.         |
| 0.5mM FFA + 17.1µM UCB               | ↓ 0.87 ± 0.16 n.s. | ↓ 0.76 ± 0.09 n.s.         |
| 1mM FFA                              | ↓ 0.97 ± 0.11 n.s. | ↓ 0.87 ± 0.07 n.s.         |
| 1mM FFA + 17.1µM UCB                 | ↓ 0.85 ± 0.13 n.s. | ↓ <b>0.74 ± 0.05 0.045</b> |

## HepG2

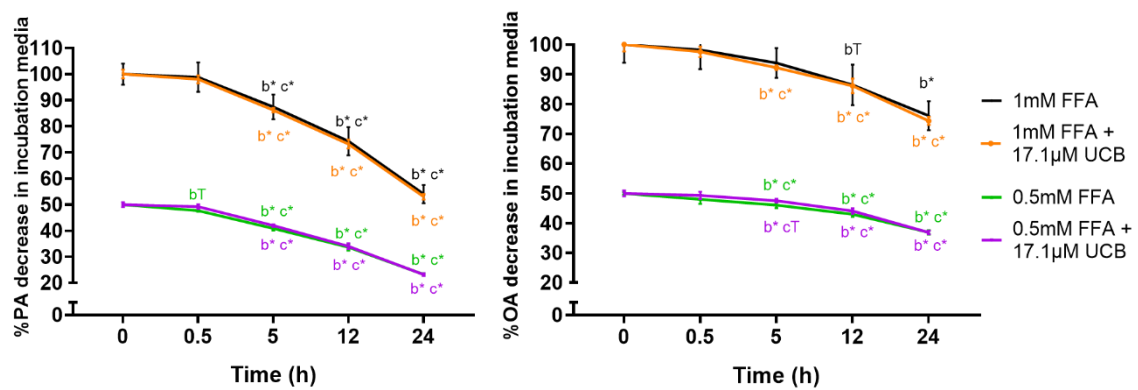
| Lipid accumulation by flow cytometry |                    |                            |
|--------------------------------------|--------------------|----------------------------|
| Treatments                           | 0.5h               | 5h                         |
| BSA                                  | 1.00 ± 0.06        | 1.00 ± 0.05                |
| 17.1µM UCB                           | ↓ 0.91 ± 0.08 n.s. | ↓ 0.98 ± 0.09 n.s.         |
| 55µM UCB                             | ↓ 0.90 ± 0.08 n.s. | ↓ 0.92 ± 0.07 n.s.         |
| 0.5mM FFA                            | ↓ 0.97 ± 0.13 n.s. | ↓ 1.09 ± 0.06 n.s.         |
| 0.5mM FFA + 17.1µM UCB               | ↓ 0.81 ± 0.13 n.s. | ↓ 0.91 ± 0.14 n.s.         |
| 0.5mM FFA + 55µM UCB                 | ↓ 0.80 ± 0.09 n.s. | ↓ 1.06 ± 0.10 n.s.         |
| 1mM FFA                              | ↓ 0.92 ± 0.11 n.s. | ↓ 1.05 ± 0.10 n.s.         |
| 1mM FFA + 17.1µM UCB                 | ↓ 0.85 ± 0.11 n.s. | ↓ <b>0.84 ± 0.08 0.096</b> |
| 1mM FFA + 55µM UCB                   | ↓ 0.77 ± 0.08 n.s. | ↓ 0.93 ± 0.15 n.s.         |

**Table S2:** Lipid accumulation in C2C12 and HepG2 single cells. Both cell lines were exposed to hypoglycaemic conditions and analysed using flow cytometry. The geometric mean fluorescence (FL2) of 50,000 NR-stained cells per treatment was compared to vehicle control. Data are mean ±SD of n=3 (each in duplicate). n.s.=non significant.





**Figure S1:** UCB slightly increased FFA uptake of C2C12 cells after 12h of incubation at hypoglycaemic conditions. FFA uptake was assessed indirectly by measuring the PA and OA concentrations in the supernatant of both cell lines by gas chromatography. Data are mean  $\pm$ SD of n=3 (each in duplicate). \* $p \leq 0.05$  and T  $p \leq 0.1$  of a = compared UCB treated vs. untreated cells, of b = compared to the preceding incubation time and of c = compared the initial concentration of the incubation media.



**Figure S2:** UCB did not change the FFA uptake of C2C12 during 24h of incubation when glucose was present. FFA uptake was assessed indirectly by measuring the PA and OA concentrations in the supernatant of both cell lines by gas chromatography. Data are mean  $\pm$ SD of n=3 (each in duplicate). \* $p \leq 0.05$  and T  $p \leq 0.1$  of a = compared to UCB treated vs. untreated cells, of b = compared to the preceding incubation time and of c = compared to the initial concentration of the incubation media.

### 3.1.2 Manuscript II

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# Towards quantitative [18F]FDG-PET/MRI of the brain: Automated MR-driven calculation of an image-derived input function for the non-invasive determination of cerebral glucose metabolic rates

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## Abstract

Absolute quantification of PET brain imaging requires the measurement of an arterial input function (AIF), typically obtained invasively via an arterial cannulation. We present an approach to automatically calculate an image-derived input function (IDIF) and cerebral metabolic rates of glucose (CMRGlc) from the [18F]FDG PET data using an integrated PET/MRI system. Ten healthy controls underwent test–retest dynamic [18F]FDG-PET/MRI examinations. The imaging protocol consisted of a 60-min PET list-mode acquisition together with a time-of-flight MR angiography scan for segmenting the carotid arteries and intermittent MR navigators to monitor subject movement. AIFs were collected as the reference standard. Attenuation correction was performed using a separate low-dose CT scan. Assessment of the percentage difference between area-under-the-curve of IDIF and AIF yielded values within  $\pm 5\%$ . Similar test–retest variability was seen between AIFs ( $9 \pm 8\%$ ) and the IDIFs ( $9 \pm 7\%$ ). Absolute percentage difference between CMRGlc values obtained from AIF and IDIF across all examinations and selected brain regions was 3.2% (interquartile range: (2.4–4.3) %, maximum < 10%). High test–retest intravariability was observed between CMRGlc values obtained from AIF (14%) and IDIF (17%). The proposed approach provides an IDIF, which can be effectively used in lieu of AIF.

## Keywords

Brain imaging, cerebral metabolic rates, [18F]FDG, integrated PET/MRI, image-derived input function

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## Introduction

In the last decade, combined PET/CT demonstrated the added value of anato-metabolic imaging<sup>1</sup> in patient management, mainly in oncology.<sup>1,2</sup> With the advent of combined PET/MR imaging, we witness a similar paradigm shift in managing patients with neurological disorders.<sup>3</sup> Specifically, fully integrated PET/MRI bodes well for the prospect of finally realizing the promise of absolute quantification of PET data in clinical routine. This migration from semi-quantitative measures to full quantification is highly desirable as it has the potential to improve the diagnostic value of molecular imaging.<sup>4</sup> To this end, routine quantification will depend on the development of automated approaches to provide clinicians with absolute quantitative values, in the same way as with standardized uptake values (SUVs) today.<sup>5</sup>

Absolute quantification in PET studies of the brain requires the knowledge of an input function (IF). In research studies, an arterial input function (AIF) can be obtained using arterial cannulation and collection of blood samples at regularly timed intervals. However, this approach is untenable for clinical routine. As a result, clinical brain studies currently apply a semi-quantitative approach based on SUVs, which lacks an absolute physiological scale. In the past, extraction of an IDIF for PET brain studies proved to be a challenge, given the presence of partial volume effects (PVEs)<sup>6,7</sup> and involuntary subject motion.<sup>6,8</sup>

Various methodological approaches have been proposed to calculate an accurate IDIF, thereby accounting for the factors above. These methods can be classified into three categories: (1) PET-only,<sup>9–19</sup> (2) standalone PET and MRI,<sup>20–25</sup> and (3) combined PET/MRI.<sup>26–29</sup> Most of the PET-only methods mandate the measurement of venous samples from a second venous line to avoid errors in IDIF originating from spill-in and spill-out effects occurring at late time points.<sup>6,30</sup> On the other hand, standalone PET- and MR-based methods are subject to logistical challenges and a sheer amount of post-processing required. A fully integrated PET/MRI addresses the main challenges for the determination of an accurate IDIF in a clinical setting, such as delineation of the internal carotid arteries<sup>20,26,27</sup> and motion correction of dynamic PET frames using MR navigators.<sup>31</sup>

Here, we introduce an MR-driven approach that allows for an automated calculation of IDIF using the synergistic information from an integrated PET/MRI. The calculated IDIF then allows for the non-invasive determination of brain metabolic rate of glucose (CMRGlc). Specifically, we employ an automated vessel segmentation algorithm and a PVC method, which accounts for the radial and circumferential

variability of the PET tracer distribution around the vessel. Our objective was to establish an automated workflow for the absolute quantification of [18F]FDG-PET/MRI brain data for clinical routine that is validated against the reference standard (AIF).

## Materials and methods

Ten healthy adults ((27 ± 7) years, (70 ± 10) kg, 5 males and 5 females) were included in this study. The study was approved by the Ethics Committee of the Medical University of Vienna (EK1960/2014) and was performed in accordance with the Declaration of Helsinki (1964), including current revisions. Subjects were confirmed to be healthy based on medical history, physical examinations and vital signs. Written informed consent was obtained from all subjects prior to the examinations.

### Imaging protocol

All subjects underwent test–retest PET/MRI brain examinations on a fully integrated PET/MRI system (Siemens Biograph mMR). All examinations were performed in the afternoon, with subjects at rest with their eyes open. Moreover, no specific task was performed by the subject. The average time difference between the two examinations was (17 ± 44) days. Prior to each scan, a venous line was established for the injection of the [18F]FDG tracer and an arterial line was established in the contralateral arm for blood sampling. A head and neck coil was used in order to ensure a high signal-to-noise ratio of the MR imaging. Foam cushions were placed inside the MR head coil to minimize involuntary head movement.

The integrated PET/MR imaging protocol included a 3D time-of-flight MR angiography (TOF-MRA) sequence to image the internal carotid arteries with the following parameters: 0.5 × 0.5 × 1 mm<sup>3</sup> voxel size, TE = 3.6 ms, TR = 21 ms, 25° flip angle, 228 × 384 matrix, 220 slices and an acquisition time of 6 min. The field-of-view of the TOF-MRA extended from the circle of Willis (CoW) to 10 slices below the branching point of the internal and external carotid arteries (ECAs). Subsequently, subjects were injected with [18F]FDG ((352 ± 66) MBq, 5.18 MBq/kg) intravenously as a slow bolus over 40 s. At the start of the injection, a 60-min list mode PET data acquisition was initiated and blood samples (1 mL each) were obtained from the radial artery using a varying time schedule (24 × 5 s, 1 × 60 s, 1 × 120 s, 1 × 300 s, 1 × 600 s, 2 × 1200 s post injection) (Supplementary Figure 1). The blood sampling was done manually using vacuum test tubes via an arterial cannula fitted with an adapter.

Prior to every arterial sample, the line was flushed with 5 mL sodium chloride solution to prevent clotting and sampling stagnant blood. To avoid dilution of the actual sample, a 1 mL of discard was drawn followed by the sampling of arterial blood sample. Whole-blood radioactivity concentrations were measured using a gamma counter (PerkinElmer, 2480 Automatic Gamma counter, Wizard<sup>23</sup>). To obtain the AIF, whole blood samples were centrifuged to separate the plasma component, followed by the measurement of radioactivity in the plasma. The measured whole blood and plasma tracer concentrations were used to calculate the dynamic plasma-to-blood ratio for each subject. This ratio was then used to convert the blood-IDIF to plasma-IDIF. Arterial sample concentrations were measured at instantaneous time-points, whereas the IDIF concentrations were measured at PET mid-scan time-points. In order to match the instantaneous blood sampling times with the PET mid-scan time, the AIF was interpolated into discrete time segments of 1 s length using a Piecewise Cubic Hermite Interpolating Polynomial.

To monitor head motion, MR navigators were used throughout the dynamic PET acquisition using the following parameters: 2D EPI  $3.0 \times 3.0 \times 3.0 \text{ mm}^3$  voxels,  $64 \times 64$  matrix, 36 slices, TE 30 ms, TR 3000 ms. Navigator volumes were obtained at fixed time intervals: 0, 2.5, 5, 7.5, 10, 14, 17, 21, 26, 33, 38, 42, 44 and 50.5 min post injection. Following the PET/MRI examination, the subjects were moved to a PET/CT system (Biograph TruPoint64, Siemens Healthcare, USA), where a low-dose CT scan (120 kVp, 50 mAs) of the brain was acquired solely for the purpose of attenuation correction (AC).<sup>32,33</sup>

The PET list mode data were re-binned into a dynamic frame sequence ( $24 \times 5 \text{ s}$ ,  $1 \times 60 \text{ s}$ ,  $1 \times 120 \text{ s}$ ,  $1 \times 300 \text{ s}$ ,  $1 \times 600 \text{ s}$ ,  $2 \times 1200 \text{ s}$  post injection) and each PET frame was reconstructed (Siemens e7 tools) into a  $344 \times 344 \times 127$  matrix (voxel size  $2.08 \times 2.08 \times 2.03 \text{ mm}^3$ ) using the ordinary Poisson ordered subset expectation maximization (OP-OSEM) 3D algorithm (3 iterations, 21 subsets, 2 mm Gaussian filter). Scatter correction along with a CT-AC was applied to all PET data.<sup>34</sup> To perform the CT-AC, the low-dose CT scan was co-registered to the T1-MPRAGE sequence (RS-1, Supplementary Figure 1) and a bilinear scaling<sup>31</sup> was applied to convert the low-dose CT image to a CT-AC map.

### Automated ICA segmentation

Data from the 3D TOF-MRA sequence were used to extract the ICA. To obtain the IDIF, the petrous region

of the ICA was chosen as the volume-of-interest (VOI). Image segmentation was performed in three steps:

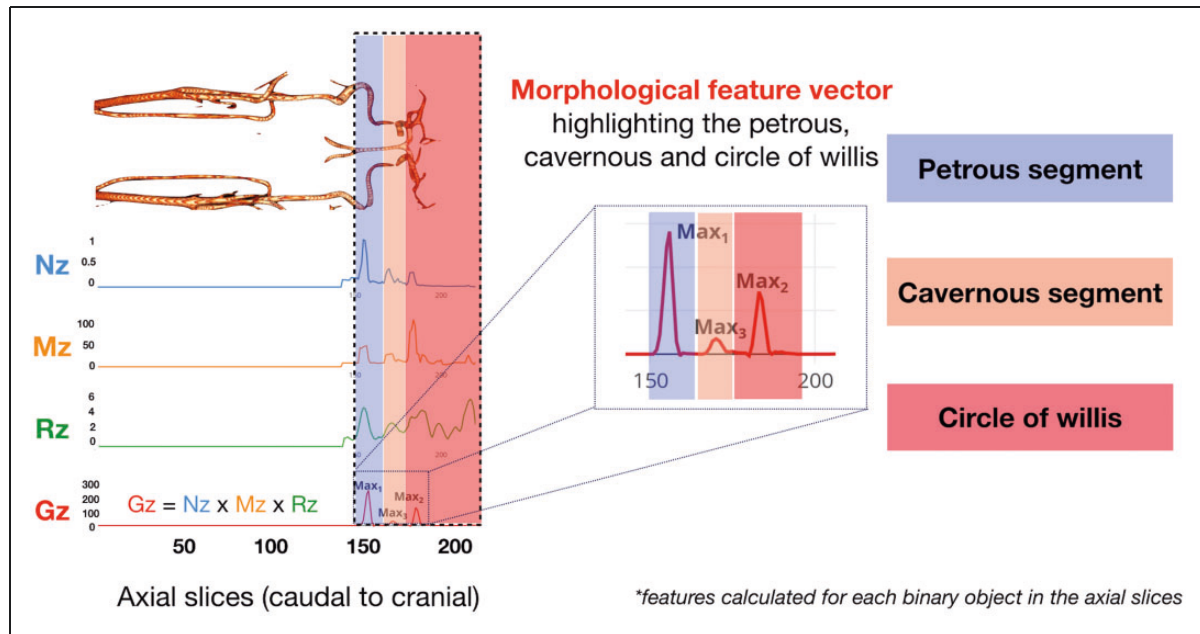
**Extraction of the carotid vasculature.** A combination of histogram-based quantile thresholding<sup>35,36</sup> and automatic seeded region growing was used to extract the entire carotid vasculature (CV) (Supplementary Figure 2). The intensity corresponding to the 0.987 quantile of the gray value distribution was chosen as the optimum threshold value following an iterative optimization procedure using the TOF-MRA datasets. To remove residual contributions of the peripheral fat, an automated seed region growing was performed with a connectedness constraint, yielding only the CV (Supplementary Figure 2).

**Extraction of the ICA.** The obtained CV consists of the ICA, ECAs and the CoW (Supplementary Figure 2). The ICA was obtained by removing the CoW first, followed by the pruning of ECA. The CoW is superior to the cavernous segment and can be removed once the cavernous segment in the vasculature is localized. This was determined based on a morphological feature vector (Gz), which characterizes the shape of the vascular tree (Figure 1). This feature curve was calculated only for the intracranial segment of the TOF-MRA volume as only segmentation of the petrous part of the ICA was of interest. The morphological feature curve incorporates features, such as mean intensity, major axis length, ellipticity as well as the orientation of vessel segments present in the transaxial slices (Figure 1). The axial slice containing the structure with an orientation of  $90^\circ$  to the vessel denotes the location of the cavernous segment of the ICA, thus, allowing for the removal of the CoW. Moreover, the ICA and ECA arise from a common carotid artery, and therefore the localization of the branching point is key to the pruning of the smaller vessel (ECA), thus, leaving the ICA as the only remaining structure.

**Segmentation of the petrous region of the ICA.** This segmentation was done based on the classification of ICA by Gibo et al.<sup>37</sup> The feature curve in Figure 1 highlights elliptical structures in the image as prominent peaks. Accordingly, the petrous segment was identified as the structure with the highest peak. These steps resulted in a petrous mask ( $P_{\text{mask}}$ ), which was later used to extract the IDIF from the dynamic PET frames.

### PET motion correction and alignment with TOF-MRA

To achieve the spatial correspondence between the PET frames and the MR-derived petrous masks, a post-reconstruction motion correction was employed.



**Figure 1.** Segmentation of the carotid vasculature (CV) features such as normalized mean intensity (Nz), major axis length (Mz) and ratio of major to minor axis length (Rz) were calculated and combined to produce a morphological feature vector (Gz), to highlight elliptical structures. The global maxima,  $Max_1$  of Gz (blue), corresponds to the petrous segment, while  $Max_2$  and  $Max_3$  correspond to the floor of circle of Willis (red) and cavernous (orange).

MR navigators interleaved between MR clinical sequences were used to monitor subject head motion (Supplementary Figure 1). The MR navigator acquired at the start of the PET acquisition (Nav-0,  $t=0$ ) was considered as the reference volume and all subsequent navigators were rigidly co-registered to Nav-0 (SPM 12, Wellcome Trust Center for Neuroimaging, UCL) to obtain the motion vectors. The resulting motion profile consisted of six parameters (three translations and three rotations; Figure 2). In early PET frames with low tracer activity, MR based motion correction was implemented. Temporal matching of MR navigators with PET frames was achieved using the least time difference between the PET frame mid-scan times and the navigator acquisition times. (Figure 3(a)). Eventually, transformation matrices obtained from the early MR navigators ( $<10$  min) with respect to the reference MR navigator will be applied to  $P_{mask}$ , thus, generating multiple resliced  $P_{mask}$  VOIs.

Subsequently, spatial correspondence between the petrous mask and the PET data ( $>10$  min) was achieved in a multi-step process using SPM 12 (Figure 3(b)). First, transformation matrices ( $T_{10-60}$ ) were derived from rigid inter-modality co-registration (Normalized mutual information) of the TOF-MRA data and the late PET frames (10 min, 20 min, 40 min and 60 min). Second, these matrices ( $T_{10-60}$ ) were applied to the petrous mask ( $P_{mask}$ ). The alignment was confirmed visually using AMIDE 1.0.5 (AMIDE's a Medical Image

Data Examiner<sup>38</sup>) without the need for further post-processing.

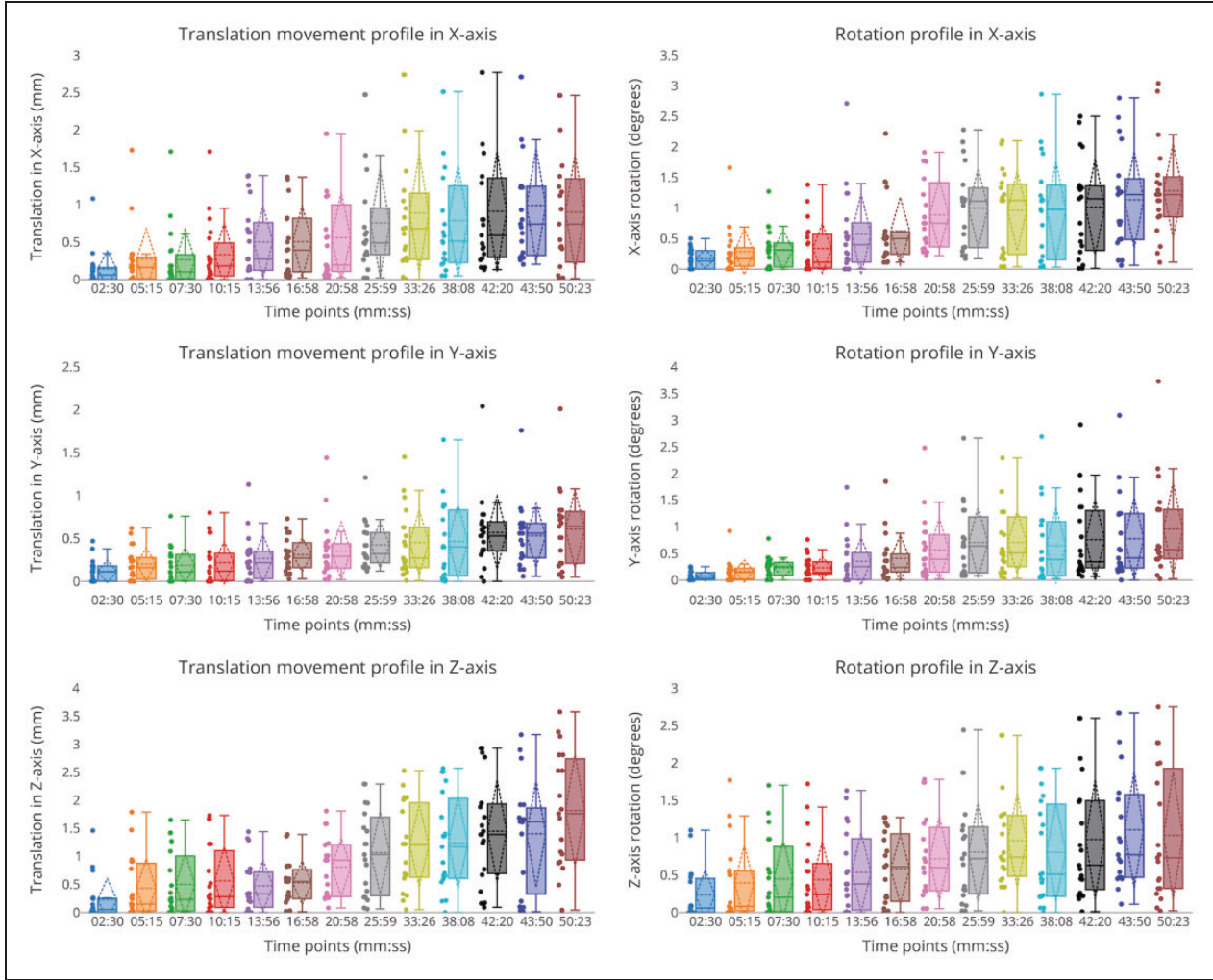
As a part of quality control, MR navigators acquired after 10 min were used to check for intra-frame motion within the individual late PET image volumes ( $1 \times 10$  min,  $2 \times 20$  min frames). Here, motion vectors never exceeded 1 mm in any direction (Supplementary Table 1), thus, indicating no prominent intra-frame motion.

### Partial volume correction

Correction of PVE was performed using a modified version of the Mueller-Gaertner method (MGM),<sup>39</sup> with the extension of accounting for radial and circumferential variability of the activity in the petrous segment's background. Calculation of the true ICA tracer concentration entails first the removal of variable background activity that spilled into the target region (spill-in correction) followed by correction of activity loss in the target region (spill-out correction) caused by the convolution of true activity distribution by the system's PSF. This procedure mandates the knowledge of both the PSF and the nature of the background activity distribution (Supplementary Figure 3).

In order to determine the PSF corresponding to the applied acquisition protocol, a 1-mL syringe (diameter 4.7 mm, cylinder length 57 mm) was filled with about 75 MBq of [ $^{18}F$ ]FDG and placed axially in the system in approximately the same off-center position





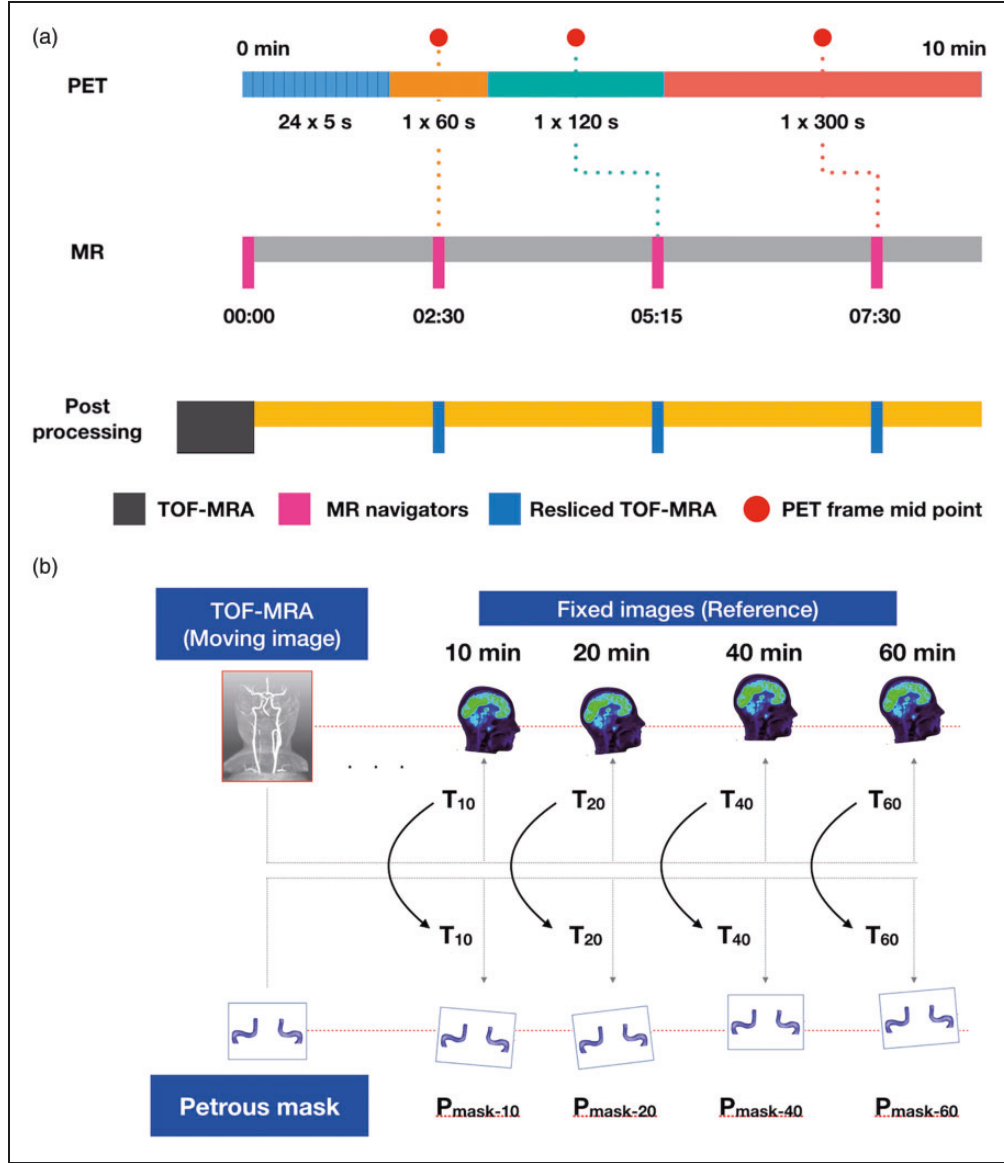
**Figure 2.** Translation (left) and rotation (right) profiles in each axis with respect to examination time (Supplementary Figure 1) for 20 datasets. Subject motion (translation and rotation) increased with time and subject translation was prominent in the z-axis. It is seen that the motion was minimal during the first 10 min of the scan (maximum translation < 2 mm, average rotation < 1 and maximum rotation < 2°).

(~50 mm) as the presumed position of the ICA. Data were acquired and reconstructed using the same protocol as used in the subjects. Subsequently, a modeled profile (using a step function with a height corresponding to the tracer concentration in the syringe at the start of the PET scans) was convolved with a 3D Gaussian PSF and compared to the measured trans-axial profile. The FWHM of the PSF was incremented in 0.5 mm steps. The convolved model function that best approximated the measured profile defined the FWHM of the PSF corresponding with our protocol. The FWHM of the PSF for the PET/MR system in use (Biograph mMR) was found to be  $(6.0 \pm 0.4)$  mm.

To obtain an initial estimate of background activity, the spill-out zone ( $Sp_{out}$ ) was defined by convolving  $P_{mask_i}$  ( $i$  – indicates the PET frame, with ‘ $i$ ’ varying from 1 to 30) with the derived PSF of the PET system

(Figure 4(a)). An initial background mantle ( $B_{mask_i}$ ) was defined 10 mm radially from the edges of the  $Sp_{out}$  (Figure 4(a)). To account for circumferential differences in background activity (Figure 4(b)), the tracer concentration in the ring-like structure was segmented into 20 background regions ( $B_{mask_{ij}}$ ) by applying Otsu thresholding<sup>40</sup> and scaled with the corresponding median activity ( $B_{ij}$ ) sampled from the PET frame. This allowed for the calculation of an initial estimate of  $ICA_i$  using equation(2), assuming that the area between each  $B_{mask_{ij}}$  and the ICA wall contains a constant tracer concentration equal to the  $B_{ij}$  values determined at the periphery.

$$PET_i = (ICA_i \cdot P_{mask_i}) \otimes PSF + \sum_{j=1}^{20} B_{ij} \cdot B_{mask_{ij}} \otimes PSF \quad (1)$$



**Figure 3.** (a) Use of MR navigator-based motion correction, in early PET frames (<10 min). Each MR navigator is assigned to a PET frame based on the time difference between the PET frame midpoint (PFM, red) and MR navigator's acquisition time (AT). Lower the time difference between PFM and AT, higher the probability of assigning the MR navigator to the respecting PET frame. The MR navigator acquired at start of the PET acquisition (time = 00:00) is considered as the reference volume (RV) and all other MR navigators (02:30, 05:15, 07:30) are registered to RV. The transformation matrices obtained from the process are transferred to the 3D TOF-MRA or  $P_{mask}$ , to achieve spatial correspondence to the PET frames. (b) Motion vectors ( $T_n$ ) were obtained by co-registering the TOF-MRA with the late dynamic PET frames (>10 min) and then applied to the petrous mask ( $P_{mask-n}$ ) to achieve spatial correspondence with the late dynamic PET frames.

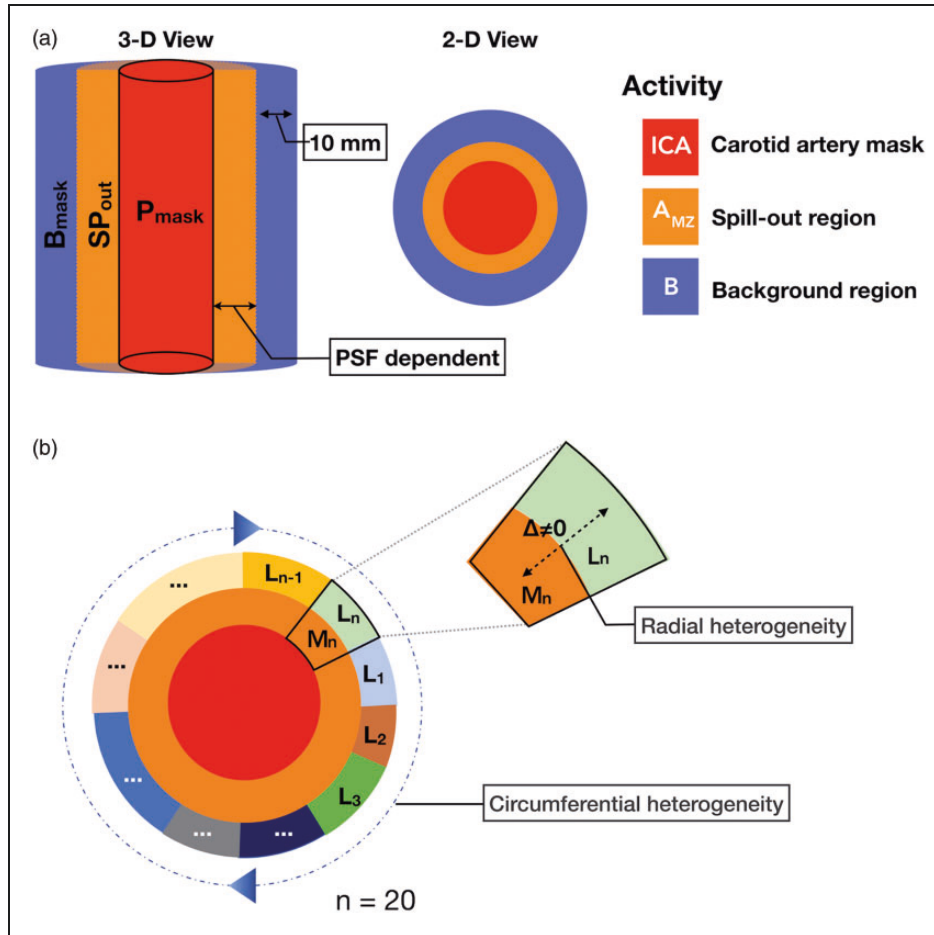
$$ICA_i = [SP_{Out_i}]^{-1} \left[ PET_i - \sum_{j=1}^{20} B_{ij} \cdot SP_{in_{ij}} \right] \quad (2)$$

$$\begin{aligned} \text{where } SP_{out_i} &= P_{mask_i} \otimes PSF \quad \text{and} \quad SP_{in_{ij}} \\ &= B_{mask_{ij}} \otimes PSF \end{aligned}$$

Radial variability in the area between  $B_{mask_{ij}}$  and  $ICA$  wall was addressed as follows. Initially, for each of the  $B_{mask_{ij}}$  regions, a mixed zone ( $MZ_{ij}$ ) was defined, which includes the area between the  $ICA$  ( $P_{mask}$ ) and  $B_{mask_{ij}}$  (Figure 4(b)). The true tracer concentration in  $MZ_{ij}$  (denoted  $A_{MZ_{ij}}$ ) was then modelled as

$$A_{MZ_{ij}} = PET_{MZ_{ij}} - ICA \cdot SP_{out_i} - B_{ij} \cdot SP_{in_{ij}} \quad (3)$$





**Figure 4.** (a) Spill-in correction: automatic delineation of a spill-out region ( $SP_{out}$ , orange), defined by convolving the petrous mask ( $P_{mask}$ , red) with the PSF (FWHM-6.00 mm), and a background region (purple) of 10 mm ( $\sim 5$  voxels) thickness defined from the edges of the spill-out region. (b) The circumferential heterogeneity of petrous segment (red) is graphically represented by labels ( $L_1-n$ ) with different colors, where  $n = 20$ . The radial heterogeneity is depicted using the sector- $n$ , where the activity,  $M_n$  in the spill-out zone/mixed-zone (orange) is not the same as the activity,  $L_n$  in the background region (green).

where  $PET_{MZ_{ij}}$  is the observed tracer concentration in  $MZ_{ij}$  and  $SP_{in}$ , the spill-in factor. Subsequently, the set of background regions was tested for radial homogeneity by calculating the differences between  $A_{MZ_{ij}}$  and  $B_{ij}$ . If  $A_{MZ_{ij}}$  was found to be within 3% of  $B_{ij}$  (equivalent to  $|B_{ij} - A_{MZ_{ij}}| < 0.03 B_{ij}$ ), it was assumed that radial uniformity was met for that particular background segment. Otherwise, a new estimate of ICA was derived iteratively until equation (2) was satisfied:

$$|\alpha_{ij} - ICA \cdot SP_{out_i}| = \min \quad (4)$$

$$\alpha_{ij} = (PET_{MZ_{ij}} - B_{ij} \cdot SP_{in_{ij}} - A_{MZ_{ij}}) \quad (5)$$

where  $\alpha_{ij}$  is the observed spill-out contribution in the mixed zone  $MZ_{ij}$  originating from the true ICA tracer concentration.

Here, the difference between the observed ( $\alpha_{ij}$ ) and modeled ( $ICA \times SP_{out}$ ) ICA spill-out contribution in

$MZ_{ij}$  was minimized for the whole set of mixed zones ( $MZ_{ij}$ ,  $j = 1, \dots, 20$ ). The resulting ICA value was adjusted for maximum agreement of radial profiles in the mixed zone, thus, accounting for radial inhomogeneity. This procedure was repeated for all PET frames, to generate a partial volume corrected IDIF. Supplementary Figure 4 summarizes the radial and circumferential variability in a real case scenario.

### Post-processing of the IDIF

Following MoCo and PVC, the IDIF was interpolated with a step length of 1 s to match the blood sampling times and was corrected for multiple effects. First, a plasma IDIF was derived based on the individual plasma-to-blood ratios obtained from sampled arterial blood of the study subjects. Second, the IDIF was scaled using the cross-calibration factor between the PET/MR and the on-site gamma counter. Third, the

delay between the AIF and the IDIF was corrected by shifting the IDIF curve to match the arrival times of the AIF. Finally, due to the difference in sampling location (ICA for IDIF and radial arteries for AIF), a mono-exponential dispersion function with a tau value of  $5\text{ s}^{41,42}$  was convolved with the IDIF to mimic the dispersion effects. Since the AIF is considered as the reference standard, all the corrections were applied to the IDIF for an unbiased comparison with the AIF.

### Assessment of IDIF-derived metabolic rate of glucose

To assess agreement between the IDIF and AIF, the area-under-the-curve (AUC) derived from both curves was used. Specifically, disparities between the AUC measurements were assessed based on the percentage AUC difference (%Diff<sub>AUC</sub>) calculated as

$$\% \text{Diff}_{\text{AUC}} = 100(\text{AUC}_{\text{AIF}} - \text{AUC}_{\text{IDIF}}) / (\text{AUC}_{\text{AIF}}) \quad (6)$$

Absolute percentage AUC differences were calculated across all studies as well as pair-wise between test and re-test acquisitions.

Finally, cerebral metabolic rate of glucose (CMRGlc) maps were calculated (PMOD 3.802, PMOD Technologies, Zurich, Switzerland) using the standard rate constant approach<sup>43</sup> (lumped constant,  $\text{LC} = 0.65^{61}$ ). Absolute percentage differences between regional CMRGlc values for whole brain and in six pre-selected large regions of the brain (cerebellum, brainstem, anterior cingulate cortex, thalamus, corpus callosum and superior frontal cortex) from the Hammersmith atlas were determined using AIF and IDIF. All comparisons were performed in a non-parametric manner, using the median difference, the interquartile range and the extremes. In order to determine whether regions are affected differentially using the IDIF, a rank-sum test was performed among all regions.

Test-retest variability for both the IFs was assessed by calculating the absolute mean % difference, after normalizing the IFs to the respective injected dose and body weight (SUV). Similarly, test-retest variability was assessed by calculating the absolute median % difference for the CMRGlc values (whole brain and four random regions – insula, caudate, mid-frontal gyrus and superior frontal gyrus) obtained from both IFs

## Results

Assessment of the percentage difference between all AUCs using the IDIF and AIF yielded values within  $\pm 5\%$  (Figure 5(a)), with a median absolute difference

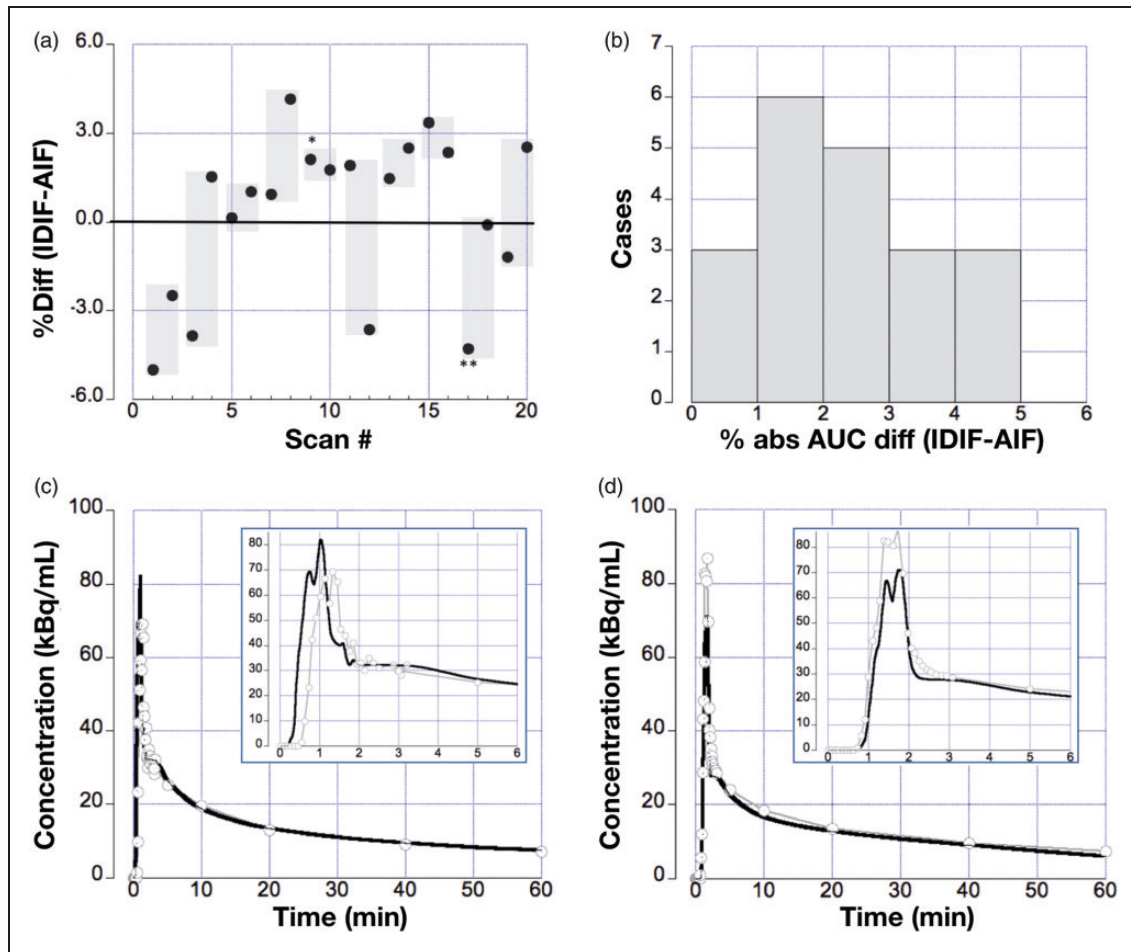
of 2.5% (interquartile range (IQR) = 2%–4%). Figure 5(b) shows the histogram of the absolute percent differences, indicating that 14/20 (70%) of the IDIF curves were found to be within 3% of the reference standard (AIF). The remaining 30% displayed a somewhat larger AUC difference in the range of 3%–5%. Moreover, 2/3 of all percent differences were determined to be positive, indicating a trend towards a possible overestimation of the AUC using the IDIF. The representative time-activity curves emphasizing the relationship between arterial blood samples and the IDIF are shown for a case with AUC difference  $< 3\%$  (Figure 5(c)) and for a case with AUC difference  $\sim 4\%$  (Figure 5(d)).

Supplementary Figure 5 depicts the range of the AUCs for AIF ( $46.2 \pm 7.6$ ), IDIF with PVC ( $46.4 \pm 8.1$ ) and IDIF without PVC ( $35.7 \pm 7$ ). Supplementary Figure 6 shows the test-retest variability in the IFs, both AIF and IDIF showed similar variability with an absolute mean % difference (test-retest) of  $9 \pm 8\%$  and  $9 \pm 7\%$ .

Figure 6(a) shows an example of a parametric image based on AIF and IDIF for a subject (HC014 retest) with 3% overestimation by IDIF. The whole brain CMRGlc values derived from AIF was found to be (mean  $\pm$  SD =  $32 \pm 6$ , median  $\pm$  IQR =  $31 \pm 9$ )  $\mu\text{mol}/100\text{ g}/\text{min}$  and for IDIF (mean  $\pm$  SD =  $32 \pm 6$ , median  $\pm$  IQR =  $29 \pm 9$ )  $\mu\text{mol}/100\text{ g}/\text{min}$  (Supplementary Figure 10). Absolute median % difference between CMRGlc values obtained using the AIF and IDIF for the whole brain was found to be 3.9%, with an interquartile range of 2.4%–5.4%. And for the six regions, the absolute mean % difference was determined as 3.2%, with an interquartile range of 2.4%–4.3% and a maximum difference of  $< 10\%$ . Figure 6(b) shows the %-differences with respect to CMRGlc values derived using the two IFs. Comparison of Figures 5(c) and 6(b) indicates an inverse relationship between the percentage difference in AUCs and CMRGlc.

Figure 6(c) shows the corresponding histogram, indicating that the most frequently observed differences are in the range of 2%–4%, with 85% of all differences being  $< 5\%$ . In all six-preselected brain regions, the CMRGlc values obtained from AIF and IDIF showed no significant difference.

The test-retest variability between AIF based CMRGlc and IDIF based CMRGlc is shown in Supplementary Figure 7. For both the IFs, CMRGlc obtained from the four regions during the retest were higher when compared to test, along with a reduction in variance during the retest. In general, retest AIF CMRGlc values were 14% higher (14% higher for insula, mid-frontal gyrus, superior frontal gyrus and 22 % higher for caudate) when compared to the test values, indicating a high intra-variability. A similar



**Figure 5.** (a) The panel displays the %-differences for all 20 scans (points); the shaded areas depict the difference range for test/retest scans obtained in the same subject. IDIF curves obtained from subject #5 (HC004 test, denoted as \*) and #9 (HC007 test, denoted as \*\*) are displayed in (c) and (d). Percent (%) differences between AUCs obtained from AIF (gray with circles) and IDIF (black) were calculated (b). Histogram displaying the distribution of absolute % AUC differences, 70% of the AUCs differ by <3% with the remaining curves differing by <5%. (c) IDIF obtained in subject #5: (HC004 test Table 1) with a good agreement between IDIF and AIF. (d) Example IDIF obtained from subject #9 (HC007 test) with a limited (tail area) agreement between IDIF and AIF.

tendency was seen in retest IDIF CMRGlc values as well, with a 17% increase (12% higher for insula, 18% for caudate, 17% for mid-frontal gyrus and 16% for superior frontal gyrus) when compared to test values.

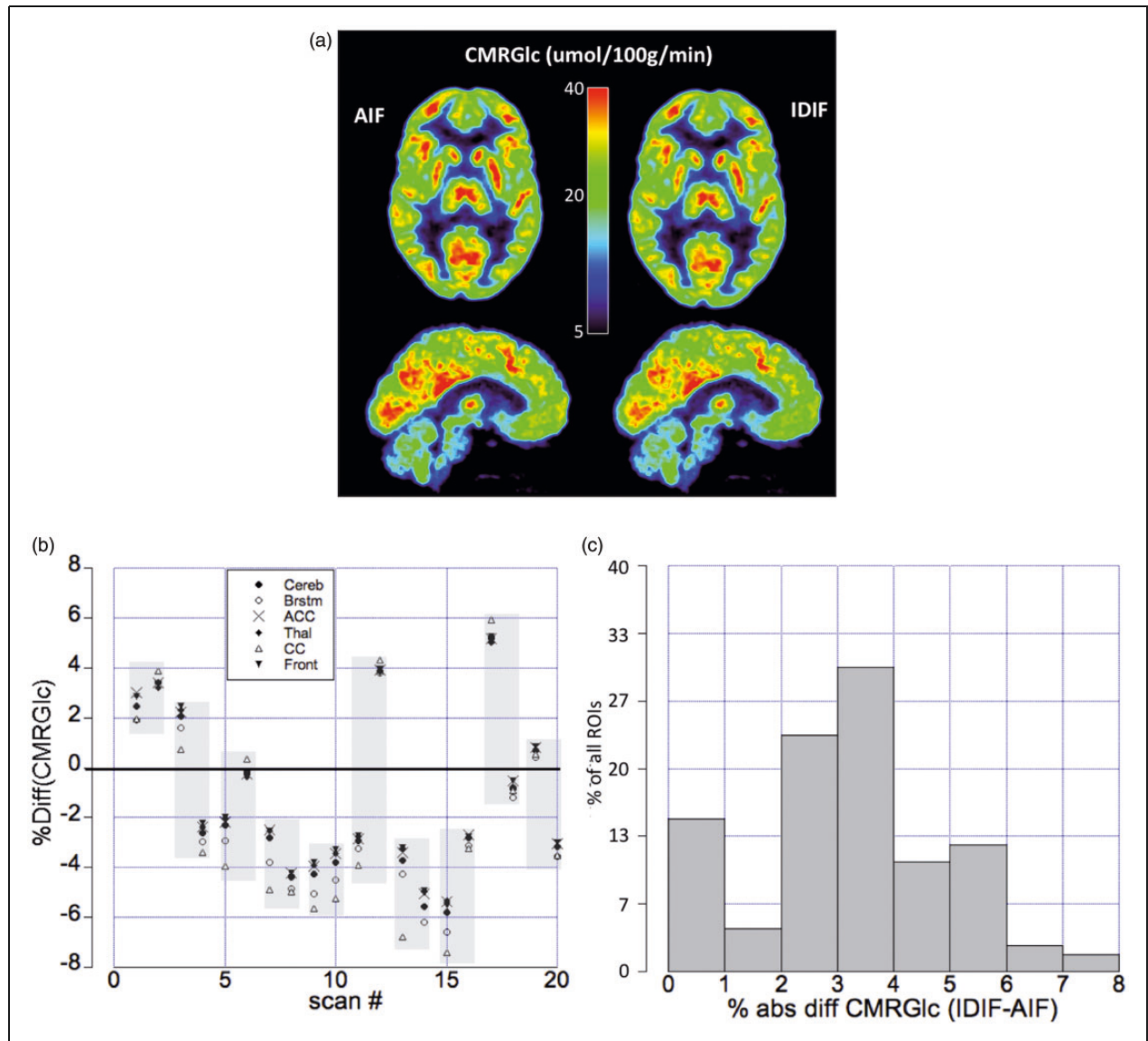
The absolute % relative difference between test and retest whole-brain CMRGlc values (Supplementary Figure 11) was found to be  $14 \pm 8\%$  for AIF (with median  $\pm$  IQR =  $15 \pm 16\%$ ) and  $15 \pm 10\%$  for IDIF (with median  $\pm$  IQR =  $14 \pm 17\%$ ).

## Discussion

We present a clinically viable automated MR-driven approach to extracting an IDIF for the non-invasive determination of CMRGlc images using an integrated [18F]FDG-PET/MRI protocol. The approach is based

on the accurate and automated extraction of an IDIF based on the combined acquisition of PET and MR data. It was validated against the reference standard of AIF. In the context of the automated IDIF calculation, a multi-factorial partial volume correction is required. Therefore, the directional and radial variability of the background region (Figure 4(b)) must be considered for an accurate correction of the spillover effects. This procedure also mandates the knowledge of the system's PSF for a given tracer and image reconstruction protocol. Any PVC must be preceded by motion correction. Our study shows that the above requirements can be effectively addressed by employing an integrated PET/MRI protocol followed by an automated post-processing pipeline.

Traditionally IDIF has been extracted from two different segments of the ICA: (1) the petrous



**Figure 6.** (a) Transaxial (top row) and sagittal (bottom row) images representing CMRGlc derived using the AIF (left) and the IDIF (right) for a subject with a 3% AUC overestimation by IDIF. Images show excellent agreement in absolute CMRGlc values. (b) Relative %-differences for six reference regions (cerebellum, brainstem, anterior cingulate cortex, thalamus, corpus callosum and superior frontal cortex) in all 20 scans. The shaded areas depict test/retest scans obtained in the same subject. (c) Histogram depicting the % absolute differences in CMRGlc values derived using the IDIF and the AIF. The graph shows a maximum for difference values in the range of 2–4%, with 85% of all differences laying within 5%.

region<sup>24–27,29,44–46</sup> or (2) the cervical region.<sup>20,47</sup> We believe that the petrous region is preferable, as it represents a rigid structure due to its placement inside the petrous canal. In contrast, the cervical segment is more elastic and prone to deformation. Since rigid transformations were used to achieve spatial correspondence between the TOF-MRA and the PET frames, the petrous region was chosen for derivation of the IDIF. The aforementioned segmentation algorithm was successful in defining the ICA irrespective of the subject-specific cerebral vasculature (Supplementary Figure 1).

Moreover, due to its modular nature, the proposed algorithm can be easily modified for other FOV acquisitions (cervical and cavernous segments). The optimum quantile value of 0.987 may change based on the quality of TOF-MRA volumes. Decreasing the quantile value will result in higher inclusion of peripheral fat, whereas an increase will result in eroded segmentation of the carotid arteries. Care should be taken in choosing an optimum quantile value as the pre-segmentation of the carotid arteries is dependent on the chosen quantile value.



**Table 1.** Demographics of the 10 healthy controls.

| Subject ID | Gender | Age (y) | Weight (kg) | Date       |            | FDG Activity (MBq) |        | Blood glucose (mmol/L) |        |
|------------|--------|---------|-------------|------------|------------|--------------------|--------|------------------------|--------|
|            |        |         |             | Test       | Retest     | Test               | Retest | Test                   | Retest |
| HC002      | M      | 23      | 75          | 06-06-2016 | 14-07-2016 | 217                | 371    | 4.55                   | 5.72   |
| HC003      | F      | 24      | 55          | 08-06-2016 | 09-11-2016 | 229                | 288    | 4.33                   | 4.73   |
| HC004      | M      | 28      | 74          | 11-07-2016 | 26-09-2016 | 384                | 367    | 4.88                   | 5.49   |
| HC006      | F      | 22      | 69          | 12-09-2016 | 07-11-2016 | 365                | 355    | 5.11                   | 5.22   |
| HC007      | F      | 22      | 56          | 14-09-2016 | 28-09-2016 | 292                | 292    | 5.38                   | 5.61   |
| HC009      | F      | 42      | 63          | 23-11-2016 | 05-12-2016 | 330                | 324    | 5.11                   | 5.83   |
| HC010      | F      | 20      | 65          | 07-12-2016 | 19-12-2016 | 337                | 338    | 5.11                   | 5.55   |
| HC012      | M      | 36      | 92          | 23-01-2017 | 13-02-2017 | 493                | 476    | 6.32                   | 6.05   |
| HC013      | M      | 24      | 70          | 15-02-2017 | 27-02-2017 | 350                | 363    | 5.05                   | 5.16   |
| HC014      | M      | 25      | 80          | 27-03-2017 | 29-03-2017 | 422                | 364    | 4.61                   | 5.88   |

The ICA has a variable background both in radial and circumferential direction. Circumferential variability is due to the presence of cortical structures with different amount of tracer accumulation around the target region, whereas radial variability is related to the sampling of the background region (Figure 4(a) and (b)). Due to the spillover of ICA activity into the neighboring background tissue, an estimate of the circumferentially dependent background tracer concentration must be obtained at a distance from the ICA where the ICA spillover can be ignored. In most implementations, this is achieved by sampling background tracer concentration at a distance where ICA tracer concentration contributes <3% to the local tracer concentration. Of note, this approach cannot account for radial changes, as the tracer concentration sampled in such a way might not be representative for the background concentration directly adjacent to the vessel wall (Supplementary Figure 4).

The importance to account for radial and circumferential variability has not been fully appreciated in previous region-based PVC methods. Rousset et al.<sup>48</sup> derived a closed solution for PVC using a geometric transfer matrix, which relates a vector of observed activities to a vector of true (PVC-corrected) activities. This linear equation system can be solved for the true activities by matrix inversion; however, the accuracy of such a solution strongly depends on the degree of ill-conditioning of the transfer matrix and, in addition to a large computational effort, might lead to unstable estimates of the corrected activities.

Obviously, the complexity of such a correction is dependent on the number of tissues believed to have different functional properties, i.e. the number and shape of homogenous regions that contribute to the PVE in each image voxel. Various investigators proposed the definition of such regions based on a

combination of information from a high-resolution anatomical (e.g. MRI) and a functional (PET) image using wavelet decomposition.<sup>49,50</sup> Our approach is similar to these methods in that it uses functionally homogenous background regions to correct for PVEs at one specific target region (ICA). Furthermore, it is accurate and computationally efficient for determining an IDIF by avoiding complex mathematical overhead by iteratively adjusting the activity in the target region so that it becomes consistent with the observed activity in the mixed zone. Although our approach is less ambitious than the general solution presented by Rousset et al., we believe that it is fitting for determining an IDIF that can be readily implemented in clinical applications.

The proposed PVC method requires the knowledge of PSF of the PET system. Since PSF varies with factors such as image reconstruction parameters and the type of tracer, a dedicated PSF measurement for the specific PET system and protocol is recommended.

Since all corrections are applied to a relatively small region, determination of an IDIF is highly sensitive to local misregistration arising from involuntary patient motion (Supplementary Figure 8). In a combined PET/MR system, MR navigator sequences can be used to derive high-temporal-resolution motion estimates along with the PET emission scan.<sup>31</sup> The resulting motion vectors can be then applied to the VOI to account for motion between frames, therefore, avoiding PET image resampling. In the current protocol, MR navigators were acquired with a reasonably fast temporal sampling rate (~2 min temporal separation between navigator), during the early phase of the scan (<10 min), thereby offering the possibility to perform motion correction using the motion fields from the MR navigators. However, due to the constraints of clinical sequences during the subsequent 50 min, MR navigators were acquired with low-temporal sampling rate

(~4–5 min temporal separation between every navigator). Therefore, a PET-MRI NMI-based co-registration was preferred, instead of using the motion fields from the late navigators (>10 min). Since MR navigators are acquired in a relatively short time duration (~5 s), there is an increased probability that the motion vectors captured by these navigators are not representative of the average motion occurring during a 5-min time frame. While post-reconstruction motion correction approaches yield satisfactory results, simultaneously acquired PET and MR data offer the possibility of an “on-the-fly” motion correction, in which the motion vector is used to rebin the PET coincidence data, so that subject motion is addressed on a very basic line-of-response (LOR) level. Several pilot approaches have been proposed to incorporate on-the-fly motion correction schemes,<sup>51</sup> yet implementation of these methods proved to be challenging due to the necessity of recalculating both normalization factors and random estimates.

Apart from comparing the CMRGlc images from the AIF and IDIF to determine the accuracy of the IDIF methodology, it is informative to compare the IFs by using metrics such as AUC. In the context of the standard rate constant approach, the AUC of the IF represents the total amount of tracer that is available to the tissue, while the metabolic rate encodes the relationship between the amount of tracer available for uptake and the actual tissue uptake. Therefore, demonstration of similarity by comparing the AUCs of AIF and IDIF provides a reasonable quality check to assess the accurateness of the method.

Analysis of AIF-derived AUC differences obtained from test/retest scans showed no significant correlation between the AUC differences and the length of time separating the two acquisitions ( $R^2=0.03$ ). These results suggest that time effects are most likely not responsible for the observed differences in IDIF-derived AUCs determined for the test/retest studies. A high test-retest variability (<20%) was seen in regional CMRGlc obtained from both the IFs (Supplementary Figure 7). A regional analysis of CMRGlc was chosen, as studies in the past have indicated the possibility of different brain regions exhibiting different amount of metabolic variability.<sup>52,53</sup> However, it has been hypothesized that this relatively high amount of regional variability is due to changes in the physiological state of the subject across time.<sup>54–57</sup>

A current limitation of our study in view of a fully automation of the workflow is the need for a separate low-dose CT scan for CT-AC, which, for brain research, still is assumed a “silver standard.”<sup>34</sup> Various accurate brain MR-based attenuation correction (MR-AC) methods<sup>58</sup> have been proposed in the recent years and there is now guarded optimism in

the field that, at least for the brain, MR-AC will soon be clinically feasible.<sup>32,33</sup> However, the impact of these methods with respect to the derivation of an accurate IDIF still needs to be investigated. In our current study, the observed motion was minimal (maximum translation <2.0 mm and maximum rotation <2.0°, Figure 2) for all subjects during the first 10 min of the study. Therefore, no motion correction was performed for the early PET frames (<10 min). Though, a practical MR navigator-based motion correction approach<sup>31</sup> was implemented (Figure 3(a)) to handle prominent motion for early PET frames (>2 mm), the performance of the method still needs to be evaluated. In our current implementation, stationary attenuation maps were used to perform AC, as motion magnitudes were minimal. However, such an approach might not be optimal in a clinical scenario where motion can be prominent. Since, CT-AC map misalignments can affect the quantification of the IDIF, the use of motion corrected CT-AC maps to reconstruct clinical PET data is advisable.

Finally, the calculation of CMRGlc was based on the standard rate constant approach since this study was performed with healthy volunteers. Though the use of standard rate constants might be less accurate than the application of kinetic modeling in case of pathology, the simplified approach might be better suited for clinical routine. Furthermore, when adopting our approach for the clinic, the obtained blood IDIF needs to be converted to plasma IDIF by using the hematocrit measured from the venous blood. However, it has been shown that the hematocrit values obtained from venous blood are ~3% higher than those obtained from arterial blood<sup>59</sup> and this difference must be considered during the conversion. In our study, we used the individual dynamic plasma to whole blood ratio to derive the plasma-IDIF. The mean ( $\pm$ SD) of this ratio derived from our study group ( $n=20$ ) was found to be  $1.06 \pm 0.01$  (Supplementary Figure 9). Therefore, in future [18]FDG studies without arterial blood samples, we intend to apply a scaling factor of 1.06 to convert the blood-IDIF to plasma-IDIF.

As discussed earlier, most PET-based methods<sup>9–19</sup> require blood samples for scaling the IDIF, while the stand-alone PET/CT and MR-based methods<sup>20–22</sup> suffer logistic and co-registration issues. Integrated PET/MR-based IDIF methods,<sup>27,60</sup> apart from providing logistic advantage, have shown to be promising in addressing the challenges associated with the calculation of IDIF. Our method extends the previous PET/MR approaches, by introducing an FOV independent robust petrous segmentation algorithm, MR navigator-based motion monitoring and a PVC algorithm, which accounts for circumferential and radial homogeneity for spill-in correction.

## Conclusion

We have developed a computational framework to automatically calculate an IDIF for the absolute quantification of [18F]FDG PET brain data using a fully integrated PET/MRI system. Calculations of the IDIF and the CMRGlc parameters have been shown to correspond well to the values derived from the invasive AIF, thus, attesting to the concept of non-invasive absolute quantification of [18F]FDG PET imaging of the brain in combined PET/MRI studies.

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## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Authors' contributions

LK and OM contributed to analysis and interpretation of data along with drafting, revising and final approval of the manuscript. TTW, EP and MP contributed to data acquisition, followed by revision and approval of the manuscript. LR, AH, MH1, IR, RL, FGF and EMK made substantial contribution with data analysis, along with revision and approval of the manuscript. MH2 contributed with revising and approving the manuscript. TB contributed with study concept, design and revised the manuscript critically and approved the final version.

## Supplementary material

Supplementary material for this paper can be found at the journal website: <http://journals.sagepub.com/home/jcb>

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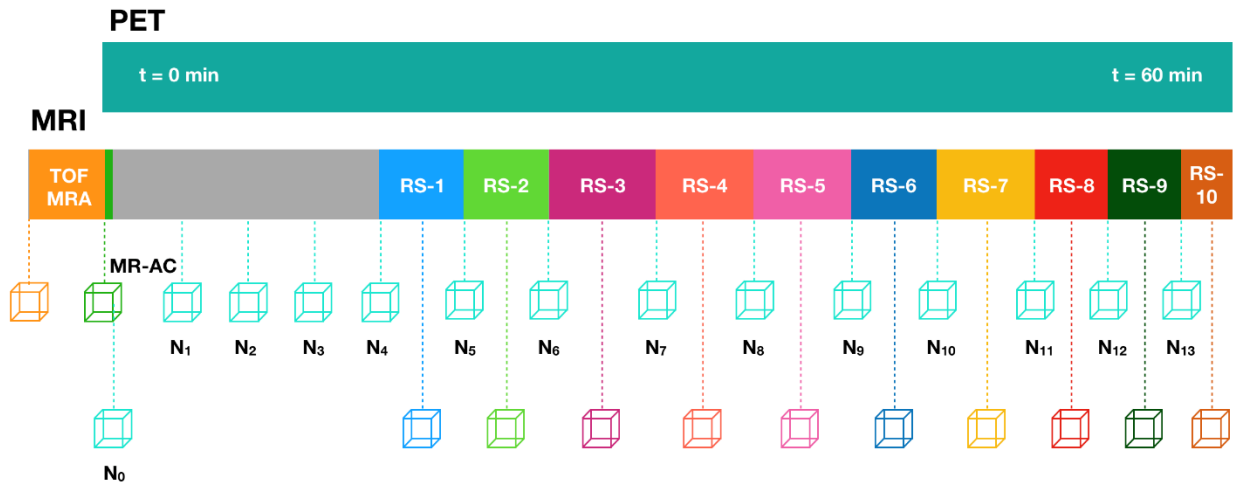
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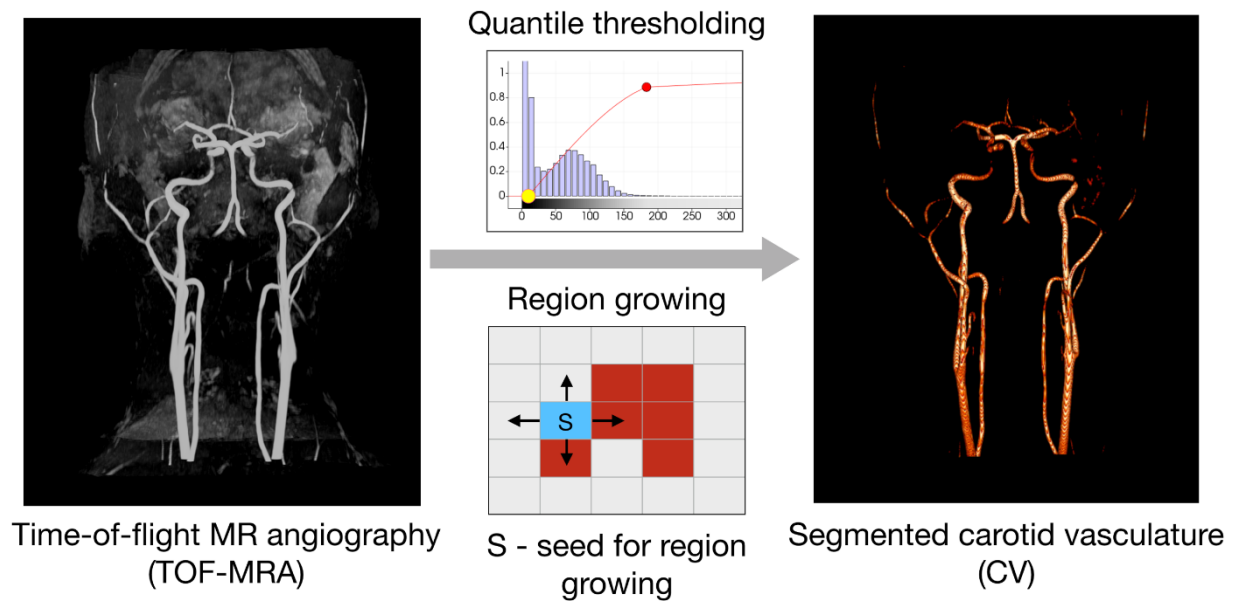


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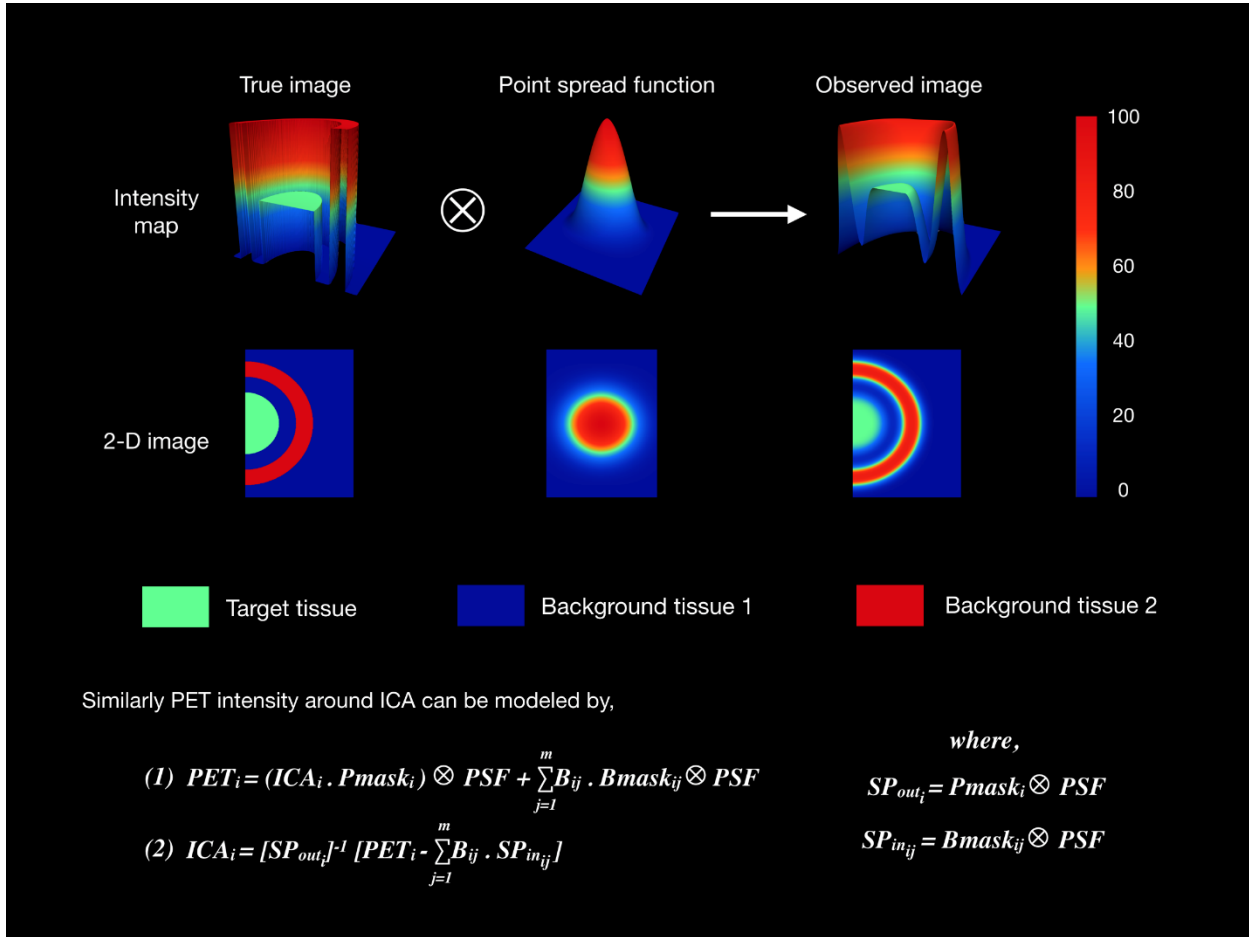
## Supplement material



**Suppl. Fig. 1** Clinical combined PET/MRI protocol: A Time-of-flight angiography (TOF-MRA) sequence was acquired prior to the start of PET acquisition followed by the acquisition of Dixon MR-attenuation map (MR-AC). A 60-min list-mode PET acquisition was initiated, with the simultaneous application of the reference MR navigator ( $N_0$ ). 14 MR navigators (cyan) were interleaved between the clinical research sequences (RS-n) throughout the dynamic study to monitor subject motion.

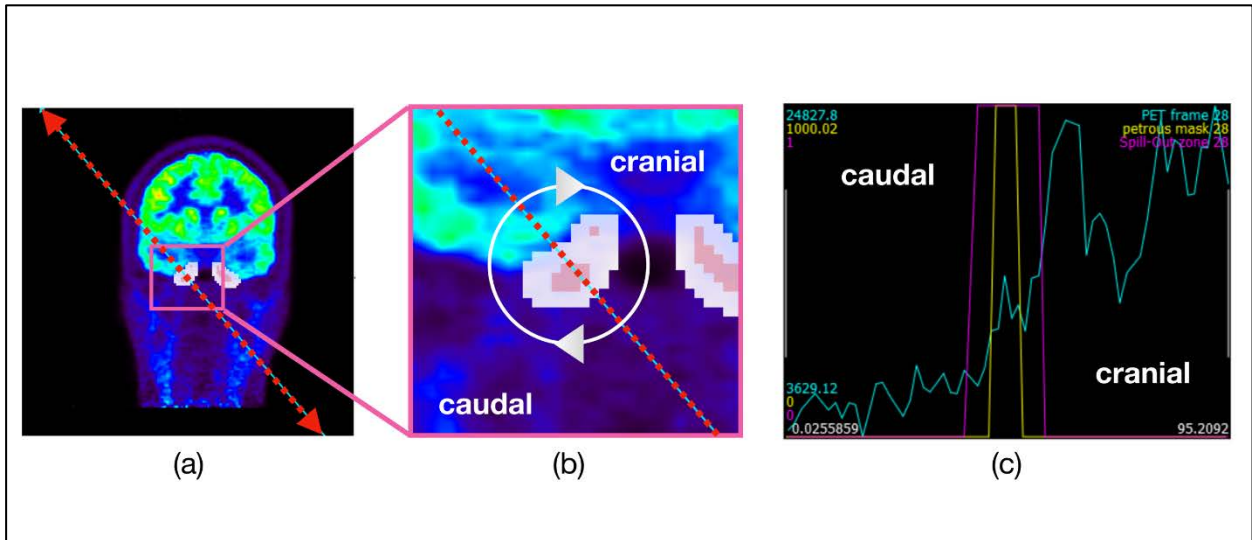


**Suppl. Fig. 2.** A combination of quantile based histogram thresholding (top-center) and automatic seeded region growing algorithm (top-bottom) is applied on the TOF-MRA dataset (left) resulting in a binary segmented carotid vasculature, CV (right).

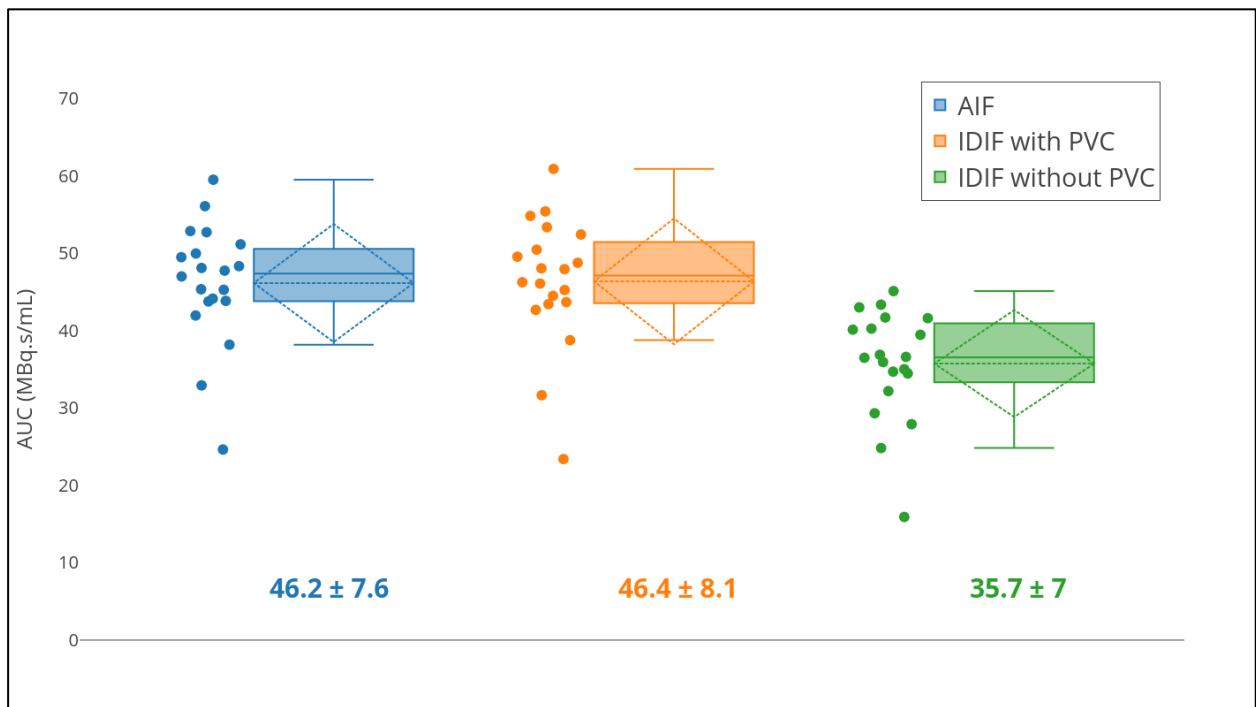


**Suppl. Fig. 3.** To emphasize the influence of PSF, synthetic 2-D images (bottom row) along with their intensity height map (top row) are shown. The observed PET image (right) can be considered as a convolution ( $\otimes$ ) of the true image (left) with the PET system's point spread function (PSF, center). The observed PET activity around the ICA can be modelled

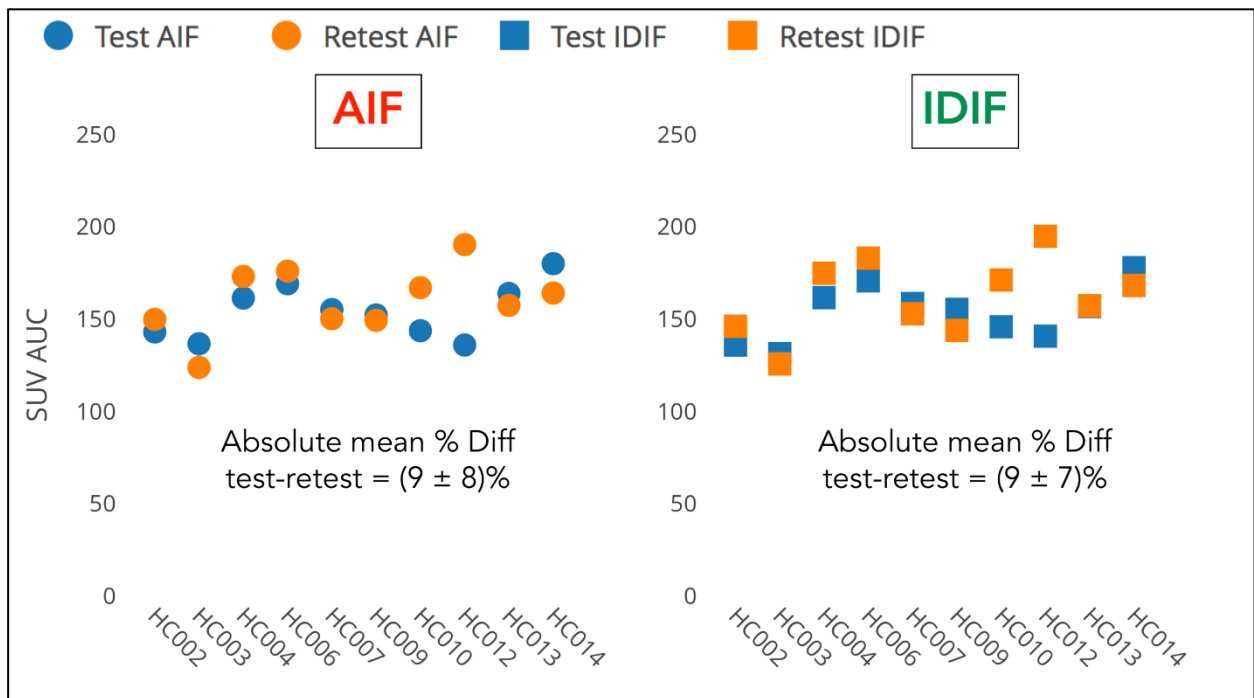
by equation (1). To obtain the true ICA activity using equation (2),  $SP_{out}$  and  $SP_{in}$  factors were calculated by using the petrous mask ( $Pmask_i$ ), background masks ( $Bmask_{ij}$ ) and PET system's PSF.



**Suppl. Fig. 4.** Circumferential and radial heterogeneity in a real case scenario: (a) PET frame fused with petrous (light red) and spill-out zone (white), along with the red dotted line where the activity profile was measured (b) zoom-in demonstrating the circumferential heterogeneity (white circle with arrows), as the petrous is encapsulated by the temporal lobe of the brain and the base of the skull, (c) radial activity line profile (PET – blue, petrous mask – yellow, Spill-out zone/mixed zone – magenta) illustrating the difference between the activity in the spill out zone and the immediate background.

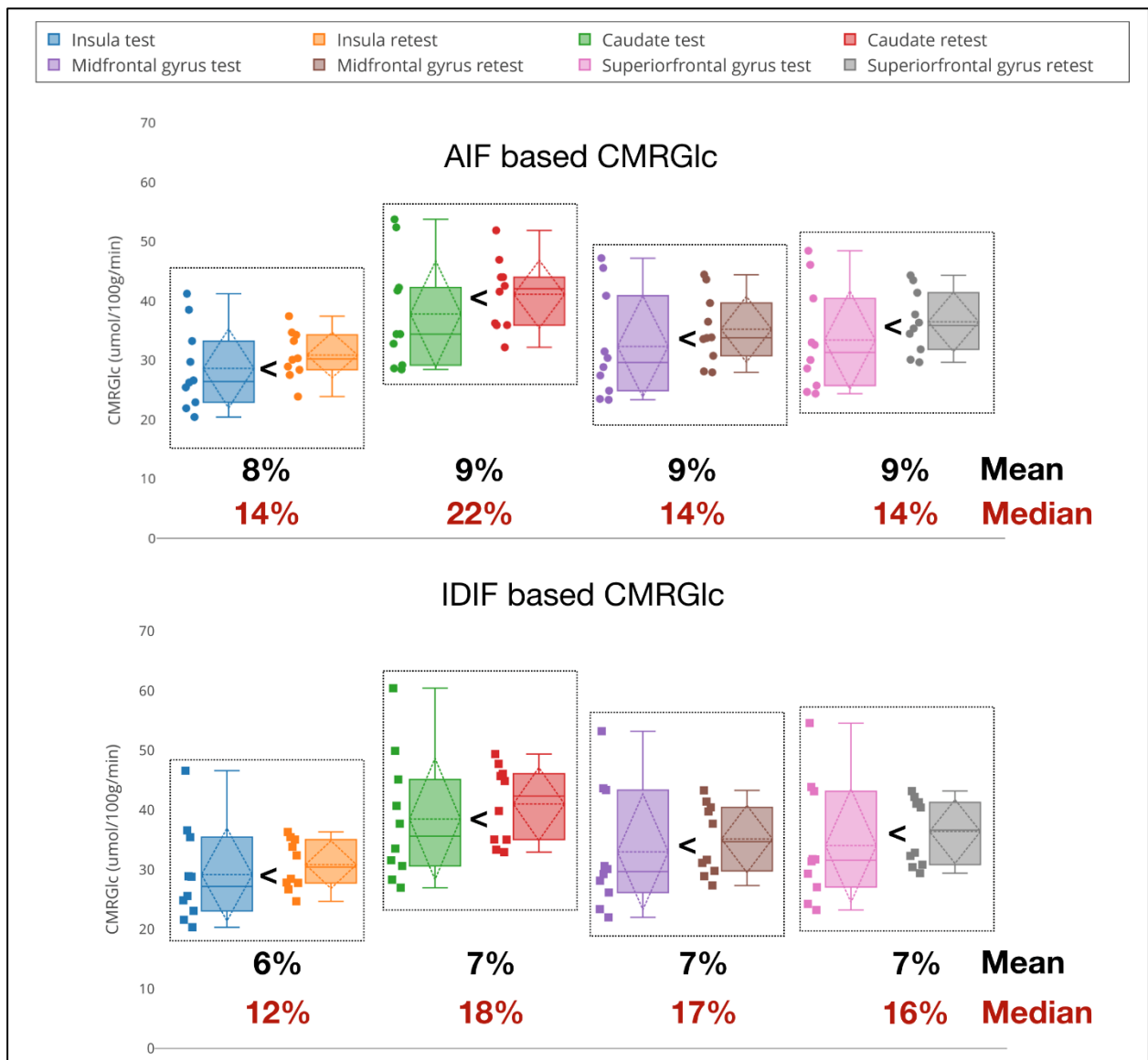


**Suppl. Fig. 5.** Box plots of the range of the AUCs (MBq.s/mL) of AIF (blue), IDIF after PVC (orange) and IDIF before PVC (green) along with the mean and standard deviation values. The dots adjacent to the box plot correspond to the AUC of each individual IF curve.



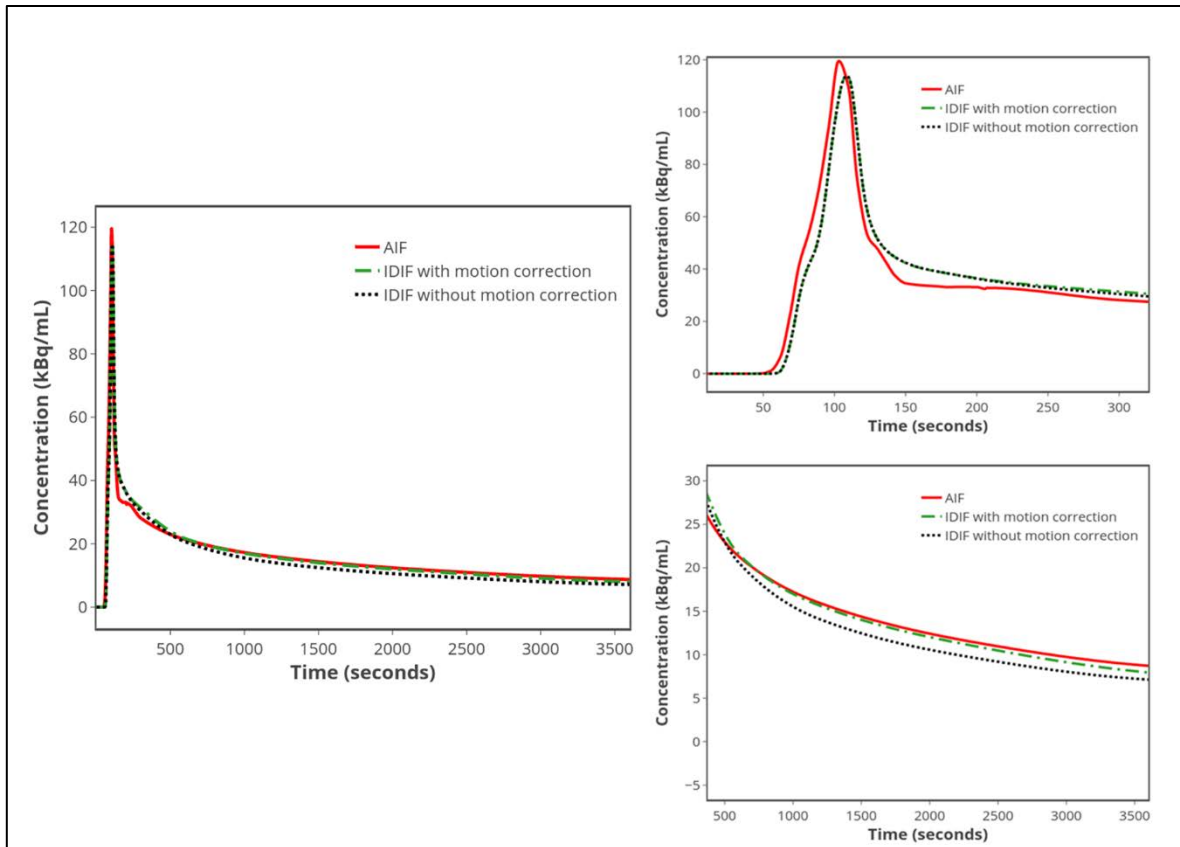
**Suppl. Fig. 6.** Test-retest variability of input functions normalized to body weight and injected dose (SUV). The area-under-the curve was used as a similarity metric. The intra-variability of AIF and IDIF SUV curves in terms of absolute mean % difference was similar at  $(9 \pm 8) \%$ , left) and  $(9 \pm 7) \%$ , right) respectively.





**Suppl. Fig. 7.** Test-retest CMRGlc variability is depicted here. Four reference regions were chosen at random (Insula, Caudate, Mid-frontal gyrus and superior frontal gyrus). AIF

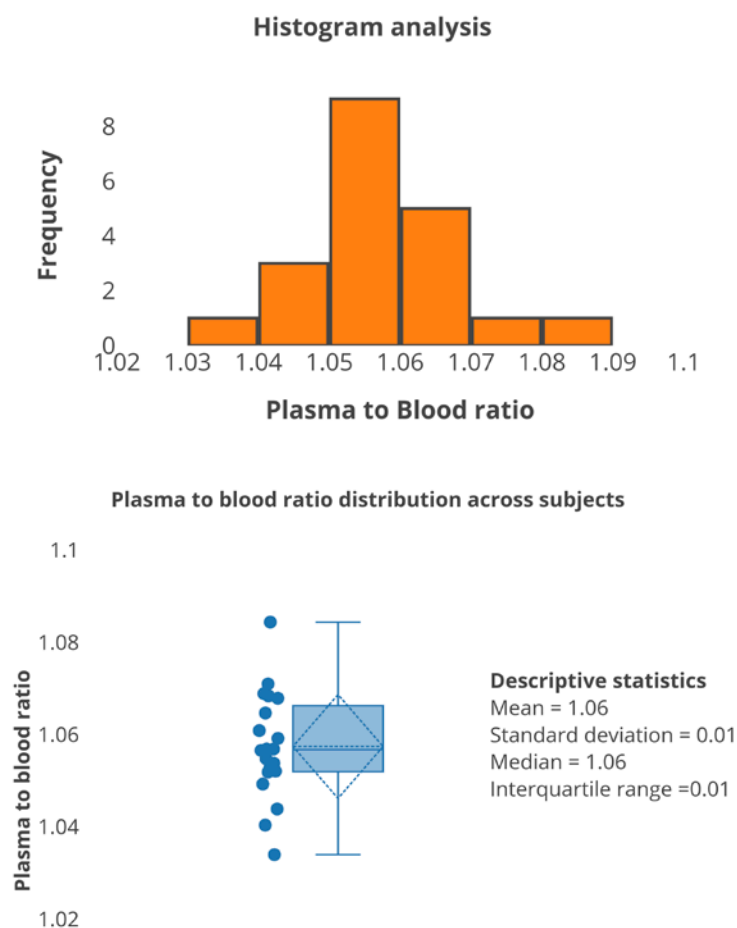
based CMRGlc values for test-retest scans were plotted for these four regions (top), it can be seen that the variance (dotted lines) is lower in the retest scans, however the mean and median CMRGlc values were higher. The increase in mean (black) and median (brown) between test and retest scans are reported in terms of absolute percentage differences. This test-retest trend is seen in the IDIF based CMRGlc values as well (bottom).



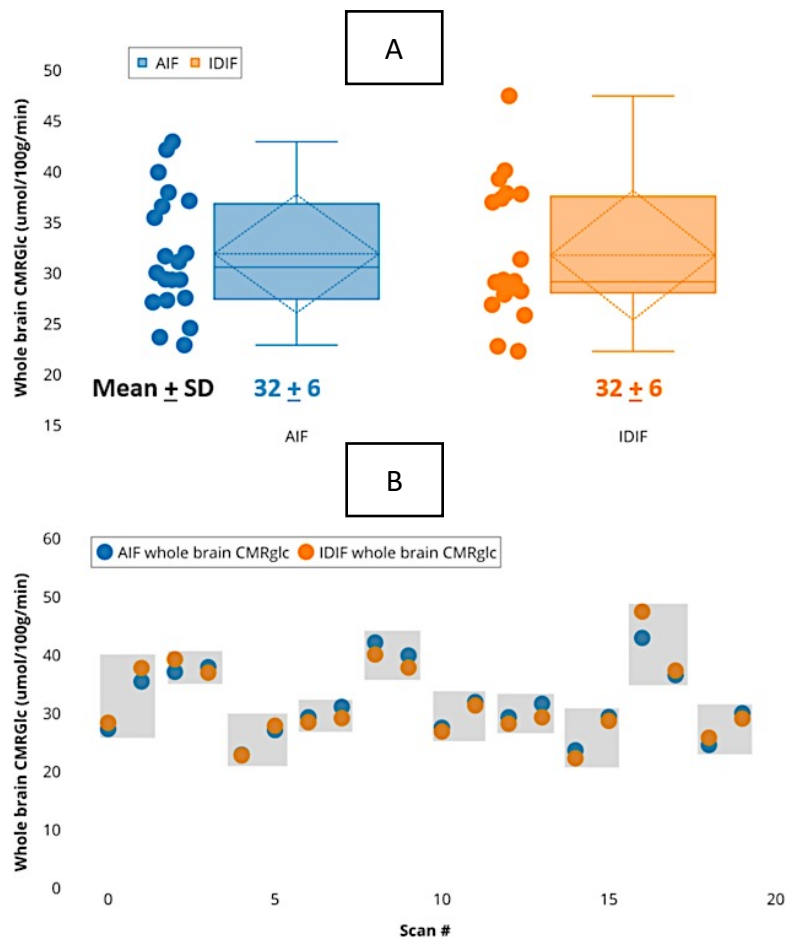
**Suppl. Fig. 8.** Influence of motion on IDIF (HC014 test scan, Table 1): AIF (red), IDIF with motion correction (MoCo IDIF, dashed green line) and IDIF without motion correction (no-MoCo IDIF, dotted black line). It can be seen that the MoCo IDIF has a better agreement with the AIF in comparison to the no-MoCo IDIF. The percentage difference between MoCo IDIF and the AIF was 1%, whereas the percentage difference between no-MoCo IDIF and the AIF was found to be 9%. The graph also indicates that by accounting for

involuntary subject motion, the agreement between the IDIF and AIF is significantly improved.

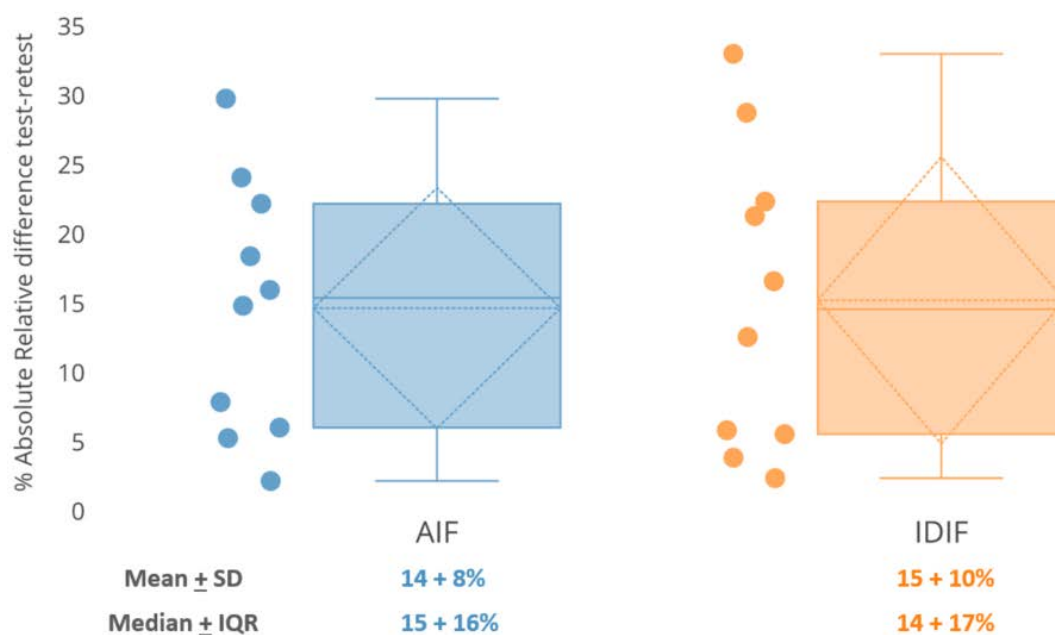
**Supplementary Figure 9.** Histogram showing the average plasma-to-blood ratio of FDG tracer concentration obtained from all the 20 subjects studied (top). Group analysis for all the 20 datasets, where the average plasma to blood ratio was found to be  $1.06 \pm 0.01$ . (bottom)



**Supplementary Figure 10.** (A) Whole brain CMRGlc for 20 datasets calculated using AIF (blue) and IDIF (orange), (B) Scatter plot depicting the individual whole brain CMRGlc values, where the shaded rectangle indicates same subject, with its height representing the difference between test and retest.



**Supplementary Figure 11.** Absolute relative % difference between test-retest Global CMRGlc calculated using AIF (blue) and IDIF (orange).



## Supplementary tables

**Suppl. Table. 1.** Intra-frame motion (IFM) in PET frames as estimated from simultaneously acquired MR navigators was found to be negligible (<1 mm). Of note, the IFM increases

with the duration of the study.

| <b>Intra-frame<br/>translation (mm)</b> | <b>10-20 min</b> | <b>20-40 min</b> | <b>40-60 min</b> |
|-----------------------------------------|------------------|------------------|------------------|
| <b>X direction</b>                      | -0.06 ± 0.25     | -0.01 ± 0.18     | -0.17 ± 0.80     |
| <b>Y direction</b>                      | 0.02 ± 0.12      | 0.02 ± 0.12      | 0.12 ± 0.45      |
| <b>Z direction</b>                      | 0.03 ± 0.22      | 0.02 ± 0.27      | 0.08 ± 0.40      |



### 3.1.3 Manuscript III

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## Article

# If It Works, Don't Touch It? A Cell-Based Approach to Studying 2-[<sup>18</sup>F]FDG Metabolism

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**Abstract:** The glucose derivative 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucose (2-[<sup>18</sup>F]FDG) is still the most used radiotracer for positron emission tomography, as it visualizes glucose utilization and energy demand. In general, 2-[<sup>18</sup>F]FDG is said to be trapped intracellularly as 2-[<sup>18</sup>F]FDG-6-phosphate, which cannot be further metabolized. However, increasingly, this dogma is being questioned because of publications showing metabolism beyond 2-[<sup>18</sup>F]FDG-6-phosphate and even postulating 2-[<sup>18</sup>F]FDG imaging to depend on the enzyme hexose-6-phosphate dehydrogenase in the endoplasmic reticulum. Therefore, we aimed to study 2-[<sup>18</sup>F]FDG metabolism in the human cancer cell lines HT1080, HT29 and Huh7 applying HPLC. We then compared 2-[<sup>18</sup>F]FDG metabolism with intracellular tracer accumulation, efflux and the cells' metabolic state and used a graphical Gaussian model to visualize metabolic patterns. The extent of 2-[<sup>18</sup>F]FDG metabolism varied considerably, dependent on the cell line, and was significantly enhanced by glucose withdrawal. However, the metabolic pattern was quite conserved. The most important radiometabolites beyond 2-[<sup>18</sup>F]FDG-6-phosphate were 2-[<sup>18</sup>F]FDMannose-6-phosphate, 2-[<sup>18</sup>F]FDG-1,6-bisphosphate and 2-[<sup>18</sup>F]FD-phosphogluconolactone. Enhanced radiometabolite formation under glucose reduction was accompanied by reduced efflux and mirrored the cells' metabolic switch as assessed via extracellular lactate levels. We conclude that there can be considerable metabolism beyond 2-[<sup>18</sup>F]FDG-6-phosphate in cancer cell lines and a comprehensive understanding of 2-[<sup>18</sup>F]FDG metabolism might help to improve cancer research and tumor diagnosis.

**Keywords:** 2-[<sup>18</sup>F]FDG; 2-[<sup>18</sup>F]FDG metabolism; PET-tracer metabolism; molecular imaging; cancer metabolism

## 1. Introduction

The radioactively labelled glucose derivative 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucose (2-[<sup>18</sup>F]FDG) is still the most used tracer in positron emission tomography (PET) [1,2]. In principle, this tracer is said to visualize the so-called Warburg Effect, which is described as a shift towards glycolysis and lactate overproduction despite a normoxic environment [2,3].

This ineffective way to generate energy causes cells to consume more glucose [3]. As this is a major feature of most tumors, PET imaging with 2-[<sup>18</sup>F]FDG, together with computer tomography (CT), is an indispensable tool for the precise localization and simultaneous metabolic characterization of malignant lesions. Aside from cancer diagnosis or staging, 2-[<sup>18</sup>F]FDG is used for the assessment of myocardial viability and the characterization and imaging of neurodegenerative diseases [1,2]. Firstly, higher glucose utilization might also occur naturally in healthy tissues such as brain (potentially complicating diagnosis), or at sites of inflammation (causing false-positive results in cancer diagnosis) [1,2]. Moreover, because of their metabolic makeup, some cancers by nature show low 2-[<sup>18</sup>F]FDG avidity [4], calling for more specific, receptor-based tracers.

Although 2-[<sup>18</sup>F]FDG has been widely used and comprehensively studied, its exact mode of action remains unclear. In a study by Li et al. with mice bearing peritoneal carcinomatosis and subcutaneous tumors, 2-[<sup>18</sup>F]FDG uptake was presented as a readout for hypoxia (anaerobic glycolysis) rather than depicting the Warburg Effect (aerobic glycolysis) [5]. In other studies with non-small cell lung cancer patients [6,7], where 2-[<sup>18</sup>F]FDG PET was combined with [<sup>13</sup>C]glucose tracing, enhanced 2-[<sup>18</sup>F]FDG uptake did not correlate to glycolytic metabolism, but to respiration [6] and to the proliferation index [7]. The latter study hence suggests 2-[<sup>18</sup>F]FDG PET-signal to be a readout for the use of glucose in pathways other than glycolysis.

Moreover, 2-[<sup>18</sup>F]FDG's own intracellular "standstill" after phosphorylation is being questioned. In general, 2-[<sup>18</sup>F]FDG is taken up via glucose transporters, but unlike glucose it is said to be trapped as 2-[<sup>18</sup>F]FDG-6-phosphate (2-[<sup>18</sup>F]FDG-6-P) after its phosphorylation by hexokinase ("metabolic trapping"). In contrast to glucose-6-phosphate, the isomerization to an analogue of fructose-6-phosphate, which would be necessary for further processing through the glycolytic pathway, is hindered by the fluoride in position 2 [1,2]. Moreover, 2-[<sup>18</sup>F]FDG PET-signal quantification nowadays still builds on the assumption that 2-[<sup>18</sup>F]FDG-6-P is the only metabolite. The refined Sokoloff compartment model used for quantification lists three or four rate constants, describing the transport of 2-[<sup>18</sup>F]FDG from blood pool to tissue, the redistribution of 2-[<sup>18</sup>F]FDG into blood, phosphorylation leading to intracellular retention and occasionally the dephosphorylation of 2-[<sup>18</sup>F]FDG-6-P by glucose-6-phosphatase (G6Pase) [8], as seen in liver tissue due to gluconeogenesis [9]. However, although 2-[<sup>18</sup>F]FDG cannot be processed through glycolysis, it can still be metabolized beyond 2-[<sup>18</sup>F]FDG-6-P [10]. Notably, the glucose analogue seems to be shuttled into glycogen metabolism or the pentose phosphate pathway (PPP) [11]. Its metabolites have mostly been analyzed using its non-radioactive analogue 2-deoxy-2-fluoro-D-glucose and <sup>19</sup>F-NMR [11–13]. However, this approach demands the application of vast amounts of non-radioactive FDG, which could—due to mass effects—result in a different outcome when compared to trace amounts used with 2-[<sup>18</sup>F]FDG. Other groups that tested metabolism of the radioactive form 2-[<sup>18</sup>F]FDG in xenograft mice and rats detected fewer metabolites, potentially due to sensitivity limitations of their HPLC system [14,15].

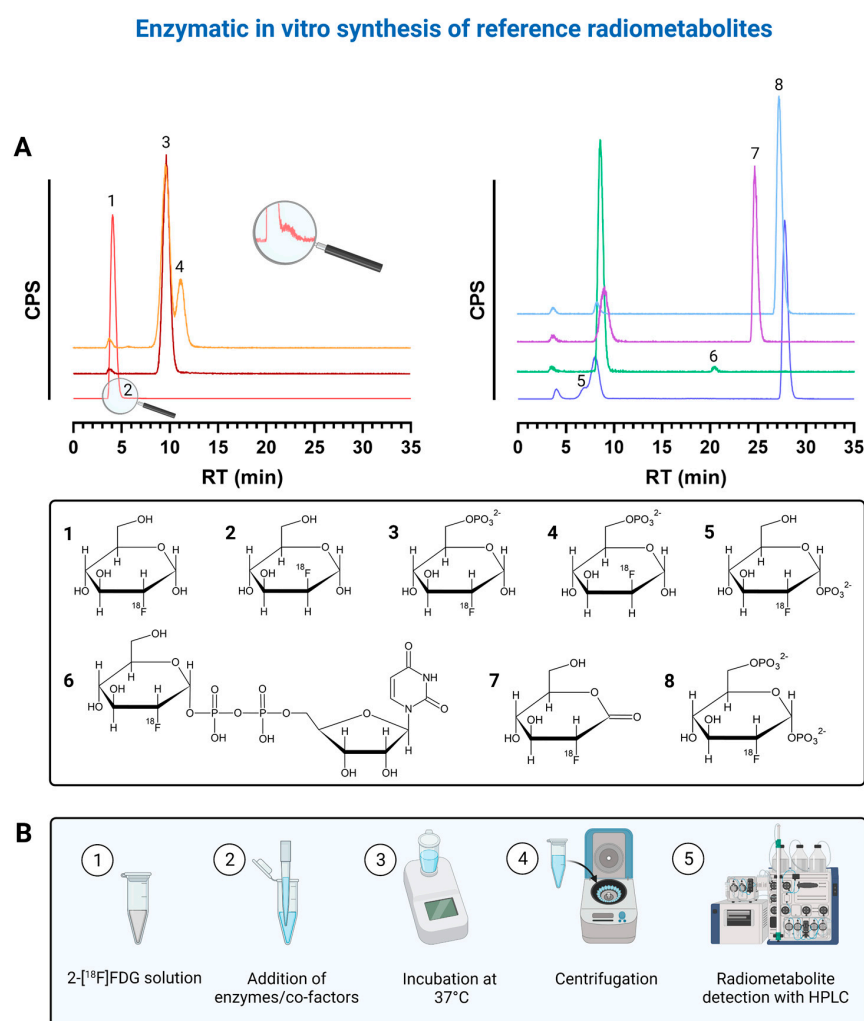
Recently, research on 2-[<sup>18</sup>F]FDG metabolism has gained new momentum with publications describing intracellular 2-[<sup>18</sup>F]FDG accumulation as specifically being a function of hexose-6-phosphate dehydrogenase (H6PD), the first enzyme of the oxidative branch of the PPP located in the endoplasmic reticulum (ER) [16,17].

Although 2-[<sup>18</sup>F]FDG is increasingly replaced by more specific PET-tracers, it is still an indispensable tool in nuclear medicine. While the simplistic model of the tracer's intracellular trapping might be sufficient for some clinical questions, an in-depth understanding of its intracellular fate could improve basic cancer research or cancer diagnosis. We hypothesized that 2-[<sup>18</sup>F]FDG metabolism would differ significantly in cancer cell lines of different tumor entities, as well as under different glucose conditions. Therefore we studied the 2-[<sup>18</sup>F]FDG metabolism in human cell lines of fibrosarcoma (HT1080), colorectal adenocarcinoma (HT29) and hepatocellular carcinoma (Huh7). We aimed to elucidate metabolic patterns and pathways involved in the tracer's metabolism to create a basis for a better understanding of cancer-relevant 2-[<sup>18</sup>F]FDG accumulation and efflux.

## 2. Results

### 2.1. Enzymatic In Vitro Preparation and Verification of Radiometabolites

To properly identify the radiometabolites of 2-[ $^{18}\text{F}$ ]FDG, we started to synthesize potential radiometabolites via enzymatic in vitro preparation. After adapting the HPLC assay by Rokka et al. [18] to our system in terms of gradient profile and run time (Table S1), we could verify the identity of the following radiometabolites: 2-[ $^{18}\text{F}$ ]FDG-1-phosphate (2-[ $^{18}\text{F}$ ]FDG-1-P, retention time (RT)~7 min), 2-[ $^{18}\text{F}$ ]FDG-6-P (RT~9.5 min), 2-[ $^{18}\text{F}$ ]FDMannose-6-phosphate (2-[ $^{18}\text{F}$ ]FDM-6-P, RT~12 min), uridine diphosphate-2-[ $^{18}\text{F}$ ]FDG (UDP-2-[ $^{18}\text{F}$ ]FDG, RT~21 min), 2-[ $^{18}\text{F}$ ]FD-phosphogluconolactone (2-[ $^{18}\text{F}$ ]FD-PGL, RT~25 min) and 2-[ $^{18}\text{F}$ ]FDG-1,6-bisphosphate (2-[ $^{18}\text{F}$ ]FDG-1,6-P<sub>2</sub>, RT~28 min). Figure 1A shows representative chromatograms of the reference radiometabolites and corresponding chemical structures, while Figure 1B shows the workflow of the enzymatic in vitro synthesis of reference radiometabolites.



**Figure 1.** (A) shows representative chromatograms of the reference radiometabolites that were prepared via enzymatic in vitro synthesis and corresponding chemical structures. Each color represents the chromatogram of a different reference compound. 1 = 2-[ $^{18}\text{F}$ ]FDG, 2 = 2-[ $^{18}\text{F}$ ]FDM, 3 = 2-[ $^{18}\text{F}$ ]FDG-6-P, 4 = 2-[ $^{18}\text{F}$ ]FDM-6-P, 5 = 2-[ $^{18}\text{F}$ ]FDG-1-P, 6 = UDP-2-[ $^{18}\text{F}$ ]FDG, 7 = 2-[ $^{18}\text{F}$ ]FD-PGL, 8 = 2-[ $^{18}\text{F}$ ]FDG-1,6-P<sub>2</sub>. (B) shows the corresponding workflow of enzymatic in vitro synthesis of reference radiometabolites. Created with BioRender.com (accessed on 4 September 2021) based on HPLC raw data and graphs from Prism 7.03 software.

We could not identify the radiometabolites at ~19.5, ~22, ~23.5, ~27 and ~29.5 min. However, the radiometabolites at ~22, ~23.5 and ~27 min never exceeded 0.1, 0.3 and 0.05%, respectively. 2-[<sup>18</sup>F]FDG-6-P and 2-[<sup>18</sup>F]FDM-6-P were not fully separable despite HPLC optimization. Rokka et al. [18] reported that UDP-2-[<sup>18</sup>F]FDG was not synthesized because when adding phosphoglucomutase (PGM), the conversion of 2-[<sup>18</sup>F]FDG-6-P to 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> via 2-[<sup>18</sup>F]FDG-1-P is always favored over UDP-2-[<sup>18</sup>F]FDG formation in the presence of hexokinase. Therefore, we filtered our 2-[<sup>18</sup>F]FDG-6-P solution containing hexokinase through a 30 kDa filter prior to the addition of PGM, uridine triphosphate (UTP) and UDP-glucose-pyrophosphorylase (UGPase). However, when we additionally added glucose-1,6-bisphosphate, ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA) to the solution after filtering, with or without UTP and UGPase, 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> was always the main metabolite after ~2.5 h. UDP-2-[<sup>18</sup>F]FDG was not produced. Interestingly, when we did not introduce any additional compounds to the filtrate mixed with UTP and UGPase, 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> was not or was only merely produced and we finally saw a metabolite at ~21 min, namely UDP-2-[<sup>18</sup>F]FDG (Figure 1A). We observed that 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub>, a natural intermediate of the conversion of 2-[<sup>18</sup>F]FDG-6-P to 2-[<sup>18</sup>F]FDG-1-P, is also produced in the absence of hexokinase. Adding glucose-1,6-bisphosphate, EDTA and BSA seemed to shift the equilibrium towards the intermediate 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> and UDP-2-[<sup>18</sup>F]FDG cannot be built. We once also detected an additional peak at ~7 min after around 2 h when synthesizing 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub>, which should be 2-[<sup>18</sup>F]FDG-1-P (Figure 1A).

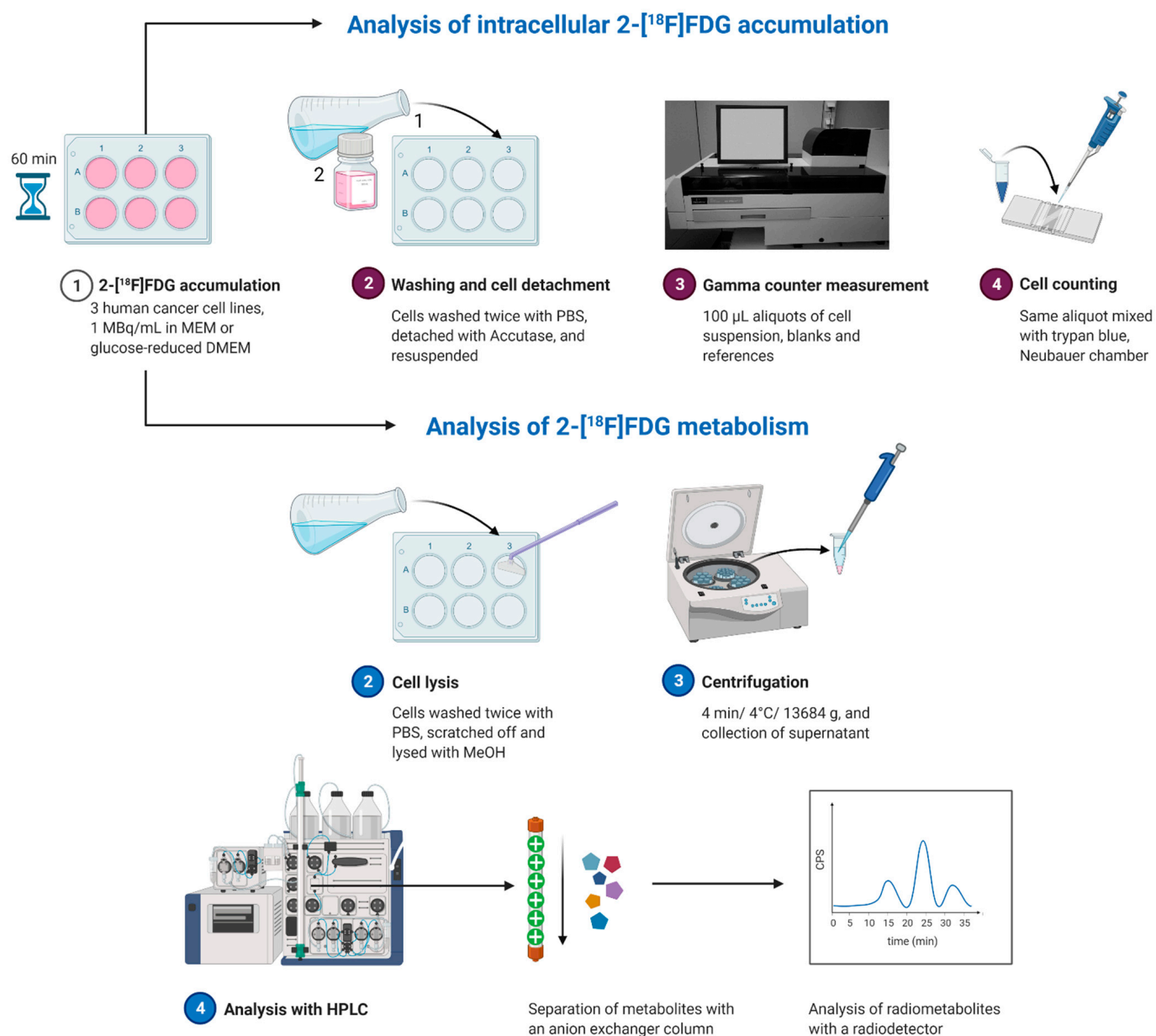
Furthermore, we identified the peak at ~12 min as 2-[<sup>18</sup>F]FDM-6-P (Figure 1A). The injection of pure 2-[<sup>18</sup>F]FDG showed an additional peak with an RT of ~6 min. This peak should be 2-[<sup>18</sup>F]FDMannose, a by-product of 2-[<sup>18</sup>F]FDG production that results from epimerization during alkaline hydrolysis (Figure 1A) [19]. Furthermore, we synthesized 2-[<sup>18</sup>F]FD-PGL using two different commercially available, recombinant glucose-6-phosphate dehydrogenases (G6PDs) (Figure 1A). As 2-[<sup>18</sup>F]FD-PGL is not stable [20], the peak at ~25 min can also be the decomposition product 2-[<sup>18</sup>F]FD-phosphogluconate or a mixture of both.

Overall, we verified the identity of three more radiometabolites (2-[<sup>18</sup>F]FDG-1-P, 2-[<sup>18</sup>F]FDM-6-P and UDP-2-[<sup>18</sup>F]FDG) with enzymatic in vitro synthesis as compared to Rokka et al. [18], facilitating future analyses of 2-[<sup>18</sup>F]FDG metabolism.

## 2.2. Intracellular Accumulation and Metabolism of 2-[<sup>18</sup>F]FDG in HT1080 (Fibrosarcoma), HT29 (Colorectal Adenocarcinoma) and Huh7 (Hepatocellular Carcinoma)

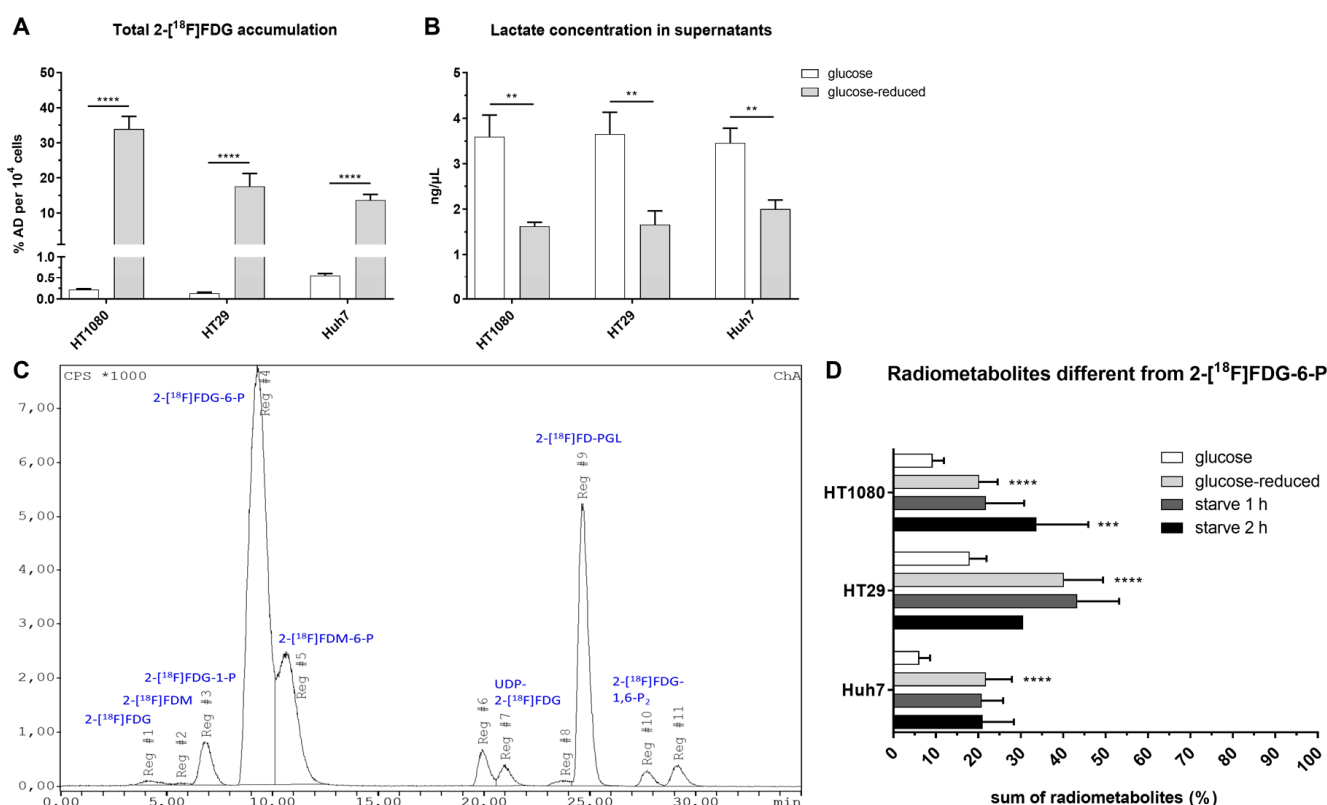
2-[<sup>18</sup>F]FDG was applied to the cells either under glucose (1.13 g/L), glucose-reduced (0.13 g/L) or starving conditions (glucose-reduced medium 1 or 2 h prior to 2-[<sup>18</sup>F]FDG addition). Figure 2 summarizes the workflow of experiments determining 2-[<sup>18</sup>F]FDG accumulation and 2-[<sup>18</sup>F]FDG metabolism in cancer cells. Under glucose conditions, the highest 2-[<sup>18</sup>F]FDG accumulation in % applied dose (% AD) per 10<sup>4</sup> cells after 1 h was seen in Huh7 (0.57 ± 0.04%), followed by HT1080 (0.22 ± 0.01%) and HT29 (0.13 ± 0.02%) (Figure 3A). Contrary to this, under glucose-reduced conditions, HT29 and Huh7 showed similar accumulation (18 ± 4% and 14 ± 2%), while there was significantly higher 2-[<sup>18</sup>F]FDG accumulation per 10<sup>4</sup> cells in HT1080 (34 ± 4%) (Figure 3A).

Analysis of lactate concentration in supernatants after the experiments showed that extracellular lactate was more than twice as high for HT29 and HT1080 under glucose conditions (HT29: 3.7 ± 0.5% ng/μL; HT1080: 3.6 ± 0.5% ng/μL) compared to glucose-reduced conditions (HT29: 1.7 ± 0.3% ng/μL; HT1080: 1.6 ± 0.1% ng/μL). In Huh7, the difference between glucose and glucose-reduced conditions was slightly smaller with 3.5 ± 0.3% vs. 2.0 ± 0.2% ng/μL (Figure 3B). These data indicate a metabolic switch in all three cell lines following glucose reduction.



**Figure 2.** Workflow of experiments determining intracellular 2-<sup>18</sup>F]FDG accumulation and metabolism. Adapted from “Protein Purification by Size-Exclusion Chromatography” by BioRender.com (accessed on 4 September 2021). Retrieved from <https://app.biorender.com/biorender-templates>.





**Figure 3.** Total intracellular 2-[<sup>18</sup>F]FDG accumulation after 1 h (A) and lactate concentration in the supernatants at the end of the experiment (\*\*  $p \leq 0.01$ ) (B). (C) shows a representative image of the radiometabolite pattern under glucose-reduced conditions in HT29 after 1 h and (D) the sum of radiometabolites other than 2-[<sup>18</sup>F]FDG-6-P after 1 h in % (\*\*\*\*  $p \leq 0.0001$ , \*\*\*  $p \leq 0.001$  compared to glucose conditions). Conditions: glucose = 1.13 g/L, glucose-reduced = 0.13 g/L, starve = glucose-reduced medium 1 or 2 h prior to 2-[<sup>18</sup>F]FDG addition. If not visible, the error bars are within the margin of the symbols.

Overall, we could detect up to 12 radiometabolites in HPLC when stopping 2-[<sup>18</sup>F]FDG accumulation after 1 h. Figure 3C shows a representative image of the metabolic pattern in HT29 using glucose-reduced medium, Figure 3D summarizes the extent of metabolism beyond 2-[<sup>18</sup>F]FDG and 2-[<sup>18</sup>F]FDG-6-P in the three cell lines. Except for the metabolite at ~27 min, which was only observed in HT1080, all radiometabolites were detected in all three cell lines. Under glucose conditions, HT29 showed the highest levels of radiometabolites other than 2-[<sup>18</sup>F]FDG-6-P after 1 h with  $18 \pm 4\%$ , followed by HT1080 ( $9 \pm 3\%$ ) and Huh7 ( $6 \pm 3\%$ ). It was the same cell line that showed the highest radiometabolite formation under glucose-reduced conditions with  $40 \pm 9\%$ , while glucose reduction led to similar results in HT1080 and Huh7 ( $20 \pm 4\%$  and  $22 \pm 6\%$ ). Starving did not significantly enhance metabolism beyond 2-[<sup>18</sup>F]FDG in Huh7 or HT29 compared to glucose-reduced conditions. However, 2 h starving significantly enhanced radiometabolite formation in HT1080 to  $34 \pm 12\%$  ( $p \leq 0.001$  compared to glucose conditions).

In summary, glucose withdrawal led to increased intracellular 2-[<sup>18</sup>F]FDG accumulation and enhanced radiometabolite formation dependent on the cell line.

Table 1 gives a more detailed overview on the change of radiometabolite formation dependent on the glucose concentration. In general, the most prominent radiometabolites rising when glucose in the medium was reduced were 2-[<sup>18</sup>F]FDG-1-P, 2-[<sup>18</sup>F]FDM-6-P and 2-[<sup>18</sup>F]FD-PGL. In contrast to that, besides parent 2-[<sup>18</sup>F]FDG and 2-[<sup>18</sup>F]FDG-6-P, levels of 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> decreased under glucose deprivation. HT29 was the only cell line with noteworthy radiometabolite formation beyond 2-[<sup>18</sup>F]FDG-6-P under glucose conditions and also the cell line with the highest 2-[<sup>18</sup>F]FD-PGL formation under glucose ( $3 \pm 1\%$ ) and glucose-reduced ( $15 \pm 7\%$ ) conditions.

**Table 1.** Mean radiometabolite formation (%) under different conditions in HT1080, HT29 and Huh7. Conditions: glucose = 1.13 g/L, glucose-reduced = 0.13 g/L, starve = glucose-reduced medium 1 or 2 h prior to 2-<sup>18</sup>F]FDG addition. Radiometabolites at ~6, ~22, ~23.5 and ~27 min were excluded due to their negligible formation.

| HT1080                                    |          |          |     |                 |      |              |      |              |      |
|-------------------------------------------|----------|----------|-----|-----------------|------|--------------|------|--------------|------|
| Radiometabolites                          | RT (min) | Glucose  |     | Glucose-Reduced |      | Starve (1 h) |      | Starve (2 h) |      |
|                                           |          | mean (%) | SD  | mean (%)        | SD   | mean (%)     | SD   | mean (%)     | SD   |
| 2- <sup>18</sup> F]FDG                    | ~4       | 11       | 3   | 0.3             | 0.1  | 0.40         | 0.09 | 0.62         | 0.28 |
| 2- <sup>18</sup> F]FDG-1-P                | ~7       | 0        | 0   | 1.6             | 0.7  | 3.18         | 2.61 | 5.7          | 3.5  |
| 2- <sup>18</sup> F]FDG-6-P                | ~9.5     | 79       | 2   | 79              | 4    | 78           | 9    | 66           | 12   |
| 2- <sup>18</sup> F]FDM-6-P                | ~12      | 0        | 0   | 9               | 5    | 6            | 5    | 12           | 1    |
| radiometabolite 5                         | ~19.5    | 0        | 0   | 0.53            | 0.44 | 0.56         | 0.25 | 2            | 1    |
| UDP-2- <sup>18</sup> F]FDG                | ~21      | 0        | 0   | 0.51            | 0.23 | 0.69         | 0.40 | 1.7          | 0.8  |
| 2- <sup>18</sup> F]FD-PGL                 | ~25      | 0.0      | 0.0 | 4               | 2    | 7            | 4    | 12           | 7    |
| 2- <sup>18</sup> F]FDG-1,6-P <sub>2</sub> | ~28      | 8        | 2   | 3               | 2    | 3            | 2    | 0.47         | 0.11 |
| radiometabolite 12                        | ~29.5    | 1.1      | 0.6 | 0.95            | 0.60 | 0.96         | 0.82 | 0.22         | 0.02 |

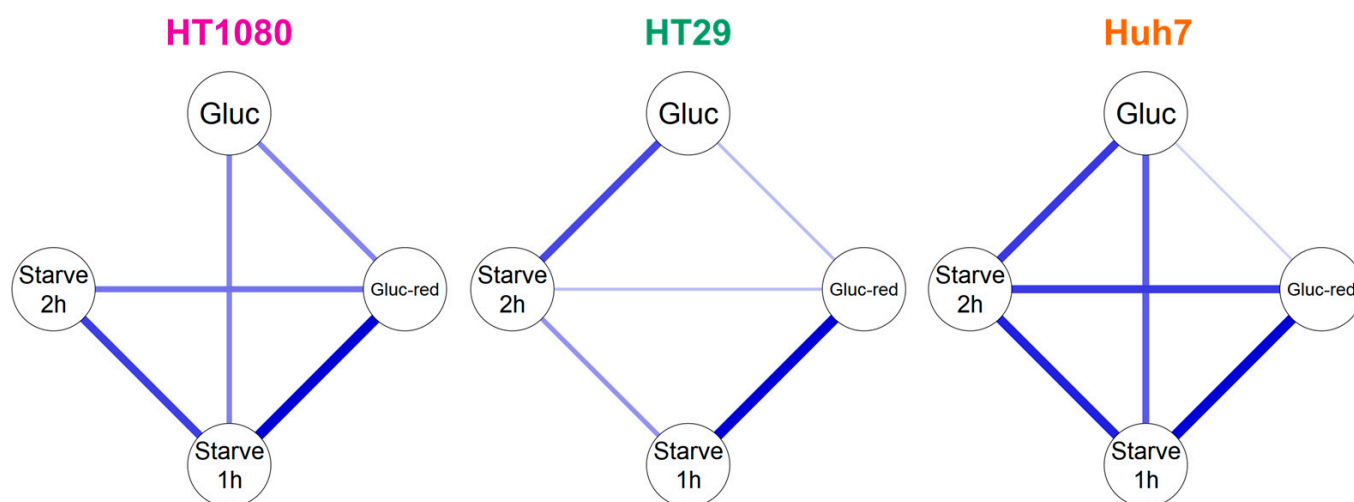
| HT29                                      |          |          |     |                 |     |              |      |              |     |
|-------------------------------------------|----------|----------|-----|-----------------|-----|--------------|------|--------------|-----|
| Radiometabolites                          | RT (min) | Glucose  |     | Glucose-Reduced |     | Starve (1 h) |      | Starve (2 h) |     |
|                                           |          | mean (%) | SD  | mean (%)        | SD  | mean (%)     | SD   | mean (%)     | SD  |
| 2- <sup>18</sup> F]FDG                    | ~4       | 3        | 1   | 0.4             | 0.2 | 0.57         | 0.14 | 0.4          | 0.1 |
| 2- <sup>18</sup> F]FDG-1-P                | ~7       | 1.2      | 0.5 | 3               | 1   | 3            | 2    | 1.2          | 0.2 |
| 2- <sup>18</sup> F]FDG-6-P                | ~9.5     | 79       | 4   | 59              | 9   | 56           | 10   | 69.0         | 0.7 |
| 2- <sup>18</sup> F]FDM-6-P                | ~12      | 7        | 2   | 14              | 3   | 17           | 3    | 19           | 1   |
| radiometabolite 5                         | ~19.5    | 0.1      | 0.1 | 1.5             | 0.9 | 2.9          | 1.0  | 2.2          | 0.4 |
| UDP-2- <sup>18</sup> F]FDG                | ~21      | 0.1      | 0.2 | 1.0             | 0.6 | 2.1          | 0.5  | 2.0          | 0.3 |
| 2- <sup>18</sup> F]FD-PGL                 | ~25      | 3        | 1   | 15              | 7   | 15           | 8    | 5            | 2   |
| 2- <sup>18</sup> F]FDG-1,6-P <sub>2</sub> | ~28      | 4        | 3   | 3               | 3   | 1            | 1    | 0.3          | 0.1 |
| radiometabolite 12                        | ~29.5    | 3        | 1   | 3               | 1   | 2            | 1    | 0.7          | 0.2 |

| Huh7                                      |          |          |     |                 |     |              |      |              |     |
|-------------------------------------------|----------|----------|-----|-----------------|-----|--------------|------|--------------|-----|
| Radiometabolites                          | RT (min) | Glucose  |     | Glucose-reduced |     | Starve (1 h) |      | Starve (2 h) |     |
|                                           |          | mean (%) | SD  | mean (%)        | SD  | mean (%)     | SD   | mean (%)     | SD  |
| 2- <sup>18</sup> F]FDG                    | ~4       | 8        | 2   | 2               | 1   | 3            | 2    | 2            | 1   |
| 2- <sup>18</sup> F]FDG-1-P                | ~7       | 0.7      | 0.4 | 4               | 2   | 3            | 2    | 4            | 2   |
| 2- <sup>18</sup> F]FDG-6-P                | ~9.5     | 86       | 3   | 76              | 6   | 76           | 5    | 77           | 6   |
| 2- <sup>18</sup> F]FDM-6-P                | ~12      | 4        | 2   | 13              | 4   | 10           | 6    | 8            | 5   |
| radiometabolite 5                         | ~19.5    | 0.2      | 0.3 | 0.5             | 0.3 | 1.5          | 0.5  | 2            | 1   |
| UDP-2- <sup>18</sup> F]FDG                | ~21      | 0.1      | 0.2 | 1.1             | 0.5 | 1.8          | 0.7  | 2.7          | 1.0 |
| 2- <sup>18</sup> F]FD-PGL                 | ~25      | 0.4      | 0.8 | 3               | 1   | 3.4          | 0.8  | 3            | 2   |
| 2- <sup>18</sup> F]FDG-1,6-P <sub>2</sub> | ~28      | 0.7      | 0.3 | 0.2             | 0.2 | 0.1          | 0.1  | 0.4          | 0.1 |
| radiometabolite 12                        | ~29.5    | 0.2      | 0.2 | 0.1             | 0.2 | 0.06         | 0.07 | 0.2          | 0.2 |

Building on Table 1, we analyzed our HPLC data with a graphical Gaussian model to identify and illustrate relevant correlations between radiometabolite formation under different glucose conditions (Figure 4). In accordance with Table 1, the graphical Gaussian model demonstrates that the cell lines handle 2-<sup>18</sup>F]FDG differently dependent on glucose concentration. Overall, positive partial correlations between radiometabolite formation under different conditions were particularly pronounced in Huh7, while they were the least pronounced in HT29. However, the highest positive partial correlation was observed between glucose-reduced (gluc-red) and starve (1 h) conditions in HT29, with a value of 0.73.

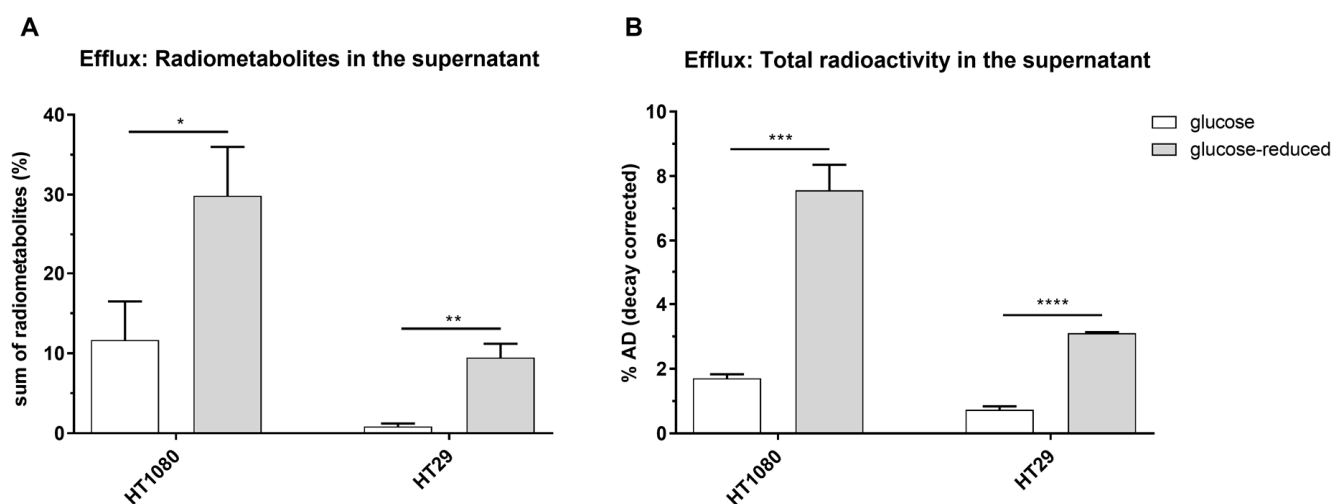




**Figure 4.** Graphical Gaussian model of 2- $^{18}\text{F}$ FDG metabolism in HT1080, HT29 and Huh7. Blue lines show positive correlations. The thicker the line, the higher the correlation.

### 2.3. Efflux of Radioactivity in HT1080 (Fibrosarcoma) and HT29 (Colorectal Adenocarcinoma)

In general, as expected, 2- $^{18}\text{F}$ FDG was the most prominent compound when analyzing the supernatant with HPLC, 1 h after medium change. However, 2- $^{18}\text{F}$ FDM and 2- $^{18}\text{F}$ FDG-6-P could be detected additionally under glucose conditions and 2- $^{18}\text{F}$ FDM, 2- $^{18}\text{F}$ FDG-1-P, 2- $^{18}\text{F}$ FDG-6-P and 2- $^{18}\text{F}$ FD-PGL when glucose content was reduced. In HT1080 cells, levels of extracellular radiometabolites were higher than in HT29, both under glucose or glucose-reduced conditions (Figure 5A). Under glucose-reduced conditions, levels of extracellular radiometabolites were significantly elevated in both cell lines 1 h after medium change.



**Figure 5.** Sum of radiometabolites (%) detected with HPLC (A) and total radioactivity in % applied dose (% AD) in supernatants of HT1080 and HT29 1 h after change to non-radioactive medium (B) (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$  and \*\*\*\*  $p \leq 0.0001$ ). Conditions: glucose = 1.13 g/L, glucose-reduced = 0.13 g/L.

Quantifying radioactivity in the supernatant 1 h after the change to non-radioactive medium, decay-corrected amounts were  $1.7 \pm 0.1\%$  AD (glucose) and  $7.6 \pm 0.8\%$  AD (glucose-reduced) for HT1080 and  $0.7 \pm 0.1\%$  AD (glucose) and  $3.1 \pm 0.3\%$  AD (glucose-reduced) for HT29 (Figure 5B).

In the lactate dehydrogenase (LDH) assay, no change of extracellular LDH activity was seen in the supernatants between time-point 1 (before medium change) and time-point 2 (1 h after medium change) (Figure S1), indicating no leakage of cell membranes.

### 3. Discussion

Rokka et al. [18] presented a suitable radio-HPLC assay for the analysis of 2-[<sup>18</sup>F]FDG metabolism identifying the radiometabolites 2-[<sup>18</sup>F]FDG-6-P, 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> and 2-[<sup>18</sup>F]FD-PGL. Building on their assay, we additionally verified the formation of the radiometabolites 2-[<sup>18</sup>F]FDG-1-P, 2-[<sup>18</sup>F]FDM-6-P and UDP-2-[<sup>18</sup>F]FDG. In particular, the formation of FDM-6-P was described in several 19F-NMR studies with FDG before [11–13,21]. It was postulated that while building an analogue of fructose-6-phosphate is prevented by the fluoride, the formation of 1,2-endiol as an intermediate and lastly FDM-6-P is still possible [22]. However, to the best of our knowledge, we are the first to verify the intracellular formation of 2-[<sup>18</sup>F]FDM-6-P, as well as of the other radiometabolites 2-[<sup>18</sup>F]FDG-1-P and UDP-2-[<sup>18</sup>F]FDG using the radiotracer 2-[<sup>18</sup>F]FDG. Furthermore, despite what is often stated [16,23], 2-[<sup>18</sup>F]FDG-6-P seems to also be processed by G6PD and not only H6PD, at least in a cell-free assay in vitro, as already proposed by Suolinna et al. [14].

Looking at the accumulation experiments, glucose reduction to one tenth led to an increase in 2-[<sup>18</sup>F]FDG uptake of around 24 times in Huh7, 130 times in HT29 and 150 times in HT1080, although the same amount of radioactivity was used. The comparatively little difference within Huh7 might be rooted in using MEM for the accumulation experiments under glucose conditions. As Huh7 was cultivated in high-glucose DMEM, the change to MEM already resulted in a large glucose reduction for these cells (4.63 g/L to 1.13 g/L). This might also explain why there was a smaller difference of extracellular lactate concentration between glucose and glucose-reduced conditions in this cell line as compared to HT1080 and HT29.

Our efflux experiments showed that radiometabolites can be found in the cell culture medium 1 h after change to non-radioactive medium. Although the results of the LDH assay indicate no leakage of cell membranes, the 2-[<sup>18</sup>F]FDG accumulation experiments were generally performed in fully supplemented cell culture medium with inherent LDH activity. Therefore, the background was high and small changes in LDH excretion might therefore be hard to detect. Moreover, the LDH assay determines the activity of the enzyme, not the mere presence of it. Hence, in contrast to HPLC measurements of radiometabolites, the results of this assay are subject to many influences and results should therefore be interpreted with caution. Moreover, up to this date, the only known transporters for sugar-phosphates are located at the membranes of intracellular microsomes [24], making a sugar phosphate transport out of the cell unlikely. Still, extracellular phosphorylated 2-[<sup>18</sup>F]FDG metabolite levels and total radioactivity in the supernatant after the efflux experiment were significantly higher in HT1080, both under glucose and glucose-reduced conditions. Interestingly, the intracellular levels of phosphorylated radiometabolites beyond 2-[<sup>18</sup>F]FDG-6-P were around twice as high in HT29 compared to HT1080 after 1 h (glucose: ~18 vs. ~9%; glucose-reduced or starved: ~40 vs. ~20%). Hence, there is less efflux in the cell line that shows higher metabolism. Cossu et al. suggested that the dephosphorylating enzyme G6Pase and H6PD compete for 2-[<sup>18</sup>F]FDG-6-P in the ER [25]. Thus, enhanced production of 2-[<sup>18</sup>F]FDG-PGL by H6PD would significantly reduce dephosphorylation and subsequent efflux of 2-[<sup>18</sup>F]FDG, which would at least explain higher extracellular 2-[<sup>18</sup>F]FDG levels of HT1080.

The main focus of this paper, however, was to investigate whether a specific pattern of 2-[<sup>18</sup>F]FDG metabolism could be observed in different human cancer cell lines, under different glucose concentrations. In comparison to Rokka et al. we could in general detect more radiometabolites with HPLC analysis. However, compared to our cell assay, where we applied relatively high activities (1MBq/mL), they analyzed tissue samples of rats and mice [18]. Hence, due to biodistribution and subsequent work steps such as organ removal and processing (homogenization, dilution), radioactivity in their samples was

most likely lower. Moreover, we saw the highest amounts of radiometabolites when we reduced glucose concentration in the medium. This set-up cannot be applied in an *in vivo* experiment.

Although the three tumor cell lines were derived from very different tumor entities (fibrosarcoma, colorectal adenocarcinoma, hepatocellular carcinoma), which in turn originate from different cell types, the radiometabolites generated were almost identical. Interestingly, we saw differences regarding the extent of radiometabolite formation and the cells' response to glucose reduction or starving (Table 1), suggesting different 2-[<sup>18</sup>F]FDG handling. This was further supported by our graphical Gaussian model, an established mathematical model for network analysis of biological systems [26,27] that we applied to visualize correlations (Figure 4).

We found that—except for HT29—metabolism beyond 2-[<sup>18</sup>F]FDG-6-P was not pronounced under glucose conditions (baseline). However, the extent of 2-[<sup>18</sup>F]FDG metabolism and especially radiometabolites reflecting glycogen metabolism (2-[<sup>18</sup>F]FDG-1-P) or the PPP (2-[<sup>18</sup>F]FD-PGL) rose under glucose reduction. This fact matches the cells' metabolic switch under glucose reduction as displayed by reduced extracellular lactate levels. Glucose withdrawal is also known to cause a stress situation for glucose-dependent cancer cells [28,29]. Cancer cells can use glycogen metabolism [28] or the PPP [30]—both interconnecting with key metabolic pathways such as glycolysis—to counterbalance (oxidative) stress and sustain growth. Furthermore, we observed enhanced 2-[<sup>18</sup>F]FDM-6-P formation when glucose content was reduced. Interestingly, McSheehy et al. showed in a study with cold FDG and 5-fluorouracil that generated FDM and its phosphorylated conjugates were representative for the treatment response to 5-fluorouracil in mice [21], emphasizing the potential of analyzing FDG metabolism.

Although we present *in vitro* data from established cancer cell lines, we propose that glucose availability could also have substantial influence on the uptake and metabolism of 2-[<sup>18</sup>F]FDG *in vivo*. While Sprinz et al. postulate that the influence of blood glucose levels on 2-[<sup>18</sup>F]FDG uptake in normal organs and tumors is negligible [31], other publications report false-negative 2-[<sup>18</sup>F]FDG tumor PET-scans due to hyperglycemia [32–34]. They also highlight the importance of fasting prior to the PET-scan [32]. A fasting period of at least 4 h is also recommended in the latest version of the tumor PET/CT guidelines of the European Association of Nuclear Medicine [35].

Furthermore, on a cellular level, factors like tumor vascularization, lymphatics or competition for nutrients with microenvironmental cells can substantially influence nutrient accessibility for tumor cells [36] and could thus influence 2-[<sup>18</sup>F]FDG handling locally.

## 4. Materials and Methods

### 4.1. General

2-[<sup>18</sup>F]FDG was synthesized in-house in a fully automated cassette-based module (FASTlab, GE Healthcare, Uppsala, Sweden) at the Vienna General Hospital, Austria and was applied to the cells after quality control, formulated for patient use. All chemicals were purchased from Merck KGaA (Darmstadt, Germany) and all consumables or cell culture supplies were bought from known suppliers, such as VWR International (Radnor, PA, USA), Merck KGaA or Thermo Fisher Scientific Inc. (Waltham, MA, USA).

### 4.2. Cell Culture

The human fibrosarcoma cell line HT1080 was kindly provided by the Institute of Inorganic Chemistry, University of Vienna. HT1080 was grown in MEM with the addition of 10% FBS and 2 mM L-glutamine. The human colorectal adenocarcinoma cell line HT29 was a generous gift from the Department of Pathology, Medical University of Vienna. This cell line was grown in Roswell Park Memorial Institute Medium (RPMI-1640) supplemented with 10% FBS and 2 mM L-glutamine. The human hepatocellular carcinoma cell line Huh7 was kindly provided by Dr. Nataliya Rohr-Udilova, Division of Gastroenterology and

Hepatology, Medical University of Vienna. Huh7 cells were grown in high-glucose DMEM with 4 mM L-glutamine, complemented with 10% FBS.

All cells were kept in a humidified incubator (37 °C, 5% CO<sub>2</sub>) at the Department of Biomedical Imaging and Image-guided Therapy.

For experiments, we used either fully supplemented MEM or glucose-free DMEM supplemented with 10% fetal bovine serum (FBS). FBS has an average glucose content of 125 mg/100 mL [37]. Supplementation therefore results in an average concentration of 0.13 g/L as compared to a glucose concentration of around 1.13 g/L in fully supplemented MEM that we used for “glucose” experiments. Glucose content was therefore reduced to approximately one tenth. For starving experiments, glucose-reduced DMEM was already applied to the cells 1 or 2 h prior to 2-[<sup>18</sup>F]FDG addition.

#### 4.3. Enzymatic In Vitro Synthesis of 2-[<sup>18</sup>F]FDG Radiometabolites

In general, reference radiometabolites were prepared via enzymatic in vitro synthesis on the basis of previously published procedures by Rokka et al. [18] and Kaarstadt et al. [15] (Figure 1). After centrifugation (22 °C, 13,684 g), the reaction mixes were analyzed with HPLC as described for the cell samples.

#### 4.4. 2-[<sup>18</sup>F]FDG-6-P

Around 20 MBq 2-[<sup>18</sup>F]FDG was added to a phosphate buffered saline (PBS) solution containing 100 µL hexokinase, 2 mg adenosine triphosphate (ATP) and 1 mg magnesium chloride. The pH was adjusted to 7–7.5 with 1 M sodium hydroxide (NaOH). The mixture was mixed in a shaker (Eppendorf, Hamburg, Germany) for 2 h at 37 °C.

#### 4.5. 2-[<sup>18</sup>F]FD-PGL

After synthesis of 2-[<sup>18</sup>F]FDG-6-P, 4.5 mg β-nicotinamide adenine dinucleotide phosphate and 35 units of G6PD (from yeast or leuconostoc mesenteroides) were added. The solution was again incubated at 37 °C for 2.5 h.

#### 4.6. 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub>

In that case, the 2-[<sup>18</sup>F]FDG-6-P solution was further processed with 20 units PGM, 1 mg α-D-glucose-1,6-diphosphate cyclohexylammonium salt, 1 mg EDTA and 1.3 mg BSA. The pH was adjusted to around 7.9 with 1 M NaOH. The mixture was again shaken at 37 °C for 2.5 h.

#### 4.7. 2-[<sup>18</sup>F]FDG-1-P

This metabolite was detected after 2 h, when synthesizing 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> under suboptimal conditions (pH < 7.9).

#### 4.8. UDP-2-[<sup>18</sup>F]FDG

After synthesis of 2-[<sup>18</sup>F]FDG-6-P, the solution was filtered with a Centrifree Ultrafiltration Device with Ultracel PL membrane (cutoff 30 kDa, Merck KGaA, Darmstadt, Germany) by centrifugation at 1520 × g (15 min, RT) in order to remove hexokinase (~100 kDa). Then, 20 units PGM, 2 µmol UTP and 10 units UGPase were added for 3 h.

#### 4.9. 2-[<sup>18</sup>F]FDM-6-P

As PBS can hamper the activity of phosphoglucose isomerase (PGI) according to the supplier, another batch of 2-[<sup>18</sup>F]FDG-6-P was produced in HEPES buffered saline solution (pH 7.5). After the formation of 2-[<sup>18</sup>F]FDG-6-P was confirmed, 200 units of PGI were added to synthesize 2-[<sup>18</sup>F]FDM-6-P. The mixture was incubated at 37 °C for another 3 h.

#### 4.10. 2-[<sup>18</sup>F]FDG Accumulation Experiments

The workflow for accumulation experiments is shown in Figure 2. Cells were seeded into 6-well plates two days prior, reaching 80–90% confluency on the day of the experiment. For the experiment, growth medium was withdrawn and cells were washed once with the respective medium, before fully supplemented MEM or glucose-reduced DMEM were added, containing 1 MBq/mL 2-[<sup>18</sup>F]FDG ( $n = 5$ ). Then, 1 h after addition of 2-[<sup>18</sup>F]FDG, aliquots of the supernatants were taken, cells were washed two times with PBS, detached with 500  $\mu$ L of Accutase and resuspended in MEM. A 100  $\mu$ L aliquot of the cell suspension was measured with a gamma counter (PerkinElmer, 2480 Automatic Gamma counter, Wizard23) and subsequently, cells were mixed with trypan blue and counted with a Neubauer chamber. Values are expressed as % applied dose (% AD) per  $10^4$  cells.

The aliquots of the supernatants were filtered through a Microcon-30 kDa Centrifugal Filter Unit (Merck KGaA, Darmstadt, Germany) to remove LDH from the medium. Then, lactate concentration was determined with a lactate assay kit (Merck KGaA, Darmstadt, Germany) following the supplier's instructions.

#### 4.11. Radiometabolite Detection with HPLC

While we used one of the anion-exchanger columns applied by Rokka et al. [18] for radiometabolite separation, we used a different HPLC system and a different experimental setup. The HPLC method by Rokka et al. [18] was therefore adapted to our system for an optimal separation of the peaks by changing the gradient profile and the run time (Table S1). The final gradient profile of solvent A (0.6 M sodium dihydrogen phosphate buffer with 3% methanol) and B (3% methanol in water) was 0–12 min 5% A, 13 min 10% A, 14–18 min 15% A, 19–32 min 50% A and 34–35 min 5% A. Table S1 shows a comparison between the HPLC method by Rokka et al. [18] and our method.

Figure 2 shows the workflow of HPLC experiments to determine intracellular radiometabolite formation. 2-[<sup>18</sup>F]FDG was applied under glucose conditions, glucose-reduced conditions or 1 or 2 h starving (addition of glucose-reduced DMEM prior to 2-[<sup>18</sup>F]FDG). In general, cells were seeded, treated and washed as described for the accumulation experiments ( $n \geq 9$  except for 2 h starve, where  $n = 7$  (HT1080), 6 (Huh7) and 3 (HT29)). However, cells were not detached with Accutase, but lysed with 250  $\mu$ L of methanol (MeOH) and scratched off the surface with a cell scraper. Lysates were then centrifuged for 4 min (4  $^{\circ}$ C, 13,684 g) and supernatants were subsequently injected into an HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a radio detector (Ramona, Elysia-Raytest, Straubenhardt, Germany) and a cooled sample table. Radiometabolites were separated with an anion-exchanger Partisil<sup>TM</sup> 10 SAX column (250 mm  $\times$  4.6 mm, Supelco<sup>®</sup> analytical, PA, USA) with a flow of 1 mL/min. Chromatograms were subsequently evaluated with the software GINA Star<sup>TM</sup> (Version 5.9 Service Pack 17, Ramona, Elysia-Raytest, Straubenhardt, Germany).

The limit of detection was defined as three times and the limit of quantification as at least five times the area under the curve of the background area as described by González, O., & Alonso, R. M. [38]. The results presented in this article were corrected for the background area by manual integration of the peaks of interest. For every chromatogram the background was quantified separately.

#### 4.12. Efflux Experiments

2-[<sup>18</sup>F]FDG was applied to the cells in either MEM or glucose-reduced DMEM as described above. Then, 1 h after 2-[<sup>18</sup>F]FDG addition, the supernatant was withdrawn and cells were washed three times with fresh medium, before 1.5 mL of new, non-radioactive medium (MEM or DMEM) was added carefully. After 1 h, 500  $\mu$ L of the supernatant was collected, centrifuged at 13,684 g and injected into HPLC ( $n = 3$  each). Another aliquot was measured in the gamma counter to quantify extracellular radioactivity. Values are again expressed as % AD (decay corrected). To ensure that the cell membranes were still intact after the washing steps and medium changes, aliquots of the supernatants were taken



before the washing steps and at the end of the efflux experiments to determine extracellular LDH activity with an LDH assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the supplier's instructions.

#### 4.13. Statistics

##### 4.13.1. General

The values are depicted as mean  $\pm$  SD. All experiments were performed in triplicate (or in duplicate in the case of radiometabolite analysis after 1 h) and repeated at least three times. Two-sided *t*-tests were performed with the software Prism 7.03 (GraphPad, San Diego, CA, USA) and *p*-values  $\leq 0.05$  were considered statistically significant.

##### 4.13.2. Gaussian Model

For a further analysis of the metabolic pattern, a network analysis was performed with a graphical Gaussian model as recently described [27] using JASP software (JASP Team, Version 0.14.1, <https://jasp-stats.org/>, accessed on 7 June 2021). This analysis uses partial correlation, i.e., the correlation between the residuals resulting from linear regression, as a measure of independence between two data points, allowing us to distinguish between direct and indirect interactions. Line thickness indicates the strength of the positive partial correlation. A detailed description of the Gaussian network model theory can also be found in the Supplementary Materials of Perišić et al. [39].

## 5. Conclusions

Overall, we could show that the extent of 2-[<sup>18</sup>F]FDG metabolism is not the same in different cancer cell lines and that glucose deprivation significantly enhances radiometabolite formation and total intracellular accumulation dependent on the cell line. Building on the publication by Kernstine K.H. et al., suggesting that 2-[<sup>18</sup>F]FDG PET predicts the use of glucose in pathways other than glycolysis [7], we claim that 2-[<sup>18</sup>F]FDG metabolism can be an important readout for the cells' metabolic (re-)wiring. From a translational point of view and bearing in mind that 2-[<sup>18</sup>F]FDG radiometabolites could be an important readout for tumor heterogeneity, we need to have a closer look at the biological processes involved. Instead of simple static quantifications of 2-[<sup>18</sup>F]FDG uptake at single time-points, the kinetic behavior of 2-[<sup>18</sup>F]FDG in each voxel over time should be assessed. New imaging technologies for whole-body PET combined with kinetic modelling and parametric analysis of the given images over time could provide an important toolset for this methodological challenge.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/ph14090910/s1>, Figure S1: LDH activity in the supernatants of HT1080 and HT29, 1 h after changing the medium., Table S1: Comparison between the HPLC method of Rokka et al. [18] and our adapted method.

**Author Contributions:** E.-M.K. and M.M. (Mahshid Mahmudi) performed the experiments. B.K.G., V.P., C.V. and T.B. helped design the experiments and analyze the data. E.-M.K., B.K.G. and M.M. wrote the manuscript. A.M., A.H., H.V., N.R.-U., M.H. and M.M. (Markus Mitterhauser) helped with data interpretation and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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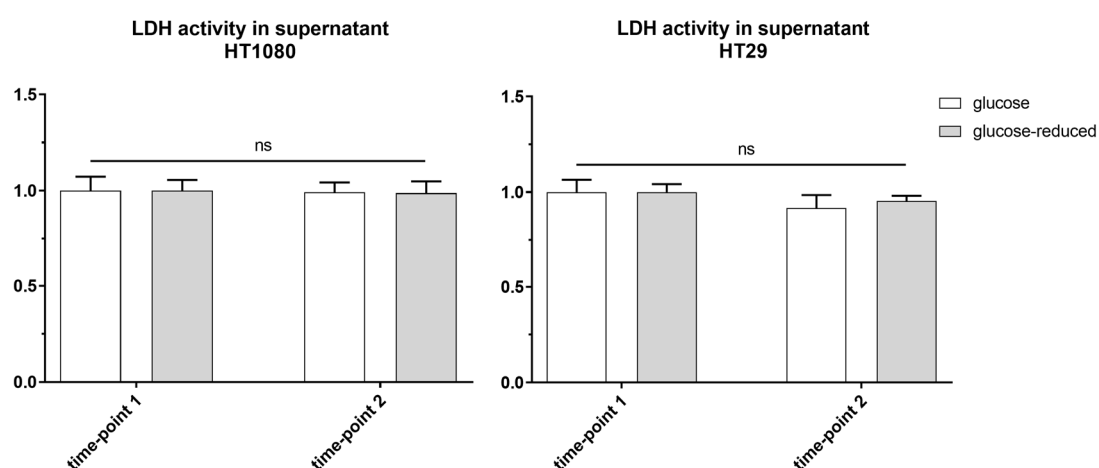
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## Supplementary materials

**Table S1.** Comparison between the HPLC method of Rokka et al. [18] and our adapted method.

| Rokka et al.                                                                                              |    |    | Our method                                                                                                |    |    |
|-----------------------------------------------------------------------------------------------------------|----|----|-----------------------------------------------------------------------------------------------------------|----|----|
| HPLC system:<br>Merck Hitachi                                                                             |    |    | HPLC system:<br>Agilent technologies                                                                      |    |    |
| Columns:<br>Radial-Pak 8P SAX 10 μm (100 mm × 8 mm) or Partisil<br>SAX 10 μm (250 mm × 4.6 mm)            |    |    | Column:<br>Partisil SAX 10 μm (250 mm × 4.6 mm)                                                           |    |    |
| Run time:<br>30 min                                                                                       |    |    | Run time:<br>35 min                                                                                       |    |    |
| Flow:<br>1 mL/min                                                                                         |    |    | Flow:<br>1 mL/min                                                                                         |    |    |
| Solvents:<br>A = 0.6 M sodium dihydrogen phosphate buffer with 3%<br>methanol<br>B = 3% methanol in water |    |    | Solvents:<br>A = 0.6 M sodium dihydrogen phosphate buffer with 3%<br>methanol<br>B = 3% methanol in water |    |    |
| Gradient profile:                                                                                         |    |    |                                                                                                           |    |    |
| Time (min)                                                                                                | %A | %B | Time (min)                                                                                                | %A | %B |
| 0-10                                                                                                      | 5  | 95 | 0-12                                                                                                      | 5  | 95 |
| 11-18                                                                                                     | 15 | 85 | 13                                                                                                        | 10 | 90 |
| 19-29                                                                                                     | 50 | 50 | 14-18                                                                                                     | 15 | 85 |
| 30                                                                                                        | 5  | 95 | 19-32                                                                                                     | 50 | 50 |
|                                                                                                           |    |    | 34-35                                                                                                     | 5  | 95 |



**Figure S1.** LDH activity in the supernatants of HT1080 and HT29, 1 h after changing the medium. Conditions: glucose = 1.13 g/L, glucose-reduced = 0.13 g/L.

### 3.1.4 Manuscript IV

**Patronas EM**, Balber T, Miller A, Geist BK, Michligk A, Vraha C, Krisch M, Rohr-Udilova N, Haschemi A, Viernstein H, Hacker M, Mitterhauser M. A fingerprint of 2-[<sup>18</sup>F]FDG radiometabolites - how tissue-specific metabolism beyond 2-[<sup>18</sup>F]FDG-6-phosphate could affect tracer accumulation. Under Revision in iScience (ISCIENCE-D-23-02552)

**A fingerprint of 2-[<sup>18</sup>F]FDG radiometabolites –  
how tissue-specific metabolism beyond  
2-[<sup>18</sup>F]FDG-6-phosphate could affect tracer accumulation**

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## Summary

Studies indicate that the radiotracer 2- $^{18}\text{F}$ fluoro-2-deoxy-D-glucose (2- $^{18}\text{F}$ FDG) can be metabolized beyond 2- $^{18}\text{F}$ FDG-6-phosphate (2- $^{18}\text{F}$ FDG-6-P), but its metabolism is incompletely understood. Most importantly, it remains unclear whether downstream metabolism affects tracer accumulation *in vivo*. Here we for the first time present a fingerprint of 2- $^{18}\text{F}$ FDG radiometabolites over time in cancer cells, corresponding tumor xenografts and murine organs. Strikingly, radiometabolites representing glycogen metabolism or the oxPPP correlated inversely with tracer accumulation across all examined tissues. Recent studies suggest that not only hexokinase, but also hexose-6-phosphate dehydrogenase (H6PD), an enzyme of the oxidative pentose phosphate pathway (oxPPP), determines 2- $^{18}\text{F}$ FDG accumulation. However, little is known about the corresponding enzyme glucose-6-phosphate dehydrogenase (G6PD). Our mechanistic *in vitro* experiments on the role of the oxPPP suggest that 2- $^{18}\text{F}$ FDG can be metabolized *via* both G6PD and H6PD, but only H6PD interferes with tracer accumulation. Overall, we propose that tissue-specific metabolism beyond 2- $^{18}\text{F}$ FDG-6-P could matter for imaging.

## Introduction

2-[<sup>18</sup>F]FDG can reportedly be metabolized beyond 2-[<sup>18</sup>F]FDG-6-phosphate (2-[<sup>18</sup>F]FDG-6-P), as shown for different cell lines<sup>1-4</sup> and individual animal organs or murine tumors<sup>5-8</sup>. According to the current literature, the main radiometabolites besides 2-[<sup>18</sup>F]FDG-6-P are 2-[<sup>18</sup>F]FDG-1-phosphate (2-[<sup>18</sup>F]FDG-1-P), 2-[<sup>18</sup>F]fluoro-2-deoxy-D-mannose and 2-[<sup>18</sup>F]fluoro-2-deoxy-D-mannose-6-phosphate (2-[<sup>18</sup>F]FDM and 2-[<sup>18</sup>F]FDM-6-P), 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucose-1,6-bisphosphate (2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub>), 2-[<sup>18</sup>F]fluoro-2-deoxy-D-gluconolactone (2-[<sup>18</sup>F]FD-PGL) and nucleotide-bound [<sup>18</sup>F]FDG (sometimes specified as 2-[<sup>18</sup>F]FDG bound to uridine diphosphate, 2-UDP-[<sup>18</sup>F]FDG). <sup>19</sup>F nuclear magnetic resonance imaging (NMR) using the non-radioactive <sup>19</sup>F-analogon of the tracer has revealed even more metabolites, such as other FDM-based radiometabolites<sup>9-11</sup>. This could be due to the use of substantially higher doses, or an analysis at later time-points, as done by Kanazawa *et al.*<sup>9</sup>.

As for 2-[<sup>18</sup>F]FDG accumulation, particular attention has been paid to the pentose phosphate pathway (PPP) during the last years<sup>12-15</sup>. The PPP, a carbohydrate metabolic pathway which branches from glucose 6-phosphate (G6P) in parallel to glycolysis, is involved in various cellular processes, as it is a significant source of pentose phosphates and NADPH required for redox balance and the synthesis of biomolecules like fatty acids. The PPP consists of an oxidative (oxPPP) and a non-oxidative branch, with glucose-6-phosphate dehydrogenase (G6PD) as the first and thereby rate-limiting enzyme of the oxPPP<sup>16</sup>. However, there exists a separate PPP in the endoplasmic reticulum (ER)<sup>17,18</sup>, the compartment where 2-[<sup>18</sup>F]FDG-6-P preferentially accumulates and where its dephosphorylation takes place<sup>13,19,20</sup>. According to literature, the intracellular accumulation of 2-[<sup>18</sup>F]FDG seems to be determined by the oxPPP enzyme hexose-6-phosphate dehydrogenase (H6PD) in the ER<sup>12-15</sup>. This enzyme is said to compete with glucose-6-phosphatase (G6Pase), thereby preventing the tracer's dephosphorylation<sup>13</sup>. In contrast, little is known about the role of the corresponding enzyme G6PD. In literature, G6PD is usually associated with the cytoplasmic oxPPP, while H6PD is often described as ER-exclusive<sup>15,18</sup>. However, public protein expression data suggest that neither G6PD nor H6PD are strictly confined to the cytoplasmic or reticular compartment<sup>21,22</sup>. Furthermore, it is generally assumed that G6PD cannot process other hexoses than glucose<sup>23,24</sup>. However, some studies show *in vitro* formation of the oxPPP radiometabolite 2-[<sup>18</sup>F]FD-PGL with isolated G6PD enzyme<sup>2,25</sup>.

While the effect of H6PD expression and activity on 2-[<sup>18</sup>F]FDG accumulation has been thoroughly studied<sup>12-14</sup>, the role of G6PD in in this context remains ill-defined. Furthermore, even though metabolism beyond 2-[<sup>18</sup>F]FDG-6-P is undeniable, interorgan or translational differences in 2-[<sup>18</sup>F]FDG metabolism have not been studied in detail, especially over time. Most importantly, it remains unclear if metabolism beyond 2-[<sup>18</sup>F]FDG-6-P is indeed relevant for *in vivo* tracer accumulation and thus routine clinical imaging.

Therefore we aimed to establish a fingerprint of 2-<sup>[18F]</sup>FDG radiometabolites in various murine organs and tumors, as well as to study differences between cultured tumor cells and corresponding tumors *in vivo* to evaluate, in a second step, the impact of downstream metabolism on tracer accumulation. As the oxPPP seems to be highly involved in 2-<sup>[18F]</sup>FDG trapping, we also aimed to further define the role of oxPPP enzymes in regulating tracer accumulation and radiometabolite formation.

## Results

### Accumulation of 2-<sup>[18F]</sup>FDG and overall metabolism beyond 2-<sup>[18F]</sup>FDG-6-P are organ-dependent

Total radioactivity (% ID/g) detected in *ex vivo* measurements was significantly different between organs at all tested time-points. Accumulation was in equilibrium after 30 min for all tissues except for the kidney and plasma, where the activity decreased, and the heart, where the activity increased steadily within the measured time frame (Figure 1A). In addition, overall metabolism beyond 2-<sup>[18F]</sup>FDG-6-P at 60 and 120 min, assessed by the sum of derived radiometabolites excluding 2-<sup>[18F]</sup>FDG-6-P, was significantly different between the measured organs. At 120 min, lowest radiometabolite levels beyond 2-<sup>[18F]</sup>FDG-6-P were detected in the kidney ( $26 \pm 2.0$  %), while lung tissue showed the highest levels ( $39 \pm 2.0$  %) and also the highest slope over 120 min (Figure 1B).

### Analysis of individual radiometabolites reveals distinct tissue-dependent patterns of 2-<sup>[18F]</sup>FDG metabolism

Concerning 2-<sup>[18F]</sup>FDG metabolism in different organs, distinct metabolic patterns were observed (Figure 2). In general, based on our previous work <sup>2</sup>, the radiometabolites 2-<sup>[18F]</sup>FDM, 2-<sup>[18F]</sup>FDG-6-P, 2-<sup>[18F]</sup>FDM-6-P, 2-<sup>[18F]</sup>FDG-1-P, 2-<sup>[18F]</sup>FDG-1,6-P<sub>2</sub>, UDP-2-<sup>[18F]</sup>FDG and 2-<sup>[18F]</sup>FD-PGL were detected in addition to 2-3 other radiometabolites that were not identified. Chemical structures of the radiometabolites and a representative HPLC chromatogram are given in the Supplemental Information (Figure S1).

2-<sup>[18F]</sup>FDM was sometimes present in *ex vivo* analysis, but without a consistent pattern and 2-<sup>[18F]</sup>FDG and 2-<sup>[18F]</sup>FDM peaks could not be accurately separated for quantitative assessments. Considering that both molecules can exit the cell, we summed the levels of 2-<sup>[18F]</sup>FDG and 2-<sup>[18F]</sup>FDM to form an “unphosphorylated pool”. This pool was highest in the kidney and liver (roughly 60 % at both 60 and 120 min), while 2-<sup>[18F]</sup>FDG-6-P and 2-<sup>[18F]</sup>FDM-6-P levels were lowest in these organs at all investigated time-points. Relevant levels of glycolytic 2-<sup>[18F]</sup>FDG-1-P were only found in the liver, where around 7 % were detected at all three time-points. Importantly, this radiometabolite was not observed in the brain and heart. The glycolytic radiometabolite 2-UDP-<sup>[18F]</sup>FDG was the main radiometabolite besides

2-[<sup>18</sup>F]FDG-6-P in the kidney, reaching  $13 \pm 2.9$  % at 120 min, but remaining below 5 % in other tissues. The oxPPP radiometabolite 2-[<sup>18</sup>F]FD-PGL remained low in all tissues except for the liver, where it reached  $11 \pm 1.9$  % at 120 min. Importantly, detected 2-[<sup>18</sup>F]FD-PGL might also have been a mixture of 2-[<sup>18</sup>F]FD-PGL and the hydrolyzed product 2-[<sup>18</sup>F]FD-phosphogluconate, as spontaneous hydrolysis can occur<sup>6,7,26</sup>. Like 2-[<sup>18</sup>F]FDG-1-P, 2-[<sup>18</sup>F]FD-PGL was not detected in the brain and heart. However, as observed for tumors, 2-[<sup>18</sup>F]FDM-6-P was the only relevant radiometabolite besides 2-[<sup>18</sup>F]FDG-6-P in those two organs, reaching  $24 \pm 3.2$  % and  $23 \pm 2.8$  % at 120 min, respectively. In contrast to cell culture, where it was the main radiometabolite beyond 2-[<sup>18</sup>F]FDG-6-P, only low levels of 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> were found *ex vivo*.

In summary, these results indicate tissue-dependent patterns of 2-[<sup>18</sup>F]FDG metabolism. 2-[<sup>18</sup>F]FDM-6-P was the most abundant radiometabolite besides 2-[<sup>18</sup>F]FDG-6-P in the brain, heart, lung and tumors. Glycogenic radiometabolites were prominent in the liver and kidney, while levels of the oxPPP radiometabolite 2-[<sup>18</sup>F]FD-PGL were highest in the liver.

#### HT1080 and HT29 xenograft tumors differ in 2-[<sup>18</sup>F]FDG accumulation and overall metabolism and show a shifted radiometabolite pattern compared to *in vitro* cell culture

Higher total radioactivity was detected in the more aggressive HT1080 tumors compared to HT29 tumors as determined by *ex vivo* gamma counting (Figure 3A), which is in line with the representative  $\mu$ PET/CT images (Figure 3B). Unexpectedly, a comparison of the *in vitro* and *in vivo* fingerprint of 2-[<sup>18</sup>F]FDG metabolism indicated that 2-[<sup>18</sup>F]FDG metabolism beyond 2-[<sup>18</sup>F]FDG-6-P was around 1.5-fold (HT1080) and 2-fold (HT29) higher *in vivo* (Figure 3C). Higher levels of unphosphorylated tracer, that can potentially exit the cell, were found in HT29 tumors (data not shown), which agreed with accumulation data. In contrast to HT1080 and *in vivo* data, overall metabolism in HT29 decreased over time *in vitro*, which was accompanied by a decrease in 2-[<sup>18</sup>F]FDM-6-P levels (Figure 3C, box). In general, the radiometabolite profile was inherently different between *in vitro* cell culture and corresponding tumors: *in vitro*, the most abundant radiometabolite besides 2-[<sup>18</sup>F]FDG-6-P was 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub>, reaching a maximum of  $13 \pm 3.0$  % of total radiometabolites beyond 2-[<sup>18</sup>F]FDG-6-P in HT1080 and  $9.8 \pm 5.0$  % in HT29 cells at 120 min. However, *in vivo* it was 2-[<sup>18</sup>F]FDM-6-P with levels up to  $17 \pm 4.6$  % and  $19 \pm 4.5$  %, respectively (Figure 3C, box).

#### Radiometabolites of glycogen metabolism and the oxPPP correlate with total radioactivity in tissues and with each other

The radiometabolite levels were correlated with total tissue radioactivity measured *via* gamma counter (% ID/g) at the time-points 30, 60 and 120 min. Comparing all measured tissues and time-

points, the radiometabolites 2-[<sup>18</sup>F]FD-PGL, 2-[<sup>18</sup>F]FDG-1-P, and the unphosphorylated pool correlated inversely with the measured % ID/g ( $r_s = -0.762$ ,  $P = 0.004$ ;  $r_s = -0.770$ ,  $P = 0.001$ ;  $r_s = -0.590$ ,  $P = 0.005$ , respectively) (Figure 4A). Considering that unphosphorylated tracer can leave the cell, the observed inverse correlation between the unphosphorylated pool and total 2-[<sup>18</sup>F]FDG accumulation can be considered as proof of principle. In contrast, the sum of all radiometabolites beyond 2-[<sup>18</sup>F]FDG-6-P did not correlate with tracer accumulation, indicating their selective function in regulating 2-[<sup>18</sup>F]FDG metabolism. As a cross-check, radiometabolite levels were further correlated with corresponding % ID/cc values of the five dynamic scans by defining volumes of interest in the organs (the approach is specified in the Supplemental Information). This cross-check revealed almost identical correlations (data not shown).

To reveal potential relationships between radiometabolites, their levels were correlated with each other, and the Spearman  $r$  was plotted in a heatmap (Figure 4B). A strong correlation was identified between the oxPPP radiometabolite 2-[<sup>18</sup>F]FD-PGL and the glycogenic intermediates 2-[<sup>18</sup>F]FDG-1-P and UDP-2-[<sup>18</sup>F]FDG ( $r_s = 0.953$ ,  $P \leq 0.0001$  and  $r_s = 0.769$ ,  $P = 0.003$ , respectively). As expected, this analysis also revealed a correlation between 2-[<sup>18</sup>F]FDG-1-P and UDP-2-[<sup>18</sup>F]FDG ( $r_s = 0.599$ ,  $P = 0.018$ ). UDP-2-[<sup>18</sup>F]FDG was the only radiometabolite that strongly correlated inversely with 2-[<sup>18</sup>F]FDG-6-P ( $r_s = -0.804$ ,  $P \leq 0.0001$ ), while a trend was also observed with 2-[<sup>18</sup>F]FDG-1-P ( $r_s = -0.550$ ,  $P = 0.064$ ). The unphosphorylated pool correlated inversely with 2-[<sup>18</sup>F]FDG-6-P and 2-[<sup>18</sup>F]FDM-6-P ( $r_s = -0.643$ ,  $P = 0.004$  and  $r_s = -0.736$ ,  $P = 0.001$ ), while no or a positive correlation was observed with other radiometabolites.

#### The oxPPP inhibitors DHEA and G6PDi-1, but not CBX enhance 2-[<sup>18</sup>F]FDG accumulation and metabolism

To test whether the first step of the oxPPP - catalyzed by G6PD/H6PD - can influence 2-[<sup>18</sup>F]FDG accumulation and metabolism, we chose a pharmacological approach and pretreated cells with G6PD/H6PD inhibitors. Unexpectedly, only dehydroepiandrosterone (DHEA) increased 2-[<sup>18</sup>F]FDG accumulation to  $187 \pm 23$  and  $326 \pm 9.9$  %, respectively, and also downstream metabolism to  $160 \pm 3.3$  and  $128 \pm 3.3$  % in both HT1080 and HT29 cells after 1 h, while carbenoxolone (CBX) showed no effect (Figure 5A,B). DHEA significantly increased 2-[<sup>18</sup>F]FDG-1-P and 2-[<sup>18</sup>F]FD-PGL radiometabolite levels in both cell lines and additionally 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> levels in HT1080 (Figure 5B, box).

To ensure inhibitory function of DHEA, an *ex vivo* G6PD/H6PD activity assay with HT29 lysates was performed. As expected, DHEA decreased the specific activity by  $\sim 80$  % using G6P as a substrate. No baseline activity was observed with FDG6P as substrate, but low specific activity was observed in the presence of DHEA (Figure 5C).



To validate this observation, DHEA effects were compared to an alternative inhibitor, G6PDi-1, using HT29 cells. Both inhibitors increased 2-[<sup>18</sup>F]FDG accumulation, when they were added 1 h before or simultaneous with 2-[<sup>18</sup>F]FDG, however, DHEA effects were more pronounced at tested concentrations (Figure 5D). As for 2-[<sup>18</sup>F]FDG downstream metabolism, G6PDi-1 reproduced the enhancing effects of DHEA on overall metabolism and specifically on 2-[<sup>18</sup>F]FDG-1-P and 2-[<sup>18</sup>F]FD-PGL (Figure 5E). Thereby the data suggested an inverse correlation of G6PD/H6PD activity with 2-[<sup>18</sup>F]FDG accumulation and showed an unexpected increase of the oxPPP radiometabolite in the presence of these inhibitors.

#### H6PD but not G6PD knockdown enhances 2-[<sup>18</sup>F]FDG accumulation and metabolism

In order to analyze the individual contributions of G6PD and H6PD to observed effects, we selectively silenced these enzymes by siRNA, which was confirmed *via* western blot (WB) (Figure 6A). A selective knockdown of H6PD in HT29 cells resulted in an increase of total 2-[<sup>18</sup>F]FDG accumulation by 36 ± 17 %, which mimicked DHEA or G6PDi-1 effects, while G6PD knockdown had no effect on tracer accumulation (Figure 6B). However, following G6PD knockdown, the oxPPP radiometabolite 2-[<sup>18</sup>F]FD-PGL was considerably reduced from 5.0 ± 0.96 % to 1.3 ± 0.15 % (Figure 6C, box). While it had no influence on the oxPPP radiometabolite, knockdown of H6PD increased overall metabolism beyond 2-[<sup>18</sup>F]FDG-6-P, particularly levels of 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> and the subsequent, unidentified radiometabolite at minute 30 of the HPLC run (Figure 6C). Based on Fedders *et al.* <sup>4</sup>, this peak could be 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucuronic acid. These results indicate a divergent role of the oxPPP enzymes G6PD and H6PD in 2-[<sup>18</sup>F]FDG accumulation and metabolism.

## **Discussion**

In general, *in vivo* metabolism beyond 2-[<sup>18</sup>F]FDG-6-P reached up to 28 % at 60 min and 39 % at 120 min, reinforcing the opinion that tracer metabolism, at least in prolonged studies, should not be dismissed and might be relevant for accurate signal quantification in imaging <sup>6,27</sup> (Figure 1). Concerning tissue-specific patterns, UDP-2-[<sup>18</sup>F]FDG levels were high in the kidney, an organ with usually low glycogen levels building glycogen under hyperglycemic conditions <sup>28</sup>. We hypothesize that this is due to hyperglycemia, which is reportedly induced by isoflurane anesthesia <sup>29</sup>. Radiometabolite data of the liver solidifies the assumption, although the other glycogenic radiometabolite 2-[<sup>18</sup>F]FDG-1-P was more prominent here. Given the proximity of the two peaks in HPLC, we acknowledge that what we described as UDP-2-[<sup>18</sup>F]FDG might also be or include the epimerized form UDP-2-[<sup>18</sup>F]fluoro-2-deoxy-D-galactose, as postulated by Fedders *et al.* <sup>4</sup>. The most abundant radiometabolite in the liver was 2-[<sup>18</sup>F]FD-PGL, which is consistent with literature describing highest PPP activity in organs performing

lipid and steroid synthesis, like the liver <sup>30</sup>, and also reflects previously reported *in vivo* data <sup>5,6</sup> (Figure 2B). As for xenograft tumors, the observed radiometabolites were different to those reported by Suolinna *et al.*, Haaparanta *et al.*, Kaarstad *et al.*, who described nucleotide-bound 2-[<sup>18</sup>F]FDG/UDP-2-[<sup>18</sup>F]FDG<sup>8,25</sup> and 2-[<sup>18</sup>F]FD-PGL/PG1 and 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub><sup>5</sup> as only radiometabolites, respectively. However, different rodents, different types of (non-continuous anesthesia), other tumor models and other tissue processing and HPLC methods were used.

2-[<sup>18</sup>F]FDM-6-P was the only other quantitatively relevant radiometabolite besides 2-[<sup>18</sup>F]FDG-6-P in all tissues except for the liver and kidney, which have a different physiological function as excretory organs (Figure 2B). Although downstream metabolism was much higher than in our study, Southworth *et al.*, using 150 mg/kg FDG and <sup>19</sup>F NMR, also demonstrated this large difference between 2-[<sup>18</sup>F]FDM-6-P levels in the rat brain and heart compared to the liver and kidney <sup>10</sup>. Interestingly, except for our preceding study <sup>2</sup>, 2-[<sup>18</sup>F]FDM-6-P was not detected in other studies specifically using the radioactive tracer and HPLC for detection <sup>5-7,25</sup>. It has been discussed if 2-[<sup>18</sup>F]FDG-6-P epimerization by phosphoglucose isomerase (PGI), the enzyme forming fructose-6-phosphate, could be used as readout for glycolytic activity <sup>31,32</sup>. Comparing 2-[<sup>18</sup>F]FDM-6-P levels in 2D cell culture and corresponding tumors, 2-[<sup>18</sup>F]FDG-6-P epimerization is only relevant in the latter (Figure 3C). This is in line with Murakami *et al.*, showing that fructose-6-phosphate formation by PGI, the first step towards glycolysis, is much higher in corresponding tumors <sup>33</sup>. We believe that considering 2-[<sup>18</sup>F]FDM-6-P formation as a general indicator of glycolysis might be an oversimplification. However, it might give an indication of how much the metabolic flux is shifted towards glycolysis in a first step, since 2-[<sup>18</sup>F]FDG-6-P can be either epimerized to 2-[<sup>18</sup>F]FDM-6-P, isomerized to 2-[<sup>18</sup>F]FDG-1-P (→ glycogen metabolism), or oxidized to 2-[<sup>18</sup>F]FD-PGL (→ PPP). Interestingly, while there was no connection between 2-[<sup>18</sup>F]FDM-6-P levels and total tracer accumulation, we found an inverse correlation between the unphosphorylated pool and the radiometabolites 2-[<sup>18</sup>F]FDG-6-P and 2-[<sup>18</sup>F]FDM-6-P. This hints at a link between 2-[<sup>18</sup>F]FDG “trapping” and the tracer being directed towards glycolysis, indicated by 2-[<sup>18</sup>F]FDM-6-P formation.

The inverse correlation between 2-[<sup>18</sup>F]FDG-1-P and 2-[<sup>18</sup>F]FD-PGL levels and total 2-[<sup>18</sup>F]FDG accumulation suggests that a) 2-[<sup>18</sup>F]FDG metabolism *via* glycogen metabolism and the oxPPP could influence tracer accumulation *in vivo*, or b) that an enhanced activity of glycogen metabolism or the oxPPP rewires the cells’ metabolism, causing altered 2-[<sup>18</sup>F]FDG accumulation through changes in the activity of other enzymes or transporters (Figure 4A). Interestingly, we also observed a correlation between the oxPPP radiometabolite 2-[<sup>18</sup>F]FD-PGL and the glycogenic radiometabolites 2-[<sup>18</sup>F]FDG-1-P and UDP-2-[<sup>18</sup>F]FDG in *ex vivo* analysis (Figure 4B). This link was consistent *in vitro*, where increased 2-[<sup>18</sup>F]FD-PGL levels following inhibitor treatment were accompanied by increased 2-[<sup>18</sup>F]FDG-1-P

levels (Figure 5). Taken together, these data underscore the close link between glycogen metabolism and the PPP previously described for macrophages and cyanobacteria <sup>34,35</sup>.

In our *in vitro* experiments to assess the impact of oxPPP activity on 2-[<sup>18</sup>F]FDG accumulation and metabolism, CBX pretreatment did not alter 2-[<sup>18</sup>F]FDG accumulation, which is in contrast to previously published *in vitro* experiments showing decreased accumulation using this indirect H6PD inhibitor <sup>12</sup> (Figure 5A). Instead, DHEA and G6PDi-1 enhanced both tracer accumulation and metabolism beyond 2-[<sup>18</sup>F]FDG-6-P in a similar way (Figure 5A,B,D,E). The stronger effect of DHEA on tracer accumulation could be due to the reported stimulation of glucose transporter translocation to the cell membrane <sup>36</sup>. As the non-steroidal G6PDi-1 has been shown to be a more potent and specific G6PD inhibitor compared to the nonspecific hormone DHEA <sup>37</sup>, the observed effects could indeed be partly attributable to a specific interaction with the oxPPP. Interestingly, the data of the *ex vivo* enzyme activity assay using saturating concentrations of either G6P or FDG6P clearly demonstrated a different regulatory effect of DHEA on substrate usage of G6PD/H6PD (Figure 5C). The observed higher specific activity of G6PD/H6PD for FDG6P upon DHEA treatment could serve as potential explanation of the unexpected increase in oxPPP radiometabolite levels although oxPPP activity is repressed. Together these data indicate that the activity of G6PD/H6PD might have effects on both 2-[<sup>18</sup>F]FDG accumulation and metabolism, however, their selective contribution remained to be resolved. For all used inhibitors it is unclear whether only G6PD, H6PD, or both enzymes are targeted. At least the most popular G6PD inhibitor DHEA seems to also inhibit 11 $\beta$ -hydroxysteroid dehydrogenase <sup>38</sup>, the same mechanism by which CBX indirectly blocks H6PD activity <sup>39</sup>. To further understand these effects, we separately knocked down both of the first oxPPP enzymes, G6PD and H6PD (Figure 6). Interestingly, only H6PD knockdown enhanced downstream metabolism and, more relevant as a readout, 2-[<sup>18</sup>F]FDG accumulation. As G6PD knockdown did not affect tracer accumulation, we hypothesize that enhanced accumulation observed with DHEA/G6PDi-1 was mainly mediated *via* H6PD. Unfortunately, *in vivo* knockdown experiments to further analyze G6PD and H6PD effects were unsuccessful, which might have been due to the density of HT29 tumors or the unmet need for prolonged repetitive injection of siRNA as reported in other studies <sup>40,41</sup> (see Supplemental Information). While the observed importance of H6PD for tracer accumulation is in line with previous studies <sup>12–15</sup>, the effect on accumulation is the opposite to what was reported by Marini *et al.* <sup>12</sup>. Instead of glucose-free cell culture medium, which could *per se* induce metabolic adaptation, we used medium with physiological glucose concentration. This might explain the divergent results regarding CBX effects, as mentioned above, and H6PD knockdown. Furthermore, although it is believed that 2-[<sup>18</sup>F]FDG metabolism *via* the oxPPP is confined to H6PD, we observed significantly reduced levels of 2-[<sup>18</sup>F]FD-PGL upon specific G6PD knockdown (Figure 6C). This agrees with previous *in vitro* studies where 2-[<sup>18</sup>F]FD-PGL was successfully synthesized using isolated G6PD <sup>2,25</sup>.

To put our results in a clinical context, we used the Stanford 2021 PRECOG database to examine G6PD/H6PD gene expression in tumors in relation to patient survival <sup>42</sup>. We observed that overall, higher H6PD expression is associated with longer survival, while higher G6PD expression is linked to shorter survival across different types of cancer (Table S1). Considering that high 2-[<sup>18</sup>F]FDG accumulation is generally associated with poor prognosis <sup>43–46</sup>, this provides an interesting bridge to our finding that higher oxPPP activity inversely correlates with tracer accumulation, likely influenced by H6PD.

In summary, we for the first time present a more extensive evaluation of 2-[<sup>18</sup>F]FDG metabolism over time in different cells and tissues, revealing a distinct, tissue-dependent metabolic pattern. Concerning 2-[<sup>18</sup>F]FDG metabolism *via* the oxPPP, our data propose that while both G6PD and H6PD might play a role in 2-[<sup>18</sup>F]FDG metabolism, only H6PD has an influence on 2-[<sup>18</sup>F]FDG accumulation. Most importantly, our data suggest that the metabolic flux of 2-[<sup>18</sup>F]FDG into the oxPPP or glycogen metabolism or an altered activity of these specific pathways could impact tracer accumulation and hence imaging. These data might provide the basis for a better understanding of less 2-[<sup>18</sup>F]FDG avid tumors beyond reduced glucose transporter or hexokinase expression. As already suggested by Kaarstad *et al.* 20 years ago, the existence of radiometabolites different to 2-[<sup>18</sup>F]FDG-6-P “*may have implications for the interpretation of estimated kinetic rate constants in terms of the enzymatic processes*” <sup>7</sup>. We propose that extended kinetic models might be needed in the future for correct image interpretation and quantification. Finally, the potential quantification of radiometabolites derived from specific glucose metabolic pathways opens the possibility to better characterize basic tissue metabolism, tumor heterogeneity, or treatment effects in a preclinical setting, complementing other approaches such as metabolomics or genomics.

## Limitations

As limitation of our experimental approach using immunodeficient mice, we cannot exclude that the subcutaneous tumors also affected the metabolism of tested healthy mouse organs. In another study, tumor growth induced a metabolic shift in non-involved organs on day 6 and day 39 post inoculation in athymic nude mice <sup>47</sup>. Although the effects were much more pronounced at the later time-point and a different mouse strain and application site was used, the tumor size in our study was closer to that on day 39 in the other study. In addition to that, we continuously anaesthetized mice until sacrificing to normalize conditions and make scan data comparable to other *ex vivo* data. As mentioned above, isoflurane anesthesia causes hyperglycemia in mice especially at levels higher than 1.5 % v/v <sup>29</sup>, potentially altering the metabolic pattern. In that context, a shift in the FDG/FDM ratio due to

phenobarbital anesthesia was previously reported in a  $^{19}\text{F}$  NMR study <sup>31</sup>. Therefore, comparability with other experimental approaches using conscious animals may be limited.

## Materials and Methods

### Radiotracer

2- $^{18}\text{F}$ ]FDG was synthesized in-house at the Department of Biomedical Imaging and Image-guided Therapy, University Hospital Vienna, and formulated for patient use.

### Animal experiments

For *in vivo* experiments 9- to 14-week-old (time of sacrificing) female Fox Chase SCID Beige mice (Charles River) were used. The animal experiments were approved by the Federal Ministry of Education, Science and Research and conducted in accordance with the Austrian laws for animal protection.

Until the day of the experiment, the animals were fed ad libitum. Approximately two weeks after arrival, the animals were subcutaneously injected with  $2 \times 10^6$  tumor cells in phosphate buffered saline (PBS) and 20 % matrigel above the right flank. The desired tumor size of 50-150 mm<sup>3</sup> (length x (width)<sup>2</sup> x 0.5) was reached after around 10 days for HT29 and after around 7 days for HT1080. On the day of the experiment, animals were fasted for 4 h. Then, the mice were injected with 12-20 MBq of 2- $^{18}\text{F}$ ]FDG *via* the tail-vein, warmed and kept under 1-2 % isoflurane anesthesia until sacrificing. After 30, 60, or 120 min, the mice were sacrificed by cervical dislocation before the respective organs or tumors were harvested and washed. The tissues were snap-frozen over liquid nitrogen to quench metabolism and stored on dry ice before analysis. Blood was withdrawn directly from the murine heart, cooled, and centrifuged (3000 g, 15 min, 4°C) to gain plasma. In summary, blood, brain, heart, kidney, liver, lung, HT1080 and HT29 tumors were collected ( $n \geq 4$  each) for gamma counter measurements (2480 Automatic Gamma counter, Wizard23, PerkinElmer) and radiometabolite analysis. In addition, five mice underwent a 120 min dynamic 2- $^{18}\text{F}$ ]FDG scan with an Inveon®  $\mu\text{PET/CT}$  device (Siemens), applying the same dose as stated before. For image generation and quantification PMOD Software (Fuse it tool, Version 3.8, RRID:SCR\_016547) was used.

Additional information concerning the animals, imaging and image quantification can be found in the Supplemental Information.

### Cell culture

The human cancer cell lines HT29 (colorectal adenocarcinoma) and HT1080 (fibrosarcoma) were cultured in Roswell Park Memorial Institute medium and Modified Eagle Medium (MEM), respectively, substituted with 2 mM glutamine and 10 % fetal bovine serum (all Gibco™, ThermoFisher) under standardized conditions (humidified atmosphere, 37°C, 5 % CO<sub>2</sub>). The cells were generous gifts of other departments as stated elsewhere <sup>2</sup>, but were originally obtained from ATCC (RRID:CVCL\_0320 and RRID:CVCL\_0317).

#### Knockdown of oxPPP enzymes

HT29 cells were transfected using 12.5 nM of G6PD, H6PD, or non-targeting SMARTpool ON-TARGETplus siRNA, DharmaFECT transfection reagent No. 1 (all Horizon, PerkinElmer) and Opti-MEM (Gibco™, ThermoFisher), following the provider's protocol. The respective sequences are listed in the Supplemental Information. The optimal knockdown was found to be reached 72 h after transfection (Figure S2). Thus, all experiments were started 72 h after siRNA application. Successful and selective knockdown was monitored with WB analyses (Figure 6A, Figure S3).

Additionally, intratumoral *in vivo* silencing of G6PD/H6PD in Fox Chase SCID Beige mice was tested. However, we could not reach a significant downregulation of oxPPP enzymes by this approach (Methods in Supplemental Information, Figure S4).

#### 2-[<sup>18</sup>F]FDG accumulation experiments

HT1080 and HT29 cells were incubated with 1 MBq/mL 2-[<sup>18</sup>F]FDG for 1 h in MEM (1 g/L glucose), and accumulation was determined as previously described <sup>2</sup>.

For the knockdown experiments, HT29 cells were also incubated with 2-[<sup>18</sup>F]FDG as stated above, 72 h after siRNA addition ( $n = 5$ ). To assess the effects of oxPPP enzyme inhibitors, 100 μM DHEA (Sigma-Aldrich), 84 (HT1080) or 130 μM (HT29) CBX (Sigma-Aldrich), or 50 μM G6PDi-1 (a generous gift of the Rabinowitz-Lab <sup>37</sup>) were added either 1 h before (all 3 inhibitors) or simultaneously with 2-[<sup>18</sup>F]FDG (DHEA, G6PDi-1) ( $n \geq 3$ ). CBX concentration was based on previous analyses of IC<sub>50</sub> concentrations, DHEA concentration was chosen according to literature <sup>37,48,49</sup> and G6PDi-1 concentration was based on recommendations by the research group providing the inhibitor.

#### Radiometabolite detection with HPLC

To study 2-[<sup>18</sup>F]FDG metabolism and its intermediates over time, radiometabolites were determined at the time-points 30, 60 and 120 min for murine organs, tumors and both cell lines ( $n \geq 4$  for all), as

described previously<sup>2</sup>. Before analysis, fresh frozen tissue was homogenized with an ULTRA-TURRAX® (Ika) in 3:1 methanol and PBS (approximately 2 mL/ 100 mg tissue) on ice and shortly sonicated. The cultivated cells were scraped off and homogenized correspondingly. Cell or tissue lysates were then centrifuged for 4 min (4 °C, 13,684 g).

To additionally analyze the influence of the oxPPP inhibitors and enzyme knockdown on the metabolism of 2-[<sup>18</sup>F]FDG, cells were treated with the respective inhibitor (HT1080, HT29) or siRNA (HT29) as stated above and metabolism was analyzed 1 h after 2-[<sup>18</sup>F]FDG addition, analogous to accumulation experiments ( $n \geq 3$ ).

### Western blot analysis

For WB analysis, RIPA-buffer (ThermoFisher) was used to lyse cells in the presence of 1x protease inhibitor (Sigma-Aldrich). Cells were scraped off, shaken on ice for 30 min and subsequently centrifuged for 20 min at 4°C and 13,684 g. The protein concentration of each lysate was determined using a bicinchoninic acid kit (ThermoFisher). Samples were loaded into 4–20 % Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) for gel electrophoresis. Then, semi-dry blotting was performed using nitrocellulose blotting membranes (Amersham) and a Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were subsequently blocked with 5 % dry milk powder in Tris-buffered saline with 0.1 % tween-20 for 1.5 h at room temperature (RT). Thereafter, membranes were cut into two halves to separately incubate them with a primary antibody overnight at 4°C against the respective enzyme (1:1000 anti-G6PD Abcam Cat# ab210702, RRID:AB\_2923527, or 1:1000 anti-H6PD, Abcam Cat# ab170895, RRID:AB\_2864314), or 1:2500 anti-β-actin (Abcam Cat# ab8227, RRID:AB\_2305186). The next day, membranes were washed thoroughly three times and the secondary antibody was applied 1:2500 for 1 h at RT (Thermo Fisher Scientific Cat# A16104, RRID:AB\_2534776). After washing the membranes again, protein bands were detected using the Clarity™ Western ECL Substrate and a ChemiDoc™ detection system (both Bio-Rad, RRID:SCR\_021693). Protein bands of the target enzymes were normalized to β-actin using Fiji software (RRID:SCR\_002285).

### Enzyme activity assays

For photometric analyses, HT29 cells were scraped off in a mixture of 50 mM Tris buffer (pH 8), 1 mM EDTA, 0.05 % Triton X-100 and 1x EDTA-free cOmplete™/PhosSTOP™ (all Sigma-Aldrich), and subsequently treated as described for WB analysis. Samples were kept at -80°C until the measurement. Enzyme activity assays were carried out with a Hitachi U-2900 spectrophotometer based on a previously described protocol<sup>50</sup>. Briefly, G6PD/H6PD activity was determined for 5 µg of crude protein in a 50 mM Tris buffer (pH 7.8) with 6.5 mM MgCl<sub>2</sub> and 0.6 mM NADP<sup>+</sup> (all Sigma-Aldrich). Immediately

after the addition of 30 mM of the substrates G6P (Sigma-Aldrich) or FDG6P (Biosynth Carbosynth®), NADPH formation was measured at 340 nm for 5 min at RT with or without addition of 100  $\mu$ M DHEA.

### Statistics

Measurements were performed in duplicates or triplicates and repeated at least 3 times. A Shapiro-Wilk test was performed to check for normality. Student's t-test and one-way ANOVA were performed in GraphPad Prism (Version 7.03, RRID:SCR\_002798), the calculation of normality distribution and correlation using Spearman correlation was carried out with IBM SPSS statistics (Version 27, RRID:SCR\_019096). A *P* of  $\leq 0.05$  was deemed significant. All values are given as mean  $\pm$  SD.

### **Author contributions**

Conceptualization, E.-M.P., T.B. and M.M.; Methodology, E.-M.P., T.B., A.Miller, B.K.G. and C.V.; Validation, E.-M.P., T.B. and A.Miller; Formal Analysis, E.-M.P., B.K.G., M.K. and N.R.-U.; Investigation, E.-M.P., T.B., A.Michligk; Resources, A.H., M.H., M.M.; Data curation, E.-M.P., B.K.G. and M.K.; Writing-Original draft, E.-M.P.; Writing-Review & Editing, T.B., A.Miller, B.K.G., C.V., M.K., N.R.-U., A.H., M.M.; Visualization, E.-M.P., B.K.G., M.K. and N.R.-U.; Supervision, M.M., H.V. and A.H., Project Administration, M.H., H.V.; Funding Acquisition, M.M. and M.H.

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### **Declaration of interests**

The authors declare no competing interests.

### **Figure titles and legends**



Figure 1: Accumulation of 2-[<sup>18</sup>F]FDG and overall metabolism beyond 2-[<sup>18</sup>F]FDG-6-P are organ-dependent. (A) Total radioactivity (% ID/g) detected in murine organs with *ex vivo* gamma counting 30, 60 and 120 min post injection and (B) corresponding overall metabolism beyond 2-[<sup>18</sup>F]FDG-6-P (\*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ).

Figure 2: Analysis of individual radiometabolites reveals distinct tissue-dependent patterns of 2-[<sup>18</sup>F]FDG metabolism. (A) Proposed pathways of radiometabolite formation including the respective enzymes based on our data (created with BioRender.com) and (B) levels of individual radiometabolites over time in examined tissues.

Figure 3: HT1080 and HT29 xenograft tumors differ in 2-[<sup>18</sup>F]FDG accumulation and overall metabolism and show a shifted radiometabolite pattern compared to *in vitro* cell culture. (A) Total radioactivity (% ID/g) detected in HT1080 and HT29 tumors over time with *ex vivo* gamma counting and (B) corresponding representative  $\mu$ PET/CT images (taken from one frame). (C) Comparison between 2-[<sup>18</sup>F]FDG metabolism *in vitro* and *in vivo* (stars refer to differences between 2D culture and tumors), the box showing most relevant radiometabolites besides 2-[<sup>18</sup>F]FDG-6-P (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ).

Figure 4: Radiometabolites of glycogen metabolism and the oxPPP correlate with total radioactivity in tissues and with each other. (A) Spearman correlation between the levels of 2-[<sup>18</sup>F]FDG-1-P, 2-[<sup>18</sup>F]FD-PGL, 2-[<sup>18</sup>F]FDG/2-[<sup>18</sup>F]FDM (unphosphorylated pool) and % ID/g tissue. (B) Heat map of correlations between radiometabolites (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ; the correct nomenclature has been omitted in 4B).

Figure 5: The oxPPP inhibitors DHEA and G6PD $\alpha$ -1, but not CBX enhance 2-[<sup>18</sup>F]FDG accumulation and metabolism. (A,B) Effects of the inhibitors CBX or DHEA on tracer accumulation and metabolism 1 h post 2-[<sup>18</sup>F]FDG addition. (C) Relative G6PD/H6PD activity with or without DHEA using G6P or FDG6P as substrate. (D,E) Comparison of DHEA and G6PD $\alpha$ -1 effects on tracer accumulation and metabolism, applying the inhibitors 1 h before (pre), or simultaneous with 2-[<sup>18</sup>F]FDG (sim). Boxes show relevant radiometabolite changes. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$

Figure 6: Knockdown of H6PD but not G6PD enhances 2-[<sup>18</sup>F]FDG accumulation and metabolism. (A) WB of HT29 cells treated with siRNA for the respective enzyme or non-targeting siRNA (NTsi) (samples from different experiments). Effects of G6PD/H6PD knockdown on 2-[<sup>18</sup>F]FDG accumulation (B) and metabolism (C), the box showing relevant radiometabolite changes (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ).

## Supplementary Information: figure and table titles and legends

Figure S1: (A) Representative chromatogram of 2-[<sup>18</sup>F]FDG radiometabolites following organ lysis (lung) and (B) corresponding structures of previously identified radiometabolites.

Table S1: Relationship between G6PD/H6PD gene expression and survival across different cancer types.

Figure S2: WB of preliminary tests with SMARTpool ON-TARGETplus siRNAs to find the optimal concentration for G6PD/H6PD knockdown in HT29 cells.

Figure S3: Representative WB that excludes compensatory overexpression of the respective other enzyme after knockdown with siRNA (samples from different experiments).

Figure S4: Attempts for *in vivo* knockdown of G6PD/H6PD were not successful. (A) WB analysis determining the most effective siRNAs within the tested pool. (B) Post-mortem IVIS® images showing successful siRNA injection and good distribution in HT29 tumor, and (C) summary of WB analyses of G6PD/H6PD knockdown (NTsi = non-targeting siRNA, AB 1 = proteintech antibody, AB 2 = abcam antibody).

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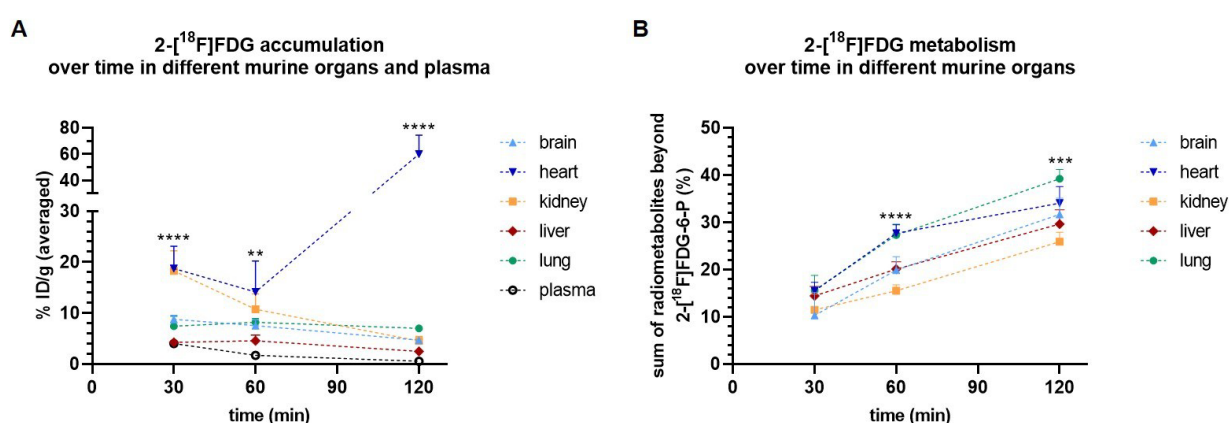


Figure 1: Accumulation of 2-<sup>18</sup>F]FDG and overall metabolism beyond 2-<sup>18</sup>F]FDG-6-P are organ-dependent. (A) Total radioactivity (% ID/g) detected in murine organs with *ex vivo* gamma counting 30, 60 and 120 min post injection and (B) corresponding overall metabolism beyond 2-<sup>18</sup>F]FDG-6-P (\*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ).



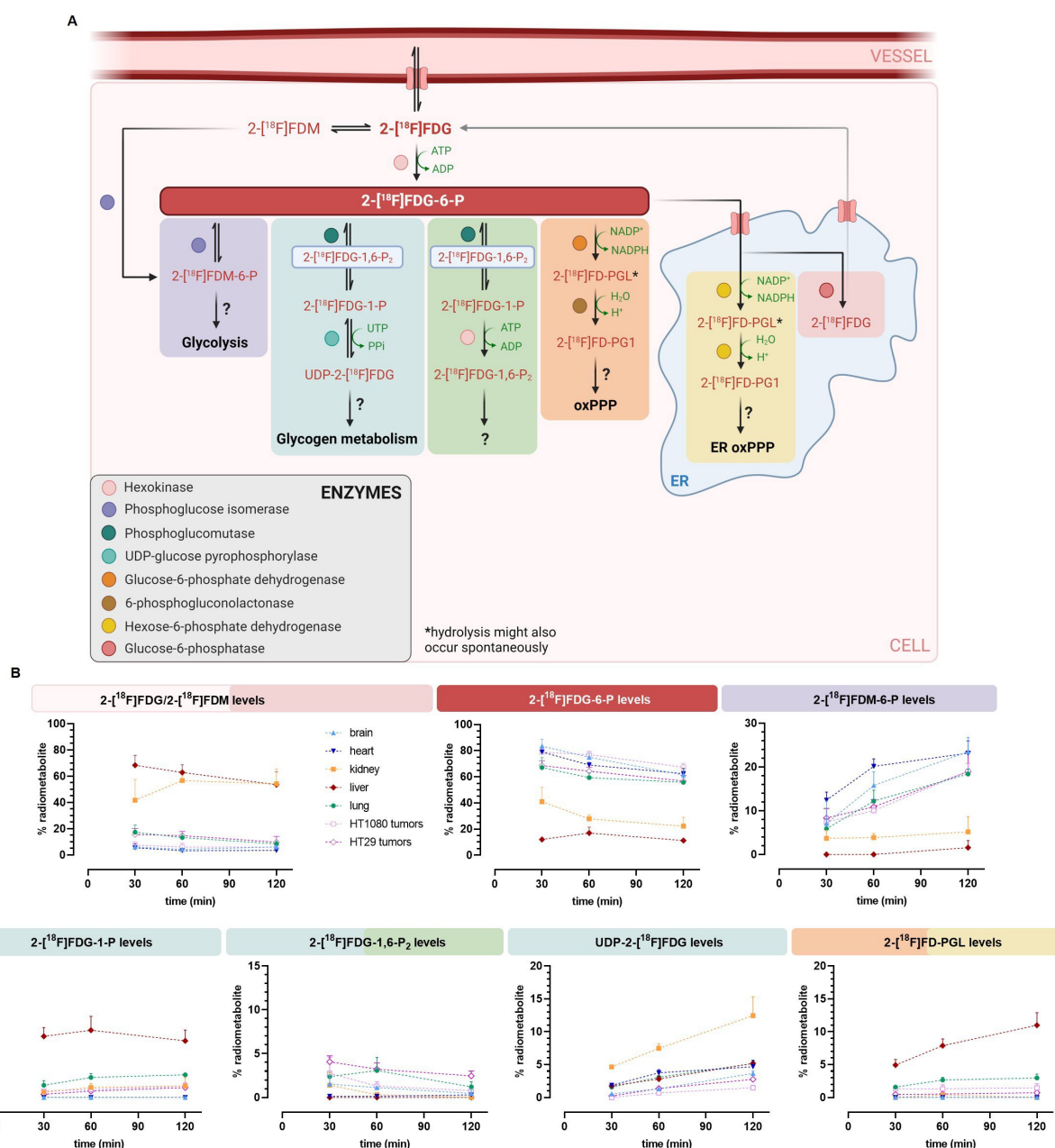


Figure 2: Analysis of individual radiometabolites reveals distinct tissue-dependent patterns of 2-[ $^{18}\text{F}$ ]FDG metabolism. (A) Proposed pathways of radiometabolite formation including the respective enzymes based on our data (created with BioRender.com) and (B) levels of individual radiometabolites over time in examined tissues.

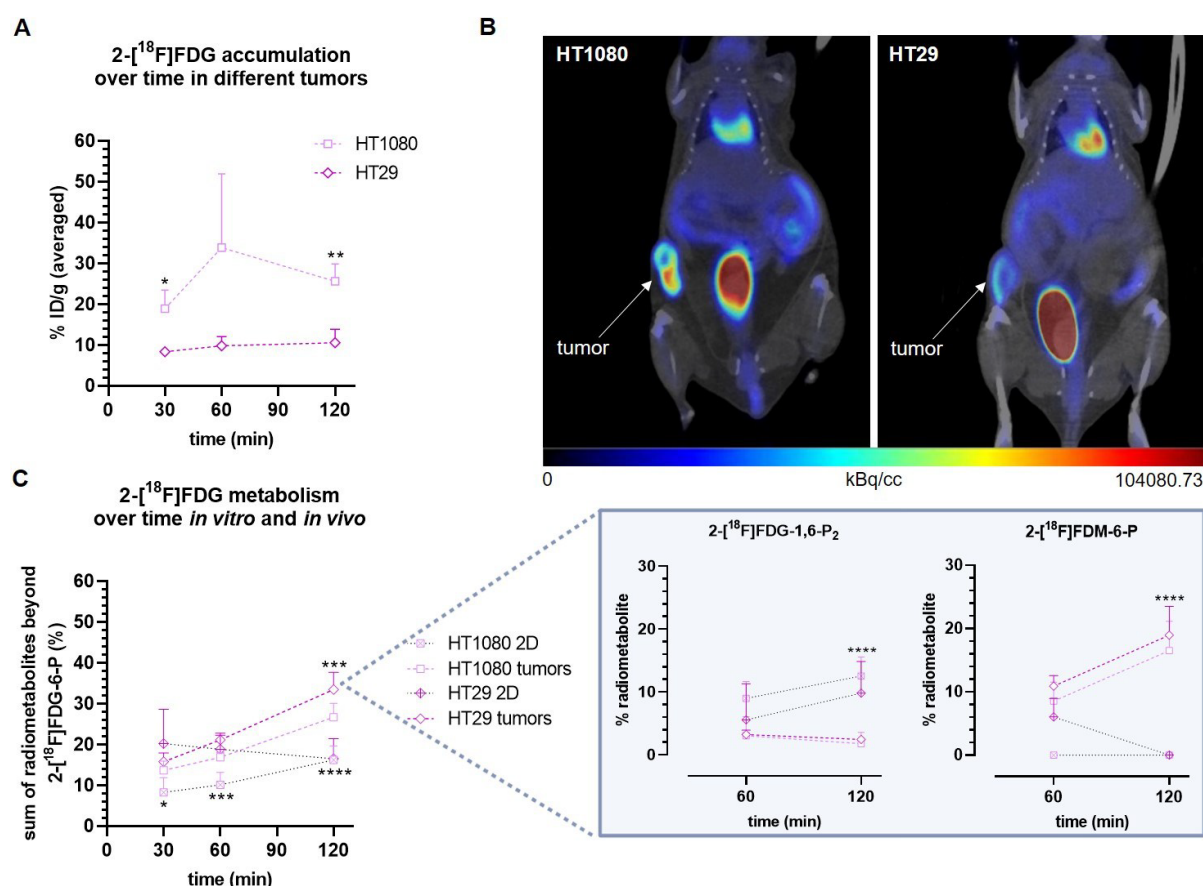


Figure 3: HT1080 and HT29 xenograft tumors differ in 2- $^{18}\text{F}$ FDG accumulation and overall metabolism and show a shifted radiometabolite pattern compared to *in vitro* cell culture. (A) Total radioactivity (% ID/g) detected in HT1080 and HT29 tumors over time with *ex vivo* gamma counting and (B) corresponding representative  $\mu\text{PET}/\text{CT}$  images (taken from one frame). (C) Comparison between 2- $^{18}\text{F}$ FDG metabolism *in vitro* and *in vivo* (stars refer to differences between 2D culture and tumors), the box showing most relevant radiometabolites besides 2- $^{18}\text{F}$ FDG-6-P (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ).



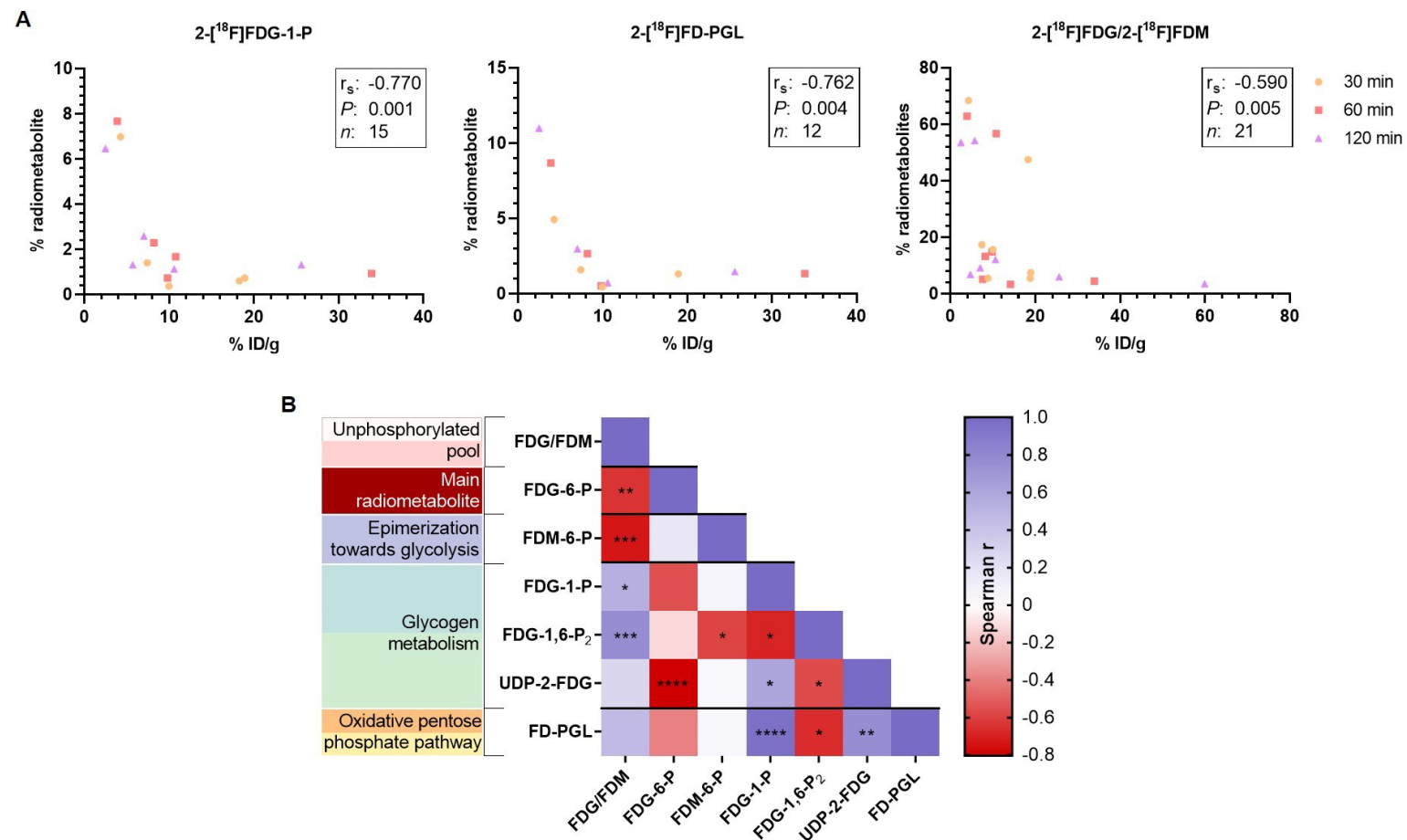


Figure 4: Radiometabolites of glycogen metabolism and the oxPPP correlate with total radioactivity in tissues and with each other. (A) Spearman correlation between the levels of 2-[<sup>18</sup>F]FDG-1-P, 2-[<sup>18</sup>F]FD-PGL, 2-[<sup>18</sup>F]FDG/2-[<sup>18</sup>F]FDM (unphosphorylated pool) and % ID/g tissue. (B) Heat map of correlations between radiometabolites (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ; the correct nomenclature has been omitted in 4B).

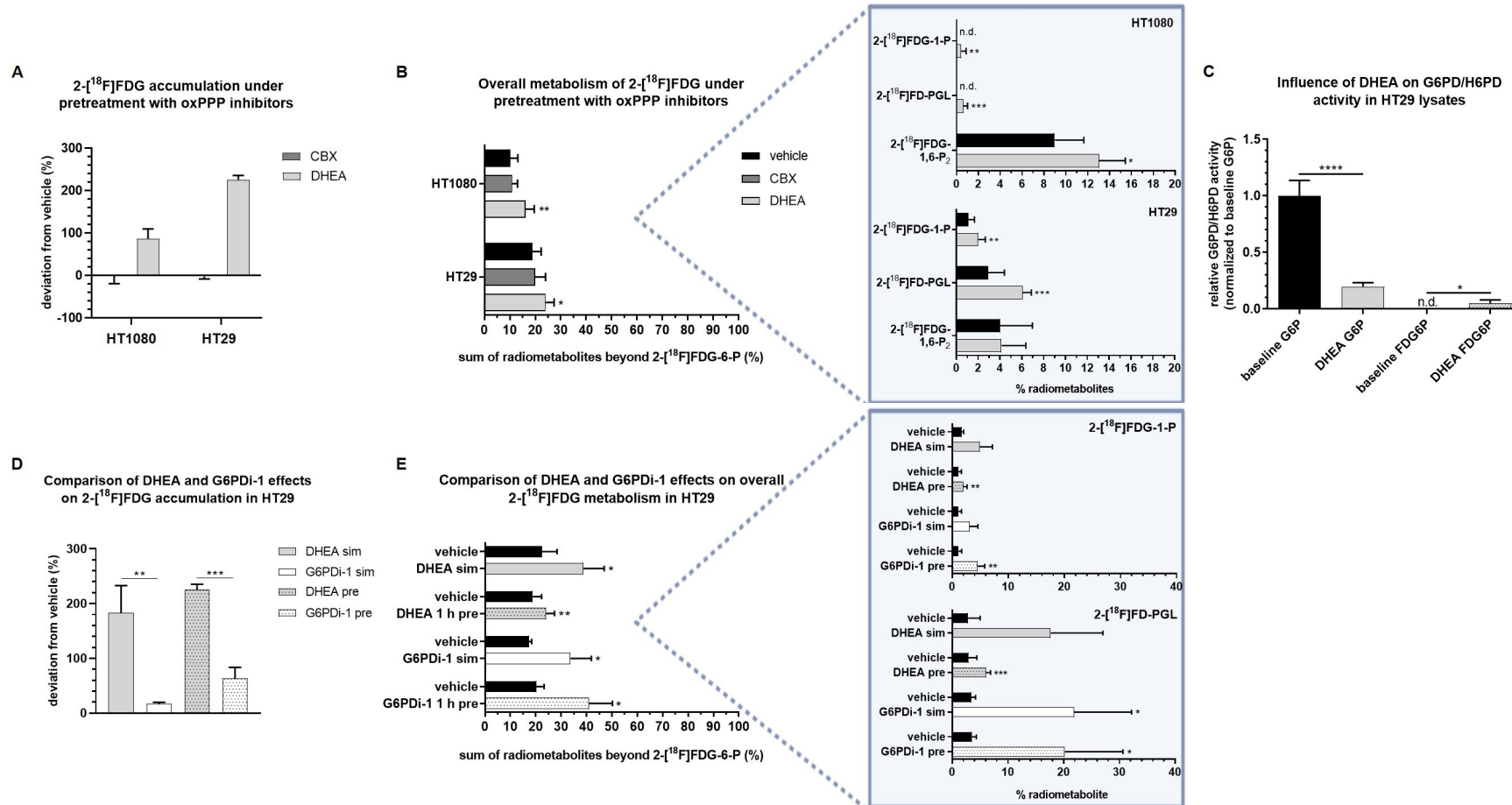


Figure 5: The oxPPP inhibitors DHEA and G6PDi-1, but not CBX enhance 2-[<sup>18</sup>F]FDG accumulation and metabolism. (A,B) Effects of the inhibitors CBX or DHEA on tracer accumulation and metabolism 1 h post 2-[<sup>18</sup>F]FDG addition. (C) Relative G6PD/H6PD activity with or without DHEA using G6P or FDG6P as substrate. (D,E) Comparison of DHEA and G6PDi-1 effects on tracer accumulation and metabolism, applying the inhibitors 1 h before (pre), or simultaneous with 2-[<sup>18</sup>F]FDG (sim). Boxes show relevant radiometabolite changes. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$

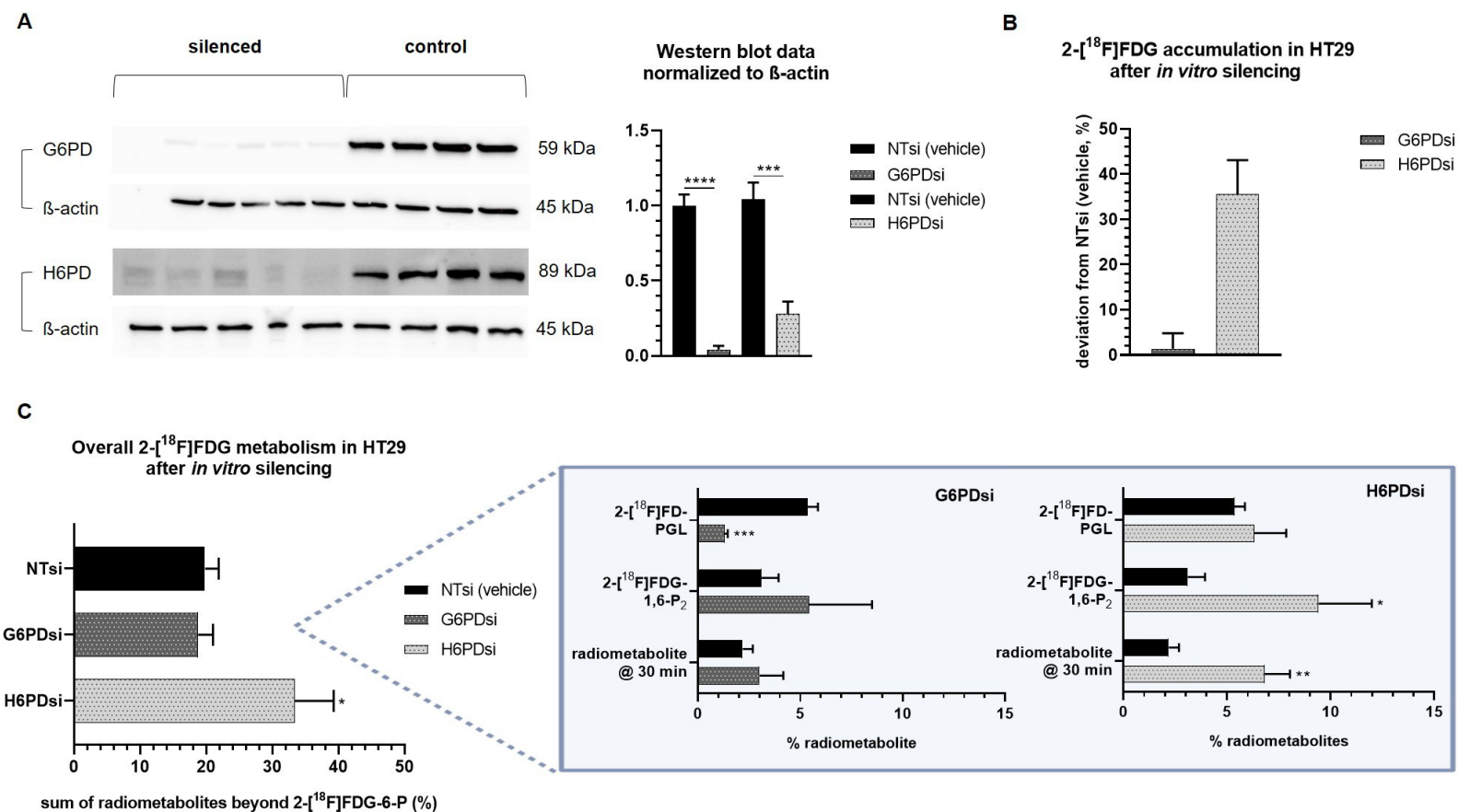


Figure 6: Knockdown of H6PD but not G6PD enhances 2- $^{18}\text{F}$ FDG accumulation and metabolism. (A) WB of HT29 cells treated with siRNA for the respective enzyme or non-targeting siRNA (NTsi) (samples from different experiments). Effects of G6PD/H6PD knockdown on 2- $^{18}\text{F}$ FDG accumulation (B) and metabolism (C), the box showing relevant radiometabolite changes (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ).

## Supplemental Information

### Detailed information concerning animal experiments

**Animal housing:** Mice were housed in Tecniplast Green Line cages with Smart Flow (up to 8 animals per cage), using LASbedding™ PG2 (LASvendi) and Sizzle-Pet, as well as egg boxes, bio-huts, nesting sheets and wooden chew as enrichment (all autoclaved). Mice were fed with LASQCdiet™ Rod16-A (LASvendi). The night-day cycle was 12/12 hours. On the day of the experiment, mice weighed between 16.5 and 23.3 g.

**Dynamic imaging:** Animals were placed in the scanner feet first in prone position for 120 min (29 frames, frame duration 5-600 sec), restrained on a heating pad (38°C) with PEHA adhesion, and covered with a custom-made blanket. Heatpad temperature and respiratory rate were monitored with a BioVET CT1 system (Siemens). Concerning anesthesia, a range of 2 L/min 2 % isoflurane in oxygen (induction) and 0.5 L/min 1 % isoflurane in oxygen (maintenance during the scan) was used.

**Image reconstruction and quantification:** Concerning the volumes of interest (VOIs), the whole tissue was delineated in the case of the brain and the lung. In the case of the kidney, the pelvis was excluded. For the liver, three small representative VOIs were placed across the organ and averaged, thereby avoiding falsification by large blood vessels and heterogeneity. As for the heart, the muscle was delineated, thereby excluding the large blood pool.

For image quantification, no smoothing was applied. PET images were co-registered (trilinear interpolation) to the CT image (reference, with reduction 2/2/2 x/y/z, final voxel size = 0.195 mm x/y/z, image size = 512 x/y/z) using automated rigid matching or manual reslicing in case of unsuccessful automated matching. CT windowing: 90-500 HU. PET images were decay-corrected, attenuation-corrected (CT-based), scatter-corrected, dead-time-corrected and the detector normalized.

For PET, the reconstruction algorithm OSEM3D/MAP was used (MAP subsets: 16, iterations: 18, beta-value: 0.0527972, ordinary Poisson type OSEM3D/MAP, voxel size = x:0.388mm, y:0.388mm, z: 0.796mm; image size = x:256, y:256, z:159). For CT, Feldkamp cone beam reconstruction was used (voxel size = 0.0975mm x/y/z, Image size = 1024 x/y/z).

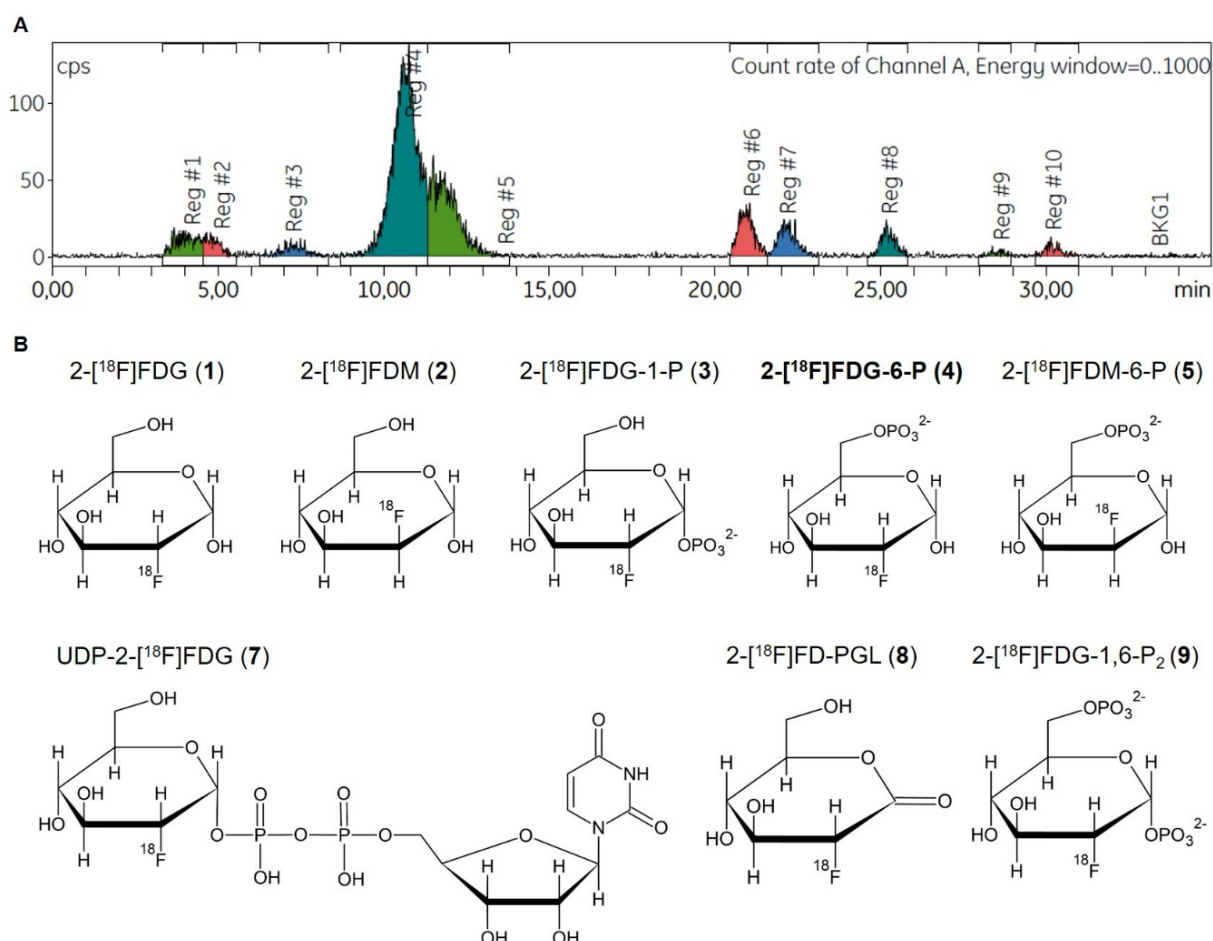


Figure S1: (A) Representative chromatogram of 2-[<sup>18</sup>F]FDG radiometabolites following organ lysis (lung) and (B) corresponding structures of previously identified radiometabolites. The 2-[<sup>18</sup>F]FDG-PGL peak could also include the hydrolytic product 2-[<sup>18</sup>F]FDG-PG1.

| Gene                                       | H6PD                                                  | G6PD               |
|--------------------------------------------|-------------------------------------------------------|--------------------|
| <b>Unweighted_meta-Z_of_all_cancers</b>    | <b>-3.028058243</b>                                   | <b>5.104754536</b> |
| Adrenocortical_cancer                      | -0.224                                                | -1.307             |
| Bladder_cancer                             | -1.095                                                | 1.584              |
| Brain_cancer_Astrocytoma                   | 2.315                                                 | 1.174              |
| Brain_cancer_Glioblastoma                  | -0.836                                                | 0.638              |
| Brain_cancer_Glioma                        | 1.687                                                 | 2.104              |
| Brain_cancer_Medulloblastoma               | 0                                                     | 0                  |
| Brain_cancer_Meningioma                    | -0.92                                                 | 0.81               |
| Brain_cancer_Neuoblastoma                  | -4.873                                                | 7.427              |
| Breast_cancer                              | -5.307                                                | 4.185              |
| Colon_cancer                               | -0.265                                                | -0.594             |
| Gastric_cancer                             | -0.638                                                | 1.069              |
| Germ_cell_tumors                           | 0.531                                                 | -1.269             |
| Head_and_neck_cancer                       | 0.27                                                  | 0.841              |
| Head_and_neck_cancer_Hypopharyngeal_cancer | -0.123                                                | 0                  |
| Head_and_neck_cancer_Oesophageal_cancer    | -0.548                                                | 0                  |
| Head_and_neck_cancer_Oral_SCC              | 0.848                                                 | 0.205              |
| Hematopoietic_cancer_AML                   | -1.654                                                | 1.814              |
| Hematopoietic_cancer_B_ALL                 | -0.273                                                | 0.442              |
| Hematopoietic_cancer_Burkitt_lymphoma      | 0.668                                                 | -0.29              |
| Hematopoietic_cancer_CLL                   | 0.072                                                 | 1.544              |
| Hematopoietic_cancer_DLBCl                 | 0.477                                                 | 0.603              |
| Hematopoietic_cancer_FL                    | -2.66                                                 | 1.595              |
| Hematopoietic_cancer_Mantle_cell_lymphoma  | 0.771                                                 | 2.301              |
| Hematopoietic_cancer_Multiple_myeloma      | -0.103                                                | 2.832              |
| Kidney_cancer                              | -0.209                                                | 0                  |
| Liver_cancer                               | -1.438                                                | -0.824             |
| Liver_cancer_Primary                       | -0.049                                                | -0.255             |
| Lung_cancer_ADENO                          | -0.861                                                | 2.156              |
| Lung_cancer_LCC                            | -0.319                                                | 0.343              |
| Lung_cancer_SCC                            | -1.922                                                | 1.712              |
| Lung_cancer_SCLC                           | 0.258                                                 | 0                  |
| Melanoma                                   | 1.114                                                 | 0.319              |
| Melanoma_Metastasis                        | 1.127                                                 | 1.193              |
| Mesothelioma                               | -0.479                                                | 0                  |
| Ovarian_cancer                             | -0.992                                                | -1.996             |
| Pancreatic_cancer                          | -1.207                                                | 0.693              |
| Prostate_cancer                            | -1.005                                                | -0.014             |
| Sarcoma_Ewing_sarcoma                      | -0.375                                                | 0.446              |
| Sarcoma_Osteosarcoma                       | -0.674                                                | 0.397              |
|                                            | higher expression is associated with longer survival  |                    |
|                                            | higher expression is associated with shorter survival |                    |

Source: PRECOG database Stanford 2021

Table S1: Relationship between G6PD/H6PD gene expression and survival across different cancer types.

Sequences of ON-TARGETplus siRNA pools

**H6PD:**

No. J-004692-09: CGUCUGUUUAAAAGCGUUA

No. J-004692-10: CGUGGUGGGCUGAGGUUAA

No. J-004692-11: GCGGGUUGUCCUUGAGAAA

No. J-004692-12: UGGACGAGAGAGUGGGCUA

**G6PD:**

No. J-008181-18: ACAGAUACAAGAACGUGAA

No. J-008181-19: CCGUGUACACCAAGAUGAU

No. J-008181-20: CAGAUAGGCUGGAACCGCA

No. J-008181-21: AUUCACGAGUCCUGCAUGA

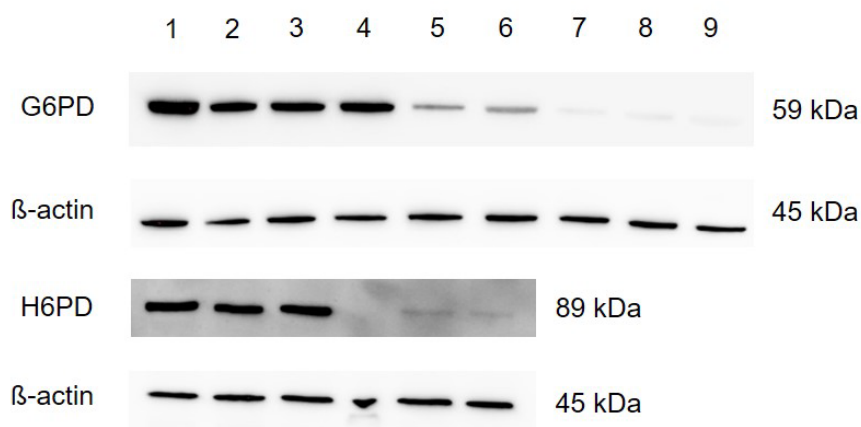
**Non-targeting:**

No. J-016083-05: CCGCAGGGCUCAUGAGUAU

No. J-016083-06: GGACAAACACCCAUGAACA

No. J-016083-07: AGGAACAAACGUUGACUUA

No. J-016083-08: CCAAUUCUCGUGAUGAAUC



**Amount of siRNA applied and time course of silencing:**

**G6PD**

1: control  
2: 12.5 nM, 24 h  
3: 25 nM, 24 h  
4: 50 nM, 24 h  
5: 12.5 nM, 48 h

6: 50 nM, 48 h  
7: 12.5 nM, 72 h  
8: 25 nM, 72 h  
9: 50 nM, 72 h

**H6PD**

1-3: control  
4-6: 12.5 nM, 72 h

Figure S2: WB of preliminary tests with SMARTpool ON-TARGETplus siRNAs to find the optimal concentration for G6PD/H6PD knockdown in HT29 cells.

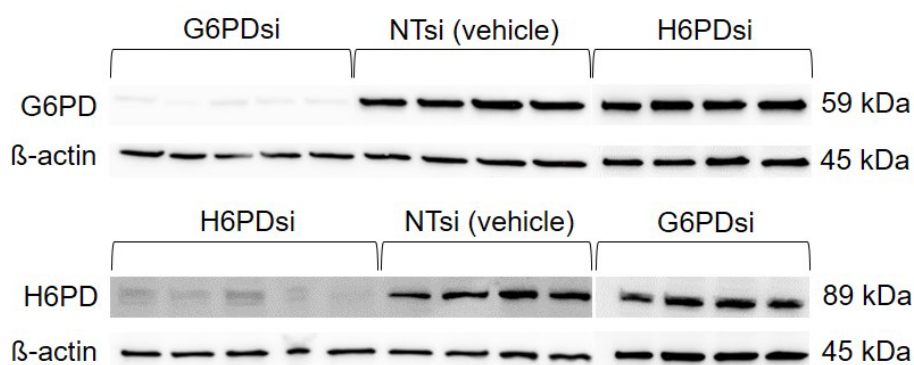


Figure S3: Representative WB that excludes compensatory overexpression of the respective other enzyme after knockdown with siRNA (samples from different experiments).

## Supplemental Methods

For *in vivo* silencing of G6PD or H6PD, the siRNA pools for *in vitro* use described in the main manuscript were tested beforehand with HT29 cells to define the most active siRNAs from the pools. HT29 xenografts were grown as stated before. For injection into mice, the respective *in vivo*-grade siRNAs (No. 19/20 for G6PD, No. 10/11 for H6PD, No. 6 non-targeting) were prepared and complexed with *in*



*vivo*-jetPEI® (Polyplus) according to the suppliers' instructions. The mice were briefly anesthetized with isoflurane and 50  $\mu$ L containing 10  $\mu$ g siRNA (N/P ratio 8) were injected directly into the tumor from different angles. For the first experiments, mice were sacrificed after 48 or 72 h (based on our *in vitro* experiments) and tumors, colon, and liver (as reference organs to exclude incorrect injection) were harvested as previously stated. As the siRNA mixture was spiked with 1  $\mu$ g fluorescently labeled non-targeting siRNA, pictures of the whole mouse, the sliced tumor and two reference organs were taken post-mortem with an optical imaging device (IVIS®, RRID:SCR\_020397) to ensure successful injection. To assess protein silencing, WB were performed with the tumor lysates as described above (see also main manuscript). In contrast to the cell experiments, analyses were performed with another H6PD antibody (1:1000 Proteintech Cat# 15255-1-AP, RRID:AB\_10642699) but later repeated with the abcam antibody mentioned in the main manuscript. In addition to the single injection, double injection within 3 days, as well as double siRNA amount and longer incubation time (96 h for G6PD only) were evaluated. The latter approach was based on a publication for G6PD knockdown in brown adipose tissue (BAT) [S1].

[S1] Sohn, J.H., Ji, Y., Cho, C.Y., Nahmgoong, H., Lim, S., Jeon, Y.G., Han, S.M., Han, J.S., Park, I., Rhee, H.W., et al (2021). Spatial Regulation of Reactive Oxygen Species via G6PD in Brown Adipocytes Supports Thermogenic Function. *Diabetes* 12, 2756-2770. 10.2337/db21-0272.

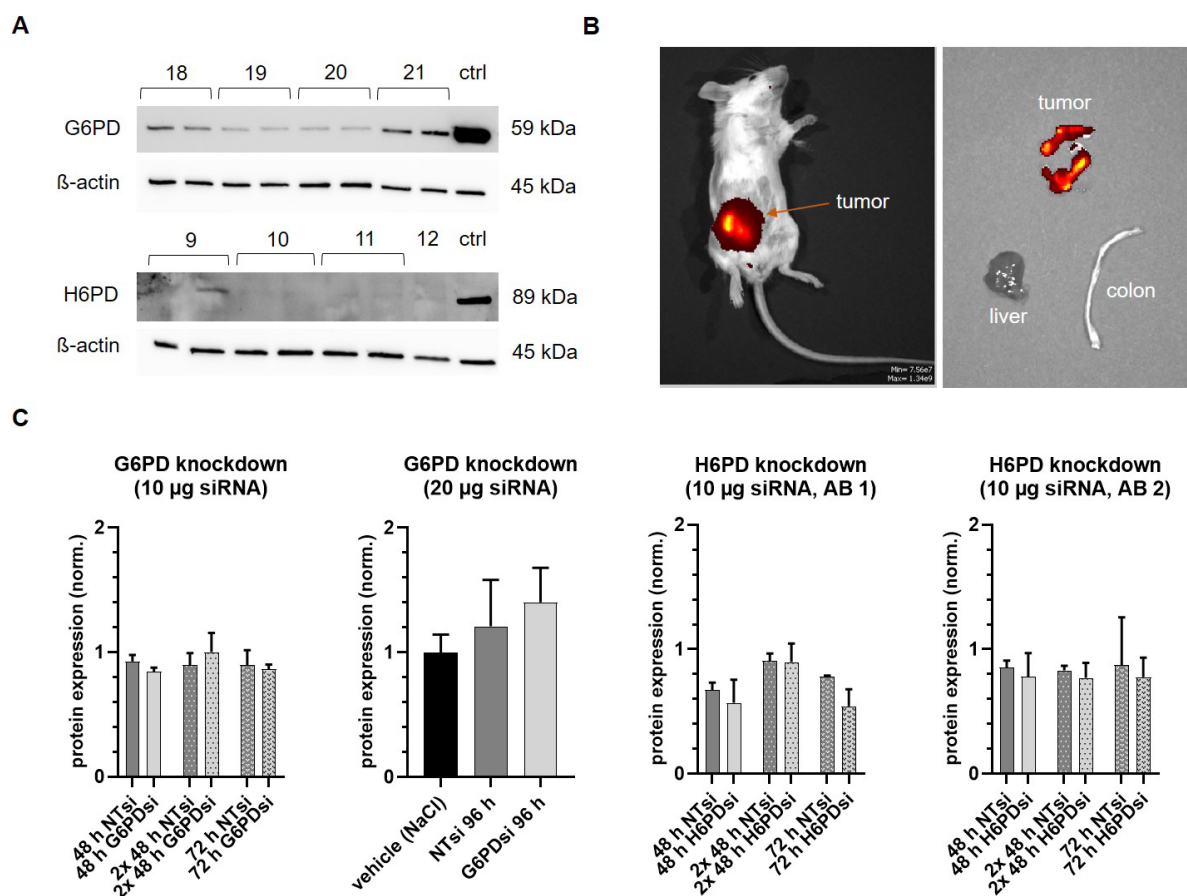


Figure S4: Attempts for *in vivo* knockdown of G6PD/H6PD were not successful. (A) WB analysis determining the most effective siRNAs within the tested pool. (B) Post-mortem IVIS® images showing successful siRNA injection and good distribution in HT29 tumor, and (C) summary of WB analyses of G6PD/H6PD knockdown (NTsi = non-targeting siRNA, AB 1 = proteintech antibody, AB 2 = abcam antibody).

### 3.1.5 Manuscript V

Philippe C<sup>a</sup>, **Klebermass EM<sup>a</sup>**, Balber T, Kulterer OC, Zeilinger M, Egger G, Dumanic M, Herz CT, Kiefer FW, Scheuba C, Scherer T, Fürnsinn C, Vranka C, Pallitsch K, Spreitzer H, Wadsak W, Viernstein H, Hacker M, Mitterhauser M. Discovery of melanin-concentrating hormone receptor 1 in brown adipose tissue. *Ann N Y Acad Sci.* 2021 Jun;1494(1):70-86.

<sup>a</sup>**These authors contributed equally to the study.**

## Original Article

## Discovery of melanin-concentrating hormone receptor 1 in brown adipose tissue

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Although extensive research on brown adipose tissue (BAT) has stimulated optimism in the battle against obesity and diabetes, BAT physiology and organ crosstalk are not fully understood. Besides BAT, melanin-concentrating hormone (MCH) and its receptor (MCHR1) play an important role in energy homeostasis. Because of the link between hypothalamic MCH neurons and sympathetic BAT activation via  $\beta$ -adrenoceptors, we investigated the expression and physiological role of the MCHR1 in BAT. MCHR1 was detected in rodent and human BAT with RT-qPCR and western blot analyses. *In vivo* imaging in rats used the glucose analog [<sup>18</sup>F]FDG and the MCHR1-tracer [<sup>11</sup>C]SNAP-7941. We found that the  $\beta$ 3-adrenoceptor (ADRB3) agonist CL316,243 increased [<sup>11</sup>C]SNAP-7941 uptake in BAT. Additionally, a pharmacological concentration of SNAP-7941—a low-affinity ADRB3 ligand—stimulated [<sup>18</sup>F]FDG uptake, reflecting BAT activation. In cultured human adipocytes, CL316,243 induced *MCHR1* expression, further supporting a direct interaction between MCHR1 and ADRB3. These findings characterized MCHR1 expression in rodent and human BAT for the first time, including *in vitro* and *in vivo* data demonstrating a link between MCHR1 and the  $\beta$ 3-adrenergic system. The presence of MCHR1 in BAT emphasizes the role of BAT in energy homeostasis and may help uncover treatment approaches for obesity.

**Keywords:** MCHR1; brown adipose tissue; PET; imaging; obesity

## Introduction

Since the rediscovery of active brown adipose tissue (BAT) in adult humans,<sup>1</sup> BAT has become a topic of high interest in physiological and medical research.

Through its ability to dissipate energy through heat generation, BAT has gained in significance as an antiobesity organ and may serve as a therapeutic target against obesity.<sup>2–5</sup> Besides cold exposure and food intake, other stimuli, including nutraceuticals and functional food, lead to an increase in norepinephrine that, among other things, activates

<sup>a</sup>These authors contributed equally to this study.

$\beta$ -adrenoceptors ( $\beta$ 1– $\beta$ 3) in BAT.<sup>6,7</sup> This signal triggers a physiological cascade in which triglycerides stored in white adipose tissue (WAT) are hydrolyzed to free fatty acids (FFAs) that, in turn, activate BAT-specific uncoupling protein-1 (UCP1) in the inner mitochondrial membrane. UCP1 activation promotes the generation of heat by uncoupling oxidative phosphorylation from ATP synthesis. This cascade can also be triggered by  $\beta$ 3-adrenoceptor (ADRB3) agonists such as CL316,243. Besides the combustion of released FFAs, other energy substrates, like glucose, are increasingly taken up from the blood by brown adipocytes during thermogenesis.<sup>6</sup>

While present in rodents throughout life, it was believed for a long time that BAT rapidly disappears in humans at a young age. This dogma was refuted in the last decade using a noninvasive imaging technique, positron emission tomography (PET).<sup>1,8–11</sup> The PET-tracer 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucose ([<sup>18</sup>F]FDG) is a surrogate for enhanced glucose utilization as observed in tumor metabolism and inflammation. Symmetrical [<sup>18</sup>F]FDG accumulation, which is highly atypical for tumors, was serendipitously observed in the neck and shoulder (supraclavicular) area of patients. Besides its main localization in the supraclavicular area, which corresponds to the interscapular BAT of rodents, it is also found in the mediastinum (paraortic), as well as in the paravertebral and suprarenal regions.<sup>1,12</sup> Unlike WAT, BAT is metabolically favorable as an endogenous combustion system, and its abundance correlates with a leaner phenotype.<sup>13</sup> Hence, the activation of BAT may represent an attractive strategy to treat obesity as well as type 2 diabetes (T2D), where active BAT might restore insulin sensitivity.<sup>14,15</sup>

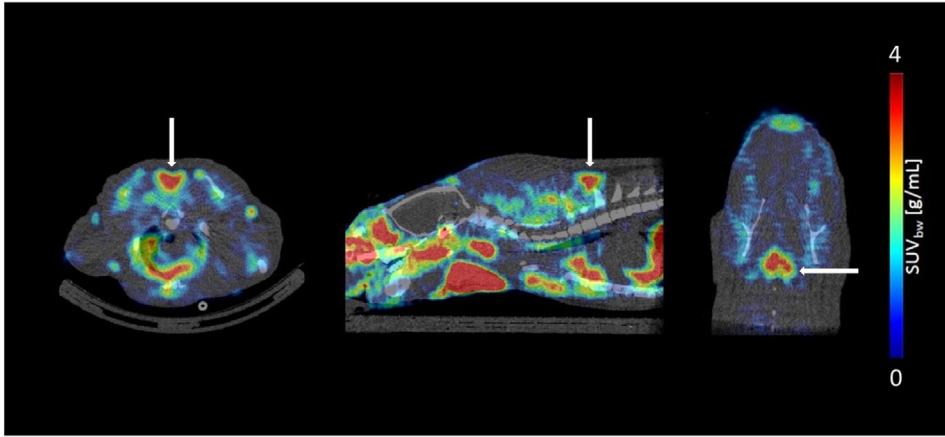
Another promising target for the treatment of obesity and T2D is melanin-concentrating hormone receptor 1 (MCHR1).<sup>16–21</sup> This G protein-coupled receptor is predominantly expressed in the brain and involved in energy homeostasis.<sup>22,23</sup> Its natural ligand, melanin-concentrating hormone (MCH),<sup>24</sup> functions as a neuropeptide in mammals and is predominantly expressed in the lateral hypothalamus,<sup>25</sup> one of the main brain regions involved in appetite regulation. Upregulation of MCH is present in obese mice and can be induced by fasting in naive mice.<sup>26</sup> Furthermore, MCH-overexpressing mice are hyperphagic, have

elevated blood glucose levels, and develop excessive obesity.<sup>27</sup> By contrast, MCH-deficient mice are leaner than wild-type mice due to hypophagia and a slight increase in energy expenditure.<sup>28</sup> MCHR1 gene knockout mice further have a leaner physique, which results from hyperactivity, increased energy expenditure, and altered metabolism.<sup>23,29</sup>

These findings stimulated the development of numerous MCHR1 antagonists as a possible treatment option for obesity. In several preclinical studies, the antagonists showed the expected effect of reducing food intake and increasing energy expenditure.<sup>23,30</sup> Moreover, Ito *et al.*<sup>21,31</sup> reported a trend toward enhanced FFA oxidation in isolated BAT after 1 month of MCHR1 antagonism. A link between BAT and the MCH system is further supported by neuronal MCH projections to brainstem regions harboring sympathetic neurons involved in BAT activation.<sup>32</sup> As a result, PET-tracers visualizing MCHR1 were developed<sup>33–42</sup> to enable its noninvasive *in vivo* quantification and monitoring of related pathologies, as well as to support drug development.

Taking advantage of the specific MCHR1 antagonist SNAP-7941,<sup>43</sup> we developed the radiolabeled analogue [<sup>11</sup>C]SNAP-7941 and its fluoroethylated derivative [<sup>18</sup>F]FE@SNAP as specific PET-tracers targeting the MCHR1 in the central nervous system.<sup>33–41</sup> However, during a preclinical evaluation, both tracers were found to be P-glycoprotein substrates.<sup>35,40,44</sup> Unexpectedly, nonnegligible MCHR1-tracer uptake in BAT of rats was repeatedly detected via small animal PET ( $\mu$ PET) (Fig. 1), pointing to the possibility of specific expression of MCHR1 in BAT. Because adrenergic receptors play an important role in BAT physiology, our next logical step was to exclude binding of the MCHR1 PET-tracers to the predominant adrenergic receptor ADRB3;<sup>4</sup> we demonstrated their affinities to ADRB3 to be in a micromolar range, which is irrelevant for PET imaging.<sup>45</sup>

Hence, the aim of our present study was to determine expression of MCHR1 in BAT, and to propose a more direct link between the MCH system and BAT physiology. Building on the discovery of this receptor in cultured murine brown adipocytes,<sup>45</sup> we investigated and verified its expression in rodent and human BAT. Additionally, we used *in vivo* imaging to better understand MCHR1 expression in BAT and the implications of this expression.



**Figure 1.** The  $\mu$ PET scan shows [ $^{11}\text{C}$ ]SNAP-7941 uptake in rat BAT. Exemplary BAT uptake of [ $^{11}\text{C}$ ]SNAP-7941 in rats shown with a transversal (left), sagittal (center), and coronal (right)  $\mu$ PET/CT summation image (0–45 minutes). White arrows indicate corresponding BAT uptake.

## Materials and methods

### Animals

Adult male mice (BALB/cAnNRj and C57BL/6N) and rats (Sprague–Dawley HIM:OFA) were obtained from the Division of Laboratory Animal Science and Genetics, Himberg, Austria. The animals were kept under standard housing conditions ( $22 \pm 1^\circ\text{C}$ ; 40–70% relative humidity) on a constant 12-h light/dark cycle with food and water supply *ad libitum*. All animals were treated according to the European Union rules on animal care, and respective experiments were approved by the Austrian Ministry of Science, Research and Economy (BMWF-66.009/0029-WF/V/3b/2015). In total, 36 HIM:OFA rats (24 for imaging and 12 for gene and protein analyses) and four mice (two BALB/cAnNRj and two C57BL/6N for gene and protein analyses) were used.

### Human adipose tissue samples

Adipose tissue samples were obtained from patients undergoing parathyroidectomy for primary hyperparathyroidism or elective thyroidectomy for benign goiter, Grave's disease, autonomous adenoma, or suspected thyroid carcinoma between March 2017 and April 2018. After providing informed written consent, all subjects who fulfilled the inclusion criteria (Supporting Information, File S1, online only) underwent thyroidectomy or parathyroidectomy at the Vienna General Hospital. This study has been approved by the Ethics Com-

mittee of the Medical University of Vienna (no. 2008/2014) and was conducted at the Department of Surgery, Division of General Surgery, and at the Division of Endocrinology and Metabolism, Department of Medicine III, Medical University of Vienna in accordance with the principles of the Declaration of Helsinki. Briefly, after the transverse skin incision in the anterior neck region, small parts of the subcutaneous fat (1–2 g) were removed. Afterward, during the preparation of the carotid artery for neuromonitoring of the nervus laryngeus recurrens (RLN, recurring laryngeal nerve), small specimens of the fat surrounding the carotid sheath were removed. During the further preparation of the RLN, deep neck fat surrounding the longus colli muscle was taken out. Finally, during the preparation of the caudal pole of the thyroid, small fat depots from the region were removed. In general, 0.5–2 g of the respective deep fat depot was excised.

### Isolation of human adipocyte precursor cells

Human adipocyte precursor cells (hAPCs) were isolated from fat specimens obtained during abdominoplasty from three different donors. Abdominal subcutaneous adipose tissue was excised from healthy female patients undergoing abdominoplastic surgery between June 2017 and May 2018. All subjects provided written informed consent. This study was approved by the Ethics Committee of the Medical University of Vienna and was conducted in accordance with the principles



of the Declaration of Helsinki (EK 1149/2011 and EK 1032/2013). hAPCs were isolated and cultured as previously described.<sup>46</sup> Confluent cells were induced (day 0) using a differentiation medium supplemented with 0.85  $\mu$ M insulin (#I9278, Sigma-Aldrich), 2 nM triiodothyronine (#I9278, Sigma-Aldrich), 5  $\mu$ M rosiglitazone (#R2408, Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (#I7018, Sigma-Aldrich), and 1  $\mu$ M dexamethasone (#D8893, Sigma-Aldrich) for 2 days, followed by postdifferentiation medium supplemented with 0.85  $\mu$ M insulin, 2 nM triiodothyronine, and 5  $\mu$ M rosiglitazone. The postdifferentiation medium was changed every other day until day 6. Cells were stimulated with CL316,243 or vehicle (H<sub>2</sub>O) during the entire adipogenic differentiation.

#### *RNA extraction and reverse-transcription quantitative PCR*

The rodent tissue and human fat depot samples were snap-frozen and stored at  $-80^{\circ}\text{C}$  before RNA isolation. For rodent *Mchr1* detection on the genome level, BAT of two BALB/cAnNRj and two C57BL/6N mice (6 and 8 weeks old, respectively), as well as of seven HIM:OFA rats (three animals aged  $\leq 40$  weeks and four aged 50, 90, 95, and 99 weeks) were analyzed. Two spleen and brain samples of BALB/cAnNRj mice and HIM:OFA rats served as the negative and positive controls, respectively. In the case of the four rats aged  $\geq 50$  weeks and all mouse tissues, organs were cut into halves to conserve a specimen for western blot (WB) analysis. All samples were ground using TRIzol<sup>®</sup> reagent (#15596026, Invitrogen). For rodent BAT samples, additional centrifugation steps at  $4^{\circ}\text{C}$  were included in the extraction protocol in order to remove fat content. Subsequent to RNA extraction, RNA concentration and purity were checked with a NanoDrop device (Thermo Fisher Scientific). Reverse transcription was run with the qScript<sup>®</sup> cDNA Synthesis Kit (#95047, Quantabio), where after two-step reverse-transcription, quantitative PCR (RT-qPCR) was performed with 2  $\mu$ g DNA each, using the Luna<sup>®</sup> Universal qPCR Kit (#M3003, BioLabs) and the CFX96<sup>™</sup> Real-Time Detection System (Bio-Rad).

In the case of human fat depot biopsies ( $n = 6$  for the carotid sheath,  $n = 19$  for the thymus,  $n = 15$  for the longus colli muscle, and  $n = 18$  for subcutaneous fat) and hAPCs ( $n = 3$ ), total

RNA was also extracted using TRIzol (#15596026, Invitrogen), and samples were treated with DNase (#EN0521, Thermo Scientific) and reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (#4374966, Applied Biosystems). RT-qPCR was performed with 3  $\mu$ g DNA each, using FastStart<sup>®</sup> Universal SYBR green (#04887352001, Roche) and a QuantStudio 6 RealTime PCR System (Applied Biosystems).

Relative mRNA expression of both rodent and human samples was determined using the  $\Delta\Delta\text{Ct}$  method, with  $\beta$ -actin (rodent) or 36B4 (human) mRNAs as a normalization control. In the case of human tissue samples, only the deep cervical fat samples with significantly higher *UCP1* expression, compared with white fat depots ( $\Delta\text{Ct} > \text{mean } \Delta\text{Ct WAT}$ ), were used for further analysis. Data were further processed with Excel 2013 (Microsoft<sup>®</sup> Office) and GraphPad Prism 6 (GraphPad Software Inc.).

All rodent and human primer sequences (eurofins) are described in the Supporting Information (online only).

#### *Protein extraction and WB*

For MCHR1 detection at the protein level, the other halves of the murine samples and four rat BATs  $\geq 50$  weeks, previously analyzed with RT-qPCR, were used. Additionally, two new brain and spleen samples and five fresh BAT samples from HIM:OFA rats aged  $\leq 40$  weeks were used. Tissues were extracted using an Ultra Turrax<sup>®</sup> tissue homogenizer (IKA, Germany) in the presence of a radioimmunoprecipitation assay (RIPA) buffer (#20-188, Merck) and the recommended amount of standard protease inhibitor cocktail (#P2714, Merck). After centrifugation ( $14,500 \times g$ ,  $4^{\circ}\text{C}$ ), protein concentration in supernatants was determined with a bicinchoninic acid (BCA) kit (#PR 23227, Thermo Scientific). To avoid imprecise results caused by high fat content, BAT was centrifuged at least two to three times to remove the fatty layer. Gel electrophoresis was performed with 20  $\mu$ g protein each on TGX<sup>™</sup> pre-cast gels (#456-1083, Bio-Rad), followed by semidry blotting and total protein staining (TPS) with Ponceau S (#P7170, Sigma-Aldrich) to ensure consistent protein loading. Subsequently, nitrocellulose membranes (#10600004, GE Healthcare Life Science) were blocked with a 5% dry milk powder for 90 min at room temperature. Incubation with the

primary antibody (rabbit polyclonal anti-MCHR1 #PA5-50705, RRID:AB\_2636157, 1:1000, Thermo Fisher Scientific) was run at 4 °C, overnight, followed by thorough washing and a 1-h incubation at room temperature with a goat anti-rabbit IgG HRP conjugate (#A16104, RRID:AB\_2534776, 1:2500, Thermo Fisher Scientific). Chemoluminescence imaging was performed with the Clarity™ Western ECL substrate detection kit (#170-5060, Bio-Rad) and the ChemiDoc™ Imaging System (Bio-Rad).

Detected protein bands were normalized to TPS with Ponceau S (Fig. S1, online only), performing pixel analysis in the free image processing program ImageJ (the National Institutes of Health, Bethesda, MD). To this end, the pixel densities of the respective MCHR1 band were divided by those of corresponding TPS lanes, allowing for relative quantification. These data were further processed with Excel 2013 and GraphPad Prism 6.

### Chemistry

The MCHR1 PET-tracer [<sup>11</sup>C]SNAP-7941 was synthesized as previously described.<sup>33</sup> [<sup>18</sup>F]FDG was prepared using a fully automated cassette-based synthesizer (FASTlab, GE Healthcare) within the clinical routine production at the Vienna General Hospital, Austria. All PET-tracers were physiologically formulated and fulfilled the quality control parameters. The corresponding unlabeled compound SNAP-7941 ((±)-methyl(4S)-3-[(3-{4-[3-(acetylamino)phenyl]-1-piperidinyl}propyl)amino]carbonyl]-4-(3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetra-hydro-5-pyrimidine-carboxylate hydrochloride)) and the precursor SNAP-acid ((±)-3-[(3-{4-[3-(acetylamino)phenyl]-1-piperidinyl}propyl)amino]carbonyl]-4-(3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetra-hydro-5-pyrimidinecarboxylate acid)) were synthesized according to Schirmer *et al.*<sup>47</sup> at the Departments of Pharmaceutical Chemistry and Organic Chemistry (University of Vienna, Austria). The ADRB3 agonist CL316,243 (#1499) was purchased from Tocris Bioscience.

### Small animal imaging

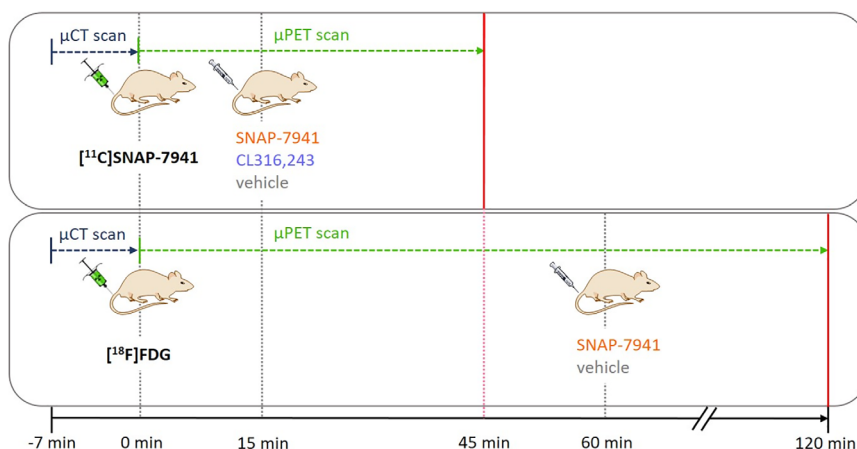
Ten- to twelve-week-old male rats (397 ± 67 g) were anesthetized using 1.5–2% isoflurane with oxygen (1.5–2 L/min) and immobilized. Animals were warmed with a 37 °C positioning bed, except for imaging with [<sup>18</sup>F]FDG. Tracers (75.56 ± 5.48 MBq [<sup>18</sup>F]FDG (radiochemical purity >98%) or 82.7 ±

8.9 MBq [<sup>11</sup>C]SNAP-7941 (molar activity: 44.33 ± 29.63 GBq/μmol; radiochemical purity >99%) as well as SNAP-7941 (15 mg/kg body weight (BW)), CL316,243 (2 mg/kg BW), or the respective vehicles were injected into the lateral tail vein. The total volume applied did not exceed 1 milliliter. After a μCT scan of 7 min, dynamic PET imaging was acquired on a Siemens Inveon preclinical μPET/SPECT/CT system. Sixty minutes after [<sup>18</sup>F]FDG injection, SNAP-7941 (*n* = 4) or vehicle (*n* = 4) was injected, and the PET scan was continued for another 60 minutes. For scans with [<sup>11</sup>C]SNAP-7941, CL316,243 (*n* = 6), SNAP-7941 (*n* = 6), or vehicle (*n* = 4) was administered 15 min after tracer application, respectively. The total PET acquisition time was 45 minutes. Differences between [<sup>18</sup>F]FDG and [<sup>11</sup>C]SNAP-7941 in PET acquisition time are due to the half-lives of the respective nuclides (carbon-11: 20.3 min; and fluorine-18: 109.8 min) and the tracers' equilibrium at 60 min ([<sup>18</sup>F]FDG) and 15 min ([<sup>11</sup>C]SNAP-7941) after application. Figure 2 shows the timeline of small animal imaging.

### Image reconstruction and data postprocessing

The CT raw data were reconstructed with a Feldkamp algorithm using a Ramp filter followed by standard rat beam-hardening correction and noise reduction (matrix size: 1024 × 1024; effective pixel size: 97.56 μm). The dedicated CT image data were calibrated to Hounsfield Units (HU). PET list mode data were sorted into three-dimensional sinograms and reconstructed using an OSEM 3D/OP-MAP scatter corrected reconstruction algorithm and a ramp filter (matrix size 128 × 128). The data were normalized and corrected for random, dead time, and radioactive decay. A calibration factor was applied to convert the activity information into absolute concentration units. Multimodal (μPET/CT) rigid-body image registration and biomedical image quantification was performed using the image analysis software PMOD 3.8 (PMOD Technologies, Switzerland) and Inveon Research Workplace (IRW; Siemens Medical Solutions). Volumes of interest were outlined on multiple planes of the CT and PET images. Time activity curves (TACs) were calculated, normalized to dose and weight, and expressed as standardized uptake values (SUV; g/mL) to facilitate the comparison.





**Figure 2.** This figure represents the timeline of small animal imaging. After a  $\mu$ CT scan of 7 min, the respective tracer was applied and the  $\mu$ PET scan started. In the case of imaging with  $[^{11}\text{C}]\text{SNAP-7941}$ , SNAP-7941, CL316,243, or vehicle was added 15 min after tracer application. The  $\mu$ PET scan was terminated after 45 minutes. As for the  $[^{18}\text{F}]\text{FDG}$  scans, SNAP-7941 or vehicle was added 60 min after tracer application and the  $\mu$ PET scan was stopped after 120 minutes.

### Ex vivo metabolite analysis

After PET scanning with  $[^{11}\text{C}]\text{SNAP-7941}$  and vehicle, anesthetized rats ( $n = 4$ ) were sacrificed by decapitation; BAT was harvested and subsequently homogenized with 250  $\mu\text{L}$  0.9% saline solution using an Ultra Turrax<sup>®</sup>. After the addition of acetonitrile (250  $\mu\text{L}$ ), the homogenate was vortexed and centrifuged ( $23,000 \times g$ , 5 min,  $4^\circ\text{C}$ ) to remove fatty tissue and precipitated proteins. The recovery of the radioparent compound and potential radiometabolite extraction was measured using a gamma-counter (2480 Wizard<sup>2</sup>, Perkin Elmer). The obtained supernatant was spiked with SNAP-7941 and analyzed by analytical high-performance liquid chromatography (HPLC) (mobile phase: (water:acetic acid = 97.5:2.5 %v/v; 2.5 g/L ammonium acetate; pH 3.5):acetonitrile 70:30 %v/v; stationary phase: Chromolith<sup>®</sup> Performance RP-18e, 100–4.6 mm, #102129, Merck; flow: 1 mL/min;  $\lambda = 254$  nm). The HPLC system (Agilent) comprised a diode array detector 1200 and a lead-shielded BGO radiodetector (Elysia-raytest GmbH, Germany). The ratio between the metabolite and intact tracer was decay corrected and calculated using quantitative HPLC analysis (GINAstar software, Elysia-raytest GmbH, Germany).

### Statistical analysis

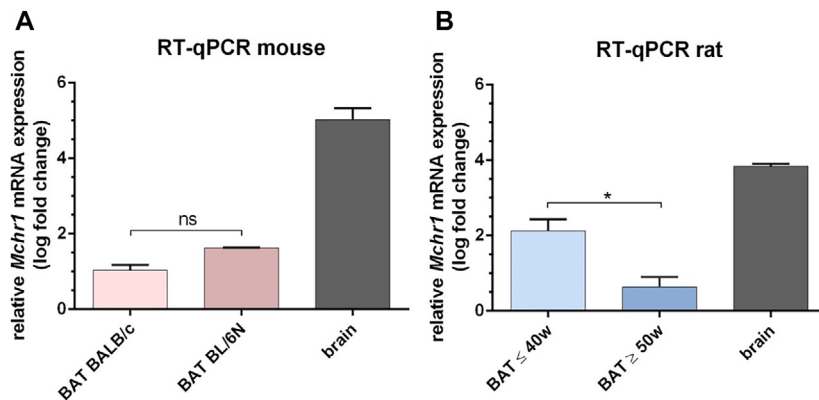
All experimental data are expressed as the mean  $\pm$  SEM. Statistical testing was performed using Graph-

Pad Prism 6. Differences among groups were proved using a two-tailed parametric *t*-test (paired in the case of small animal imaging data), and values of  $P < 0.05$  were considered as statistically significant. Small animal imaging data were obtained using different batches of the radioligand. Multiple comparisons testing was performed using either ordinary one-way ANOVA with Tukey's correction or ordinary two-way ANOVA with Sidak's correction.

## Results

### *Mchr1* mRNA is expressed in mouse, rat, and human BAT

Referring to Kokkotou *et al.*,<sup>48</sup> the brain and spleen were chosen as the positive and negative controls, respectively. *Mchr1* mRNA was quantified in the brain and spleen of BALB/c mice and BAT of two different strains of mice (Fig. 3A), as well as in the brain, spleen, and BAT of HIM:OFA rats (Fig. 3B). In both species, high relative *Mchr1* mRNA levels were found in the positive reference brain (mean log fold change  $5.02 \pm 0.29$  ( $n = 2$ ) and  $3.81 \pm 0.06$  ( $n = 3$ ), respectively). Lower levels were found in the BAT of rats ( $n = 7$ ) and all tested mice ( $n = 4$ ), with C57BL/6N having slightly higher levels ( $1.62 \pm 0.01$ ,  $n = 2$ ) than BALB/cAnNRj ( $1.07 \pm 0.10$ ,  $n = 2$ ) and rats showing a very heterogeneous expression dependent on their age (mean log fold-change  $2.12 \pm 0.30$  for  $\leq 40$  weeks old ( $n = 3$ ) and  $0.63 \pm 0.27$  for  $\geq 50$  weeks old ( $n = 4$ )). *Mchr1*



**Figure 3.** *Mchr1* mRNA is expressed in rodent BAT. Relative *Mchr1* expression in BALB/c brain and BAT of two different mouse strains (A, two animals each) and in the brain and BAT of HIM:OFA rats (B, three and four animals, respectively). Data are represented as the mean  $\pm$  SEM from independent experiments. The Ct values were normalized to  $\beta$ -actin, relative mRNA expression was calculated with respect to the negative control tissue spleen using the formula  $\log(2^{-\Delta\Delta C_t})$ . *Mchr1* expression on the genome level is significantly lower in rats aged  $\geq 50$  weeks ( $*P < 0.05$ ).

mRNA was not detectable in the spleen of any of the tested mice and rats. Melting curve analysis confirmed the formation of a specific product corresponding to the positive control (Figs. S2 and S3, online only).

Receptor gene expression was further evaluated in several human biopsies of subcutaneous adipose tissue and fat depots surrounding the carotid sheath, thymus, and longus colli muscle (Fig. 4A). For the human samples, subcutaneous adipose tissue served as reference, known to express *MCHR1*<sup>49</sup> but low to no *UCP1*, as it mostly consists of white adipocytes. Generally, average  $\Delta C_t$  values (*MCHR1/B6B4*) of the thymus ( $12.14 \pm 1.00$ ,  $n = 19$ ) and longus colli muscle ( $11.94 \pm 1.15$ ,  $n = 15$ ) were in the range of the positive control ( $14.06 \pm 0.82$ ,  $n = 18$ ), but the mean  $\Delta C_t$  was significantly lower for carotid sheath ( $9.63 \pm 1.63$ ,  $n = 6$ ,  $P < 0.05$ , see Fig. S4, online only). Highest *MCHR1* levels were found in the carotid sheath samples (mean log fold change  $1.33 \pm 0.49$ ,  $n = 6$ ) and lower ones in the thymus and longus colli muscle fat depots ( $0.58 \pm 0.30$  ( $n = 19$ ) and  $0.64 \pm 0.34$  ( $n = 15$ ), respectively). Mean relative mRNA expression of *UCP1* of these samples was  $3.48 \pm 0.61$  for the carotid sheath,  $2.93 \pm 0.29$  for the thymus, and  $2.74 \pm 0.31$  for the longus colli muscle, when compared with subcutaneous adipose tissue with very low *UCP1* levels. Detected *MCHR1* mRNA expression could not be correlated with *UCP1* mRNA levels, as a fit was not reasonable with given data points (Fig. 4B–D). However, trend

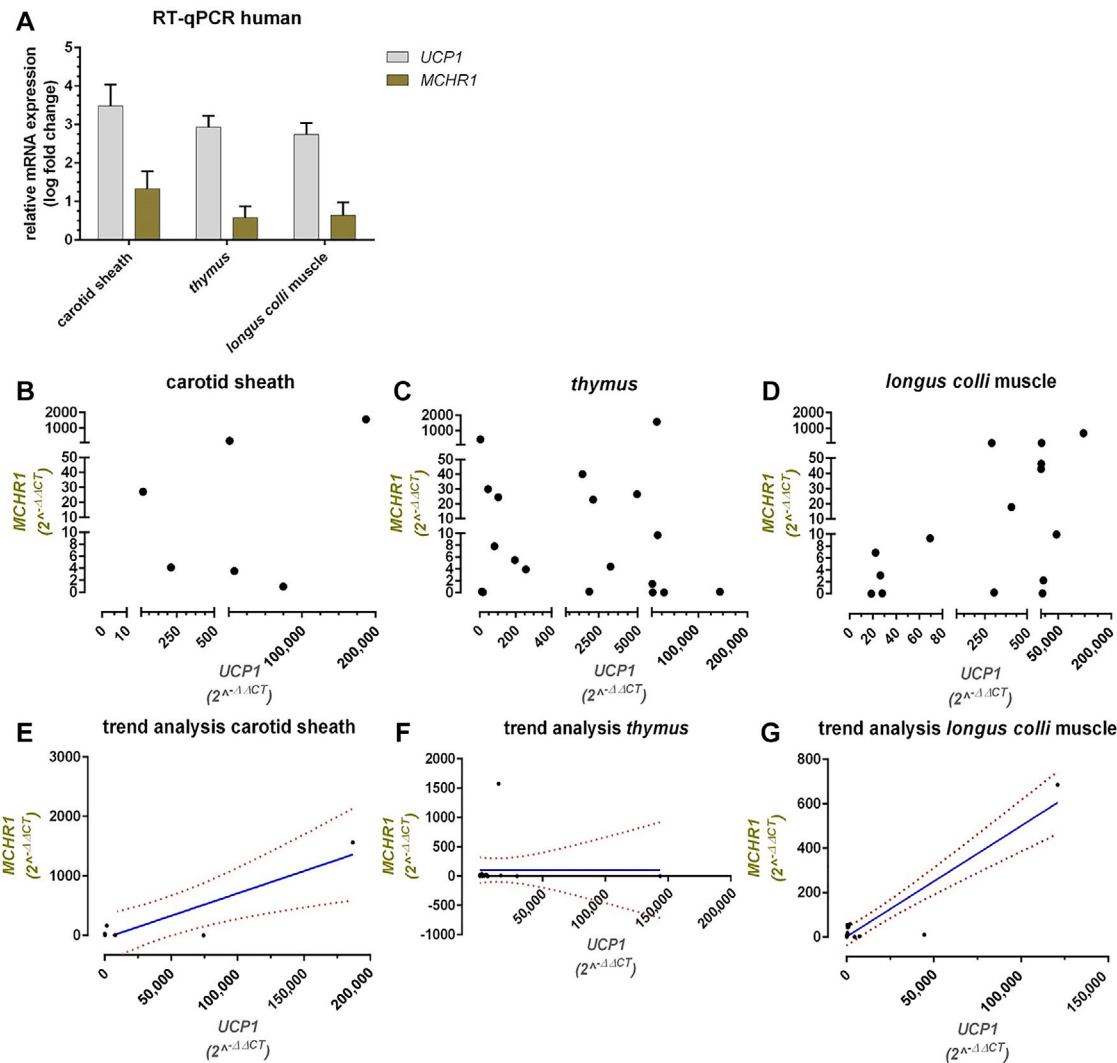
analysis (Fig. 4E–G) revealed a significant positive trend for the carotid sheath ( $R^2 = 0.8196$ ) and longus colli muscle samples ( $R^2 = 0.8529$ ).

#### *MCHR1 is present in human adipocytes, and its expression is induced by chronic stimulation with CL316,243*

In order to study if MCHR1 is present on hAPCs and to investigate the influence of the ADRB3 agonist CL316,243 on MCHR1 gene expression, cells were isolated from three different donors. hAPCs were differentiated for 6 days using a standard adipocyte differentiation protocol. In order to obtain a BAT-like phenotype, cells were stimulated with either control or CL316,243 (1  $\mu$ M) during adipogenic differentiation. Cells were harvested before the start of differentiation (day 0), during differentiation (day 3), and when they were fully differentiated (day 6). Cell differentiation increased both *UCP1* and *MCHR1* gene expression over time (Fig. 5A and B). Interestingly, chronic stimulation with 1  $\mu$ M CL316,243 determined a significant increase ( $P < 0.05$ ) in *MCHR1* gene expression on day 6 (Fig. 5A). These findings demonstrate that *MCHR1* mRNA is expressed in undifferentiated hAPCs as well as in fully *in vitro* differentiated adipocytes, and that its expression increases with CL316,243 stimulation.

#### *MCHR1 protein is expressed in rodent BAT*

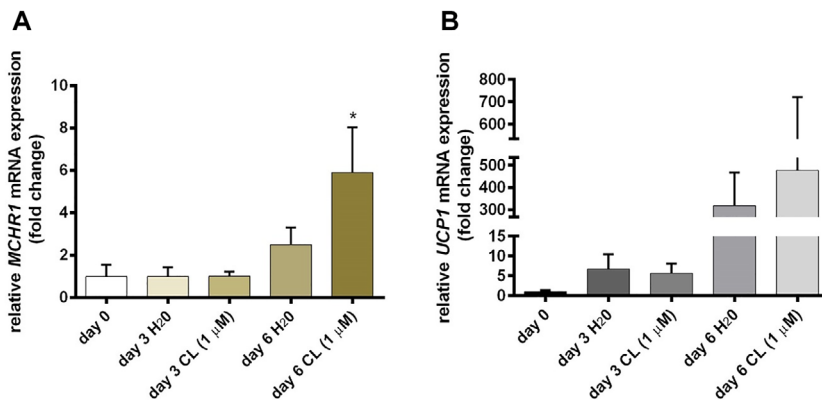
At the protein level, MCHR1 was discovered in all analyzed rodent brain and murine BAT



**Figure 4.** *MCHR1* mRNA is expressed in human BAT, but does not correlate with *UCP1* mRNA expression. Mean relative *MCHR1* and *UCP1* expression ( $\pm$ SEM) in human fat depot biopsies of the carotid sheath, thymus, and longus colli muscle (A). The Ct values were normalized to *36B4*, relative mRNA expression was calculated with respect to a subcutaneous WAT as the positive control with the formula  $\log(2^{-\Delta\Delta C_t})$  (see Fig. S4 for the  $\Delta C_t$  values, online only). The respective  $2^{-\Delta\Delta C_t}$  values for *MCHR1* were plotted against *UCP1* levels of the carotid sheath (B), thymus (C), and longus colli muscle (D) fat depots. Trend analyses (E–G) show a significant positive trend with regard to *MCHR1* versus *UCP1* for the carotid sheath ( $R^2 = 0.8196$ ) and longus colli muscle samples ( $R^2 = 0.8529$ ), but not for the thymus. Dotted red lines correspond to the confidence interval (95%). Only deep cervical fat samples with a significantly higher *UCP1* expression compared with white fat depots were used for analysis ( $n = 6$  for the carotid sheath,  $n = 19$  for the thymus, and  $n = 15$  for the longus colli muscle).

samples (Fig. 6). Rats older than 50 weeks, in general, showed low (BAT 2, 4, and 6) or no *MCHR1* protein (BAT 3) expression in BAT. However, *MCHR1* was detected in all BAT samples of rats aged  $\leq 40$  weeks (Fig. 6C–E). Relative expression levels in BAT samples of younger rats were heterogeneous, with two samples showing a very high

expression (BAT 1 and 8) comparable to or even exceeding expression *MCHR1* levels found in the brain, and three samples (BAT 5, 7, and 9) had lower expression. All relative *MCHR1* expression levels in both BALB/cAnNRj and C57BL/6N BAT were similar to those of the brain (Fig. 6A and B). In accordance with RT-qPCR results, *MCHR1*



**Figure 5.** *UCP1* and *MCHR1* mRNA expression increases during differentiation of hAPCs and is induced by CL316,243. The mean relative *MCHR1* (A) and *UCP1* (B) mRNA expression ( $\pm$ SEM) on day 0 (before differentiation), day 3 (during differentiation), and day 6 (fully differentiated). *MCHR1* significantly ( $*P < 0.05$ ) increases with CL316,243 stimulation.

was not detected in the spleen. Generally, two bands (35–40 and ~55 kDa) were detected in the tested BAT samples; in the brain, the MCHR1 band was always found at ~55 kDa regardless of age, strain, and species. As for BAT, BL/6N mice exhibited an immunoreactive band at the same height, whereas BALB/c and HIM:OFA showed a lower molecular weight band. In some BAT samples from rats, we observed an additional weak band at ~55 kDa.

#### *[<sup>18</sup>F]FDG uptake in BAT is increased by SNAP-7941, while [<sup>11</sup>C]SNAP-7941 uptake is enhanced by SNAP-7941 and CL316,243*

Given that SNAP-7941 binds to ADRB3 in higher concentrations, we aimed to study its effect on BAT activation with [<sup>18</sup>F]FDG imaging in rats. The administration of 15 mg/kg BW SNAP-7941 ( $n = 4$ ) 60 min after [<sup>18</sup>F]FDG injection induced a significantly increased [<sup>18</sup>F]FDG uptake in BAT ( $P = 0.0044$ ,  $df = 3$ , Fig. 7A). No effect was seen after addition of vehicle ( $P = 0.3277$ ,  $df = 3$ ,  $n = 4$ , Fig. 7B).

Subsequently, in order to investigate MCHR1 *in vivo*, we performed imaging experiments with [<sup>11</sup>C]SNAP-7941 using SNAP-7941 for competition studies. Furthermore, as CL316,243 induced MCHR1 gene expression in hAPCs, we also investigated CL316,243 administration in  $\mu$ PET studies. The injection of 2 mg/kg BW CL316,243 ( $n = 4$ , Fig. 8A), as well as 15 mg/kg (BW) SNAP-7941 ( $n = 4$ , Fig. 8B) 15 min after radiotracer appli-

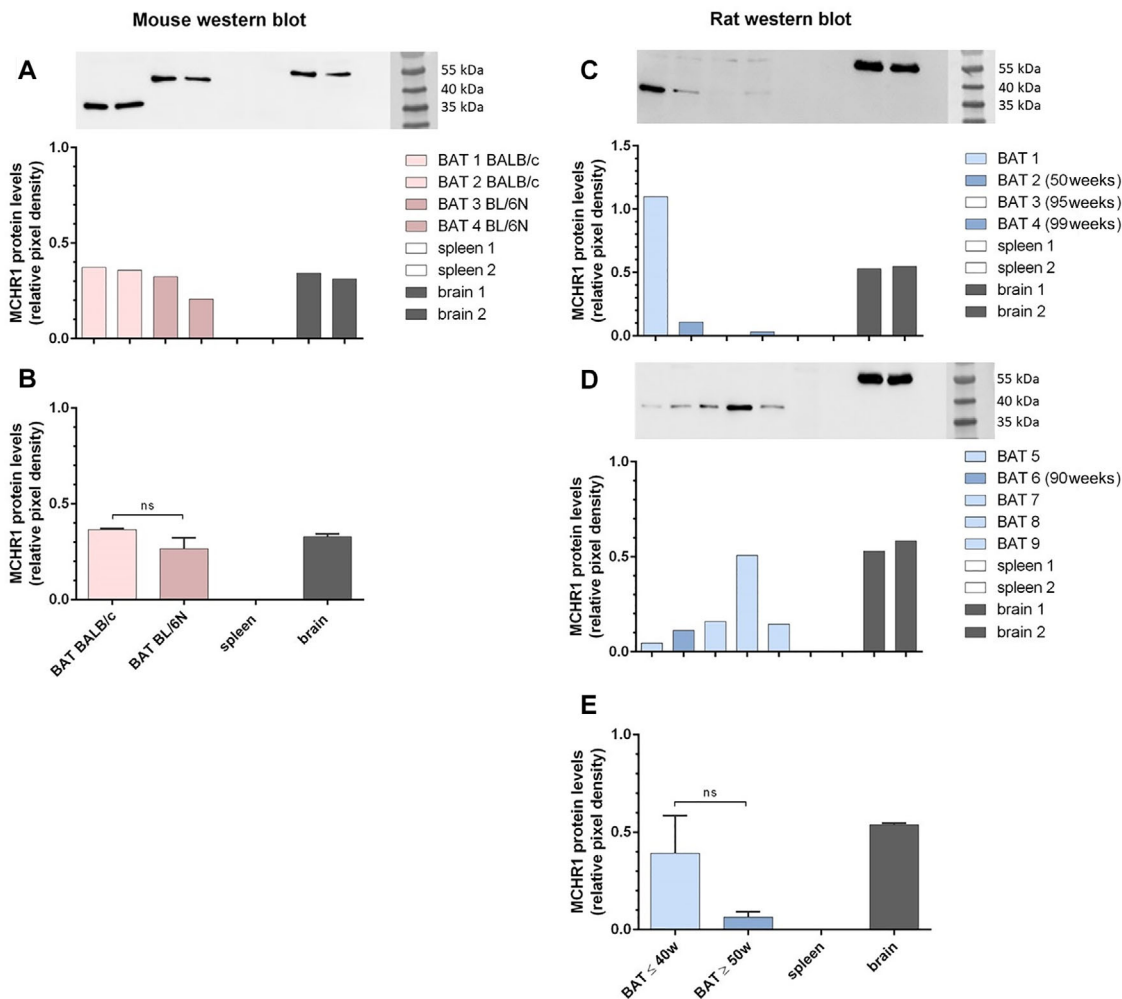
cation significantly enhanced BAT uptake in a similar range ( $P = 0.0287$ ,  $df = 2$ ). Again, the administration of vehicle ( $n = 4$ ) had no influence on [<sup>11</sup>C]SNAP-7941 BAT uptake ( $P = 0.9469$ ,  $df = 3$ , Fig. 8C). Four rats, however, had a high initial uptake of [<sup>11</sup>C]SNAP-7941 ( $SUV > 2.5$ ) and were excluded. In those rats, the administration of SNAP-7941 ( $n = 2$ ) or CL316,243 ( $n = 2$ ) after 15 min caused either displacement or had no effect (data not shown).

#### *[<sup>11</sup>C]SNAP-7941 is metabolically stable in BAT*

In order to ensure the detected PET signal resulted from intact radiotracer, we determined potential radiometabolites of [<sup>11</sup>C]SNAP-7941 in BAT using HPLC. Two hydrophilic radiometabolites at 2- and 4-min retention time occasionally appeared in very limited quantities. The intact [<sup>11</sup>C]SNAP-7941 ( $n = 4$ ;  $94.0 \pm 5.2\%$ ) was still present 45 min after tracer application. The recovery of the radioparent compound and radiometabolites after tissue preparation was  $>90\%$ . A representative chromatogram is shown in the Supporting Information (Fig. S5, online only). This proves the stability of [<sup>11</sup>C]SNAP-7941 during the scan period.

## Discussion

Collectively, our results prove presence of MCHR1 in rodent and human BAT and high-light [<sup>11</sup>C]SNAP-7941 as a potential alternative PET-tracer for BAT imaging. Since serendipitous



**Figure 6.** MCHR1 expression is comparable in two different mouse strains and dependent on age in HIM:OFA rats. MCHR1 expression in the brain and spleen of BALB/c mice and in BAT of two different mouse strains (A and B), as well as in the brain, spleen, and BAT of HIM:OFA rats of different age (C–E). (A, C, and D) The original western blots and matching relative pixel densities of the respective protein bands, normalized to Ponceau S (see Fig. S1, online only). In (C) and (D), all rats aged  $\leq 40$  weeks are shown in light blue and the ones aged  $\geq 50$  weeks in darker blue. Relative pixel densities of the protein bands of rat BAT probes in (C) were calculated for the 35–40-kDa bands, not for the weaker ones visible at  $\sim 55$  kDa.

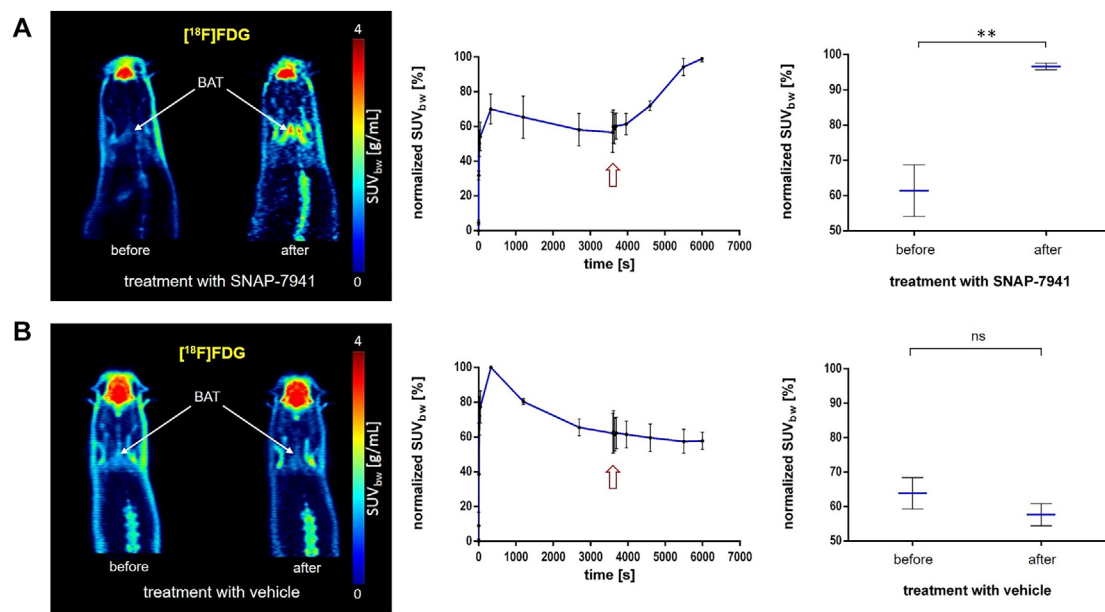
observations with PET led to the rediscovery of BAT in adult humans, it may be seen as a coincidence that the same technique finally led to the detection of MCHR1 in BAT.

After we unexpectedly observed MCHR1-tracer uptake in this tissue, we investigated an eventual binding to the ADRB3, the receptor predominantly involved in BAT activation. The sequence similarity between  $\beta$ -adrenoceptors and MCHR1 already suggests the off-target effects of MCHR1

antagonists.<sup>50</sup> Both MCHR1 antagonists, SNAP-7941 and FE@SNAP, were shown to bind to the ADRB3 only in a micromolar range (SNAP-7941:  $K_i = 14.5 \pm 0.3 \mu\text{M}$ , FE@SNAP:  $K_i = 65.1 \pm 2.9 \mu\text{M}$ ),<sup>45</sup> demonstrating a high selectivity against the target protein. As PET-tracers are applied in a pico- to nanomolar range, PET imaging with [ $^{11}\text{C}$ ]SNAP-7941 only depicts MCHR1 binding.

Since we had established the superiority of [ $^{11}\text{C}$ ]SNAP-7941 in terms of metabolic stability,





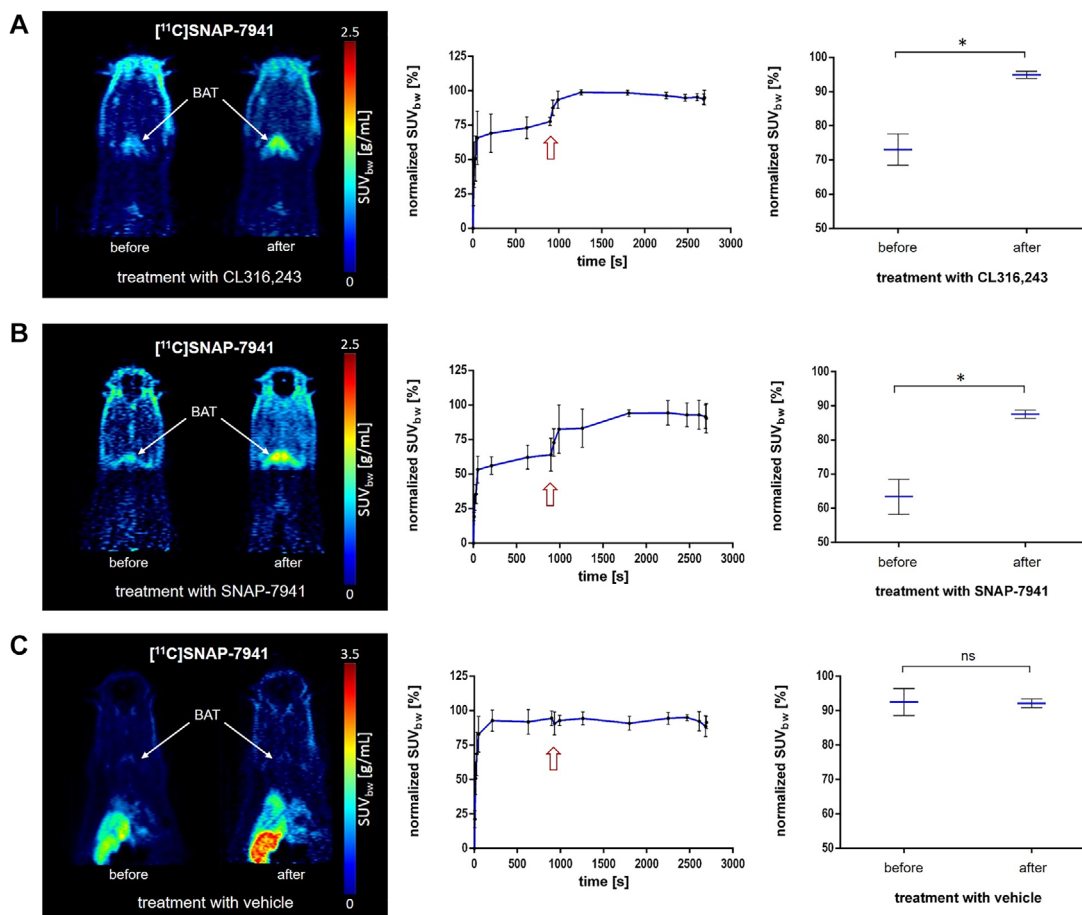
**Figure 7.**  $[^{18}\text{F}]\text{FDG}$  uptake in BAT is significantly induced through SNAP-7941. A representative BAT uptake of  $[^{18}\text{F}]\text{FDG}$  shown in axial rat  $\mu$ PET summation images before and after treatment with 15 mg/kg SNAP-7941 (A) and the dedicated vehicle (B), respectively. Corresponding TACs (center) and paired test statistics (right) are presented for both treatments with 15 mg/kg SNAP-7941 (A) and the dedicated vehicle (B). Data are plotted as the mean  $\pm$  SEM from independent experiments ( $n = 4$ ). Differences among groups were tested using a two-tailed parametric paired  $t$ -test (\*\* $P \leq 0.01$ ). If not visible, error bars are within the margin of the symbols. Red arrows indicate the time point of SNAP-7941 or vehicle addition.

affinity, and selectivity in a previous study,<sup>40</sup> we chose the carbon-11 tracer to further investigate the *in vitro* uptake in murine brown adipocytes.<sup>45</sup> In uptake experiments with immortalized and differentiated murine brown adipocytes, SNAP-7941 led to an  $\sim 23\%$  blocking of  $[^{11}\text{C}]\text{SNAP-7941}$  uptake, whereas ADRB3 agonist CL316,243 had a significantly lower effect. Hence, these data suggested that MCHR1 is expressed in these cultured murine brown adipocytes.

In order to put these findings into a more physiological context, we examined MCHR1 expression in rodent and human BAT. To also take strain differences into account, we analyzed BL/6N as well as BALB/c mice, and found that BL/6N had slightly higher levels of *Mchr1* mRNA than BALB/c. As for HIM:OFA rats, *Mchr1* mRNA in BAT was generally detected to a greater extent than in mice (Fig. 3A), although significantly lower gene expression correlated with increasing age (Fig. 3B). BAT function is known to decrease with age not only in humans but also in rodents. It has been reported

that substantial morphological remodeling of BAT takes place in 6-month-old mice, resulting in the formation of hypertrophied brown adipocytes with larger lipid droplets.<sup>51</sup> Consequently, one could suggest a transformation of BAT as a combustion system toward becoming an energy-storing organ. This might explain why older animals are more susceptible to obesity. Owing to this age-related morphological change, *Mchr1* mRNA expression could be affected as well.

Despite the comparably low gene expression of BAT, we detected relatively high MCHR1 protein levels comparable to, or exceeding, those in the brain in some cases (6 out of 13 BAT samples, Fig. 6). This might be due to either high mRNA turnover or high MCHR1 protein stability. Furthermore, MCHR1 gene expression was in a similar range in both strains of mice aged 6–8 weeks, despite different mRNA levels (Fig. 6A and B). MCHR1 was found to be heterogeneously expressed in rats of various ages (Fig. 6C–E). The two different immunoreactive bands (35–40 kDa and  $\sim 55$



**Figure 8.**  $[^{11}\text{C}]\text{SNAP-7941}$  uptake in BAT is significantly induced through SNAP-7941 and CL316,243. Representative BAT uptake of  $[^{11}\text{C}]\text{SNAP-7941}$  shown in axial rat  $\mu\text{PET}$  summation images before and after treatment with 2 mg/kg CL316,243 (A), 15 mg/kg SNAP-7941 (B), and the dedicated vehicle (C). Corresponding TACs (center) and paired test statistics (right) are presented for the BAT uptake under treatment with 2 mg/kg CL316,243 (A), 15 mg/kg SNAP-7941 (B), and the dedicated vehicle (C), respectively. Data are plotted as the mean  $\pm$  SEM from independent experiments ( $n = 4$ ). Differences among groups were tested using a two-tailed parametric paired  $t$ -test ( $*P \leq 0.05$ ). If not visible, error bars are within the margin of the symbols. Red arrows indicate the time point of SNAP-7941, CL316,243, or vehicle addition.

kDa) detected in BAT samples may be explained by alternate splicing variants of the pre-RNA as described for human *MCHR1*.<sup>52</sup> More importantly, there is also substantial posttranslational modification of the protein. Saito *et al.*<sup>53</sup> previously reported posttranslational N-terminal glycosylation of *MCHR1*, resulting in bands between 35 and 65 kDa molecular weight. Depending on the glycosylation pattern, the uppermost or lower bands can be absent. Because of tissue differences, loading controls based on housekeeping genes were found to be inappropriate; therefore, nor-

malization to Ponceau S was performed (Fig. S1, online only).

In human samples, *MCHR1* and *UCP1*, an indicator for BAT, were divergently expressed among the tested samples (Fig. 4A–D). Owing to this heterogeneity, as well as the fact that most data points represent either comparatively low or very high *UCP1* expression, the fit we performed using Pearson's correlation was distorted and, therefore, not deemed valid (data not shown). Nevertheless, trend analysis confirmed a positive correlation of *UCP1* and *MCHR1* expression in the carotid sheath

and longus colli muscle fat depots (Fig. 4E–G). However, as the expression of *UCP1* alone is not representative of BAT activity,<sup>54</sup> the correlation between *UCP1* and *MCHR1* might not be relevant. Antonacci *et al.*<sup>55</sup> measured BAT thermogenesis by hyperpolarized <sup>129</sup>Xe MR thermometry after adrenergic stimulation of *UCP1*-KO mice and showed significant, *UCP1*-independent activation of thermogenesis. In addition, Long *et al.*<sup>56</sup> described another protein (PM20D1) as a mitochondrial uncoupler protein distinct from *UCP1* in BAT, again indicating that thermogenesis in BAT is more complex than anticipated.

Since we verified the expression of the receptor in both rodent and human BAT and had a promising MCHR1 PET-tracer at hand, we performed preclinical *in vivo* imaging studies to better understand the physiological role of MCHR1 in BAT as well as to visualize the activating effect of MCHR1 antagonists. To investigate the latter, the most used PET-tracer for BAT imaging, [<sup>18</sup>F]FDG<sup>57</sup> was applied. Interestingly, we observed an enhanced [<sup>18</sup>F]FDG uptake in BAT after administration of SNAP-7941 (15 mg/kg (BW)) in rats (Fig. 7A). [<sup>18</sup>F]FDG uptake in BAT as a measure for BAT activity is also enhanced by the ADRB3 agonist CL316,243, as reported by Mirbolooki *et al.*<sup>58</sup> Consequently, one could assume that both ADRB3 agonism and MCHR1 antagonism have the same activating effect on BAT. The mechanism of BAT activation through MCHR1 antagonists has been previously postulated by Ito *et al.*<sup>31</sup> However, since SNAP-7941 also binds to the ADRB3 when administered in a high concentration,<sup>45</sup> we cannot exclude that the observed BAT activation is at least partially attributed to ADRB3 agonism.

As the uptake of glucose in BAT is strongly enhanced during thermogenesis, activation of BAT is a prerequisite for [<sup>18</sup>F]FDG uptake. BAT mass and activation can be modulated by various factors, such as sex, age, and body mass. Most importantly, BAT is stimulated by low temperatures.<sup>13</sup> Thus, in preclinical studies, which investigate BAT with [<sup>18</sup>F]FDG, rodents are usually cooled down to 4–8 °C for several hours before the  $\mu$ PET scan to enable [<sup>18</sup>F]FDG uptake in BAT. As we did not aim to study cold-induced BAT activation, animals were not cooled down prior to the [<sup>18</sup>F]FDG scan. However, in order to provoke enough [<sup>18</sup>F]FDG uptake to study the effects of SNAP-7941 addition, animals were

not actively warmed during the dynamic  $\mu$ PET scan.

Regarding the experiments with [<sup>11</sup>C]SNAP-7941, we observed tracer uptake in BAT of rats warmed with a 37 °C positioning bed during the scan. In contrast to [<sup>18</sup>F]FDG, the uptake of [<sup>11</sup>C]SNAP-7941 is based on specific receptor binding and does not reflect glucose turnover. Moreover, *ex vivo* metabolite analyses demonstrated the stability of [<sup>11</sup>C]SNAP-7941 in BAT, therefore, imaging results are not affected by radiometabolites.

Even though the previously mentioned *in vitro* preblocking experiments showed a significant reduction in [<sup>11</sup>C]SNAP-7941 uptake by SNAP-7941, *in vitro* competition experiments showed only marginal displacement (unpublished data). Nevertheless, we decided to conduct competition experiments to determine specific binding *in vivo* in order to observe effects within the same individual, thereby avoiding any influence of biodiversity. Paradoxically, in these studies, SNAP-7941 did not decrease [<sup>11</sup>C]SNAP-7941 uptake in BAT, as one would anticipate, but led to a binding enhancement (Fig. 8B). Furthermore, an enhancement of [<sup>11</sup>C]SNAP-7941 uptake was not detected if the basal uptake in BAT was already high (initial SUV > 2.5). The same pattern of [<sup>11</sup>C]SNAP-7941 uptake in BAT was observed after the administration of CL316,243 (Fig. 8A). ADRB3 agonism is known to increase blood flow, and BAT is highly vascularized. However, the plateau of *in vivo* [<sup>11</sup>C]SNAP-7941 uptake after SNAP-7941 or CL316,243 addition (Fig. 8A and B) is more likely to reflect specific binding as there are no redistribution effects. In the case of high basal uptake, the addition of nonradioactive SNAP-7941 led to either slight displacement or had no effect (data not shown). When comparing those animals' age, weight, time of measurement, molar activity of the tracer, and temperature of the positioning bed, no apparent differences were observed. However, we observed heterogeneous MCHR1 expression in BAT among the tested rats (Fig. 6C–E). Such diverging expression levels could explain a varying initial [<sup>11</sup>C]SNAP-7941 uptake. Additionally, even though the *in vivo* measurements were following a standardized protocol, we cannot exclude that the animals were in different physiological conditions causing these diverging effects. Regarding the shape of TACs, an enhancement of both [<sup>18</sup>F]FDG and [<sup>11</sup>C]SNAP-7941



uptake after SNAP-7941/CL316,243 addition is clearly visible at a first glance. Yet, if one looks closer, SNAP-7941 causes a comparatively slow and continuous increase of [ $^{18}\text{F}$ ]FDG uptake in all studied rats, which probably reflects BAT activation triggered by SNAP-7941 binding to the ADRB3. In the case of [ $^{11}\text{C}$ ]SNAP-7941, however, SNAP-7941/CL316,243 addition causes a sharp increase in BAT uptake that is rapidly reaching a plateau and can only be observed in animals with low basal uptake. The difference in TACs could be explained by [ $^{18}\text{F}$ ]FDG uptake reflecting metabolism (BAT activation) and [ $^{11}\text{C}$ ]SNAP-7941 uptake depicting receptor binding. One cannot exclude that the enhancement of [ $^{11}\text{C}$ ]SNAP-7941 BAT uptake after SNAP-7941 or CL316,243 addition might root in some coupling between the ADRB3 and MCHR1, given that SNAP-7941 binds to the ADRB3 in higher concentrations and has the same enhancing effect as CL316,243, a known ADRB3 agonist. This supposed link could be supported by our RT-qPCR data of cultured human precursor adipocytes, where chronic treatment with CL316,243 significantly increases *MCHR1* mRNA expression (Fig. 5A). Although this strongly suggests a connection between BAT activation and MCHR1 expression, the observed mechanism cannot explain the prompt increase in [ $^{11}\text{C}$ ]SNAP-7941 uptake in BAT through ADRB3 agonism since transcriptional effects can be excluded in this short time frame. However, MCHR1 is not only localized in the cell membranes; also MCHR1-containing vesicles have been reported after receptor internalization.<sup>59,60</sup> The rapid nature of the observed effects of SNAP-7941 and CL316,243 on [ $^{11}\text{C}$ ]SNAP-7941 BAT uptake could, therefore, be explained by MCHR1 resurfacing. Furthermore, neither the exact binding mechanism of SNAP-7941 nor an interaction of SNAP-7941 and CL316,243 with other BAT-specific receptors is known. Additionally, preliminary competition experiments with a structurally different MCHR1 antagonist GW-803430 showed similar effects on [ $^{11}\text{C}$ ]SNAP-7941 uptake in rat BAT (unpublished data), proposing a general link between MCHR1 antagonists and the  $\beta$ -adrenergic system. BAT physiology is influenced by a plethora of factors, including volatile anesthetics like isoflurane<sup>61</sup> applied in this study, and the identification of the underlying mechanism provoking these effects remains an open challenge.

In conclusion, the previously presumed MCHR1 expression in BAT was more directly demonstrated in rodents and humans. This points to a direct action of MCH in BAT and emphasizes the physiological role of BAT as an endocrine organ. Although MCHR1 is present in BAT, we do not yet fully understand its involvement in BAT physiology and its effect on the organism's metabolic network. That, as well as the link between MCHR1 and the metabolically relevant adrenergic receptors in BAT, should be subject to further investigations. In the era of precision and personalized medicine, whole-body PET can help unwind the physiological connections of BAT and organ crosstalks. To our knowledge, [ $^{18}\text{F}$ ]FDG can fail as a surrogate of BAT thermogenesis and is, as a tracer reflecting glucose metabolism in general, highly unspecific. Therefore, an alternative PET-tracer for BAT imaging is of interest. As BAT has become a pharmacological target to treat metabolic diseases, the detection of MCHR1 in BAT may be another component that could help to curb the obesity pandemic.

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## Author contributions

C.P., E.M.K., and M.M. conceived the study and designed the experiments. C.P., E.M.K., T.B., O.C.K., M.D., C.V., and M.Z. performed the experiments. C.P. performed the radiosyntheses of [ $^{11}\text{C}$ ]SNAP-7941. G.E. designed the primers for RT-qPCR, helped with the establishment of the protocol for RT-qPCR, and interpretation of the data. C.T.H. and C.S. provided the human fat depot samples. K.P. and H.S. synthesized the precursor compound for radiolabeling and standard compound. M.Z. did the data postprocessing of the  $\mu\text{PET}/\text{CT}$  scans and statistical analysis. C.P., E.M.K., and O.C.K. analyzed the data and composed the draft. M.M.,

G.E., W.W., F.W.K., C.F., T.S., M.H., and H.V. helped with outcome interpretation and reviewed the manuscript. All authors have given approval to the final version of the manuscript.

## Supporting information

Additional supporting information may be found in the online version of this article.

**Figure S1.** Total protein staining with Ponceau S corresponding to western blots depicted in Figure 6.

**Figure S2.** Example of rat RT-qPCR melting curves.

**Figure S3.** Example of mouse RT-qPCR melting curves.

**Figure S4.** The  $\Delta C_t$  values (*MCHR1-36B4*) for human subcutaneous fat probes ( $n = 18$ ) and probes of adipose tissue surrounding the carotid sheath ( $n = 6$ ), thymus ( $n = 19$ ), and longus colli muscle ( $n = 15$ ).

**Figure S5.** A representative HPLC chromatogram of rat BAT 45 min after [ $^{11}\text{C}$ ]SNAP-7941 application (top: UV channel, bottom: radioactivity channel). The analyzed sample was spiked with the reference compound SNAP-7941.

## Supplementary Material File S1.

## Competing interests

The authors declare no competing interests.

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## Supporting Information

### Primer sequences – rodent

#### ***$\beta$ -actin***

mActb\_fo: GAAGATCTGGCACCACACCT

mActb\_re: GTACATGGCTGGGGTGTGTA

rActb\_fo: CACGGCATTGTCACCAACTG

rActb\_re: GTACATGGCTGGGGTGTGTA

#### ***Mchr1***

mMchr1\_fo: GCATGGATCTGCAAGCCTCG

mMchr1\_re: TGGTACCAAACACTGAAGGCA

rMchr1\_fo: AATGCCAGCAACATCTCCGA

rMchr1\_re: CTGCACCAGTGTAGCTTGGA

### Primer sequences – human

#### ***36B4***

h36B4\_fo: CAACCCAGCTCTGGAGAAAC

h36B4\_re: GTGAGGTCCTCCTTGGTGAA

#### ***UCP1***

hUCP1\_fo: GTGTGCCCAACTGTGCAATG

hUCP1\_re: CCAGGATCCAAGTCGCAAGA

#### ***MCHR1***

hMchr1\_fo: CTGATGGCCCCGATAACCTC

hMchr1\_re: TTCTTCACGACCGCGAAGAT

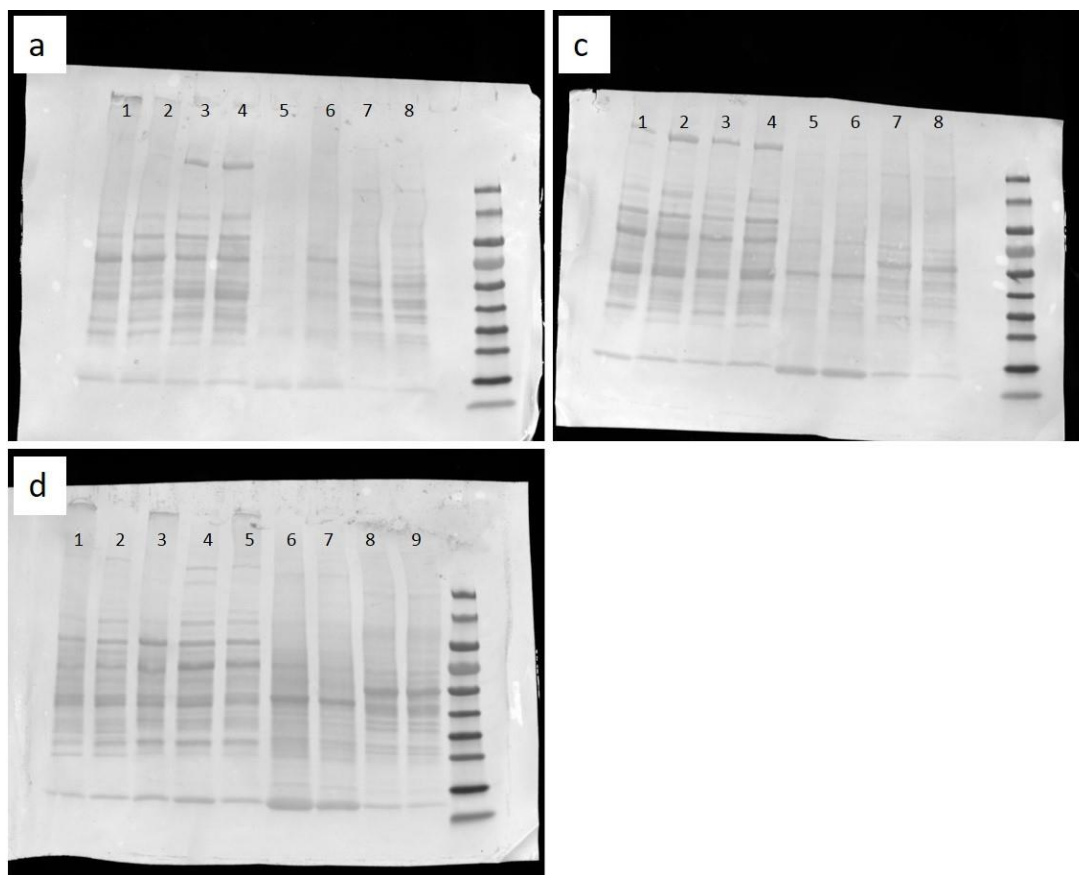
## **Human biopsy material**

### **Inclusion criteria:**

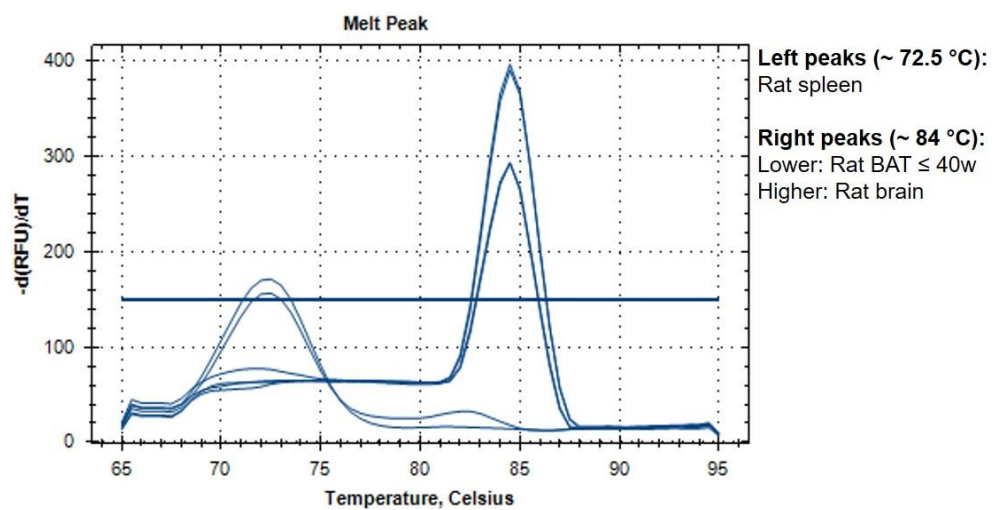
- Primary hyperparathyroidism requiring parathyroid surgery or elective thyroidectomy because of benign goiter, Graves' disease, autonomous adenoma or suspected thyroid carcinoma.
- Age 20 – 55 years

### **Exclusion criteria:**

- Untreated hyper- or hypothyroidism
- Current oncologic disease (other than thyroid carcinoma)
- Pregnancy
- Inflammatory bowel disease
- Active infection or use of antibiotics in the past 3 months
- Autoimmune or rheumatologic diseases that require immunosuppressive treatment
- For subjects participating in PET/CT studies additional exclusion criteria apply:
  - Use of medications which might influence BAT activity such as beta-blockers, alpha-blockers, or rilmenidin
  - Diabetes with antidiabetic medication or fasting glucose concentrations > 120 mg/dl

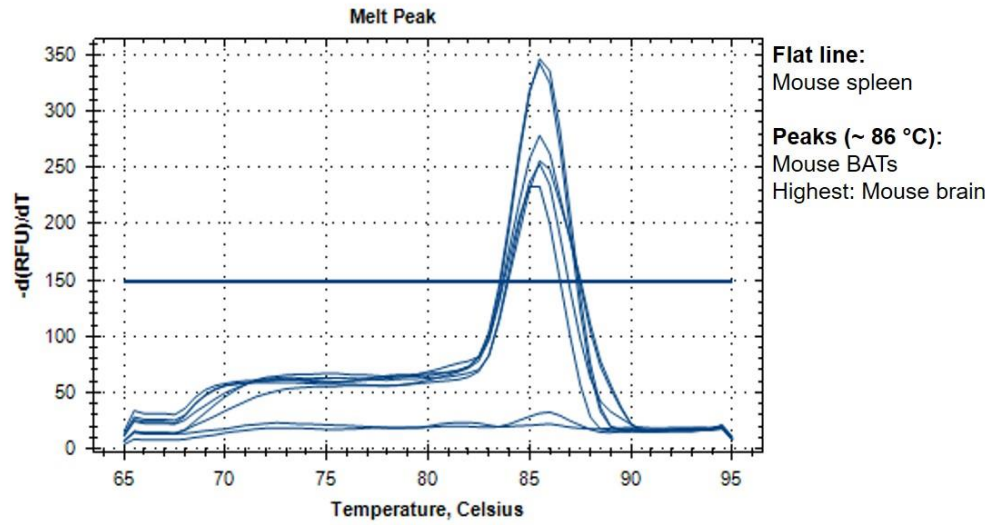


**Figure S1.** Total protein staining with Ponceau S corresponding to western blots depicted in Figure 6.

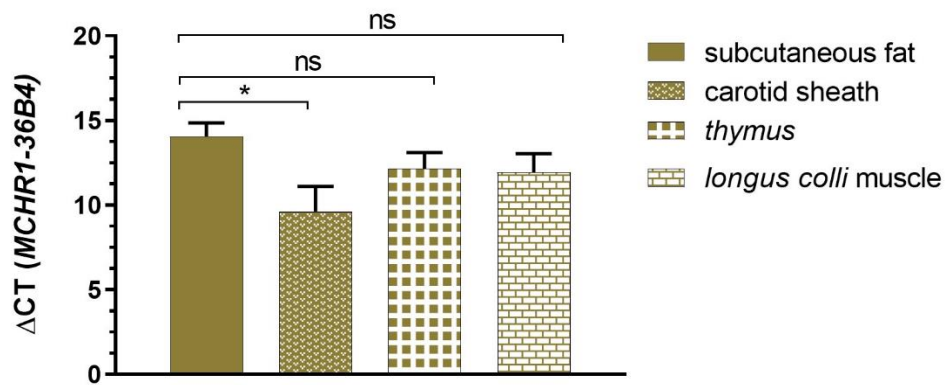


**Figure S2.** Example of rat RT-qPCR melting curves.



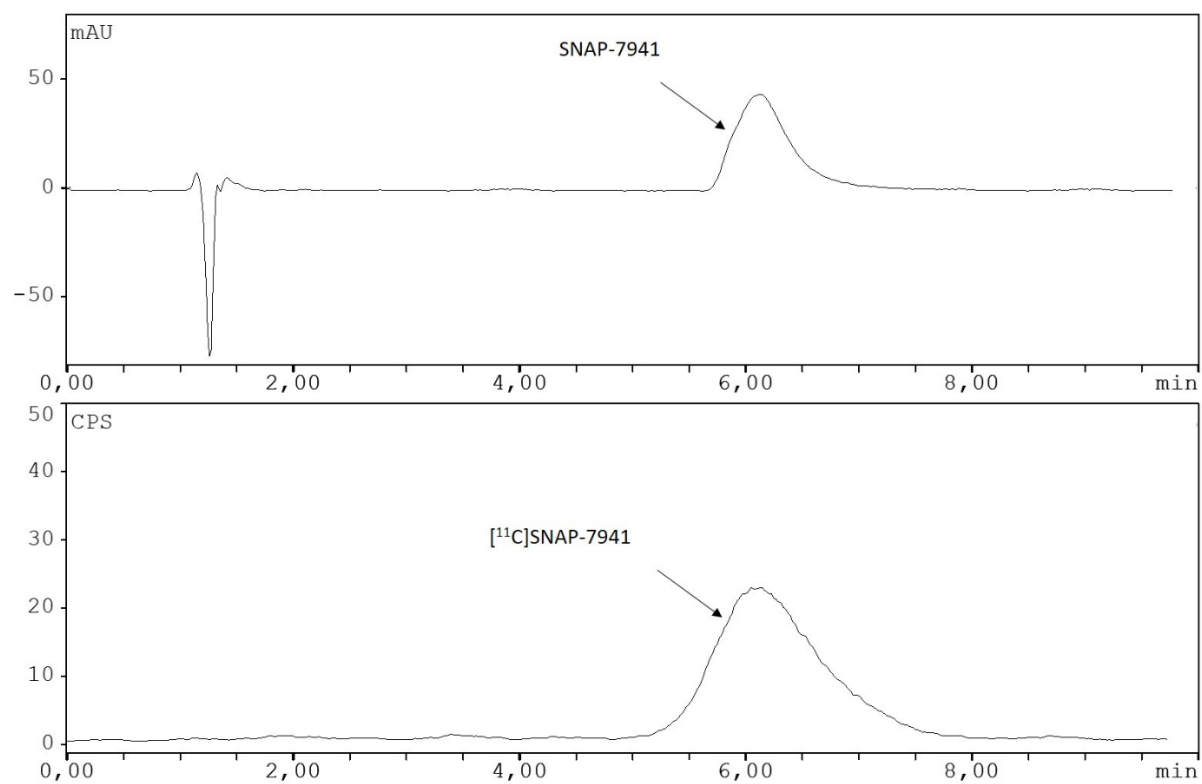


**Figure S3.** Example of mouse RT-qPCR melting curves.



**Figure S4.** The  $\Delta Ct$  values (*MCHR1-36B4*) for human subcutaneous fat probes ( $n = 18$ ) and probes of adipose tissue surrounding the carotid sheath ( $n = 6$ ), thymus ( $n = 19$ ), and longus colli muscle ( $n = 15$ ).





**Figure S5.** A representative HPLC chromatogram of rat BAT 45 min after [<sup>11</sup>C]SNAP-7941 application (top: UV channel, bottom: radioactivity channel). The analyzed sample was spiked with the reference compound SNAP-7941.

## 4. SUMMARY AND DISCUSSION

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This thesis describes new advances in the use of 2-[<sup>18</sup>F]FDG for the assessment of *in vitro* and *in vivo* energy metabolism, radiometabolites beyond 2-[<sup>18</sup>F]FDG-6-P and implications of downstream metabolism for imaging, as well as receptor-based alternatives for 2-[<sup>18</sup>F]FDG.

### 4.1 2-[<sup>18</sup>F]FDG for studying cellular metabolism and new approaches for absolute quantification using an IDIF

Gilbert's syndrome, a condition of mild hyperbilirubinemia, has been shown to reduce the risk for diabetes mellitus type 2 (DMT2) (261–264) and has been associated with a beneficial health profile including a lower body mass index and reduced blood lipids (265), as well as a lower fasting blood glucose level and reduced insulin resistance (266,267).

In the manuscript “Inhibition of lipid accumulation in skeletal muscle and liver cells: A protective mechanism of bilirubin against diabetes mellitus type 2”, 2-[<sup>18</sup>F]FDG was used as a tool to assess the energy metabolism of skeletal muscle and hepatoblastoma cells treated with unconjugated bilirubin (UCB), mimicking mild hyperbilirubinemia. 2-[<sup>18</sup>F]FDG uptake, as evaluated with accumulation experiments and real-time kinetic measurements using LigandTracer® technology, was mildly increased in both cell lines upon UCB treatment. Furthermore, to our knowledge, this was the first mechanistic assessment of UCB effects on glucose uptake. While FFA uptake was also enhanced upon treatment, lipid accumulation in the cells was reduced, suggesting that UCB reduces ectopic lipid deposition which promotes DMT2 (268,269). This could be due to the fact that UCB is reportedly an activator of the peroxisome proliferator-activated receptor alpha (270), suggesting that the divergence in lipid uptake and accumulation is based on increased lipid oxidation. In general, these *in vitro* results represent a new, cell-based approach to mechanistically study the beneficial effects of mild hyperbilirubinemia on blood glucose profile and ectopic lipid deposition in humans. Of note, the research question needs to be addressed in an *in vivo* setting in the future to validate the effects observed *in vitro*.

The next manuscript “Towards quantitative [<sup>18</sup>F]FDG-PET/MRI of the brain: Automated MR-driven calculation of an image-derived input function for the non-invasive determination of cerebral glucose metabolic rates” presented a new approach for a non-invasive absolute

quantification of cerebral  $MR_{gluc}$  from 2-[ $^{18}F$ ]FDG PET using an IDIF from the internal CA. While semiquantitative methods are convenient and frequently applied, the SUV is a highly simplified parameter as elaborated in the introduction. Hence, results might be sub-optimal (70,271) or deceptive (272), fostering interest in easily applicable, standardized approaches for absolute quantification of  $MR_{gluc}$ . In this study, a test-retest dynamic 2-[ $^{18}F$ ]FDG scan was performed with ten healthy individuals on a fully-integrated PET/MRI scanner and blood sampling was carried out to determine the AIF as reference. The area under the curves of the IDIF and AIF, as well as cerebral  $MR_{gluc}$  across all examined brain regions determined with either of the IFs were shown to be comparable.

#### 4.2 2-[ $^{18}F$ ]FDG metabolism beyond 2-[ $^{18}F$ ]FDG-6-P and its impact on tracer accumulation

The manuscripts “If it works, don't touch it? A cell-based approach to studying 2-[ $^{18}F$ ]FDG metabolism” and “A fingerprint of 2-[ $^{18}F$ ]FDG radiometabolites - how tissue-specific metabolism beyond 2-[ $^{18}F$ ]FDG-6-phosphate could affect tracer accumulation” provide a detailed description of metabolism beyond 2-[ $^{18}F$ ]FDG-6-P *in vitro* and *in vivo*, questioning the exact trapping mechanism of 2-[ $^{18}F$ ]FDG. On the basis of a method by Rokka *et al.* (132), who had detected the radiometabolites 2-[ $^{18}F$ ]FD-PGL and 2-[ $^{18}F$ ]FDG-1,6-P<sub>2</sub> with a new high pressure liquid chromatography (HPLC) method with good resolution based on Fedders *et al.* (135), three additional radiometabolites in the HPLC chromatogram were identified, enabling a more comprehensive view on 2-[ $^{18}F$ ]FDG metabolism in future studies.

*In vitro* 2-[ $^{18}F$ ]FDG metabolism was demonstrated to be highly dependent on the glucose content of the media and the origin (tumor entity) of human cancer cell lines. To study it in a physiological context, 2-[ $^{18}F$ ]FDG metabolism was then evaluated at three time-points in various organs and two different tumors of a murine xenograft model, using two of the cancer cell lines studied *in vitro*. Therefore, also translational aspects could be assessed. For example, metabolism beyond 2-[ $^{18}F$ ]FDG-6-P was higher in tumors than in corresponding cells cultured *in vitro* and other radiometabolites were dominant. In general, 2-[ $^{18}F$ ]FDG was metabolized substantially beyond 2-[ $^{18}F$ ]FDG-6-P *in vivo*, the extent of metabolism increased with time and the pattern depended on the tissue studied. Most importantly, levels of the radiometabolites 2-[ $^{18}F$ ]FDG-1-P, produced in glycogen

metabolism, and 2-[<sup>18</sup>F]FD-PGL/PG1, produced in the oxPPP, correlated inversely with total tracer accumulation across all examined time-points and tissues. This main finding hints at the importance of specific pathways for 2-[<sup>18</sup>F]FDG accumulation beyond phosphorylation by hexokinase. As the relevance of the oxPPP enzyme H6PD for 2-[<sup>18</sup>F]FDG accumulation was suggested in recent studies (99,100,145,172), we also performed mechanistic *in vitro* research on the role of the oxPPP enzyme H6PD and corresponding G6PD. Our data from various assays suggest that only H6PD contributes to total 2-[<sup>18</sup>F]FDG accumulation as postulated elsewhere (100,145,172), but the tracer might also be metabolized by G6PD. An inverse relationship between H6PD expression and 2-[<sup>18</sup>F]FDG accumulation was observed in our study, which is in particular contrast to Marini *et al.* who reported similar knockdown studies using siRNA (172). Although other reasons cannot be ruled out, the group used glucose-free medium, which could per se induce metabolic alterations, whereas we wanted to maintain a more “physiologic” setting in all *in vitro* assays. In general, given our multilayered *in vitro* data, the central role of the PPP in cell metabolism and the ill-defined intracellular location of G6PD and H6PD (162,163), we propose that the relationship between the oxPPP and 2-[<sup>18</sup>F]FDG trapping might be more complex than originally thought and requires further experimental evaluation.

Our studies provide a detailed description of 2-[<sup>18</sup>F]FDG metabolism over time combining tumor cell lines, corresponding tumors and rodent tissue specifically using the radioactive form of the tracer and hence trace amounts that do not interfere with metabolism itself. In line with other *in vitro* studies using human endothelial cells (134), human monocyte-derived macrophages (131), or human thyroid cells (138), 2-[<sup>18</sup>F]FDG-1,6-P<sub>2</sub> was a dominant radiometabolite in two out of three tested human cancer cell lines in our *in vitro* studies. *Ex vivo*, the most striking difference to other studies explicitly using the radiotracer was the high abundance of 2-[<sup>18</sup>F]FDM-6-P in the majority of studied tissues. To our knowledge, this radiometabolite was not described in any of the preceding *in vitro* or *in vivo* studies on 2-[<sup>18</sup>F]FDG metabolism in human cells or animals, whereas 2-FDM-6-P and other 2-FDM-based metabolites were prominent in <sup>19</sup>F NMR studies. The reason is difficult to decipher, as a plethora of parameters like the quantity of the probe (especially 2-FDG vs. 2-[<sup>18</sup>F]FDG), the rodent species or strain, the feeding status, the studied time-points, tissue processing or anesthesia might change the metabolic pattern of the tracer. Still, for some studies, it

seems clear that this radiometabolite was simply not characterized and hence not described (135,139).

Based on our main finding for the first time correlating individual 2-[<sup>18</sup>F]FDG radiometabolites and total tracer accumulation, assessing the influence of downstream metabolism (indicated by the presence of specific radiometabolites) on tracer kinetics was the logical next step. In more recent studies on the importance of the ER and H6PD for 2-[<sup>18</sup>F]FDG accumulation, suggesting a 3-compartment model, actual radiometabolites were not determined (99,100). To our knowledge, the only other study specifically looking into the effect of the radiotracer's metabolism beyond 2-[<sup>18</sup>F]FDG-6-P on tracer kinetics was done by Bender *et al.* in pig liver. They presented 2-[<sup>18</sup>F]FD-PGL/PG1 as a quantitatively relevant radiometabolite, tentatively suggesting an extended pharmacokinetic model for studies  $\geq 90$  min (126) in resonance with previous <sup>19</sup>F NMR studies that had suggested that the current kinetic models might be inaccurate for certain applications (115,116). However, in our case, only a limited number of  $\mu$ PET/CT scans could be performed at that time, thus the generated imaging data was too little to perform robust calculations in that context. Concerning future studies, we further acknowledge that anesthesia may have critically altered the observed pattern and that the provided “fingerprint” of radiometabolites likely does not translate to other rodent species or strains. For example, metabolism beyond 2-[<sup>18</sup>F]FDG-6-P assessed with HPLC was previously shown to be approximately 6 times higher in mouse than in rat liver (132). Anesthesia can sensitively alter metabolism (273) and an influence of phenobarbital on the 2-[<sup>18</sup>F]FDG metabolic pattern was previously reported for mouse brain (117). Still, these discussion points in turn imply that a comprehensive understanding of downstream 2-[<sup>18</sup>F]FDG metabolism might also enable the preclinical assessment of differences between species and metabolic effects of anesthesia *via* the 2-[<sup>18</sup>F]FDG radiometabolic pattern.

While our results do not present a causal relationship, the inverse correlation between 2-[<sup>18</sup>F]FDG accumulation and metabolism *via* glycogen metabolism or the oxPPP could add to a better understanding for less 2-[<sup>18</sup>F]FDG avid tumors beyond GLUT and HK expression. Differences in tumor metabolism beyond 2-[<sup>18</sup>F]FDG-6-P were already previously described for two different murine tumor allograft models (137). Building on that, our results further suggest that by identifying and characterizing 2-[<sup>18</sup>F]FDG radiometabolites, 2-[<sup>18</sup>F]FDG might be used to study tumor metabolism in a preclinical setting, which opens up new

possibilities for getting more specific with an unspecific tracer. With increasingly sensitive detectors and advanced, complementary methods, more radiometabolites might be detected and identified, enabling to better characterize the phenotype of tumors and tissues complementing “omics techniques”, such as transcriptomics or metabolomics. As the determination of radiometabolites in *ex vivo* human tissue is hardly possible on a large scale, “omics” techniques could also be used in terms of translating our findings to humans. Biopsy material of tumors could be characterized following 2-[<sup>18</sup>F]FDG imaging using, e.g., “omics” techniques and activity assays of enzymes involved in 2-[<sup>18</sup>F]FDG metabolism to further link 2-[<sup>18</sup>F]FDG accumulation to specific metabolic conditions and specific cells. Additionally, understanding 2-[<sup>18</sup>F]FDG metabolism might also help to further differentiate between malignant lesions and inflammation.

#### 4.3 [<sup>11</sup>C]SNAP-7941 as an alternative to 2-[<sup>18</sup>F]FDG for BAT imaging

Following  $\mu$ PET/CT-scans with the previously described MCHR1-tracer [<sup>11</sup>C]SNAP-7941 (255–258), the expression of the MCHR1 was demonstrated for the first time in BAT of rodents and humans in the manuscript “Discovery of melanin-concentrating hormone receptor 1 in brown adipose tissue”. After the discovery of BAT in adults through 2-[<sup>18</sup>F]FDG PET/CT, this is another example of how PET imaging can drive discoveries, as well as the understanding of physiologic processes. Furthermore, it was shown that MCHR1 expression in cultured human adipocytes was inducible upon stimulation with CL316,243, an ADRB3 agonist, suggesting a link between BAT activation *via* ADRB3 and MCHR1. [<sup>11</sup>C]SNAP-7941  $\mu$ PET/CT-scans with healthy rats showed that BAT imaging is also feasible with warmed, anaesthetized animals. In addition to its specific binding to a receptor, this represents another difference to BAT imaging with 2-[<sup>18</sup>F]FDG. It was previously shown that warmed mice under isoflurane anesthesia, fasted or non-fasted, had no noteworthy 2-[<sup>18</sup>F]FDG uptake in BAT. Only animals that were not actively warmed or anaesthetized showed higher uptake (274). While we generally also observed higher [<sup>11</sup>C]SNAP-7941 uptake upon BAT stimulation, mice could in principle be imaged at “baseline conditions”, representing the possibility to also image inactive BAT. In addition to its possible use as a new BAT tracer, [<sup>11</sup>C]SNAP-7941 might also be a candidate for imaging of inflammatory bowel disease, where the MCHR1 is reportedly overexpressed (275,276).

Despite successful imaging, the addition of SNAP-7941 for displacement surprisingly did not reduce, but enhance [ $^{11}\text{C}$ ]SNAP-7941 uptake in our study. In contrast, tracer binding could be significantly reduced upon pre-blocking with the cold equivalent in previous *in vitro* studies with murine brown adipocytes (277). SNAP-7941 was also shown to bind to ADRB3 when applied in micromolar amounts (277), which could be the reason why in our study also 2- $^{18}\text{F}$ FDG uptake was increased upon SNAP-7941 addition, but the described phenomenon observed with [ $^{11}\text{C}$ ]SNAP-7941 can still not be fully explained. Pretreatment with (S)-propranolol, a nonspecific antagonist of  $\beta$ -adrenoceptors (particularly  $\beta_1$  and  $\beta_2$ ), did not reduce binding of [ $^{11}\text{C}$ ]SNAP-7941 *in vitro* (277). Still, off-target binding of [ $^{11}\text{C}$ ]SNAP-7941 to other receptors *in vivo* cannot be entirely excluded, nor can effects of anesthesia or increased tracer uptake due to enhanced blood flow upon activation of the  $\beta$ -adrenergic system.

## 5. CONCLUSION AND OUTLOOK

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2-[ $^{18}\text{F}$ ]FDG, the most studied PET-tracer, is undoubtedly a suitable tool for the synoptic assessment of energy metabolism in tissue and indispensable for diagnostic imaging, especially for tumors, inflammation and infection. Firstly, regarding new possibilities of using 2-[ $^{18}\text{F}$ ]FDG, the tracer was used as a tool to study metabolic changes in Gilbert's syndrome, a bilirubin metabolism disorder, thereby presenting a new field of application for 2-[ $^{18}\text{F}$ ]FDG imaging. Furthermore, advances in the non-invasive absolute quantification of glucose demand in the brain were described. The use of an IDIF instead of an AIF will facilitate the implementation of future complex and dynamic studies.

Secondly, to provide new insights in the molecular mechanisms of 2-[ $^{18}\text{F}$ ]FDG trapping, we performed research on the tracer's intracellular metabolism. Importantly, our studies reinforce previous reports that the currently valid description of 2-[ $^{18}\text{F}$ ]FDG accumulation is incomplete and metabolism beyond 2-[ $^{18}\text{F}$ ]FDG-6-P might influence imaging. Most importantly, higher levels of radiometabolites representing glycogen metabolism or the oxPPP correlated inversely with tracer accumulation. This suggests that metabolism beyond 2-[ $^{18}\text{F}$ ]FDG-6-P matters and new kinetic models including glucose metabolic pathways beyond glycolysis might be needed. Given that many radiometabolites can be easily detected with HPLC, we propose that in a preclinical setting, 2-[ $^{18}\text{F}$ ]FDG metabolism could be used in the future as a tool to study the activity of different pathways of carbohydrate metabolism in cellular systems or animal organs. Furthermore, changes in 2-[ $^{18}\text{F}$ ]FDG metabolism might be used as an indicator for treatment response, as already suggested in previous studies using 2-FDG and  $^{19}\text{F}$  NMR. Its broader applicability in this context, as well as the influence of its metabolism on tracer kinetics shall be addressed in future studies. Given the interest in the PPP in oncology research, we further aim to develop an alternative carbohydrate-based PET-tracer to image the non-oxPPP, complementing 2-[ $^{18}\text{F}$ ]FDG imaging.

Lastly, to also deal with the limitations of 2-[ $^{18}\text{F}$ ]FDG imaging, [ $^{11}\text{C}$ ]SNAP-7941 was presented as an alternative tracer for BAT imaging. Since the MCHR1 was described in BAT for the first time, [ $^{11}\text{C}$ ]SNAP-7941 could serve as an additional, receptor-targeting PET-tracer for this tissue. However, its exact binding mechanism in BAT, especially addressing the increase in [ $^{11}\text{C}$ ]SNAP-7941 uptake in displacement studies, needs to be elucidated in a



follow-up preclinical study. Furthermore, the relevance of [ $^{11}\text{C}$ ]SNAP-7941 for BAT imaging in humans should be assessed in a first-in-human study that is currently being planned. As the MCHR1 is a central regulator for energy metabolism, TBP using [ $^{11}\text{C}$ ]SNAP-7941 could be used to study organ axes involved in, e.g., obesity, diabetes, or appetite regulation.

Despite the tracer's limitations, the demand for 2- $^{18}\text{F}$ FDG will stay high regardless of the emergence of specific, receptor-based radiopharmaceutical given its high worldwide accessibility and versatility. Recent advances in PET/CT scanning technology provide more sensitive scanners that cover the entire body. This opens up new opportunities to study organ crosstalk, furthering the characterization and monitoring of diseases, including metabolic disorders such as Gilbert's syndrome. In this context, approaches to non-invasively absolutely quantify 2- $^{18}\text{F}$ FDG accumulation and a better understanding of the impact of tracer metabolism could improve the accurate interpretation and quantification of 2- $^{18}\text{F}$ FDG imaging.

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## LIST OF ABBREVIATIONS

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|                          |                                                               |
|--------------------------|---------------------------------------------------------------|
| AIF                      | Arterial input function                                       |
| ADRB3                    | $\beta$ 3-adrenoceptor                                        |
| APS                      | Atypical parkinsonian syndromes                               |
| ATP                      | Adenosine triphosphate                                        |
| BAT                      | Brown adipose tissue                                          |
| BNL                      | Brookhaven National Laboratory                                |
| CA                       | Carotid arteries                                              |
| CT                       | Computed tomography                                           |
| DHEA                     | Dehydroepiandrosterone                                        |
| DMT2                     | Diabetes mellitus type 2                                      |
| DTPI                     | Dual-time-point imaging                                       |
| 2-[ <sup>14</sup> C]DG   | 2-deoxy-D-[ <sup>14</sup> C]glucose                           |
| ER                       | Endoplasmic reticulum                                         |
| $\varphi$                | Fraction of glucose introduced into glycolysis                |
| FFA                      | Free fatty acids                                              |
| [ <sup>18</sup> F]FTHA   | 14(R,S)-[ <sup>18</sup> F]Fluoro-6-thia-heptadecanoic acid    |
| <sup>19</sup> F NMR      | Fluorine-19 nuclear magnetic resonance spectroscopy           |
| F6P                      | Fructose-6-phosphate                                          |
| 2-FDG                    | 2-fluoro-2-deoxy-D-glucose                                    |
| 2-FDG-1-P                | 2-fluoro-2-deoxy-D-glucose-1-phosphate                        |
| 2-FDG-6-P                | 2-fluoro-2-deoxy-D-glucose-6-phosphate                        |
| 2-FDG-1,6-P <sub>2</sub> | 2-fluoro-2-deoxy-D-glucose-1,6-bisphosphate                   |
| 2-FDM                    | 2-fluoro-2-deoxy-D-mannose                                    |
| 2-FDM-6-P                | 2-fluoro-2-deoxy-D-mannose-6-phosphate                        |
| 2-FD-PGL/PG1             | 2-fluoro-2-deoxy-6-phosphogluconolactone/<br>phosphogluconate |
| GLUT                     | Glucose transporter                                           |
| G3P                      | Glyceraldehyde-3-phosphate                                    |
| G6Pase                   | Glucose-6-phosphatase                                         |
| G6PD                     | Glucose-6-phosphate dehydrogenase                             |

|                    |                                                                             |
|--------------------|-----------------------------------------------------------------------------|
| HK                 | Hexokinase                                                                  |
| HPLC               | High pressure liquid chromatography                                         |
| H6PD               | Hexose-6-phosphate dehydrogenase                                            |
| IDIF               | Image-derived input function                                                |
| IF                 | Input function                                                              |
| k                  | Rate constant for pharmacokinetic processes                                 |
| keV                | Kiloelectron volt                                                           |
| K <sub>i</sub>     | Net influx rate of the radiotracer                                          |
| k <sub>loss</sub>  | Net efflux rate of the tracer                                               |
| K <sub>m</sub>     | Michaelis-Menten constant for glucose phosphorylation                       |
| K <sub>m</sub> '   | Michaelis-Menten constant for 2-[ <sup>14</sup> C]DG phosphorylation        |
| λ                  | Ratio of the distribution volume between 2-[ <sup>14</sup> C]DG and glucose |
| LC                 | Lumped constant                                                             |
| MCH                | Melanin-concentrating hormone                                               |
| MCHR               | Melanin-concentrating hormone receptor                                      |
| MR <sub>FDG</sub>  | FDG metabolic rate                                                          |
| MR <sub>gluc</sub> | Glucose metabolic rate                                                      |
| MRI                | Magnetic resonance imaging                                                  |
| mTOR               | Mammalian target of rapamycin complex 1                                     |
| NADP <sup>+</sup>  | Nicotinamide adenine dinucleotide phosphate                                 |
| NADPH              | Reduced NADP <sup>+</sup>                                                   |
| NDP-2-FDG          | FDG bound to nucleoside diphosphates                                        |
| NIH                | National Institutes of Health                                               |
| non-oxPPP          | Non-oxidative branch of the pentose phosphate pathway                       |
| NST                | Non-shivering thermogenesis                                                 |
| oxPPP              | Oxidative branch of the pentose phosphate pathway                           |
| PET                | Positron emission tomography                                                |
| PGI                | Phosphoglucose isomerase                                                    |
| PGM                | Phosphoglucomutase                                                          |
| P-gp               | P-glycoprotein                                                              |
| PPP                | Pentose phosphate pathway                                                   |

|                     |                                                            |
|---------------------|------------------------------------------------------------|
| 6PGL                | 6-phosphogluconolactonase                                  |
| ROI                 | Region of interest                                         |
| SUV                 | Standard uptake volume                                     |
| SUV <sub>max</sub>  | Highest voxel value of a region of interest                |
| SUV <sub>mean</sub> | Mean voxel value of a region of interest                   |
| TBP                 | Total-body PET                                             |
| UCB                 | Unconjugated bilirubin                                     |
| UCP1                | Uncoupling protein 1                                       |
| UDP-2-FDG           | FDG bound to uridine diphosphate                           |
| UP                  | University of Pennsylvania                                 |
| V <sub>max</sub>    | Maximum velocity of glucose phosphorylation                |
| V <sub>max</sub> '  | Maximum velocity of 2-[ <sup>14</sup> C]DG phosphorylation |
| WAT                 | White adipose tissue                                       |

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