Executive summary:

Drug resistance is an important clinical problem. Globally, 50,000,000 people have epilepsy: current treatments are ineffective in 30%. Drug resistance is associated with higher rates of: unemployment, disadvantage, injury, somatic co-morbidity (affecting many body systems), psychiatric disorder, cognitive/memory decline, sudden unexplained death and other premature mortality, making chronic epilepsy pervasive and pernicious, with seizures being just one 'tip-of-the-iceberg' manifestation. Availability of several new AEDs has not solved drug resistance. We do not understand drug resistance.

One concept that has arisen for drug resistance is the transporter hypothesis, which maintains that broad resistance to antiepileptic drugs might relate to over-activity of non-specific transporter mechanisms that reduce the local concentration of AEDs within the brain tissue below a therapeutic level. The aims of EURIPIDES were to develop in-vivo imaging biomarker of multidrug transporter function as a generic tool for the prediction, diagnosis, monitoring and prognosis of major CNS diseases. EURIPIDES involved a multidisciplinary approach, based on integration of the clinical and basic science domains and on the synergistic combination of neurophysiological and biomolecular imaging methods.

In a comprehensive study of P-glycoprotein (Pgp) inhibitor based positron emission tomography (PET) tracers, we clarified that [11C]laniquidar, [11C]elacridar and [11C]tariquidar in tracer concentrations are recognised by Pgp and/or BCRP as substrates. We developed and validated, at least in epileptic animal models, eight [11C]-and three [18F]-PET radiotracers aimed to be either Pgp substrates or inhibitors, with further development of longer-lived PET radionuclides aimed at novel ways of imaging Pgp functionality. Biodistribution studies were performed in animal models of naïve, transporter knock-out and epileptic rodents. Even though these highly potent and selective radiolabelled Pgp inhibitors afforded only very low PET signals, three PET tracers ([11C]laniquidar, [11C]tariquidar, [11C]phenytoin) entered trials in humans. Using the established PET radiotracer [11C]verapmail as a model Pgp substrate and the PET receptor ligands and Pgp substrates [18F]MPPF and [11C]flumazenil, we could show that partial inhibition of the blood-brain-barrier (BBB) by administration of half-maximum inhibitor dose allows detection of regional differences in Pgp functionality at the rat BBB.

We successfully translated our findings in animal models to the human condition. Studies with [11C]VPM PET in healthy controls showed that TQD-induced Pgp modulation at the human (BBB) appeared to be transient and its magnitude directly proportionate to serum drug exposure. The optimal analytical model for baseline studies was less suitable for blocking studies, where an additional compartment improves the quality of the fits. Analysis with additional models showed small, but significant differences between patients with Alzheimer's Disease and healthy controls. This is the first direct evidence that Pgp transporter at the BBB are dysfunctional in sporadic AD and suggests that decreased Pgp function may be involved in the pathogenesis of AD. In patients with temporal lobe epilepsy (TLE), the model derived K1 (influx rate constant) values from drug-sensitive patients were higher than those from drug-resistant patients falling, which suggests more efficient Pgp function in drug-refractory patients resulting in lower drug concentrations in the brain. This difference was restricted to the temporal lobes both on the

ipsilateral and contralatral sides. After tariquidar administration, we observed smaller K1 increases in the drug-resistant patients compared to healthy volunteers supporting over expression of Pgp function, with the ipsilateral epileptogenic hippocampus being most significantly affected. Functional in-vivo evidence of over active transporters in drug-resistant compared to drug-sensitive epileptic patients was complimented by histological assays of post-mortem brains and surgically resected epileptic tissue from drug-resistant patients. The change in VPM uptake after Pgp inhibition with TQD in the hippocampus correlated inversely with Pgp-immunopositive labeling in pharmaco-resistant TLE patients who underwent anterior temporal lobe resection for surgical treatment.

In summary, our findings provide evidence supporting the hypothesis of multidrug transporter overexpression as an important mechanism for developing pharmacoresistance in epilepsy. The availability of such imaging biomarkers will support the development of new treatment strategies targeted at multidrug transporter and aimed at reversing pharmacoresistance with selection of optimal patients and assessment of molecular targets. This will lead to improved health care through individualised treatment strategies, and at the same time to a reduction of health care costs by discontinuing ineffective therapies. The stage is thus set for direct translation to clinical trials. We have a tool to identify patients in whom P-glycoprotein overactivity could conceivably contribute to drug resistant epilepsy; the same tool can act to determine whether P-glycoprotein activity has been inhibited in practice as might be intended with treatment with a P-glycoprotein inhibitor such as TQD. Thus EURIPIDES has a lasting legacy, with potential for further research and clinical application.

Project Context and Objectives:
1.2 Summary description of project context and objectives:
Background and Aims
Resistance to drug treatment is an important hurdle in the therapy of many diseases of the central nervous system (CNS). Consequently, there is a pressing need to develop new and more effective treatment strategies. Whilst drug resistance in CNS diseases is multifactorial, there are two broad likely mechanisms:
(1) resistance to the desired pharmacological effects of CNS drugs occurs from alteration in the postulated target(s) – the 'target hypothesis';
(2) inadequate access of CNS drugs to their targets across the blood-brain barrier (BBB) due to the overexpression or overactivity of multdrug transporters that efflux foreign chemicals from the brain – the 'transporter hypothesis'.

Although the brain is among the best perfused organs in the body, drug entry into the brain is limited by the BBB, which is formed by brain endothelial cells that are closely linked by tight junctions (Reichel 2006). Substances can enter the brain either by passive diffusion across the endothelial cell membranes or by selective carrier transport. High lipophilicity is an essential characteristic of a substance to cross the BBB by diffusion. However, there are several examples of lipophilic drugs (e.g. anticancer drugs, antiepileptic drugs, anti-HIV drugs) that have poor access to brain tissue. The presence of multidrug efflux transporters, such as P-glycoprotein (Pgp), breast cancer resistance protein (BCRP) and multidrug resistance proteins (MRPs), at the luminal side of the endothelial cells of the blood capillaries is believed to be the basis for these observations (De Lange 2004, Löscher and Potschka 2005). These transporters are mainly, but not exclusively, members of the ABC (ATP-Binding Cassette) superfamily, including ABCG2 (BCRP), ABCB1 (Pgp), ABCC1 (MRP1), ABCC2 (MRP2).

Multidrug efflux transporters can contribute to drug resistance in brain diseases in two ways:
- by their constitutive expression in the BBB and blood-CSF barrier, they restrict the brain access of many drugs by enhancing drug extrusion from the brain, so that the levels of drugs in the brain cannot become sufficiently high for therapeutic efficacy.
- by intrinsic or acquired over-expression of multidrug transporters in the BBB or brain target tissue limits drug penetration into that tissue. This can result from alterations (such as polymorphisms) in genes that encode multidrug transporters, or from the effects of disease or drug treatment on expression of these transporters. The consequences of intrinsic or acquired over-expression of multidrug transporters depend on the affinity of respective substrates for the efflux transporters.

Over-expression of multidrug transporters at the BBB and beyond will prevent CNS drugs from reaching sufficiently high concentrations in critical cerebral regions despite substantial plasma levels (Sisodya 2003). This may, therefore, account for the failure of multiple diverse CNS drugs in some patients.

Several lines of evidence suggest that changes in Pgp expression and/or function might also play a key role in the causation and pathogenesis of certain neurological disorders, such as AD (Vogelsang et al. 2004, Lee et al. 2004) and epilepsy (Löscher 2002). For instance, regional overexpression of Pgp in the brains of epilepsy patients might contribute to drug resistance by impeding the access of antiepileptic drugs (AED) to

the seizure focus (Löscher & Potschka 2002). These results suggest that Pgp is an attractive pharmacologic target to increase intracerebral penetration of certain drugs, such as anticancer and antiepileptic drugs (Bates 2002, Sisodiya & Bates 2006).

It has not been investigated whether expression of other transporters in the BBB is affected by Pgp inhibition, but the conformation of Pgp may be affected by substrate inhibitors, possibly in a genotype-dependent fashion (Kimchi-Sarfaty et al, 2007). A chronic study in a model of pharmacoresistant epilepsy with a combination of phenobarbital and the Pgp inhibitor TQD did not indicate that tolerance development may be an issue (Brandt et al., 2006). In contrast, development of tolerance to a combination with phenytoin and TQD was described in another chronic epilepsy model (Van Vliet et al., 2006). At present, it cannot be excluded that this is due to an effect on expression of other transporters, or genotype-dependent dynamic alterations in Pgp itself (Kimchi-Safarty et al, 2007).

Overexpression of other transporters in response to Pgp inhibition may occur and remains to be determined. Overexpression of MRP or others in response to blocking Pgp would only be relevant, if the therapeutic drug in question were also transported by these overexpressed transporters. This is unlikely, as for example many antiepileptic drugs (AEDs) are transported by Pgp, but not by MRP1. By combining AEDs with TQD, improved seizure control for several days was evident in the studies of Brandt et al. (2006) and van Vliet et al. (2006).

In this project, we focused initially on Pgp, which is the most-widely studied multidrug transporter, is found in and contributes to the normal BBB, and as efflux transporter, actively transports substrates (including multiple CNS drugs) against concentration gradients from the brain to blood and cerebrospinal fluid. This hampers the build-up of adequate tissue levels of these drugs in the brain, greatly limiting their therapeutic efficacy. As such, the 'transporter hypothesis' of drug resistance is applicable to a broad range of CNS drugs and patients with a variety of CNS diseases who critically depend on these drugs accessing the brain tissue.

Our aim was to develop a generic tool for the prediction, diagnosis, monitoring and prognosis of major CNS diseases, as well as to provide support and guidance for therapeutic interventions.

The project aims were twofold:
(1) to develop radiotracers to image Pgp function and distribution to modulation of Pgp expression,
(2) to validate the use of Pgp radiotracers as in-vivo imaging biomarker by determining the contribution of Pgp overexpression to impaired brain uptake of drugs (pharmacoresistance) for the prediction of therapeutic response, or the contribution of impaired Pgp function to reduced clearance of toxic substances (e.g. ß-amyloid) for the early in-vivo diagnosis of Alzheimer's Disease (AD).

Work strategy and general description

Circumvention of pharmacoresistance, or increasing clearance of drugs from the brain tissues, may involve inhibitors of Pgp transporters or sophisticated alternative therapies, but demonstration of overexpression or underactivity of transporter function is an essential and necessary

first step. An in-vivo imaging biomarker of Pgp function is essential for identifying altered transporter activity in individual patients. If a relation between overexpression and therapy resistance, or in the case of AD underactivity, can be demonstrated, such a biomarker will provide unique and new pathophysiological information, the means for predicting treatment response, or early diagnosis, in individual patients. The availability of a radiotracer for imaging Pgp function in healthy volunteers does not make it a biomarker for drug resistance. In order to validate the usefulness of a biomarker, studies have to be designed for relevant and sensitive clinical populations, e.g. comparative studies in patients with refractory vs responsive CNS diseases.

The sine qua non for transporters to have a role in mediating resistance is that they have an appropriate anatomical distribution in human brain tissue. The mere presence of transporters alone is not a sufficient proof of the transporter hypothesis. Proof is needed that overexpressed transporters can and do efflux CNS drugs away from their site of action to a greater extent in drug-resistant patients than in drug-responsive patients. The inevitable lack of surgically-resected brain tissue from patients with drug-responsive CNS conditions prevents ex-vivo (immunohistochemical) comparison between drug-resistant and drug-responsive patients. Information on increased functionality of efflux transporters across the clinical spectrum compared to normal subjects can only be obtained with experiments in humans in-vivo, to derive conclusive data on the importance of the transporter hypothesis.

Against this background, the availability of non-invasive in-vivo imaging methods that allow for assessing the distribution and function of Pgp in the brain is of vital importance. Based on its unrivalled sensitivity (picomolar concentrations), the most promising approach to address these questions in-vivo in animals and humans are the non-invasive molecular imaging techniques, positron emission tomography (PET) and single photon emission tomography (SPET), together with radiolabelled substrates and inhibitors of Pgp. Many radiotracers have been specifically proposed for studying multidrug transporter protein function in-vivo with PET (Hendrikse et al. 2002, Elsinga et al. 1996, Sun et al. 2003). Radiotracers based on known Pgp substrates have been developed and characterized in-vitro and in-vivo in laboratory animals, including [11C]-daunorubicin, [11C]-colchicine, [18F]-paclitaxel, [11C]-carvedilol, [11C]-loperamide and [11C]-VPM (Elsinga et al. 2004). [11C]-VPM is the best validated PET tracer that has already been used in humans (Kortekaas et al. 2005, Sasongko et al. 2005, Brunner et al. 2005, Toornvliet et al. 2006). Initially, a racemic mixture of [11C]-VPM was used, but more recently enantiomerically pure (R)-[11C]-VPM was developed enabling quantification of its rate of transport into the brain (Lubberink et al. 2007). In addition, several of the commonly used PET radiotracers developed to probe various brain receptor systems have been found a posteriori to be modulated by Pgp (Ishiwata et al. 2007).

Animal tumour models have demonstrated that the PET signal of [11C]-VPM is decreased in Pgp expressing tumours (Hendrikse et al. 1999). Conversely, Hendrikse and colleagues also demonstrated a 10-fold increased brain uptake of [11C]-VPM in Pgp-knockout as compared to wild-type mice (Hendrikse et al. 1998). A quantitatively similar effect has been described in mice by administration of the Pgp inhibitor Cyclosporine A (CsA) (Ishiwata et al. 2007). In humans, Sasongko and co-workers have found a 80% increase in brain concentrations of [11C]-VPM following CsA infusion as compared to baseline scans, which confirmed

that [11C]-VPM is transported by Pgp at the human BBB (Sasongko et al. 2005). Hence there is sufficient evidence that [11C]-VPM is a sensitive tracer of these efflux pumps that are believed to be involved in drug resistance, although at present no distinction can be made between changes in expression and activity. One limitation of [11C]-VPM is its extensive peripheral metabolism, which gives rise to radiolabelled metabolites that are also transported by Pgp. Because this metabolism is variable, they confound the interpretation of the [11C]-VPM PET signal in brain (Luurtsema et al. 2005, Lubberink et al. 2007).

None of the above-mentioned available radiotracers is ideal for PET imaging due to their relatively low sensitivity to observe increased P-gp transporter functionality as they have a low brain uptake already, while moreover their pharmacokinetics and metabolism are complex. A promising alternative to the use of radiolabelled P-gp substrates would be the use of radiolabelled P-gp inhibitors that bind with nanomolar affinity to P-gp without being transported. Such P-gp PET tracers would give a signal increase rather than a reduction in brain regions that have an increased P-gp functionality. The validity of such an approach is exemplified by the utility of PET tracers based on inhibitors for the quantitative visualization of other transporter proteins in the brain, such as dopamine- or serotonin transporters (Volkow 1996, Ginovart et al 2001). From the perspective of bio-mathematical modelling of PET data, the use of an inhibitor is more straightforward than the use of a substrate as the principles of simple ligand-receptor interactions can be applied to the kinetics of inhibitors. Hence we under took the strategy of using [11Claniquidar and [11C]TQD as high affinity imaging ligands of the Pgp transporter.

In addition to using high-affinity Pgp inhibitors as templates for the development of new radiotracers, the development of a low-affinity Pgp substrate tracer would be of considerable interest. The rationale behind this approach is that currently available high-affinity substrate radiotracers ([11C]-VPM, [11C]-loperamide) possess an overall low brain uptake. The resulting low signals hampers the delineation of brain regions with increased transporter expression. Conversely, a radiotracer that is only weakly transported by Pgp and thus possesses per se an appreciable brain uptake, would in principle allow for better delineation of brain regions with reduced substrate uptake due to transporter overexpression. For this approach, phenytoin, an old-generation AED that is still widely used in the clinic would be an interesting candidate compound. It is well established that phenytoin is a Pgp substrate. (Potschka & Loescher 2001, van Vliet et al. 2007b) Phenytoin was labelled with [11C] and evaluated in drug-responsive and drug-sensitive epilepsy patients about 30 years ago. (Baron et al 1983). However, the results of this early study were limited due to the then-available technical standards (low-spatial-resolution PET camera, lack of MR-guided definition of regions of interest, lack of input function and mathematical model for quantification of PET data). In addition to that, radiolabelling of phenytoin, which proceeded via [11C]-phosgene, was not straightforward and afforded the radiotracer in very low radiochemical yields, which made it difficult to scan a sufficient number of subjects. Nowadays, with new radiochemistry techniques (via [11C]CO) higher radiochemical yields and specific activities are expected to be obtained for [11C]-phenytoin.

Management structure and procedures

The Project Coordinator ensured the smooth operation of the project and guaranteed that all efforts were focused towards the objectives. He submitted all required progress reports, deliverables, financial statements to the European Commission, and, with the assistance of GABO he was responsible for the proper use of funds and their transfers to participants. The EURIPIDES office was established by and based at the coordinator in London and at GABO in Munich. The Project Office at the Coordinator was concerned with the scientific management and the co-ordination of all research activities. The Project Office at GABO was responsible for administrative, financial and contractual management and the organisational co-ordination of the project activities.

The Project Governing Board was in charge of the political and strategic orientation of the project and acted as the arbitration body. It met once a year unless the interest of the project required intermediate meetings. The Project Coordination Committee consisted of all work package leaders and the Coordinator and was in charge of monitoring all activities towards the objective of the project in order to deliver as promised, in due time and in the budget. The Project Coordination Committee met every six months during the funding period. Furthermore, a scientific advisory board was implemented to ensure a high standard of research and monitor the progress of the project by taking part in the annual Governing Board Meetings

Objectives of EURIPIDES:

In line with this call's main aim 'to develop highly sensitive agent(s) for established in-vivo imaging modalities able to report and quantify cellular and/or molecular events in several major disease states or disease processes for early in-vivo diagnosis and/or evaluation of response to therapy', using positron emission tomography (PET) and single-photon emission tomography (SPET) in a multidisciplinary approach integrating radiochemistry, molecular and cellular biology, physics, pharmacology and pathology, our main objectives are:

- to discover and characterise novel PET and SPET radiopharmacological tracers, which are markers for the function and the expression of cerebral multidrug transporters that form part of the BBB (WP 01 Key milestones (KM) 1.5,14,15,22,23).
- to characterise and identify compounds / potential radiotracers in-vitro that are substrates for Pgp and acts as inhibitors of drug efflux across the BBB (WP03 KM3.3,4)
- to evaluate, determine kinetics and develop quantitative methodology to measure transporter expression and/or functionality of existing radiotracers known to be either Pgp substrates ([11C]-VPM) or Pgp inhibitors ([11C]-laniquidar) in naïve animals (WP02 KM2.3).and in healthy human volunteers (WP05 KM5.4,5), and to later apply these methods to novel radiotracers emerging from WP01, in naïve animals (WP02 KM2,5,7,8,12,15) and in healthy human volunteers (WP05 KM5.10-12).
- to carry out proof-of-concept studies with existing radiotracers known to be either Pgp substrates ([11C]-VPM, [11C]-flumazenil, [18F]-MPPF) or Pgp inhibitors ([11C]-laniquidar) to image Pgp function, and determine the efficacy of existing and novel Pgp modulators to enhance cerebral penetration of substrate tracers in CNS disease models, comparing drug-resistant and drug-responsive animals (WP02 KM2.4; WP04, KM4.3,4) and humans (WP 04 KM4.3,4; WP05 KM5.4,8; WP06 KM6.4,6-8), and similarly with novel radiotracers emerging from WP01 (WP02 KM2.9-11,13,14,16-18)
- to carry out proof-of-concept studies with [11C]-VPM to image Pgp function for early diagnosis of Alzheimer's Disease (AD) (WP07 KM7.3,4)

- to determine the anatomical distribution of transporter proteins in brain tissue from patients with epilepsy and AD (WP08 KM8.5-7)

Project Results:
1.3 Description of the main S&T results/foregrounds of EURIPIDES
WP01: Development of new radiotracers
Objectives
The main objective of this WP01 was to develop new radiotracers for in-vivo imaging of cerebral multidrug transporters (P-glycoprotein, Pgp; multidrug resistance proteins, MRPs; breast cancer resistance protein, BCRP) with PET and SPET. We initially followed two different approaches. First, we developed radiotracers based on known third-generation Pgp inhibitors (laniquidar, elacridar and TQD). These compounds were expected to bind with nanomolar affinity to Pgp at the blood-brain barrier (BBB) and provide higher PET signals than available Pgp substrate radiotracers such as (R)-[11C]VPM. Moreover, radiolabelled Pgp inhibitors were expected to allow for mapping of Pgp expression levels at the BBB and to afford signal increases rather than signal decreases (as radiolabelled substrates) in epileptic brain regions overexpressing Pgp.

As a second approach, we developed two PET tracers based on antiepileptic drugs (AEDs), i.e. [11C]phenytoin and [11C]mephobarbital. The rationale behind this approach was our expectation that radiolabelled AEDs would be only weakly transported by Pgp at the BBB and thereby afford higher PET signals than the high-affinity Pgp substrate (R)-[11C]VPM. This should enable an improved visualisation of regional Pgp activity in the epileptic brain as compared with (R)-[11C]VPM. In addition, it was expected that mapping the regional brain distribution of a radiolabelled AED should be clinically more meaningful in predicting drug resistance than using a surrogate marker of Pgp activity such as (R)-[11C]VPM.

Results
[11C]Radiolabelling
After the synthesis of appropriate radiolabelling percursors, [11C]laniquidar, [11C]elacridar, [11C]TQD and [11C]mephobarbital were synthesised by [11C]methylation of phenolic OH functions ([11C]elacridar and [11C]TQD), a carboxylic acid function ([11C]laniquidar) or an imide function ([11C]mephobarbital) using [11C]methyl triflate or [11C]methyl iodide. Moreover, a presumably BCRP-selective TQD analogue (Kühnle et al, 2009)) was labelled by [11C]methylation of a carboxylic acid function.

[11C]Phenytoin was synthesised by reaction of 2,2-azido-diphenylamide with [11C]carbon monoxide. All radiotracers were obtained in good radiochemical yield, with acceptable radiochemical purity (greater than98%) and specific activity (greater than50 GBq/µmol). In addition, 3H-labelled versions of laniquidar and elacridar were synthesised by [3H]methylation of the respective desmethyl-precursor molecules with [3H]methyl nosylate.

Radiopharmacological characterisation of [11C]radiotracers
[11C]Laniquidar was tested in biodistribution experiments in rats with and without pretreatment with cyclosporine A or valspodar and in PET experiments in rats with and without pretreatment with TQD. [11C]Elacridar and [11C]TQD were tested in paired small-animal PET scans in naïve rats, wild-type mice and three different transporter knockout mouse models (Mdr1a/b(-/-), Bcrp1(-/-) and Mdr1a/b(-/-)Bcrp1(-/-)), each before and after administration of unlabelled compound. Moreover, in-vitro autoradiography was performed on rat or mouse brain slices with [11C]elacridar and [11C]TQD, with and without co-incubation with an excess of unlabelled compound (1 µM). The 11C-labelled BCRP-selective TQD analogue was characterised in small-animal PET scans in wild-type,

Mdr1a/b(-/-), Bcrp1(-/-) and Mdr1a/b(-/-)Bcrp1(-/-) mice. [11C]Phenytoin was tested in PET scans in rats before and after administration of either TQD as Pgp inhibitor or the MRP inhibitor probenecid. [11C]Mephobarbital was tested in paired PET scans in rats, wild-type, Mdr1a/b(-/-) and Mrp1(-/-) mice, before and after administration of TQD or the MRP inhibitor MK571. In-vivo metabolism of all [11C]radiotracers was assessed in naïve rats or mice by analysis of plasma samples with a combined solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) assay.

Generally, brain uptake of [11C]laniquidar, [11C]elacridar and [11C]TQD was very low (standardised uptake value, SUV less than0.5) in naïve rats and in wild-type mice. Following pretreatment of animals with unlabelled TQD or elacridar brain uptake of these radiotracers was increased by several-fold. Moreover, PET experiments in wild-type and transporter knockout mice revealed for [11C]elacridar, [11C]TQD and the 11C-labelled BCRP-selective TQD analogue the following rank order of brain activity uptake: Mdr1a/b(-/-)Bcrp1(-/-) greater thangreater than Mdr1a/b(-/-) greater than Bcrp1(-/-) greater than wild-type (exemplarily shown for [11C]TQD).

Metabolite analysis showed that at 20 min after radiotracer injection into rats, 85% and 96% of total radioactivity in plasma were in the form of unchanged parent tracer for [11C]elacridar and [11C]TQD, respectively. For [11C]laniquidar, 68% unchanged parent was found in rat plasma at 30 min after tracer injection. Taken together, these experiments suggested that [11C]laniquidar, [11C]elacridar, [11C]TQD and the 11C-labelled BCRP-selective TQD analogue were transported by Pgp and/or Bcrp at the rodent BBB. The characteristic pattern of brain activity uptake seen in wild-type, Mdr1a/b(-/-), Bcrp1(-/-) and Mdr1a/b(-/-)Bcrp1(-/-) mice is in line with the behaviour of dual transporter substrates, which only gain brain access when both Pgp and Bcrp1 are simultaneously knocked out. Interestingly, the in-vitro autoradiography experiments pointed to displaceable binding of [11C]elacridar and [11C]TQD to Pgp and possibly Bcrp1.

Both radiolabelled AEDs showed higher brain activity concentrations in rats and mice than [11C]laniquidar, [11C]elacridar and [11C]TQD. For [11C]mephobarbital, peak brain activity concentration was in the order of SUV 3-4, whereas for [11C]phenytoin peak brain activity concentration was in the order of SUV 1. Pretreatment of naïve rats with TQD (15 mg/kg) resulted in a 45% increase in cerebral volume of distribution (VT) of [11C]phenytoin whereas no such effect was observed for [11C]mephobarbital. Metabolite analysis showed that for [11C]mephobarbital greater than85% of total radioactivity in plasma was in the form of unchanged radiotracer, whereas this value was approximately 20% at 45 min after radiotracer injection for [11C]phenytoin. In brain; greater than80% of total activity was in the form of unchanged parent for both radiotracers.

Radiopharmacological characterisation of [3H]radioligands
In order to gain a better understanding of the interaction of laniquidar, elacridar and TQD with Pgp and Bcrp1, in-vitro uptake assays were performed with the respective 3H-labelled radioligands in wild-type cells and in cells overexpressing human MDR1, murine Mdr1a and murine Bcrp1 in presence and absence of a Pgp-specific inhibitor (valspodar, PSC833) or a Bcrp1-specific inhibitor (Ko143) (results exemplarily shown for [3H]TQD).

At nanomolar concentrations, cellular uptake of [3H]elacridar and [3H]TQD but not [3H]laniquidar was found to be lower in transporter overexpressing as compared with the respective wild-type cells. Moreover, addition of PSC833 increased cellular accumulation of [3H]elacridar and [3H]TQD in Pgp-overexpressing cells whereas addition of Ko143 increased cellular accumulation in Bcrp1-overexpressing cells.

Transporter inhibition had no influence on cellular accumulation of [3H]laniquidar. These results confirm our in-vivo PET results by showing that at nanomolar tracer concentrations [3H]elacridar and [3H]TQD but not [3H]laniquidar are transported by Pgp and Bcrp1. At micromolar concentrations, however, Pgp transport of [3H]elacridar and [3H]TQD was saturated and only Bcrp1 transport was partially visible. In addition, Bcrp1 transport of [3H]elacridar was confirmed in a concentration equilibrium transport assay (CETA) using monolayers of Bcrp1-overexpressing cells.

In contrast, unlabelled mephobarbital was found not to be transported by Pgp or MRP1 in CETA experiments, which was consistent with in-vivo data. In earlier experiments, unlabelled phenytoin was characterised as Pgp substrate by P05-TIHO in CETA assays (Luna-Tortós et al, 2008), Baltes et al 2007)

Development of longer-lived radiotracers for PET imaging
A 18F-labelled analogue of elacridar, 1-[18F]fluoroelacridar was synthesised and tested in small-animal PET scans in naïve rats, before and after administration of unlabelled elacridar and in wild-type and Mdr1a/b(-/-)Bcrp1(-/-) mice. The in-vivo behaviour of 1-[18F]fluoroelacridar was found to be similar to [11C]elacridar suggesting that both tracers were effluxed by Pgp and Bcrp1 at the BBB making them unsuitable to assess Pgp expression levels.

As the PET results obtained with [11C]laniquidar, [11C]elacridar and [11C]TQD did not suggest that these molecules will provide a sufficiently high signal to allow for imaging of transporter distribution at the BBB, we decided to follow a new approach for the imaging of cerebral multidrug transporters (Pgp, MRP1). The metabolite extrusion method relies on the use of a radiotracer which has no affinity to cerebral multidrug transporters and therefore shows good brain uptake. Once inside the brain, the radiotracer (prodrug) is quantitatively metabolised into a transporter specific substrate (drug) that is subsequently extruded from brain by active transport. The efflux rate constant of activity is expected to be a sensitive parameter for transporter activity. This approach may be a very elegant method for signal amplification in the imaging of cerebral multidrug transporters and is expected to overcome the problem of low PET signals encountered with radiolabelled transporter substrates ((R)-[11C]VPM) as well as inhibitors ([11C]laniquidar, [11C]elacridar, [11C]TQD) In particular, MRP1 activity may be assessed by exploiting the metabolic conversion of 6-halopurines (prodrug) into the respective glutathione conjugates (drug) inside the brain. 6-bromo-7-([18F]2-fluoroethyl)purine, a 18F-labelled analogue of the recently published 6-bromo-7-[11C]methylpurine (Okamura et al., J Cereb Blood Flow Metab. 29(3):504-11 (2009)), was found to display acceptable brain uptake in wild-type mice (4 %ID/g) and relatively rapid washout from brain over time. Moreover, brain tissue homogenate studies showed that the pro-drug was rapidly and quantitatively converted into its glutathione conjugate. The characterisation of 6-bromo-7-([18F]2-fluoroethyl)purine in Mrp1(-/-) mice is currently in progress.

Moreover, we attempted to extend the metabolite extrusion method to the imaging of cerebral Pgp by synthesising two 18F-labelled ester prodrugs of the antihistamine drugs fexofenadine and cetirizine. Using an in-vitro ATPase assay the respective unlabelled ester prodrugs were shown to lack Pgp-transport whereas the respective free acid forms showed Pgp transport activity. Biodistribution studies in wild-type mice showed that the 18F-labelled fexofenadine derivative had higher initial brain uptake than the 18F-labelled cetirizine derivative. However, high activity uptake in bone pointed to in-vivo defluorination. Therefore, another 18F-labelled fexofenadine derivative was synthesised in which the 18F-label was located in a more stable position (i.e. the phenyl ring). This phenyl-ring 18F-labelled fexofenadine derivative is currently the most promising candidate and awaits in-vivo characterisation.

Development of radiotracers for SPET imaging

We synthesised a series of iodinated elacridar/TQD analogues in order to come up with a Pgp inhibitor based SPET tracer. Unfortunately, none of these maintained the biological activity of elacridar/TQD itself as shown by in-vitro transport inhibition assays. One derivative (iodoelacridar-urea) was found to be only approximately 6 times less potent than elacridar and therefore labelled with iodine-125. A biodistribution study was performed in wild-type mice with [125I]iodoelacridar urea. The compound was shown to possess low brain uptake (less than0.5 %ID/g) which was increased after pretreatment of animals with cold TQD. Due to the similar in-vivo behaviour of [125I]iodoelacridar urea and [11C]elacridar this compound was not further pursued.

WP02: Biological evaluation of new radiotracers

Objectives

The main objective was to evaluate the feasibility of new radiotracers developed in WP01 for in vivo imaging of cerebral multidrug transporters with PET and SPET. These radiotracers have been used to study in vivo the distribution and function of multidrug transporters in animals and this had been related to actual brain uptake and distribution of CNS active drugs.

The general strategy for WP02 was to use (R)-[11C]-VPM ([11C]-VPM) PET studies as the starting point to measure the expression and distribution of the P-glycoprotein efflux transporter (Pgp), as a reference point for comparison to the newly developed PET and SPET radiotracers. In addition, the actual blood-brain barrier (BBB) transport and brain distribution kinetics were studied with the Pgp substrate quinidine (QUIN) by utilizing intracerebral microdialysis. Studies were performed in rats under normal conditions and in rats exposed to status epilepticus (SE) to trigger the epileptogenic process towards pharmacoresistant mesial temporal lobe epilepsy (mTLE), the latter experiments covering the complete time span from the initial silent period to the chronic epileptic end state. The studies were harmonized by choosing TQD as the universal Pgp modulator for all studies. SE was deliberately generated by various methods (kainate, pilocarpine or electric stimulation) to allow identification of robust disease-related effects separate from model-specific changes.

Pgp expression and activity in naive rats evaluated with (R)-[11C]-VPM and quinidine and modulation of uptake by Pgp inhibitors (task 1 + 2)

PET studies

The (R)-[11C]-VPM PET scans have been performed in a microPET scanner (AIT/TIHO) as well as in an HRRT scanner (VUA/LACDR) in female (AIT/TIHO) and male (VUA/LACDR) Sprague-Dawley rats. Essentially these studies have demonstrated that the results were not critically dependent on methodological aspects. In fact, the results were very similar: [11C]VPM had very low brain uptake at baseline conditions. For comparison with human PET data, kinetic modelling was performed to estimate the rate constants of tracer transport across the rat BBB.

PET studies on [11C]-VPM were performed with or without TQD (15 mg/kg, bolus dose at -30 min). TQD concentrations were determined in blood and at approximately 2 h post administration the concentration were 1706 ± 333, and 1560 ± 312 (average ± s.d) for the naïve and epileptic rats, respectively (see also below). Complete metabolite correction was used in the analysis of the raw data (best fit). The uptake of [11C]-VPM was rather homogenous in the whole brain. The increase in brain uptake after TQD treatment was of the same magnitude in all parts of the brain. TQD treatment resulted in a more than 11-fold increase in the Kp value, estimated through Logan analysis. The time-activity profiles in the cerebellum showed a somewhat faster uptake as well as a faster washout than in the whole brain.

Furthermore PET studies were performed to compare dose-response relationship of two potent Pgp inhibitors (TQD and elacridar) and to investigate if increased brain uptake of [11C]-VPM mediated by Pgp inhibition can be used to assess regional differences in Pgp activity. Two groups of Sprague-Dawley rats (n=12) underwent single [11C]-VPM PET scans at 120 min after administration of different doses of the Pgp inhibitors TQD or elacridar. In 6 additional rats, paired [11C]-VPM PET scans were performed before and after administration of 3 mg/kg TQD. Inhibitor administration resulted in up to 11-fold increased [11C]-VPM brain distribution volumes (DV, or Vd, or also called blood-to brain partition coefficient Kp) with ED50 values of 3.0±0.2 and 1.2±0.1 mg/kg for TQD and elacridar, respectively. In paired PET scans, 3 mg/kg TQD resulted in regionally different enhancement of brain activity distribution, with lowest DV in cerebellum and highest DV in thalamus

Intracerebral microdialysis

Parallel studies with PET and microdialysis were too complex to be performed in the same animal. QUIN pharmacokinetics was therefore determined separately following i.v. administration at 2 different dosages. The 8 hr study covered a 1 hr blank period, a 4 hr infusion period and a 3 hr elimination period. Plasma and microdialysate concentrations, as well as total brain concentrations at termination of the experiment, were analyzed by HPLC and fluorescence detection.

Results obtained with i.v. infusions of 10 and 20 mg/kg/240 min, with and without a 15 mg/kg bolus dose of TQD at -30 min. Plasma and brain concentrations of QUIN were dose-dependent, while TQD selectively increases brain microdialysis QUIN concentrations (approximately 10-fold). This indicates that QUIN brain distribution provides an excellent reflection of specific inhibition of Pgp functionality at the BBB. A pharmacokinetic model on QUIN brain distribution has been developed using population modelling (NONMEM). Initial modelling of plasma PK data of QUIN so far obtained suggest the following plasma PK parameters: clearance CL = 25.1 ml/min; volume of distribution of the central compartment V1 = 461 ml; intercompartmental clearance Q= 59.3 ml/min; and volume of distribution of the peripheral compartment V2=1650 ml.

Additional studies
A range of studies have been performed to support the studies on Pgp functionality and expression. Population modelling with NONMEM has been introduced and covariate analysis has allowed to demonstrate which parameters of brain uptake and distribution were influenced by TQD, epilepsy and other factors. For example, this approach confirmed that TQD specifically and concentration-dependently affects efflux from the brain. Moreover modelling techniques have been exchanged between institutes. Also rat data were compared with values from the human dose-escalation study, showing that TQD-induced increase in [11C]VPM uptake is distinctly stronger in rats than in man, whereas EC50 values were nearly identical.

Studies with knockout mice (Mrp1(-/-)-, Mdr1a/b(-/-)-, Bcrp1(-/-)- and Mdr1a/b(-/-)Bcrp1(-/-)-mice) have shown that [11C]VPM solely interacts with Pgp. Furthermore, In vitro transport of VPM has been studied in cells overexpressing Pgp. It has been found that the efflux transport of VPM by Pgp decreases with concentration.

Characterization of newly developed radiotracers in naive rats (Task 3)

The most frequently used pharmacokinetic model for analysing (R)-[11C]VPM brain pharmacokinetics in humans assumes that the brain distribution of [11C]D617, which is the main metabolite of (R)-[11C]-VPM, displays the same pharmacokinetics as (R)-[11C]VPM. Although the brain distribution was similar to the brain distribution of (R)-[11C]-VPM at baseline (before Pgp inhibition), the increase after Pgp inhibition was much lower compared to (R)-[11C]-VPM. [11C]D617 appears to be a weaker substrate for Pgp with a 2.3-fold increase in brain uptake at complete Pgp inhibition, compared to (R)-[11C]-VPM (10-fold increase). Thus, the presence of [11C]D617 might confound the quantification of the Pgp function when analysing (R)-[11C]-VPM data.

Isatin has been successfully labelled with 18F at the 4- and 6-position at low but reliable yield. Both compounds exhibit high instability. Addition of Tween-80 stabilizes the formulation and 10% Tween-80 has little effect on Pgp function. In naive rats there is an indication of Pgp inhibitor properties of Isatin. This needs further exploration.

Two iodinated tracers have been developed. The [125I]elacridar-urea derivative (tracer X) was stable and has been injected into normal mice pretreated with TQD (injected interbrain, 30 minutes before the radioiodinated derivative's intravenous injection). Diversification of the biodistribution was observed: Clearance became much slower in the presence of the inhibitor and radioactivity was retained in the muscular system, the radiolabelled species being eliminated mainly through the hepatobiliary tract. [125I]Curcumin (Tracer Y) could not be evaluated further, because it was found to be insoluble in water and diluted ethanolic solutions and could not be injected in vivo.

Biological evaluation of radiotracers in animal models for chronic epilepsy (Task 4)

PET studies
MicroPET scans were performed with [11C]-VPM before and after administration of TQD (3 mg/kg and 15 mg/kg) in rats 48 h after status epilepticus (SE) and respective control rats. For analysis of µPET scans after TQD administration, different brain regions of interest were

outlined on magnet resonance images which were co-registered with the PET images. For comparison with the present µPET data, computer-assisted quantification of Pgp expression in immunohistochemically stained rat brain sections was performed in respective brain regions. Striking changes in [11C]-VPM brain uptake were obvious after administration of 3 mg/kg TQD (ED50) in animals 48 h after pilocarpine-induced SE which vanished after maximal Pgp inhibition (15 mg/kg). In rats 48 h post SE, the DV (=Kp) of cerebellum was decreased by 37.5% whereas DV of frontal motor cortex displayed an increase by 18.9% compared to control rats. In thalamus, a tendency towards DV decrease was found. The decrease of [11C]-VPM uptake in the cerebellum as well as the tendency for decrease in thalamus indicate higher Pgp activity induced by SE which is reflected by increased Pgp-labelled areas in respective brain regions. In conclusion, a µPET protocol suitable to quantify SE-induced changes in Pgp activity in distinct rat brain regions was established.

Similarly, rats were treated with kainic acid to induce SE. At 1 week after treatment, each rat underwent a PET study with [11C]-VPM. Pretreatment with TQD (15 mg/kg, 30 min pre dosing) convincingly increased the brain [11C]-VPM uptake and resulted in a 10-fold increase in Kp (=DV). Between the (vehicle treated) naïve and epileptic rats there was no difference in the K1(Logan). However, the PET scans were performed 1 week after induction of SE. This does not rule out the possibility that there are differences other time points after induction of SE.

TQD had a huge effect on brain distribution of [11C]-VPM in both naive (task 1+2) and epileptic rats. The K1 derived from Logan analysis was 0.74 ± 0.12 and 0.54 ± 0.12 in naïve and epileptic rats, respectively. This indicates that the influx of [11C]-VPM in epileptic-TQD-treated rats was lower than in naïve-TQD-treated rats. Data analysis has been performed by using conventional kinetic models as well as by population modelling using NONMEM. Covariate analysis in NONMEM allowed identification of a specific influence of TQD on transport across the BBB and of kainate treatment on the (pharmacological) volume of distribution in the brain.

In conclusion the increase in efflux transport, correlated with the increase in expression of Pgp, at 2 days after pilocarpine-induced SE and at 7 days after kainate-induced SE were very similar. Differences compared to control animals were most prominent after half-maximal Pgp inhibition. At 4 months after SE, only in the cerebellum a significant decrease in [11C]VPM uptake was observed. As the arrival of some of the new tracers coincided with the availability of truly epileptic rats selected as responders and non-responders, testing ofnew tracers directly in responder/non-responder animals was given priority over testing shortly after SE.

Intracerebral microdialysis

Remarkably, kainate-treated rats tended to have a lower total brain concentration but a higher brain ECF concentration of quinidine than saline-treated rats. This suggests that the Pgp function in kainate-treated rats is altered at the parenchymal level rather than at the BBB. TQD pre-administration increased the brain ECF brain concentration 7.2-fold but the total brain concentration about 40-fold, supporting an important role for Pgp in intra-brain distribution. After kainate-treatment alone however there was no difference in transport compared to control rat

Biological evaluation of radiotracers in animal models of drug resistance (Task 5)

Rats were selected which did or did not respond to treatment with the antiepileptic drug phenobarbital in the pilocarpine model of TLE. Similar to the observations in the basolateral amygdala stimulation model, epileptic rats in the pilocarpine model showed a pronounced variability in individual response to treatment with phenobarbital. 33% of epileptic rats did not respond appropriately to treatment with the maximal tolerable dose of phenobarbital. As previously described part of the animals in the pilocarpine model did also not respond to treatment with the common antiepileptic drug levetiracetam. As a definition of pharmacoresistance in animal models asks for refractoriness to monotherapy at tolerable doses with at least two current antiepileptic drugs, these findings support the suitability of the pilocarpine model for the investigation of pharmacoresistant epilepsy. Selected responders and nonresponders have been used to evaluate if differences in Pgp activity between pharmacosensitive and –resistant rats can be detected and quantified after TQD administration using small-animal positron emission tomography with [11C]VPM.

Thus, in the context of TIHO/LMU/LACDR/VUA/AIT collaboration epileptic rats were generated by exposure either to pilocarpine- or to electrically-induced status epilepticus (SE) and after development of spontaneous recurrent seizures rats were screened with respect to their response to prolonged treatment with phenobarbital. During the selection procedure, rats were continuously monitored (video/EEG 24 h/day over 48 days) to compare seizure frequency in the pre-drug, treatment and post-drug period.

[11C]VPM uptake after half-maximal blockade of Pgp was significantly increased in frontal motor cortex, temporal hippocampus, and piriform/entorhinal cortex of non-responders compared to controls but not to responders. Controls and responders did not differ significantly. With [11C]mephobarbital and [11C]TQD no group differences in regional tracer brain uptake were detected.

[11C]-labelled laniquidar, quinidine and phenytoin were initially tested in a multitracer-design study. [11C]Phenytoin was also investigated alone in controls, responder and non-responder rats. PET scans were performed blinded, with respect to the division into responder and non-responder groups.

Before TQD treatment, [11C]laniquidar and [11C]quinidine hippocampal uptake reached significantly higher levels in the group of non-responders as compared to controls in the early phase of the scan (0-2.25 min). Following TQD pre-treatment [11C]laniquidar brain uptake (hippocampus, occipital and parietal cortex) was comparable in all groups (electrode-implanted non-epileptic controls, responders and non-responders). In contrast, [11C]quinidine uptake in non-responders during the early phase of the scan (0-2.25 min) in the hippocampus and occipital cortex exceeded that in controls and responders. Regarding a correlation of respective PET data with the pharmacoresponse to phenobarbital, statistical analyses revealed a correlation between brain uptake of [11C]laniquidar in the early phase of the scan and the percentage seizure reduction during phenobarbital treatment. Analyses of Pgp expression rates in all groups of animals demonstrated higher levels of expression in the CA1-region of responders and non-responders as compared to controls. In contrast to

previous studies no robust differences in Pgp expression were observed between responders and non-responders.

In rats scanned with [11C]phenytoin alone following complete inhibition of Pgp by TQD no group differences in regional tracer brain uptake were detected.

Additional studies
Some animals were repeatedly scanned in a (human) 3T MRI scanner during epileptogenesis. These data were used together with available brain region MRI atlases to create atlases that reflect specific developments in responder and non-responder rat brains.

A time course study on Pgp expression was undertaken in relation to time-after SE, covering a period of 5 weeks. Results showed considerable variability between animals but as a general trend there was a transient early increase peaking at 2 days after SE and a second broader peak between 7-14 days after SE.

SE induction by unilateral intrahippocampal kainate injection did not reveal clear ipsilateral to contralateral differences in Pgp expression. However correlating quinidine brain pharmacokinetics before and after TQD treatment, with Pgp expression and seizure severity during SE in individual animals, hinted to a clear and highly interesting uncoupling of Pgp expression and function strongly influenced by seizure severity.

WP03: In vitro characterisation
Objectives
The main objective is to determine the most specific and potent inhibitor of Pgp for use as in-vivo molecular imaging tracer in patients with refractory neurological and psychiatric conditions.

Task 1: In-vitro characterisation of inhibition of transport by Pgp to identify compounds with the best inhibitory profile to be used in-vivo

The objective of this task was to rank inhibitors on the basis of inhibitory potency in an effort to identify an optimal inhibitor for the subsequent in-vitro / in-vivo experiments. We have tested and determined IC50 values for seven Pgp inhibitors to validate the use of these compounds as in-vivo probes for PET imaging. The P12-DRC group has produced 5 batches of elacridar derivates labeled with iodine and we have determined the efficacies of the derivatives compared to the parent compound. Ten novel elacridar derivatives in total have been tested but most have markedly reduced efficacy compared to the parent compound which strongly suggests that they will not be suitable for use as in-vivo probes. A 5th batch of compounds was tested with the 3-iodo derivative showing an interaction with Pgp. This compound has now being taken forward for in-vivo imaging experiments.

An aim of WP03 is to test novel pro-drug ligands of Pgp generated from the lab group of E. Arstad, UCL utilising an in-vitro Pgp assay. Seventeen novel ligands have been tested in a validated assay and this has lead to the identification of an optimal ligand pair. This optimal ligand pair will now be further validated in-vivo as a novel imaging probe (E. Arstad, UCL).

Task 2: Comparison of inhibition of wild-type and variant forms of ABCB1 by model inhibitors

The primary aim of this task was to characterise the inhibition of transport of wild type and variant forms of Pgp (i.e. containing combinations of 3 single nucleotide polymorphisms [SNPs] in the ABCB1 gene) by inhibitors. To validate the Xenopus oocyte expression system, two model Pgp substrates, digoxin and imatinib were shown to be transported by human ABCB1 expressing oocytes. This Pgp mediated transport was shown to be dependent on ATPase activity and was inhibited by the Pgp inhibitor PSC-833. Site directed mutagenesis has been performed on human Pgp cDNA to generate the three SNP variant haplotype corresponding to C1236T, G2677T and C3435T. No differences in transport were observed between the wild type and triple SNP variant, indicating that the triple SNP variant does not have a direct functional effect for transport or inhibition for the two tested model substrates.

An additional sub-task was to characterise the transport of AEDs by Pgp. To address this question seven transport systems expressing Pgp have been developed and utilised in this work package to study the transport of three major AEDs. The Pgp mediated transport of phenytoin was observed utilising the equilibrium method in both Pgp stably over expressing cell lines. This suggests that phenytoin is a weak substrate of Pgp that requires non-physiological (or high) protein expression of Pgp for transport. The transport of carbamazepine and lamotrigine by Pgp was also investigated with no Pgp mediated transport observed in the in-vitro systems tested suggesting that lamotrigine and carbamazepine are not transported by Pgp. This adds to the evidence that there is no unifying mechanism of drug transporter resistance and emphasises the need for a systems approach of AED transport at the BBB.

Task 3: Characterisation of the mechanism of induction of ABCB1 by drugs
The induction properties of AEDs of ABCB1 have been investigated in an in-vitro model of the BBB. No induction was observed of ABCB1 over a timecourse and with different concentration of AED. This suggests that at least in the in-vitro model tested, that AEDs do not induce ABCB1 mRNA expression.

Task 4: Systems approach for studying drug transport at the BBB
The aim of this task was to investigate the overall transport, influx and efflux, at the blood-brain barrier (BBB), and not only focus on Pgp mediated drug transport. This systems approach is going to be essential in understanding the success of drugs used for CNS diseases, and the development of novel ligands. Identifying these mechanisms of transport at the BBB will lead to the identification of imaging probes. We have investigated lamotrigine as the mechanisms that underpin the passage of this important AED at the blood-brain barrier to its site of action in the brain is poorly understood. Lamotrigine has been postulated to be delivered to its site of action in the brain favourably despite its physicochemical properties.

WP04: Evaluation of existing radiotracers for imaging Pgp function
Objectives
The main objective is to determine the contribution of Pgp activity to uptake and binding of existing PET tracer [18F]-MPPF, and [11C]-FMZ that are Pgp substrates.

[18F]-MPPF:
Naïve and epileptic rats (Task 1-4, P06-LMU)

First PET scans, examining the impact of Pgp modulation on [18F]-MPPF brain kinetics, were performed in naïve Sprague Dawley rats. Following optimization of the quantification procedure, the initial uptake of [18F]-MPPF in brain was used as a surrogate marker of the unidirectional blood-brain clearance (K1). Based on the consortiums decision to focus on the Pgp inhibitor TQD (TQD), the effect of TQD on the K1-surrogate was determined. TQD had a significant and dose-dependent effect, with an IC50 close to 5 mg/kg, and a 2.5-fold increase at the highest TQD dose. Additional PET scans have been performed with the Pgp modulator cyclosporine A (CsA) resulting in an increase of 30-40% for the K1-surrogate. These data clearly substantiate transport of [18F]-MPPF by Pgp further supporting its suitability for non-invasive Pgp imaging.

Epileptic rats have been identified in a chronic post-status epilepticus model of temporal lobe epilepsy by continuous 24h-EEG-video-monitoring. [18F]-MPPF PET scans following vehicle or TQD administration have been performed in these epileptic rats. The comparison between pre-medication with glucose-containing vehicle and TQD in epileptic rats revealed significant differences on [18F]-MPPF brain kinetics. Treatment with TQD increased the magnitude of K1 by approximately 94% in the hippocampus and globally in other brain regions by 66-101%. We also obtained an estimate for the rate constant of the washout of [18F]-MPPF from hippocampus (k2'; min-1), which value was used for the voxelwise constrained fitting of BPND, the main parameter of interest in the non-invasive Gunn method. In contrast TQD did not affect the hippocampal [18F]-MPPF k2. TQD treatment in epileptic rats evoked no difference in the normalized FDG uptake in the hippocampus and all other brain regions compared to vehicle treated epileptic rats. TQD increased the magnitude of BPND in the hippocampus by 22% and by 19-60% in other brain binding regions. The frequency of seizures (detected during the three days before the scans) positively correlated with [18F]-MPPF K1. These data indicate that recent seizure activity might affect tracer kinetics based on transient disturbance of blood-brain barrier integrity, and might therefore bias conclusions about Pgp expression and function. As expected, seizure frequency tended to show a positive correlation with the impact of TQD on K1 data, but these data failed to reach significance. P-glycoprotein expression was analyzed in the parietal cortex. A significant increase in the optical density of labeled capillaries was observed in epileptic rats as compared to control animals at -2.3 mm from Bregma. In contrast, the area labeled for P-glycoprotein did not differ between epileptic rats and control rats in this brain region.

In a subsequent experiment, rats that developed spontaneous recurrent seizures during the pre-screening period were used for the selection of responders and non-responders by prolonged treatment with phenobarbital. During the selection procedure, rats were continuously monitored (video/EEG 24 h/day). Before the onset of phenobarbital treatment, baseline seizure frequency was determined over a period of 16 days (pre-drug control period). Phenobarbital treatment (16 days) was followed by a post-drug control period. [18F]-MPPF PET scans with and without TQD pre-treatment were completed in subgroups of responders and non-responders. In response to phenobarbital treatment, complete control of seizures was achieved in three out of twelve animals, and substantial reduction of seizure frequency was seen in two further animals (-65% and -88%). Together, these five animals were categorized as phenobarbital responders. The other seven animals not showing a relevant anticonvulsant response were considered non-responders. Analysis of plasma revealed phenobarbital concentrations within the therapeutic range (10 60 µg/ml)

in all rats. The mean parametric maps of [18F]MPPF K1 in the vehicle condition did not differ between responder and non-responder groups nor did the VOI analysis reveal any significant differences in mean K1 in the vehicle condition. However, the comparison between responders and non-responders revealed significant differences in the impact of the P-glycoprotein modulator TQD on [18F]MPPF kinetics in the hippocampus. Treatment with the P-glycoprotein modulator TQD increased the magnitude of K1 in hippocampus by 77-103% in responders and by 103-178% in non-responders such that mean TQD-evoked increases in K1 in non-responders significantly exceeded that in responders.

The percent reduction of seizure frequency in response to phenobarbital negatively correlated with the impact of TQD on the hippocampal [18F]MPPF K1 ($r = -0.5940$; $p?0.05$). Mean estimates of the magnitude of [18F]MPPF k2 in the hippocampus of responders and non-responders were similar in the vehicle condition. TQD significantly decreased the mean hippocampal [18F]MPPF k2 by 27% in non-responders, whereas it did not exert a comparable effect in responders.

Patients and healthy controls (Task 5, P07-HCL):
Cyclosporine A (CsA) was used throughout the project by partner P07-HCL. Fifteen patients with drug-resistant focal epilepsy of temporal lobe origin with or without hippocampal sclerosis were originally selected for this protocol. Twelve of the 15 scanned patients (mean age ± SD = 43.9 ± 7 years) had paired 60-minute dynamic [18F]MPPF PET scans with and without concurrent cyclosporine A (CsA) IV infusion. The three remaining patients underwent only one [18F]MPPF PET (without CsA) and were then excluded from the protocol for various reasons. A few patients reported mild central nervous system side effects, such as vertigo, during the 24 hours following CsA infusion, suggesting that the latter promoted the activity and adverse events of concurrent anti-epileptic drugs. For the PET associated with CsA infusion, the latter was started one hour prior to [18F]-MPPF injection at a dosage of 2.5 ml/kg/h over 2h resulting in a steady plasma concentration of CsA during the entire PET study at or above the target level (= 2980 µg/l). A simplified reference tissue model was used to generate parametric images of 5-HT1A receptor binding potential (BP=Bmax/Kd) and K2 values. The cerebellar white matter and the thalamic region were both used as reference regions. The same protocol was used in eight healthy subjects. All controls also gave their informed consent to participate in this study. Seven of the 8 scanned patients (mean age ± SD = 41.8 ± 7.2 years) had paired 60-minute dynamic [18F]MPPF PET scans with and without continuous cyclosporine A (CsA) IV infusion. The last control recruited had to be excluded from the study after completion of the first PET scan because the occurrence of a deep venous thrombosis in the leg.[18F]MPPF PET data obtained in the 12 patients were analyzed using a set of pre-defined regions of interests and paired non-parametric Wilcoxon test. At baseline, all patients showed a major reduction of the binding potential (BP) of [18F]MPPF for 5-HT1A receptors in their epileptogenic temporal lobe.

The concurrent infusion of CsA was associated with:
1) a significant reduction of [18F]MPPF k2 in the majority of brain regions. The average k2 reduction amounted to 14% using the thalamic reference region versus 23% using the cerebellar reference region
2) a significant increase of [18F]MPPF BP in selected regions. The average BP increase was 9% using the thalamic reference region versus 16% using the cerebellar reference region. However, we found no impact of CsA

infusion on [18F]MPPF BPND asymmetry index (AI) whatever the reference region used.

The lack of impact of CsA on the asymmetry index might partly result from the large interindividual variability that might obscure specific blockade of over-expressed Pgp in the epileptic focus of a subgroup of patients. Conversely, it might reflect the fact that the magnitude of Pgp overexpression in Human mesial temporal epileptic tissue remains too limited to be detectable with our method. Overall, our aim was to establish [18F]MPPF as an in vivo tool for the study of the mechanism underlying pharmacoresistance in patients with TLE and more specifically putative over-expression of Pgp in the epileptogenic zone. Our main hypothesis was that cyclosporine A (CsA) will block Pgp function in the brain, and that this should significantly interact with Pgp mediated [18F]MPPF efflux from the brain, and more indirectly with its binding potential. Our data confirm this main hypothesis by showing that CsA infusion is associated with a significant decrease in [18F]MPPF efflux (k2) in most brain regions and with a more restricted, though still significant, increase in [18F]MPPF BP. Differences in absolute values according to the reference region might indicate variable Pgp expression within the cerebellum and thalamus. Our second hypothesis was that the presence of focal over-expression of Pgp within the epileptogenic temporal lobe will result in CsA sensitive asymmetry in [18F]MPPF BP or k2. So far, this hypothesis has not been confirmed.

Healthy subjects (D4.9): As in epileptic patients, the concurrent infusion of CsA was associated with:
1) a significant reduction of [18F]MPPF k2 in the majority of brain regions. The average K2 reduction amounted to 13% using the thalamic reference region versus 17% using the cerebellar reference region (as compared to 14% and 23% in patients, respectively)
2) a significant increase of [18F]MPPF BP in selected regions. The average BP increase was 14% using the thalamic reference region versus about 17% using the cerebellar reference region (as compared to 9% and 16% in patients, respectively).

As expected, the [18F]MPPF BPND asymmetry indices were close to zero in controls, with no impact of CsA infusion on this parameter. Overall, data obtained in healthy subjects fully confirm those described above in patients, by showing that CsA infusion is associated with a significant decrease of [18F]MPPF efflux (k2) in most brain regions and with a more restricted increase of [18F]MPPF BP. In contrast to patients, the impact of the reference region was minimal in healthy subjects. The magnitude of CsA-induced changes in [18F]MPPF K2 and BP was globally comparable between patients and controls, being almost similar for K2 using the thalamic reference region (14% and 13% respectively), and for BP when using the cerebellar reference region (16% and 17%, respectively).

[11C]-FMZ:
Naïve and epileptic rats (Task 1-4, (P02-VUmc, P10-LACDR, P11-SEIN, P06-LMU)
The quantification procedures have been successfully optimized in naïve rats. The data of the [11C]FMZ PET scans have been modelled according to the Lassen and the Liefaard approach and the estimated parameters were remarkably similar. The values for Bmax were 31 ±13 and 34 ±5 ng/mL, respectively, and for the KD 7.5 ±1.7 and 5.8 ±0.9 ng/mL. This validates the full saturation approach as a reliable and actually more precise method for the assessment of GABAA receptor density and affinity.

Moreover, as the method requires only a single scan it is cheaper, more time-efficient and causes less inconvenience and discomfort. Therefore this method has been selected for further experiments.

Fourteen naïve rats and fifteen rats exposed to kainate-induced status epilepticus were used for [11C]FMZ PET scanning before and after administration of 3 or 15 mg/kg TQD. The KA-exposed rats were scanned 7 days post-SE. GABAA receptor density, Bmax, was estimated as (average and SD) 44 ± 2 ng mL-1 in the hippocampus and as 33 ± 2 ng mL-1 in the cerebellum with intermediate values in the occipital cortex, parietal cortex and caudate putamen. Bmax was decreased by 12% in kainate treated rats compared to controls. The receptor affinity, KD, was similar in both rat groups and all brain regions and was estimated as 5.9 ± 0.9 ng mL-1. There was no difference in flumazenil transport across the blood-brain barrier between control and kainate treated rats and the effect of TQD treatment was similar in both rat groups. TQD treatment also decreased flumazenil transport out of the brain by 73%, increased the volume of distribution in the brain by 24%, and did not influence Bmax or KD compared to baseline. As Bmax estimates were not influenced by TQD, this suggests that [11C]flumazenil scanning is not confounded by alterations in Pgp function.

Ten Pgp dKO mice and nine WT mice have been PET scanned after administration of [11C]FMZ. Six mice of each group have been scanned again after administration of 15 mg/kg TQD. Scans in dKO mice revealed that these animals exhibit 70% higher [11C]flumazenil uptake in the brain than WT mice. After administration of TQD, brain [11C]flumazenil uptake in WT mice increased by about 80%, while it remained the same in dKO mice. The increase after TQD in WT mice and in rats is comparable. This shows that [11C]flumazenil is a Pgp substrate in rodents. Consequently, altered cerebral [11C]flumazenil uptake, as observed in epilepsy, may not reflect solely GABAA receptor density changes but also changes in Pgp activity. In conclusion, all studies in animals (in WT and dKO mice and rats) have indicated that flumazenil is a weak Pgp substrate. It proved to be possible to estimate changes in receptor density (Bmax) and in Pgp mediated transport induced by kainate-induced status epilepticus independently.

Moreover to complete this study, an additional group of responder and non-responder epileptic rats has been selected by partner P06-LMU for testing of [11C]flumazenil in responders and non-responders in a chronic, electrical post-status epilepticus model with spontaneous recurrent seizures. Baseline [11C]flumazenil SUV data calculated for the complete scan and for the first 2.25 min of the scan did not differ between electrode-implanted control rats as well as epileptic responder and non-responder rats. In addition, no significant differences were identified between the three groups of animals when respective SUV data were analyzed following TQD pre-treatment. Thus, the data suggest that neither differences in Pgp function nor differences in GABAA receptor subunit composition can be detected with [11C]flumazenil PET between the three groups of animals. Immunhistological analyses of Pgp expression have been completed. The analyses did not reveal major differences in most of the brain regions, except from the caudal part of the hippocampal CA1 subregion, in which the optical density of Pgp labelling in non-responders exceeded that in controls whereas no such difference to controls was observed in responders. Thus, the failure to identify differences in the response of [11C]flumazenil to TQD might also be related to the fact that differences in Pgp expression between epileptic

and non-epileptic rats proved to be limited to one hippocampal subregion in this experiment. The reasons for differences in Pgp regulation as compared to previous studies are unknown, however, a genetic drift in the outbred rat strain might have contributed.

Epilepsy patients (Task 5 P02-VUmc, P10-LACDR, P11-SEIN)
The study has been performed successfully in ten patients with unilateral MTLE. MRI and PET images were co-registered (Vinci), grey and white matter were segmented (SPM5) and 45 ROIs were automatically delineated (PVElab). Metabolite corrected plasma levels were used for the input function and the Pons was used as the reference tissue. Modelling was performed with ROI analysis. Treatment with 2 mg/kg TQD caused a reduction in Binding Potential, an increase in the brain to plasma ratio VT and an increase in the k1/k2 ratio (15-19%). The changes in [11C]flumazenil in response to TQD did not vary much between regions. There was no evidence for changes in cerebral blood flow after TQD, as assessed by PET scans with [15O]H2O. Taken together these data indicate that flumazenil is a weak substrate for Pgp in humans, consistent with the preclinical studies in rats and mice. In summary, the investigations in pharmacoresistant patients with unilateral MTLE are consistent with the animal studies in the sense that they indicate that flumazenil is a weak Pgp substrate. However, the influence is considered too weak to be of clinical usefulness for the assessment of changes in Pgp mediated transport or to confound assessment of GABAA-receptor density and focus localisation. Therefore the data did not justify continuing the investigations in healthy volunteers.

WP05: Quantitative imaging of Pgp function in healthy controls
Objectives
The main objective is to characterize Pgp tracers first in healthy controls and to develop tracer kinetic models for quantifying Pgp related pharmacokinetic parameters.

At the start of the EURIPIDES project, only one tracer for in vivo assessment of Pgp function, i.e. (R)-[11C]VPM, was available within the consortium. At the end of the project, within WP05 three others, developed in WP01 and preclinically evaluated in WP02, have been fully evaluated in human studies and other tracers are about to enter into clinical studies. In addition, within WP05, understanding of (R)-[11C]VPM kinetics has improved significantly following extensive studies to probe Pgp function at the blood-brain barrier. This understanding was essential for probing Pgp function in epileptic and Alzheimer's disease patients (WP06 and WP07).

Task 1: Development of tracer kinetic models for (R)-[11C]-VPM and newly developed Pgp PET tracers
Just prior to the start of EURIPIDES, a tracer kinetic model for (R)-[11C]VPM had been published. This single tissue compartment model provides the volume of distribution as outcome measure and a pilot study in normal subjects had shown excellent test-retest variability. However, the model assumed that one of the labelled metabolites, [11C]D617, enters the brain with kinetics similar to that of (R)-[11C]VPM itself. To assess whether this assumption is correct, [11C]D617 was synthesized and preclinical studies were performed to study its kinetics in the brain. These preclinical studies involved in vivo PET scans and ex vivo biodistribution studies, both before and after pre-treatment with TQD. The main finding was that the volume of distribution of [11C]D617 was comparable to that of (R)-[11C]VPM, but the increase after TQD pre-

treatment was substantially lower. Although above mentioned assumption underlying the (R)-[11C]VPM model is not entirely correct, effects on quantification of baseline (R)-[11C]VPM studies is expected to be very small. A secondary finding was that [11C]D617 itself could be used as a substrate tracer of Pgp, but given its lower sensitivity to TQD pre-treatment there appears to be no clear advantage over (R)-[11C]VPM itself.

Although the single tissue compartment model appeared to be the method of choice for baseline (R)-[11C]VPM studies in healthy subjects, several studies performed within the consortium showed that the model was not ideal under all conditions. Using spectral analysis to analyse (R)-[11C]VPM studies in Alzheimer patients clearly showed a second (small) compartment and especially this second compartment differed from that in normal controls. In addition, it was shown that after TQD inhibition, the second compartment clearly became evident. Therefore, the model was adapted to include a second compartment by fixing the K1/k2 ratio to the value for whole brain grey matter. This constraint was applied to keep the degrees of freedom to a minimum and avoid convergence problems. The constrained two tissue compartment model was used in various subsequent studies.

As preparation for [11C]laniquidar brain studies, first a whole body study was performed to determine radiation dose. Following this radiation dosimetry study, actual studies on cerebral kinetics of [11C]laniquidar were performed. In plasma, [11C]laniquidar was metabolized rapidly with a parent fraction less than30% after 30 minutes. In addition, the first pass extraction was very low, i.e. 2-3%. When developing a tracer model to describe [11C]laniquidar kinetics it first appeared that a single parameter model was sufficient, which could be expected for a true Pgp inhibitor. Unfortunately, detailed analysis showed that a better model was one where labelled metabolites entered the brain, indicating that [11C]laniquidar is not an ideal tracer for studies in patients (as originally planned in WP06).

Preparatory whole body studies for radiation safety purposes were also performed for [11C]elacridar and [11C]TQD. To evaluate both [11C]elacridar and [11C]TQD as P gp tracers, they were used in paired scan protocols with (R)-[11C]VPM. Sixty minutes after injection of [11C]elacridar or [11C]TQD, 3 mg/kg TQD was administered over 30 minutes, which was followed by a further 30 minutes of data acquisition. Following a similar protocol in naïve rats (WP02), one hour after the end of the [11C]elacridar or [11C]TQD scan, an (R)-[11C]VPM scan was acquired. In general, brain uptake of [11C]elacridar and [11C]TQD was very low at baseline, i.e. approximately 3 times lower than that for baseline (R)-[11C]VPM scans. In contrast to previous (R)-[11C]VPM results, there was no change in brain and blood time-activity curves of both [11C]elacridar and [11C]TQD in response to TQD administration. A possible explanation may be that both radiolabelled inhibitors are dual Pgp and BCRP substrates and that TQD only blocks Pgp at the dose of 3 mg/kg used in this study. Both tracers showed good metabolic stability with greater than80% of total radioactivity in plasma being unchanged parent up to 60 min after injection. Different kinetic models were tested for both [11C]elacridar and [11C]TQD. A single tissue compartment model was identified as the model of choice for quantification of both tracers. Measured volumes of distribution of both [11C]elacridar and [11C]TQD positively correlated with those of (R)-[11C]VPM, indicating that both

[11C]inhibitors and (R)-[11C]VPM behave as transporter substrates at the human blood-brain barrier.

Task 2: (R)-[11C]VPM and newly developed Pgp PET tracers test-retest studies in healthy controls

Just prior to the start of EURIPIDES, preliminary (R)-[11C]VPM test-retest variability for the single tissue compartment model had been published for a rather small series of healthy subjects. Although whole brain test-retest was excellent, the subject population was too small to address test-retest variability at a regional level, which is important, as it forms the basis for determining the significance of changes seen in (intervention) studies in patients (e.g. within the epileptic focus). In addition, during the course of EURIPIDES, it was discovered that a constrained two tissue compartment model is to be preferred, especially for studies in patients. To increase statistical power, further test-retest data were acquired in healthy subjects. For regional analysis, PVElab, an automatic atlas-based approach developed by one of the partners, was used and test-retest variability was assessed for various outcome measures derived from different models used in the literature. In addition, the effects of partial volume correction were investigated. As mentioned above (task 1), the constrained two-tissue compartment model provided the best fits to the data. In addition, test-retest variability of the volume of distribution was comparable for single-tissue (6% for global cortical region) and constrained two-tissue (9% for global cortical region) compartment models, indicating that the latter model can indeed be used for assessing changes over time and for monitoring response after treatment with disease-modifying drugs. Interestingly, test-retest variability was similar for partial volume corrected data.

Test-retest variability was also assessed for [11C]laniquidar studies. Initially, data were analysed with a K1 (influx) model only and a large systematic difference between morning and afternoon scans was observed. After standardising lunch, i.e. making sure that lunch and breakfast were similar, this difference disappeared, indicating the importance of dietary control in studies of Pgp. At a later stage, it was established that a model with cerebral uptake of radiolabelled metabolites provided better fits to the data (see description task 1). Nevertheless, test-retest variability of K1 remained good for those studies where lunch was standardised.

Task 3: Effects of Pgp modulators on cerebral (R)-[11C]VPM

The optimal TQD dose for modulating (R)-[11C]VPM kinetics was determined using a TQD dose escalation study. First, in a pilot study, healthy subjects had undergone paired (R)-[11C]VPM scans before and after administration of 2 mg/kg TQD (given iv over 30 min). In this study only a small increase (+24%) in the volume of distribution of (R)-[11C]VPM in whole brain was observed. Next, in a second study, subjects were scanned with (R)-[11C]VPM after administration of 3, 4, 6 or 8 mg/kg TQD. Measured increases in the volume of distribution of (R)-[11C]VPM in whole brain were fitted to a sigmoidal dose-response curve, which showed that half-maximum effect concentrations (EC50) of TQD were similar in humans and rats (EC50 561±24 ng/ml in humans and 544±32 ng/ml in rats, respectively), corresponding to a TQD dose of 3 mg/kg. Based on this dose escalation study, the dose of 3 mg/kg was considered to be optimal for assessing Pgp function with (R)-[11C]VPM both in humans and in rats. Interestingly, the maximum increase in (R)-[11C]VPM after the highest TQD dose (8 mg/kg in humans and 15 mg/kg in rats) was different between humans and rats. In humans there was only a 3-fold increase, whereas in

rats a 12-fold increase was observed, pointing to species differences in Pgp between humans and rats.

In another study, paired [15O]H2O and (R)-[11C]VPM scans were acquired in healthy subjects both under baseline conditions and after either 2 or 3 mg/kg of TQD. The [15O]H2O scans were performed in order to measure perfusion and to exclude any confounding perfusion effects in case of changing (R)-[11C]VPM kinetics. Both doses of TQD, however, did not result in a change in perfusion, so flow effects can be excluded as the cause of TQD induced changes in (R)-[11C]VPM kinetics. As the main purpose of these studies was to differentiate TQD induced changes in epilepsy patients from those in healthy subjects, these (R)-[11C]VPM data are reported in WP06.

Task 4: Effects of age and gender on cerebral (R)-[11C]VPM
The known small global age effect was confirmed in a second, relatively small, series of young and elderly healthy subjects, showing that the volume of distribution of (R)-[11C]VPM increased in amygdala, insula and cerebellum by 30, 26 and 25%, respectively. To study age effects in more detail and to investigate possible gender effects, the original (male) data set was extended with a third (middle aged) group and additional studies in females. It was confirmed that the volume of distribution of (R)-[11C]VPM increases with age in several cortical brain regions, strongly suggesting a progressive decrease in blood-brain barrier Pgp function with age. Interestingly, this age effect was only seen in males.In contrast, Pgp function in younger females was similar to that in older women, which in turn was similar to older men. Findings were similar for partial volume corrected data. The different aging patterns between men and women highlight the need to include both males and females in aging studies.

Task 5: Effects of partial volume correction on pharmacokinetic parameters derived from (R)-[11C]VPM and newly developed Pgp PET tracer studies
Several partial volume correction methods were investigated and implemented. These included partial volume correction based on both MRI segmentation techniques and novel reconstruction algorithms that take into account the point spread function of the scanner. These methods should be generally applicable, i.e. independent of the actual tracer being used. Both methods were validated by scanning subjects on two different scanners with different spatial resolution.

Finally, the existing software package PVElab was extended by including the so-called Hammer's volume of interest (VOI) template. This template includes 67 VOIs, incorporating all brain voxels, and is now available within the software for partial volume correction. The software can be downloaded by registering at http://nru.dk/pveout.

WP06: Quantitative imaging of Pgp function in temporal lobe epilepsy
Objectives
The main objective is to validate radiotracers for Pgp activity as biomarkers for drug resistance using drug-resistant and drug-responsive epilepsy as a biological model. The required proof-of-concept of a non-invasive molecular imaging-based tool is the ability to differentiate drug-resistant from drug-responsive patients. Epilepsy is the ideal condition for testing suitability of these tracers, as either surgical specimens or whole brains are available for direct in-vivo / ex-vivo comparative analysis (WP08).

At the beginning of EURIPIDES, Pgp function had recently been measured in vivo using PET and the radiolabelled Pgp substrate (R)-[11C]VPM (VPM) in a small group of seven patients with temporal lobe epilepsy (TLE) (Langer et al, 2007 Epilepsia 48: 1774-84). Comparing the ipsilateral to the contralateral side of the seizure focus, this pilot study measured reductions in the volume of distribution (VT) in several temporal lobe regions and not in extra-temporal regions, providing evidence for regionally enhanced Pgp activity at least in some TLE patients. Due to the high tracer uptake in the adjacent choroid plexus, Pgp function could not be measured in the epileptogenic region of the hippocampus. These interesting results raised a number of important questions, whether

- Pgp activity was even more enhanced in the epileptogenic region
- Pgp overexpression was also present in the side contralateral to the seizure focus
- Pgp inhibition could increase delivery of Pgp substrates to the brain to overcome Pgp overexpression
- Pgp expression is related to exposure to AEDs and/or to seizure activity

The workpackage set out to address these questions by scanning a larger number of epilepsy patients, comparing patients refractory with those responsive to medication and against healthy volunteers, under baseline condition and after Pgp inhibition or discontinuation of AEDs. To overcome the limitation of the contamination of the hippocampus by the choroid plexus, TLE patients would be scanned in a high resolution brain PET camera and the images reconstructed and processed to further optimise the spatial resolution in order to gain access to the epileptogenic region. Patients with focal cortical dysplasia (FCD) where the seizure focus is located far away from the choroid plexus would also be studied. [11C]Laniquidar did not emerge as successful from WP05 and hence PET studies in epilepsy patients with this new Pgp inhibitor radiotracer were not conducted. All studies in WP06 reverted to using VPM to answer the clinical questions.

Methodology development

New methodology was developed to underpin the clinical studies of this workpackage. A comprehensive comparison of kinetic models was undertaken for VPM and the one-tissue compartment model using a total plasma input function was found to be the most robust to alteration in peripheral metabolism, which is more extensive in epilepsy patients taking AEDs than in healthy volunteers, and to increased brain uptake after Pgp inhibition. Considering reproducibility and consistency in effect size after Pgp inhibition, the transport rate constant from plasma to brain, K1, was selected over the total volume of distribution, VT, as the parameter of choice to quantify Pgp function measured with VPM. When fitting the first 10min of dynamic data, thereby limiting the contamination of brain crossing radiolabelled metabolites, using the VPM fraction in plasma or total plasma as the input function gave similar K1 estimates.

The choroid plexus is difficult to delineate on conventional magnetic resonance (MR) images but the contrast agent gadolinium leaks into the choroid plexus due to its lack of blood-brain barrier and high vascularity. A protocol for MR scanning was developed and the choroid plexus was segmented from the contrast enhanced MR images for use with MR-based partial volume correction (PVC) methods. Using dynamic contrast-

enhanced MRI, only part of the choroid plexus in the lateral ventricles but not in the temporal horn adjacent to the hippocampus could be kinetically segmented in the parametric maps of the contrast transfer coefficient (Ktrans) due to the poorer spatial resolution. Automatic segmentation of the choroid plexus separately from blood vessels proved difficult from the higher spatial resolution post-contrast T1-weighted images. When manually segmented, the choroid plexus was reproducible and displayed good correspondence with the VP.

However, small misregistration errors between the PET and MR images led to erroneously estimated VPM uptake in the hippocampus after MR-based PVC. It was nonetheless possible to recover the VPM uptake in the hippocampus by subsampling the part of the hippocampus located away from the choroid plexus.

Task 1: Test-retest studies in patients with epilepsy (P01-UCL, P08-UNIMAN)

The test-retest reproducibility (calculated as mean absolute difference) of VPM K1 in temporal lobe of epilepsy patients was found to be 5.6% (n=6) in pharmaco-sensitive TLE patients who underwent paired baseline PET scans on the brain HRRT camera. Test-retest scans were performed on the same day, in the morning and in the afternoon, similarly to the paired baseline-blocking scans with TQD (in this fixed order). There is no order effect of the time of day when the scans were performed, as indicated by the mean difference between test-retest scans of -0.8% in the temporal lobe. The reproducibility compares favourably to that determined for the corresponding region in healthy volunteers using the same kinetic model and outcome parameter on the lower spatial resolution HR+ camera (8.6% – see WP05).

Task 2: Effect of chronic active epilepsy (P01-UCL, P03-MUW, P08-UNIMAN)

In a voxel-based comparison of VPM K1 ratio images to global mean using statistical parametric mapping (SPM8), VPM uptake was found to be significantly reduced by 14% in pharmaco-resistant (n=14) compared to pharmaco-sensitive patients (n=8) in ipsilateral amygdala, and bilateral parahippocampal, fusiform, inferior and medial temporal gyrus, suggesting Pgp overexpression in pharmaco-resistant mTLE exclusively in temporal lobe regions, but both ipsi- and contralateral to the epileptogenic focus. There was no area of significant difference, neither reduced nor increased VPM uptake, between pharmaco-sensitive patients and healthy controls (n=13). Pharmaco-sensitive TLE patients, unlike healthy controls but like pharmaco-resistant TLE patients, were treated with AEDs. In contrast with pharmaco-resistant TLE patients, pharmaco-sensitive TLE patients did not have seizures for many years. There was a negative correlation globally and within the ipsilateral hippocampus (Spearman r = -.604, p= 0.029) of VPM uptake in pharmaco-resistant patients with seizure frequency, implying that pharmaco-resistant TLE patients with frequent seizures had lower VPM uptake and hence elevated Pgp function

After resective brain surgery, the antiepileptic drug load was reduced in all 7 pharmaco-resistant TLE patients (4 selective amygdalahippocampectomy and 3 anteromesial temporal lobe resection). Five patients were completely seizure free after the surgery (Wieser's classification class 1a), one patient had auras (class 2) and one patient had maximally 3 seizure days a year (class 3) over a follow-up period of two years after surgery. A regional analysis revealed that on average across patients VPM uptake (K1) was reduced by 2 to 15% with large

variations between patients (-46% to +39%). VPM uptake increased in the 2 patients that were medication and seizure free after surgery, suggesting that Pgp function was reduced after seizures and drug exposure ceased. The clinical study was initiated using a dose of TQD of 2 mg/kg which had been safely administered in clinical trials with the Pgp inhibitor. The dose escalation study later conducted in healthy controls for WP05 indicated that the optimal dose of TQD for modulating VPM kinetics would be 3 mg/kg, corresponding to the half maximum effective dose (ED50). The lower dose had already been used when scanning the first half of the drug-refractory TLE patients; it was changed to the higher dose for the second half of the patient cohort. A second cohort of healthy controls was scanned at the higher dose in addition to the original cohort scanned at the lower dose.

Task 3: Effect of Pgp inhibitors on ligand uptake in patients with epilepsy (P01-UCL, P08-UNIMAN)

A voxel-based analysis of VPM K1 images compared the response to two doses of TQD in pharmaco-resistant TLE patients with healthy controls and found significantly lower increases of VPM uptake in the ipsilateral hippocampus in patients (n=14, 24.3%) compared to controls (n=13, 62.4%). After 2mg/kg TQD, the hippocampal increase was lower (n=7, 16.5%) compared to 3mg/kg (n=7, 32.4%) but still significantly attenuated compared to the increases observed in healthy controls, both after 2mg/kg (n=7, 63.0%) and 3mg/kg (n=6, 66.8%), suggesting functionally elevated Pgp function in pharmaco-resistant mTLE in the epileptogenic hippocampus above all regions. Five of the 14 pharmaco-resistant TLE patients who had paired PET scans with VPM underwent anterior temporal lobe resection for surgical treatment of their epilepsy. The surgical specimens were analysed quantitatively by immunohistochemistry with antibodies for measurement of Pgp expression. There was a negative correlation (Spearman r = -.900, p= 0.034) between presurgical increases in VPM uptake after TQD in the hippocampus compared to the ipsilateral inferior and medial temporal gyrus and the corresponding difference in the percentage area of Pgp-immunopositive labelling, demonstrating a correspondence between ex-vivo Pgp expression and in-vivo Pgp function measured by PET.

Old Task 4: Influence of AEDs and acute seizures on brain Pgp function (P03-MUW)

After scanning the first patient twice with a scan-to-scan interval of three hours, it was realised that it is not possible to keep patients off medication for long enough to achieve significant reduction of plasma AED levels without increasing the risk of seizures. It was not feasible to assess the influence of AEDs on Pgp function with this approach. The technical annexe was amended and the study was replaced by the new task 4. Twenty patients with refractory epilepsy due to FCD or tumours would be scanned with VPM before and after TQD administration. As these patients were expected to be difficult to recruit, it was originally envisaged that joint efforts would be undertaken by MUW and UniMan/UCL. In the end, scanning only took place at UniMan/UCL with the consequence that data in fewer patients than anticipated were acquired. Instead, MUW recalled the pharmaco-resistant TLE patients previously scanned with VPM prior to surgery to undergo PET scanning again after surgical treatment in order to assess the effects of seizures of Pgp function (see Task 2).

New Task 4: Effect of TQD on brain Pgp function in patients with FCD and tumours (P01-UCL, P08-UNIMAN)

FCD is a congenital maldevelopment of the cortical tissue and one of the most common causes of refractory epilepsy. Unlike in TLE where several

studies have demonstrated the overexpression of Pgp in brain tissues, studies on the neuronal expression and function of Pgp in patients with FCD are scarce. Unlike TLE where the hippocampus is contaminated by spillover of radioactivity from the adjacent choroid plexus, FCD, where the seizure focus is located far away from the choroid plexus, may allow analysis of epileptogenic brain regions without the need for partial volume correction. Compared individually against the group of 13 healthy controls, all 3 pharmaco-resistant FCD patients had reduced VPM uptake in close proximity to the area of FCD identified by MRI and the reduction extended further to other ipsilateral regions. This pilot study confirms that in FCD patients there is Pgp overexpression, not only in the epileptogenic area but also the distribution of Pgp overexpression is spread across to other cortical regions.

High-grade gliomas (HGG) are currently treated with radiotherapy in combination with the DNA-methylating agent temozolomide but HGG are also treatment resistant. Four patients with suspected HGG (3 grade III and 1 grade IV, confirmed by targeted biopsies) underwent dynamic PET scanning after injection of [15O]H2O followed by VPM before and after administration of TQD. At baseline, cerebral blood flow (CBF) in gray and white matter was normal in grade III patients but reduced in the grade IV patient. CBF in glioma was heterogeneous within tumour and between patients and did not always correspond to areas of gadolinium leakage in the MR images. The uptake of VPM paralleled that of CBF at baseline. Pgp inhibition minimally altered CBF but differentially increased VPM uptake into brain tissue and glioma, and preferentially into grade III but not grade IV regions. PET with a radiolabelled Pgp substrate is a promising tool to guide the potential use of Pgp inhibitors as adjuvant chemotherapy in HGG in order to increase chemotherapeutic efficacy.

WP07: Quantitative imaging of Pgp function in Alzheimer's Disease
Objectives
Alzheimer's disease (AD) is the most common form of dementia. A major pathological hallmark of AD is the deposition of amyloid-ß plaques in the brain. The origin of amyloid-ß depositions in Alzheimer's disease is unclear. The amyloid hypothesis proposes that this may be caused by an imbalance between amyloid-ß production and clearance. The hypothesis we set out to test at the beginning of WP07 was that P-glycoprotein function is reduced in patients with Alzheimer's disease, so that an increase in binding potential (BPND) of (R)-[11C]VPM is expected in patients with Alzheimer's disease compared with healthy controls.

Mechanisms involved in clearing amyloid-ß from the brain include degradation by a variety of proteases, removal through the interstitial fluid-CSF bulk flow into the bloodstream, perivascular lymphatic drainage and transport across the blood-brain barrier. Although the exact cause of reduced clearance of amyloid-ß from the brain in sporadic Alzheimer's disease remains unclear, several leads point towards a regulatory role for the blood-brain barrier. It has been suggested that P-glycoprotein is also involved in the clearance of amyloid-ß from brain. The evidence that amyloid-ß is also a substrate for P-glycoprotein is based on several studies. For instance, it has been shown in vitro that P-glycoprotein transports amyloid-ß and that blocking P-glycoprotein function decreases transport of amyloid-ß. Furthermore, amyloid-ß depositions are inversely correlated with P-glycoprotein expression in the brain of elderly nondemented humans. In addition, in an Alzheimer's disease mouse model, knocking out blood-brain barrier P-glycoprotein expression increased amyloid-ß depositions, whilst restoring blood-brain barrier P-

glycoprotein expression and transport activity reduced brain amyloid-ß levels. At the start of this WP, there was no in vivo information on P-glycoprotein function in sporadic Alzheimer's disease.

P-glycoprotein function can be assessed in vivo using the PET tracer [11C]VPM. [11C]VPM enters the brain through passive diffusion and, at the low concentrations used in PET, it is a substrate for P-glycoprotein. The validity of [11C]VPM as a PET tracer for assessing P-glycoprotein function is discussed in WP05 of this report.

Fifteen subjects with probable Alzheimer's disease and fourteen age-matched healthy controls were included in this study. Patients with Alzheimer's disease with mild to moderate disease (Mini-Mental State Examination scores = 20) were recruited from the out-patient Memory Clinic of the Alzheimer Center of the VU University Medical Center, Amsterdam. Clinical diagnosis of probable Alzheimer's disease was established by consensus in a multidisciplinary meeting according to the criteria proposed by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA).

To confirm the presence of Alzheimer's disease pathology in the brain, increased cortical accumulation of [11C]PIB PET was required for patients with Alzheimer's disease. All subjects also underwent structural MRI. PET scans were performed on an ECAT EXACT HR+ scanner (Siemens/CTI). All subjects received an indwelling radial artery cannula for arterial blood sampling and a venous cannula for tracer injection. A dynamic emission scan in 3D acquisition mode was started simultaneously with a bolus injection of (R)-[11C]VPM. Using an on-line blood sampler (Veenstra Instruments), arterial blood was withdrawn continuously and manual samples were withdrawn at several time points. These manual samples were used to determine plasma to whole blood radioactivity concentration ratios. In addition, fractions of parent (R)-[11C]VPM and its radioactive polar metabolites in plasma were determined using a combination of solid-phase extraction and high-pressure liquid chromatography.

On the same day, patients with Alzheimer's disease underwent a second PET scan following injection of [11C]PIB in order to confirm amyloid-ß pathology in the brain. All PET data were corrected with standard algorithms. Regions of interest were defined based on the segmented MRI using a probabilistic template. PET data were not corrected for partial volume effects. First, (R)-[11C]VPM data were analyzed using spectral analysis, a technique that produces a spectrum of the kinetic components that are needed to relate tissue response to plasma input function without making a priori assumptions regarding the number of compartments. Based on the results of spectral analysis, (R)-[11C]VPM data were also analysed using non-linear regression of the standard two tissue compartment model including a blood volume component. To obtain robust BPND values, the non-specific distribution volume K1/k2 was first determined for a whole brain grey matter region of interest and then assumed to be the same for all grey matter regions of interest. BPND of frontal (volume-weighted average of orbital frontal, medial inferior frontal, and superior frontal), parietal, temporal (volume-weighted average of superior temporal and medial inferior temporal), occipital, medial temporal lobe (volume-weighted average of entorhinal cortex and hippocampus) and cerebellar cortices and posterior and anterior cingulate was used. In addition, a global cortical region was defined consisting of

the volume-weighted average of frontal, parietal, temporal and occipital cortices and anterior and posterior cingulate.

Of the 15 patients with Alzheimer's disease, 13 were classified as being PIB positive and 2 as PIB negative. The 2 PIB-negative patients were excluded from the Alzheimer's disease group for further analysis. Controls who underwent a [11C]PIB scan were PIB negative. There were no differences between groups with respect to age, sex, injected dose and specific activity of (R)-[11C]VPM. Spectral analysis of (R)-[11C]VPM data clearly showed the presence of two components in both patients with Alzheimer's disease and healthy controls.

In healthy controls these two components had clearance rates of 0.039 ± 0.010min-1 and 0.43 ± 0.42min-1 for global cortical region. In patients with Alzheimer's disease, for global cortical region clearance rates of 0.040 ± 0.008min-1 and 0.77 ± 0.49min-1 were found. For the latter component a significantly faster clearance rate was found in Alzheimer's disease ($P = 0.0003$). Because of these spectral analysis findings, a two tissue compartment model was implemented and used for further quantitative data analysis. Global cortical BPND of (R)-[11C]VPM was higher in patients with Alzheimer's disease compared with age-matched healthy controls.

In fact, for the parietal, temporal, occipital and posterior cingulate region of interest, significantly higher BPND values were found in Alzheimer's disease compared with healthy controls. Also for frontal and anterior cingulate region of interest, significantly higher BPND values were found in Alzheimer's disease, though the differences for these regions were smaller. There were no between-group differences for medial temporal lobe and cerebellum. In the two PIB-negative patients, (R)-[11C]VPM BPND values were in the range of the PIB-positive patients with Alzheimer's disease with global BPND values for (R)-[11C]VPM of 2.18 and 2.15, respectively. No significant associations were found between BPND of (R)-[11C]VPM and BPND of [11C]PIB.

The main finding of this study is an increased (R)-[11C]VPM BPND in the brain of patients with Alzheimer's disease as compared with values in healthy age-matched controls. For the global cortical region this increase was ~23% and in some smaller regions like posterior cingulate there was an even larger increase of 33%. As the direction of change in BPND in the patients with Alzheimer's disease in this study is similar to that induced by P-glycoprotein blocking in healthy subjects, the corresponding increase in BPND can be interpreted as a measure of decreased P-glycoprotein function. This suggests reduced P-glycoprotein function at the blood-brain barrier in patients with Alzheimer's disease, not only at a regional, but also at a global level. To the best of our knowledge, this is the first in vivo evidence that blood-brain barrier P-glycoprotein transporter dysfunction occurs in sporadic Alzheimer's disease.

The pathophysiology underlying decreased P-glycoprotein function at the blood-brain barrier in Alzheimer's disease is not known and it is unknown whether P-glycoprotein function decreases prior to the occurrence of amyloid depositions, or whether amyloid plaques have a destructive effect on the blood vessel wall resulting in secondary P-glycoprotein dysfunction. Longitudinal in vivo imaging studies of both amyloid deposition and P-glycoprotein function, preferably in combination with

other imaging techniques to measure the degree of small vessel disease, could shed some light on this interesting question. Decreased blood-brain barrier P-glycoprotein function may be a crucial factor in the pathogenesis of Alzheimer's disease. Probing P-glycoprotein function at the blood-brain barrier in the very early stages of amyloid depositions is of crucial importance. If decreased P-glycoprotein function is involved in the pathogenesis of amyloid deposition in Alzheimer's disease, this would implicate that P-glycoprotein may be a potential treatment target and a possible target to modulate disease progression. In a broader sense, P-glycoprotein dysfunction in Alzheimer's disease may be a (surrogate) marker for more widespread blood-brain barrier dysfunction in Alzheimer's disease, involving also other amyloid-ß transporters, such as LRP1.

In a second project in WP07, we studied the relation between amyloid-beta accumulation in cerebral blood vessel walls, known as cerebral amyloid angiopathy (CAA) and P-glycoprotein function. CAA is present in nearly all AD brains, although severity amongst individuals varies strongly. Microbleeds (MBs), which can be observed using gradient echo weighted magnetic resonance imaging (MRI) supposedly, reflect underlying CAA. Recently, decreased BBB Pgp function in sporadic AD patients was found using positron emission tomography (PET) with the radiolabelled Pgp substrate (R)-[11C]VPM. At the beginning of this project, no studies had been performed to assess differences in BBB Pgp function between AD patients with and without signs of advanced CAA in the brain. As such, the purpose of this project was to investigate global and regional associations between MBs and Pgp function in AD patients.

Eighteen patients with probable AD in a mild to moderate disease stage were included in this study. Patiens were the same as included in the first project. To assess presence of MBs and superficial siderosis, which is also a common MRI finding associated with CAA, a susceptibility weigted imaging (SWI) MRI scan was performed in all patients. MBs were identified on the SWI sequence by an experienced neuroradiologist and defined as rounded, hypointense homogeneous foci up to 10 mm in size. Lesions in sulci probably representing flow voids from vessels and lesions in the globus pallidus, supposedly representing iron or calcium deposits, were not considered. Choroid plexus and pineal calcifications were also not considered, as were lesions suggestive of partial volume effects (PVE).

There were no significant differences in (R)-[11C]VPM BPND between AD MB- and AD MB+ groups for any of the regions investigated. Results were essentially the same after PVE correction. Distribution volumes (VT) did not differ between the AD MB- and MB+ groups for any of the regions investigated (e.g. for the global cortical brain region, VT = 0.84 ± 0.16 and 0.89 ± 0.17 for MB- and MB+ groups, respectively; p 0.35). In addition, the non-specific volume of distribution (K1/k2 ratio) did not differ (for the global cortical brain region: AD MB- 0.27 ± 0.08; AD MB+ 0.29 ± 0.07, p 0.71) between the AD MB- and AD MB+ group for any of the regions investigated. No differences were found in BPND of (R)-[11C]VPM between AD patients with MBs and those without.

These results indicate that there is no evidence of additional Pgp dysfunction at the BBB in support of the hypothesis of additionally impaired Pgp function in AD MB+ patients compared with AD MB- patients. There may be multiple explanations for these findings. First, it is possible that decreased Pgp function in AD MB+ patients could not be

demonstrated due to lack of statistical power (small group sizes). Second, it could be due to the inclusion criteria used in this study. Autopsy studies have shown that nearly all AD patients show some degree of vascular amyloid-beta. Still, only a minority of AD patients shows signs of CAA on MRI such as microbleeds or superficial siderosis during life. It is possible that only patients with severe CAA show MBs on MRI, but conclusive evidence is missing. An alternative explanation would be that severity of CAA pathology is only weakly related to the presence and number of MBs on MRI. In addition, it is also possible that additional Pgp dysfunction does occur, but at a more locoregional level, e.g. directly around MB locations. This would, however, be beyond the spatial resolution of PET, given the relatively low target to background ratio of (R)-[11C]VPM uptake. It should be noted that it is also possible that AD MB+ and AD MB- patients really do not differ from each other in terms of Pgp function, since almost all AD patients do have some degree of CAA and all have amyloid-beta accumulation in the brain. Because both groups suffer from the same pathology, which very likely is present in the brain for several years or even decades before clinical symptoms occur, the possible destructive effects of these pathological processes will have taken place already.

As a final project in WP07 we studied the effects of polymorphisms in the ABCB1 gene on P-glycoprotein function at the blood-brain barrier. Pgp is encoded in the ABCB1 gene (formerly known as the multidrug resistance (MDR1) gene), which spans 28 exons that code for 1280 amino acids and is located on chromosome 7q21. The ABCB1 gene is highly polymorphic and to date, over a hundred single nucleotide polymorphisms (SNPs) have been discovered in this gene. The majority of SNPs are either intronic or non-coding; only a minority of variants in the coding region led to a change in amino acids. In Caucasian subjects, high SNP frequencies in ABCB1 were observed for 3435T in exon 26 (54%) and 2677G in exon 21 (56%). High SNP frequencies were also found for 2677T (42%) and 1236T in exon 12 (46%). Of the fifteen most frequently occuring SNPs, the synonymous variant (no amino acid exchange) C1236T (rs1128503), the non-synonymous variant G2677T/A (amino acid exchanges Ala893Ser or Ala893Thr, respectively) (rs2032582) and the synonymous variant C3435T (rs1045642) are most widely studied regarding their consequences in Pgp function and/or expression. For example, altered transport function of Pgp was observed for the C3435T SNP, as the 3435T allele was associated with decreased Pgp expression in the duodenum, and increased oral bioavailability of the Pgp substrate digoxin. However, others could not replicate these findings and even opposite effects of this SNP have been shown. While in vitro studies did not find effects of the G2677T/A SNP, another study has shown an association with decreased Pgp expression in human placenta. Highly significant linkage disquilibrium was shown among exons 12 C1236T, 21 G2677T and 26 C3435T, which could account for most of the haplotypes (combinations of SNPs) seen in ABCB. To date, two studies have assessed effects of ABCB1 haplotypes on BBB Pgp function using PET and [11C]VPM. In these studies, healthy subjects with the homozygous TTT haplotype (1236T, 2677T and 3435T) did not differ in brain distribution of [11C]VPM from subjects with the homozygous CGC haploptype (1236C, 2677G, 3435C). However, as Pgp is involved in amyloid-beta clearance at the BBB, genetic variations in ABCB1 might be related to an inherited increase or decrease in the risk for developing AD. Analysis of ABCB1 SNPs in combination with measuring BBB Pgp function in vivo in AD is needed to further evaluate this hypothesis. Therefore, we set out to assess the effects of C1236T, G2677T/A and C3435T SNPs on BBB Pgp function in both AD patients and healthy subjects. Thirty-two healthy controls, of which fourteen females

(44%), with a mean age of 47 ± 17 years and seventeen AD patients, of which five females (29%), with a mean age of 64 ± 7 years were included in this study. Selection criteria, scanning and data analysis methods are described earlier in this document.

When subjects were homozygous for the 1236T SNP, 94% was also homozygous for the 2677T SNP, and when subjects were homozygous for the 3435T SNP, 73% was also homozygous for the 2677T SNP. First, healthy controls and AD patients were dichotomized into groups with no T present, or with 1 T or more present for the C1236T, G2677T and C3435T SNPs and (R)-[11C]VPM BPND was compared between these two groups for each SNP. In healthy controls, no differences in BPND of (R)-[11C]VPM were found between the groups. In AD patients however, significant differences in BPND of (R)-[11C]VPM were found between patients without, and patients with one or more T present in C1236T, G2677T and C3435T SNPs, with higher BPND values in the patients with one or more T present . In addition, the effect of T dose (0 T, 1 T, or 2T present) in C1236T, G2677T and C3435T SNPs was assessed. In healthy controls, no effect of T dose on (R)-[11C]VPM BPND was found.

Again, in AD patients, an effect of T dose in C1236T and G2677T SNPs on (R)-[11C]VPM BPND was found (p less than 0.05), while for T dose in C3435T SNP a trend was observed (p 0.14), with higher BPND values as T dose increases. In healthy controls, no effects of SNPs in C1236T, G2677A/T and C3435T on Pgp function in vivo at the BBB were found. In AD patients, both the presence of T as well as T dose in C1236T, G2677A/T and C3435T were found to be related to increased BPND of (R)-[11C]VPM, suggesting decreased Pgp function at the BBB. Two previous PET studies that have assessed effects of ABCB1 haplotypes on BBB Pgp function in healthy subjects, also did not find differences in brain distribution of [11C]VPM.

However, before the present study, no studies have been performed assessing effects of genetic variations in ABCB1 on in vivo BBB Pgp function in AD patients. The present study shows a selective effect of the C1236T, G2677T and C3435T SNPs in ABCB1 on BBB Pgp function in AD patients. The present data suggest that when Pgp function is already compromised in AD, then, genetic variations in the ABCB1 gene might influence Pgp function or expression at the BBB. The prevalence of different variants in C1236T, G2677T/A and C3435T in this study was found to be comparable to prevalences reported previously. Furthermore, linkage disequilibrium between the three SNPs was found in in our study, which has also been described before and supports previous findings by others. It could very well be that the effects of the different SNPs in ABCB1 that we have found in AD patients are physiologically effectuated through the linkage disequilibrium via the G2677T SNP, which is a non-synonymous variant thus changing amino acid sequence, while the mutations in C1236T and C3435T are synonymous. In the present study, the prevalence of different variants in the C1236T, G2677T and C3435T SNPs in ABCB1 were roughly comparable between AD patients and healthy controls.

A previous pilot study in relatively small groups of demented patients and healthy subjects revealed no significant differences regarding the prevalence of C1236T, G2677T/A and C3435T. However, a large population based study would be needed to assess possible differences in the prevalence of genetic variations in ABCB1 between healthy controls and AD patients, in order to gain further insight into the role of SNPs in ABCB1 and ABCB1 haplotypes as a possible risk factor for developing AD.

WP08: Neuropathological foundations of Pgp radiotracer binding
Objectives
The main objective was to establish, using resected and post mortem brain tissue, the neuroanatomical parameters that are essential for the interpretation of the in-vivo imaging data, and to determine the role and mechanisms of transporter-encoding gene variation in these parameters and in the neuroimaging patterns observed.

We made use of opportunities provided by surgically-resected tissue from patients with acute insult caused by the placement of intracranial depth electrodes and post mortem brain tissue from patients with (drug-resistant or drug-sensitive) mesial temporal lobe epilepsy and unilateral hippocampal sclerosis (henceforth referred to as 'epilepsy') to examine: (i) inflammation and changes in blood–brain barrier in acute, subacute and chronic insults in epilepsy; (ii) the expression of multidrug transporters in epileptogenic and non-epileptogenic brain regions of patients with drug-resistant or drug-sensitive epilepsy to formally test the inherent assumptions of the transporter hypothesis and (iii) the neuropathological consequences of the clinical use of invasive intracranial electrode recording, which have not been studied in detail using markers of vascular integrity, inflammation and multidrug transporters. We hypothesized that there would be localized blood–brain barrier vascular and inflammatory changes in epilepsy with acute and chronic insults and that there would be a higher expression of multidrug transporters in the epileptogenic, sclerotic hippocampus than in other non-epileptogenic brain regions of the drug-resistant epilepsy brains, but not in the drug-sensitive epilepsy and control brain, in support of a role for multidrug transporters in drug resistance in epilepsy. Our results have recently been published (Liu JY, Thom M, Catarino CB, Martinian L, Figarella-Branger D, Bartolomei F, Koepp M, Sisodiya SM. Neuropathology of the blood-brain barrier and pharmaco-resistance in human epilepsy. Brain 2012 Jul 14. [Epub ahead of print] PMID: 22750659).

We showed that:
(i) there is a highly localized overexpression of P-glycoprotein in the epileptogenic hippocampus of patients with drug-resistant epilepsy, which is compatible with the current hypothesis of multidrug resistance;
(ii) this overexpression appears specific to P-glycoprotein and does not affect other transporters, such as BCRP and MRP1;
(iii) the 'double cuff' expression of P-glycoprotein is likely to be associated with ongoing, chronic seizures, being absent in sustained remission;
(iv) a single, acute insult from intracranial electrode recording causes localized inflammation, increased blood–brain barrier permeability and structural changes to blood vessels (such as the irregular expression of claudins and caveolins), but not P-glycoprotein overexpression;
(v) exposure to P-glycoprotein inducers is associated with increased P-glycoprotein expression in human brain, though the number of cases studied is small; and
(vi) chronic epilepsy is associated with inflammation, enhanced blood–brain barrier permeability and increased P-glycoprotein expression, particularly in perivascular glia of the epileptogenic hippocampus.

We are aware that our study is a cross-sectional study, necessarily limited to small case numbers, and requiring the comparisons to be interpreted cautiously. Fixed autopsy materials also restrict the type of experiments that may be performed. Without functional studies, we cannot

conclude that P-glycoprotein causes drug resistance, but our data support assumptions implicit in the transporter hypothesis and give it further strong plausibility. Our study also provides information on the distribution and location, at high resolution, of P-glycoprotein in controls and brain tissue from drug-resistant and drug-sensitive mesial temporal lobe epilepsy, which are critical for in vivo imaging studies of P-glycoprotein (WP06). We have also examined and quantified various aspects of blood–brain barrier disruption and multidrug transporter expression in the same human tissue across the whole brain from patients with drug-resistant and drug-sensitive epilepsy, and we have explored the temporal relationship of blood–brain barrier disruption and multidrug transporter expression in response to a specific insult, neither of which have been investigated in previous human studies due to limited tissue resources. Thus, our findings have potential clinical impact because they directly improve our understanding of blood–brain barrier disruption and transporter expression in humans.

Blood–brain barrier dysfunction may contribute to drug resistance in epilepsy. Evidence comes from animals studies and from human studies using surgically resected specimens from patients with drug-resistant epilepsy, which show plasma leakage and increased expression of multidrug transporters at the blood–brain barrier, and this has contributed to the 'transporter hypothesis' of drug resistance. It has been challenging to progress further partly due to the limited availability of human tissue, especially from patients with drug-sensitive epilepsy, and the lack of appropriate controls, and also because, in general, human tissue can only provide limited data about temporal patterns of change. Findings from animal and in vitro studies in blood–brain barrier disruption and drug resistance although valuable, still require confirmation in the human brain. We acknowledge that electrode-related injury and chronic epilepsy may induce different severity and types of brain response, but the use of surgical tissue with electrode-related injuries provides an opportunity to examine blood–brain barrier integrity, inflammation and P-glycoprotein expression at known intervals after a defined, sterile initial insult. Electrode placements with a defined follow-up (i.e. up to 330 days after injury) may lead to inflammation, blood–brain barrier damage and angiogenesis; new vessels have a reduced expression of P-glycoprotein and BCRP per vessel, and this localized insult is not sufficient to generate blood–brain barrier leakage and inflammation more widely, nor the 'double cuff' expression pattern of P-glycoprotein around blood vessels seen in chronic active epilepsy. This suggests a long history of accumulated insults, such as persistent seizures, local inflammation and breaches of blood–brain barrier, may be required to upregulate P-glycoprotein expression in epileptogenic brain regions. Thus, there may be a therapeutic window after an initial acute injury (at least of this type) that may be of use in prevention of increased P-glycoprotein expression and thus, potentially, prevention of at least one putative mechanism of development of drug resistance. A more comprehensive synthesis of our results is also possible. The asymmetrical increase in 'double cuff' redistribution of P-glycoprotein is seen in brain tissue from patients who had active epilepsy up to the point of death. Indeed, we found that a higher seizure frequency is associated with increased asymmetry of P-glycoprotein expression in the hippocampus. This 'double cuff' pattern of P-glycoprotein expression is seen in a specific distribution in epileptogenic rather than other brain regions, is not seen in the brain tissue of people with the same type of epilepsy who have been seizure-free at death, in brain tissue of people who do not have seizures, or in injured but non-epileptogenic brain tissue. The more comprehensive

interpretation is that the specific, local upregulation of P-glycoprotein expression seen occurs in the vicinity of chronic seizures. The use of particular anti-epileptic drugs may also play a role. Previous studies have shown that tumour necrosis factor alpha and interferon alpha may increase P-glycoprotein expression and activity in cultured cells. The administration of the anti-inflammatory cyclo-oxygenase-2 inhibitor, celecoxib, before seizure induction in animals decreases inflammation, reduces the frequency and severity of seizures, prevents the increase in P-glycoprotein expression and restores the efficacy of anti-epileptic drugs in animal models of mesial temporal lobe epilepsy. While more research is required into the effect of pro-inflammatory cytokines on regulation of P-glycoprotein, these data suggest inflammation may modulate P-glycoprotein expression and activity. If, subsequently, P-glycoprotein expression patterns do contribute to drug resistance, our findings further emphasize the importance of achieving prompt seizure control after seizure onset. Indeed, a previous study has found P-glycoprotein-expressing astrocytes cultured from surgical epileptic human tissue to be more functionally active at extruding the P-glycoprotein substrates, phenytoin and doxorubicin, compared with P-glycoprotein expressed on control astrocytes. More studies are still needed to substantiate the function of P-glycoprotein expression on astrocytes in epileptogenic regions of the epileptic human brain. Overall, our studies have provided important information on the blood-brain barrier and multidrug transporters in epilepsy, laying the foundation for clinical trials.

Potential Impact:
1.4 The potential impact
Socio-economic impact and the wider societal implications of the project

Contribution to Community and social objectives

Epilepsy is the most common serious chronic neurological condition, associated not only with major comorbidities including mood disorders and cognitive dysfunction, but also with a reduced life expectancy and a mortality rate from seizure-related death as high as 1 per 100 patients p.a. among patients with uncontrolled seizures. In Europe, epilepsy affects 6 million people of all ages, and the costs attributable to epilepsy exceed approximately 20 billion EUROS p.a. The lifetime risk for epilepsy is 2%, but as high as 10% for a single seizure. The majority of these costs are indirect, in relation with the socio-economic consequences of uncontrolled seizures and side-effects. Healthcare costs are likely to fall, and EU will benefit from avoidance of wastage on AEDs likely to be ineffective for individual patients. Economic development and scientific competitiveness will benefit from the potential for commercialisation of our findings. The societal context of research was established through regular connections with patient and support organisations, exemplified by the Epilepsy Society UK co-funding to a large extent the epilepsy research activity of the co-ordinator at UCL.

Over 20 so-called 'antiepileptic drugs' (AED) with different modes of actions are available to suppress seizures, but none has been demonstrated to prevent or cure epilepsy, and approximately 30-40% of epilepsy patients do not respond to antiepileptic drug (AED) therapy and are considered to have pharmacoresistance. The International League against Epilepsy defines pharmacoresistance as failure of two tolerated and appropriately prescribed AEDs, to achieve sustained seizure freedom. After failing two AEDs, the chance of achieving long term seizure control with any further medical treatment is less than5%. Accordingly, the development of many AEDs over the last 20 years has not significantly modified the overall burden of refractory epilepsy. Thus, one of the main challenges is to develop novel treatment approaches which would prevent the development of pharmaco-resistant epilepsy or at the early stage of the seizure disorder predict who is likely to develop pharmacoresistance.

We don't know the exact mechanisms pertinent to the development of pharmaco-resistant. In only about 50% of patients with chronic epilepsy, we are able to identify a potentially epileptogenic lesion, but even then we cannot quantify the capacity of this lesion to generate seizures (=epileptogenicity). Currently available tools (e.g. routine MRI and EEG assessments),are not accurate predictors for the risk of future seizures or the response to specific AED treatments

One proposed mechanism for pharmacoresistance in epilepsy is encapsulated by the 'transporter hypothesis' postulating that overactivity of drug transporter(s) at the blood–brain barrier (BBB) prevents AEDs from reaching their targets. To evaluate Pgp activity, we developed novel PET tracer and performed dynamic PET scans before and after partial blockade with the Pgp inhibitors, such as TQD or CsA. We tested the hypotheses that (i) there is Pgp overactivity in pharmacoresistant, but not pharmacosensitive epilepsy patients, (ii) that Pgp activity is altered most significantly in the epileptogenic region and (iii), that Pgp overactivity measured with PET correlates with Pgp expression measured ex vivo using immunohistochemistry.

We show that there are differences in Pgp activity between pharmacoresistant and pharmacosensitive epilepsy patients in the epileptogenic temporal lobe that Pgp activity is associated with frequent seizures that Pgp function can be modulated with the Pgp inhibitor TQD to maximum effect in the sclerotic hippocampus, and that Pgp overactivity correlates with Pgp expression. These findings recapitulate those in rodent TLE, establish a basis for Pgp modulation in carefully-selected patients with pharmacoresistant epilepsy and lay the foundation for novel treatment strategies overcoming pharmacoresistance.

Treatment decisions in epilepsy, like in many other diseases, are largely based on 'trial and error'. Our research has lead to the development of a potentially clinically useful tests to stratify patients for specific interventions. We have provided tools to individualize treatment choices for individual patients by determining a relevant mechanism of drug-resistance in the brain of individual patients. Patients, in whom this mechanism contributes to drug-resistance, will benefit from specific novel treatments aimed at specifically block the relevant pump, and so improving the chances for rapid seizure control and minimising risk of chronicity, which is related at least partly to early seizure density.

Prediction of response to treatment is the main indications for clinical neurophysiology and neuroimaging investigations. Our results have a much wider impact than just 'pre-surgical' assessments, and ultimately will be of benefit to patients by:
(1) allowing more rapid identification of the most efficacious and best tolerated treatment options;
(2) identifying patients in whom unnecessary drug treatment can be safely discontinued;
(3) providing the knowledge base to develop newer and more effective that are not multidrug transporter substrates; and
(4) permit development, testing and monitoring of novel therapies designed to prevent or revers pharmacoresistance.

At a basic level, pathways for new drug development can now be identified, generating new treatment targets and concepts. Our program has extended the knowledge base relevant to improving human health as multi-modal imaging combined with experimental and human in-vivo and ex-vivo imaging studies. This offered a unique opportunity to understand the functional anatomy and neuropharmacology of the brain. Our project was truly translational, as the knowledge from pre-clinical studies on drug-resistance due to transporter overexpression lead to experiments tried in humans. These aspects will be of potential benefit to the pharmaceutical industry during drug development and characterization of novel compounds in view of the high costs involved in dose-finding studies. Pathways for pharmacological mechanisms may be identified, generating new treatment targets and concepts. The pharmaceutical industry relies increasingly on the development of therapeutic products that depend on the use of a diagnostic test to meet their labeled safety and effectiveness claims. The FDA only recently (14th July 2011) issued their comments on the 'development of drug-companion tests, encouraging the development of such therapeutic products that depend on the use of approved or cleared companion diagnostic devices and technologies'. Projecting that if there is a case for translating some of the research procedures into more routine diagnostic tests, this would stimulate industry to provide the tracers/ligands on a regular commercial basis. Hence, the finding within the EURIPIDES program that the transporter is altered in Alzheimer

Disease could warrant a new important line of investigations, in response to recent announcements that pharmaceutical industries are pulling out research and therapeutic developments in Alzheimer Diseasae due to poor trial results of drugs that remove the amyloid plaques.

We anticipate that the discovery of potential imaging biomarkers for mechanism of drug resistance will help predict the treatment response and outcome in those subjects with Pgp-overexpression, and will have a fundamental impact not only on the way we treat epilepsy, but also across several aspects of medicine (neurodegeneration, inflammatory diseases, stroke, oncology) and beyond. As an example to illustrate the wider applications of the proposed technical developments, BBB dysfunction have been found in patients with stroke, were associated with disease severity in MS patients and in some psychiatric disorders (e.g. schizophrenia, depression) as well, and have been proposed as biomarkers in Alzheimer's disease and aging. Our studies in epilepsies will thus be applicable to a broad range of CNS diseases,including oncology, depression and HIV.

Main dissemination activities and exploitation of results

The main conclusion from WP01 is that the Pgp inhibitor based PET tracers [11C]laniquidar, [11C]elacridar and [11C]TQD are unexpectedly in tracer concentrations recognised by Pgp and/or Bcrp as substrates, which results in low brain uptake and renders them unsuitable to visualise Pgp expression levels at the BBB. For TQD and elacridar, in-vivo PET results were confirmed in a series of in-vitro experiments using the respective 3H-labelled molecules. For laniquidar, in-vitro data suggest, in contrast to the in-vivo PET data, absence of Pgp/Bcrp transport. Despite these apparent limitations of [11C]laniquidar, [11C]elacridar and [11C]TQD to visualise cerebral Pgp with PET, the experiments conducted in this work package and WP02 have provided important insight into the pharmacology of these widely used reference Pgp inhibitors. Due to the fact that [11C]laniquidar, [11C]elacridar and [11C]TQD have not provided sufficiently high PET signals in rodents for the imaging of transporter distribution, the preparation of longer-lived versions of these molecules for PET and SPET imaging was not further pursued. Nevertheless [11C]laniquidar, [11C]elacridar and [11C]TQD have all entered WP05 to characterise their behaviour in healthy human subjects. In the future, we plan to assess if any of these tracers might be suitable to visualise Pgp expression levels in peripheral organs. Apart from a thorough in-vivo/in-vitro characterisation of laniquidar, elacridar and TQD, we have obtained first promising results with a 18F-labelled fexofenadine ester prodrug, which might allow for visualising cerebral P gp activity and overcome the limitation of low brain uptake obtained with (R) [11C]VPM, [11C]laniquidar, [11C]elacridar and [11C]TQD. We have also synthesised two 11C-labelled AEDs ([11C]phenytoin and [11C]mephobarbital) expecting that these would be weak Pgp substrates and be better suited to visualise regional Pgp activity in the epileptic brain than the high-affinity Pgp substrate (R)-[11C]VPM. In line with these initial assumptions [11C]phenytoin was shown to behave as a weak Pgp substrate in-vivo at the rodent BBB. Brain distribution of [11C]mephobarbital, however, was unexpectedly found to be not influenced by Pgp activity at the BBB. [11C]Phenytoin will be further characterised in healthy human subjects and epilepsy patients.

We have synthesised 4-[18F]fluoroisatin-4-methoxyphenyl-thiosemicarbazone, a selective Pgp inhibitor, and assessed its ability to image Pgp in rats. Logan analysis clearly showed that the brain uptake of 4-[18F]fluoroisatin-4-methoxyphenyl-thiosemicarbazone, expressed as VT,

is not influenced by TQD pretreatment and thus 4-[18F]fluoroisatin-4-methoxyphenyl-thiosemicarbazone might prove to be a Pgp binding PET tracer which is not transported by Pgp. We have also extended our efforts to visualise Pgp to other important efflux transporters expressed at the BBB, i.e. the MRPs and BCRP. We have synthesised an 18F-labeled purine derivative which shows promise to measure cerebral Mrp1 activity based on the metabolite extrusion method. As an additional approach, a series of thiosemicarbazones, which were reported in the literature as potent and Pgp specific inhibitors (Hall et al., 2009), were selected as new lead structures to develop a Pgp inhibitor based PET tracer. 4- and 6-[18F]Fluoroisatin-4-methoxyphenyl-thiosemicarbazone were synthesised.

First in-vivo studies showed a distinctly different behaviour as compared with [11C]laniquidar, [11C]elacridar and [11C]TQD, in that brain uptake in rats was higher and cerebral VT essentially unchanged after pretreatment of animals with cold TQD.

[11C]elacridar ([11C]-tracer C) and [11C]TQD ([11C]-tracer D) have been tested in paired microPET scans in naïve rats, wild-type mice and three different transgenic mouse models (Pgp KO, BCRP KO, Pgp/BCRP KO), each before and after administration of unlabelled compound. In vivo metabolism of [11C]elacridar and [11C]TQD has been assessed in naïve rats by analysis of arterial plasma samples with a combined solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) assay. Brain uptake of [11C]elacridar and [11C]TQD in naïve rats was low (about 0.07 percent of the injected dose per gram tissue, %ID/g). In rats, brain uptake of [11C]elacridar and [11C]TQD was increased by factors of 4 and 3 following pretreatment with unlabelled elacridar (5 mg/kg, i.v.) or TQD (15 mg/kg, i.v.), respectively. Using knockout mice models it was found that brain uptake of [11C]elacridar and [11C]TQD was about 3-fold higher in Pgp KO mice as compared to wild-type mice. Brain uptake of [11C]elacridar and [11C]TQD was not higher in BCRP KO mice compared to wild-type mice. [11C]elacridar and [11C]TQD appear to display good metabolic stability in rats. For [11C]elacridar and [11C]TQD, 85% and 96% of activity in plasma at 20 min after tracer injection represents unchanged parent. In addition, it has been found that [11C]TQD and [11C]elacridar were dose-depently transported by both Pgp and Bcrp. Furthermore, using in vitro uptake and transport assays in cells overexpressing Pgp or Bcrp1 it was found that at low concentrations they were transported by both Pgp and Bcrp, but at high concentration mainly by Bcrp.

Finally, the dual Pgp/Bcrp1 substrate [11C]TQD may be used to measure cerebral Bcrp1 activity, when Pgp at the BBB is inhibited by pretreating animals with cold TQD. As we have shown in WP05 that TQD can be safely administered to humans at doses which completely inhibit Pgp at the BBB, such a protocol can in all likelihood be translated to humans. Such a use of [11C]TQD is of particular interest as there are currently no radiotracers available to measure BCRP activity at the BBB. Studying (R)-[11C]-VPM uptake with different scanners and in rats of different gender has yielded quite similar results. In all studies TQD strongly enhanced uptake of [11C]-VPM, irrespective of the methodology. One of the highlights of the project was the development of a novel imaging protocol composed of VPM–PET scans after half-maximal Pgp inhibition by TQD in control rats and rats 48 h after SE allowing quantification of group differences in regional Pgp function. It was also demonstrated that changes in outcome parameters of kinetic model analysis correlated with disease-induced changes in Pgp expression. An equally important result

was the excellent agreement between the half-maximum effective concentration of TQD found in rats (EC50 = 544±32 ng/ml) and humans (EC50 = 561±24 ng/ml). This finding is noteworthy because for several other Pgp inhibitors species-dependent differences in inhibitory effects have been reported, both in vitro and in vivo. Yet, when looking at the maximum effect of TQD on VPM brain kinetics, a most striking interspecies difference was found in the concentration–response relationships for TQD; there was a 2.7-fold maximum increase in brain activity uptake relative to baseline in humans versus an 11.0-fold increase in rats.

Another major finding was that two of the new radiotracers, [11C]laniquidar and [11C]quinidine, exhibited a small but significant increase in uptake during the early phase of the PET scan in non-responder animals compared to controls and responders. For [11C]laniquidar there was a relation with the percentage reduction in seizure frequency in response to phenobarbital. These aspects certainly deserve further study in the future. Furthermore, [11C]phenytoin was found to have good tracer properties and potential for further evaluation. This tracer is taken further for clinical studies. In vitro uptake assays in Pgp- and Bcrp1-overexpressing cell lines indicate that microdoses can behave pharmacokinetically different from multi-drug transporter inhibitory doses if the compounds interact with multidrug transporters, indicating that conclusions.

The intracerebral microdialysis study with quinidine has shown that full inhibition of Pgp with TQD leads to the same increase in quinidine uptake as with (R)-[11C]-VPM. This provides independent evidence for the reliablity of the results obtained with (R)-[11C]-VPM PET for assessment of Pgp functionality. Importantly, the microdialysis study also revealed that in kainate-treated rats Pgp inhibition by TQD increased ECF by a factor 7, but the total brain concentration 40-fold This strongly suggests that not only BBB transport but also intra-brain distribution is important for the pharmacokinetics of Pgp-sensitive drugs and that this even more so in the epileptic brain. This aspect certainly deserves further study.

Since phenobarbital is a Pgp substrate, it was argued that [11C]mephobarbital as an analogue might be a good tracer to assess Pgp function and expression. Surprisingly, it was found that uptake in rat brain was not affected by TQD and in vitro transport assays were negative as well. [11C]Laniquidar (tracer A), [11C]phenytoin (tracer B) and [11C]quinidine have been tested in a multitracer experiment. [11C]Laniquidar and [11C]quinidine were found to behave as Pgp substrates. [11C]Laniquidar was moderately and [11C]quinidine extensively metabolised during scan time. Neither tracer appeared to be superior to [11C]VPM. [11C]Phenytoin was a weak substrate and metabolism was very limited. Although it is not the ideal tracer, [11C]phenytoin emerged as the best possible candidate to detect changes in Pgp expression or functionality. [11C]Phenytoin will therefore be tested in humans. The multitracer design is a novelty. Three tracers were tested in the same animal before and after TQD treatment. It still has to be confirmed rigorously that carry-over effects did not confound the results, but there are no indications in that direction. The method is very time-efficient and allows within-animal comparison of different tracers, giving at least a first impression of difference in behaviour between tracers.

In WP3, we determined in an in-vitro model of the human BBB that lamotrigine is actively transported and that a panel of drug transporter inhibitors suggests an involvement of the organic cation transporters. Using additional inhibitors against OCT1, OCT2 and OCT3 we have shown an involvement of OCT1 in the transport of lamotrigine at the BBB . The kinetics of this OCT1 (prazosin inhabitable) transport was determined in the human brain endothelia cells (K(m) = 62 ± 14 µM; V(max) = 385 ± 30 pmol/min/million cells). The use of a stably transfected OCT1 cell line has determined that lamotrigine is a substrate and an inhibitor of OCT1. A putative pharmacokinetic drug-drug interaction (DDI) between quetiapine and lamotrigine was recently reported in patients and we found that quetiapine is a potent inhibitor of the OCT1-mediated transport of lamotrigine. This is the first time that a specific influx transporter has been shown to transport lamotrigine with the clinical implications of these findings with respect to the efficacy of lamotrigine and its potential for DDI require further investigation.

Experimental data from WP04 obtained in naïve rats, non-selected epileptic rats as well as drug-resistant and drug sensitive epileptic rats suggest [18F]MPPF PET in combination with TQD pretreatment as a tool to evaluate P-glycoprotein mediated efflux in individuals, thus allowing the identification of those individuals in whom P glycoprotein over-expression contributes to therapeutic failure. This imaging approach provided a basis to further explore the clinical relevance of P-glycoprotein over-expression.

However, clinical data obtained with the [18F]MPPF tracer do not confirm major alterations in tracer kinetics in the epileptic brain. These data might suggest that Pgp expression is not regulated in the human epileptic brain to clinically relevant levels detectable by in-vivo imaging. On the other hand we can not exclude that the combination of [18F]MPPF with CsA is not optimal to analyze Pgp function in patients considering recent data that [18F]MPPF interaction with Pgp might significantly differ between the species.

Experimental and clinical data obtained with [11C]flumazenil in combination with the Pgp modulator TQD confirmed that [11C]flumazenil must be considered a Pgp substrate, but rather argued against a suitability of this combination to detect differences between drug-responders and non-responders.

The results from the clinical studies performed as part of EURIPIDES have provided answers to the questions initially raised on pharmaco-resistance in epilepsy and demonstrated the utility of PET with a radiolabelled Pgp substrate as to study Pgp activity in vivo in humans:
1) Compared to pharmaco-sensitive patients and healthy controls at baseline, pharmaco-resistant patients have significantly reduced VPM uptake exclusively in the temporal lobes but the reduction is not restricted to ipsilateral epileptogenic regions but also extends to contralateral temporal lobe regions. Similarly in patients with FCD, VPM uptake is reduced not only in close proximity to the area of FCD identified by MRI but also the reduction extends further to other ipsilateral regions, supplementing the findings in TLE patients that Pgp overexpression is not limited to the epileptogenic seizure-onset zone but also spreads across to other cortical regions.
2) After partial blockade of Pgp with TQD, pharmaco-resistant TLE patients have attenuated increases of VPM uptake in the whole brain and more so in the ipsilateral epileptogenic area of the hippocampus compared

to healthy controls, implicating functionally elevated Pgp function primarily in the epileptogenic hippocampus in pharmaco-resistant epilepsy.
3) Pharmaco-resistant TLE patients with good surgery outcome have higher VPM increases in the post-surgery PET scan than patients with poor outcome, pointing to diminished Pgp activity which may be caused by reduced seizure frequency and lower antiepileptic drug load in patients with good outcome. Furthermore, pharmaco-resistant TLE patients who have frequent seizures have the lowest VPM uptake, substantiating the contribution of seizures to the induction of Pgp overexpression in epilepsy.
4) In pharmaco-resistant TLE patients who underwent anterior temporal lobe resection for surgical treatment, the difference in percentage change in VPM uptake after Pgp inhibition with TQD in the hippocampus compared to a reference temporal lobe region is inversely correlated with the corresponding difference in percentage area of Pgp-immunopositive labelling, demonstrating that imaging with PET, a radiolabelled Pgp substrate and a Pgp inhibitor at a half saturating dose is a suitable tool to assess Pgp function in vivo in humans.
5) In high grade glioma patients, Pgp inhibition minimally alters perfusion but differentially increases VPM uptake into brain tissue and glioma, and preferentially into grade III but not grade IV regions, supporting the use of PET with a radiolabelled Pgp substrate as a promising tool to select patients in whom Pgp inhibitors could potentially increase chemotherapeutic efficacy.

The results of WP07 indicate that BBB Pgp function in AD is decreased, supporting the hypothesis that Pgp plays a role in the pathogenesis of AD. Although no significant correlations were found between amyloid load, as measured with [11C]PIB PET, and BBB Pgp function, this does not mean that there is no relationship between them. There could be a ceiling effect for [11C]PIB, as [11C]PIB uptake appears to behave as an on/off phenomenon in AD patients, [11C]PIB retention does not reflect disease severity and [11C]PIB binding does not increase substantially over time. Furthermore, (R)-[11C]VPM and [11C]PIB binding showed substantial spatial overlap, although also some inconsistencies were observed: relatively high [11C]PIB BPND in anterior cingulate and frontal cortex, while (R)-[11C]VPM BPND was only moderately increased. As such, there appears to be a regional distribution in the severity of Pgp dysfunction in AD and studies in larger samples are necessary to further address these regional differences and its relation to amyloid depositions. The increase in (R)-[11C]verapamil BPND in AD patients compared to age-matched healthy controls was confirmed in a second cohort from Vienna of 5 AD patients and 6 control subjects. No differences were found in BBB Pgp function between AD patients with MBs and AD patients without. These results indicate that there is no evidence of additional Pgp dysfunction at the BBB in support of the hypothesis of additionally impaired Pgp function in AD patients with compared with AD patients without MBs. However, this could be due to small groups or due to inclusion criteria as used in the present study. Autopsy studies have shown that nearly all AD patients show some degree of vascular amyloid deposition. Furthermore, in AD patients but not in healthy controls, SNPs (C1236T, G2677A/T and C3435T) in the Pgp-encoding ABCB1 gene were found to be related to changes in Pgp function at the BBB. As T dose in these SNPs increased, this leads to a decrease in BBB Pgp function in AD patients. As such, certain variations in the ABCB1 gene might contribute to the risk of developing AD and might influence disease progression once having amyloid depositions in the

brain. These findings however, first need to be replicated in larger groups of patients.

The EURIPIDES project has enabled important progress to be made in better understanding drug resistance in epilepsy.

The major achievements of this WP, in concert with the Consortium overall, are:
1. Demonstration that robust studies of human brain tissue, both post mortem and post surgical, can be of enormous value in taking understanding forwards; identification of a new paradigm for the study of the response of human brain tissue to injury (using intracranial electrode tracks as a 'model' for injury).
2. Development of robust reproducible methodology for quantitative analyses in drug resistance-related studies of fixed human brain tissue. These methods can continue to be used in these and other studies
3. Development of robust reproducible methodology for quantitative analyses in drug resistance-related studies of fixed human brain tissue. These methods can continue to be used in these and other studies
4. Determination that some antigens cannot be studied in material that has been fixed for longer periods of time. This important quantitative observation sets new standards in quantifying and interpreting immunohistochemical data.
5. Determination of the spatial and temporal patterns of expression of a variety of proteins putatively involved in drug resistance, or maintenance of blood-brain barrier integrity in the human brain.Several proteins were studied.
6. Substantiation of major assumptions implicit in the drug transporter hypothesis of drug resistance: that overexpression of P-glycoprotein is spatially localised, focussed on epileptogenic tissue, and correlates with active seizures, declining with seizure freedom and being absent from brain that is simply injured but not epileptogenic (i.e. intracranial electrode tracks)
7. Correlation of in vivo activity of P-glycoprotein with ex vivo demonstration of degree of expression of P-glycoprotein. This is the first such study and elegantly demonstrates that P-glycoprotein can, at structural and functional levels, indeed play a role in drug resistance in epilepsy.
8. Demonstration that P-glycoprotein, and not other transporters studied such as BCRP and MRP1, has the appropriate pattern of expression to potentially mediate resistance.

Compromise of the human blood–brain barrier is associated with several neurological disorders, including mesial temporal lobe epilepsy. Dysfunction of the blood–brain barrier in such conditions may be causal or consequential or both, but in any case, dysfunction of the blood–brain barrier may potentially contribute further to disease biology. Dysfunction of the blood–brain barrier may be due to several reasons including inflammation, endothelial apoptosis, abnormal endothelial–glial interaction, increased expression of permeability factors, loss of tight junction proteins and/or altered expression of multidrug transporters. Previous studies have shown that multidrug transporters normally located at the vascular endothelium, may contribute to drug resistance. Multidrug transporters are important in regulating the transcellular movement of various molecules across the tightly sealed blood– brain barrier. According to the transporter hypothesis of drug resistance, altered activity of multidrug transporters may mediate resistance by increased efflux and reduction of anti-epileptic drug concentration below their

effective threshold at their targets. Implicit in this hypothesis are the assumptions that there is an altered (over)expression of multidrug transporters in the brains of patients with drug-resistant epilepsy, which is not observed in drug-sensitive patients or control subjects, and that this (over)expression is localized to the seizure onset region(s). While previous studies have shown that the well-studied transporter P-glycoprotein is ectopically expressed in surgically resected specimens from patients with drug-resistant epilepsy with a variety of structural abnormalities, including hippocampal sclerosis, there are few opportunities in humans to examine the global patterns of expression across the brain and critically, no neuropathological studies have examined multidrug transporter expression in the brains of patients with drug-sensitive epilepsy as such patients do not in general undergo neurosurgical removal of brain tissue as a treatment.

Outlook and future research

There is now considerable evidence in support of the 'transporter hypothesis':
(1) relevant multidrug transporters are overexpressed in animal models of drug-resistant epilepsy. Whilst there has been partial evidence in favour of overexpression in human drug-resistant epilepsy, we have recently finally confirmed appropriate regional patterns of overexpression across the human brain in drug-resistant epilepsy compared to drug-responsive epilepsy using a unique post mortem human brain resource
(2) there is evidence that relevant multidrug transporters are capable of transporting many AEDs, in both animal models and ex vivo models using human brain cells;
(3) in animal models of epilepsy and drug-resistant epilepsy, temporary inhibition of the multidrug transporter, P-glycoprotein, most strongly implicated in mediating drug resistance through the transporter mechanism, leads to a transient, but definite, reduction in seizure frequency. This is the current position of the field.

Future research has to show that inhibition of P-glycoprotein function in the brain in humans with multidrug-resistant epilepsy will lead to a reduction in seizure frequency. Many workers have advocated such studies in the recent past, but now we have the tools for such a study, because there are no markers to determine how active P-glycoprotein or related transporters might be in vivo in the epileptogenic human brain. Thus, it is now possible to independently determine whether and how much P-glycoprotein function might have been reduced when inhibitors were given. EURIPIDES has led to the generation of a positron emission tomography (PET) paradigm with paired scans (baseline and post-P-glycoprotein blocking) of the substrate verapamil (VPM) that can monitor Pgp activity in vivo. With access to the latest generation, best-in-class P-glycoprotein inhibitor (tariquidar, TQD), which has already had first-in-man administration, we are now in a position to undertake careful, interpretable, proof-of-concept studies of Pgp inhibition in drug-resistant epilepsy. Careful attention is needed for individual patients to undertake paired studies on the same day to assess repeatability and the effects of blocking of the Pgp transporter. Dietary control will be essential along with withdrawing and careful monitoring of arterial blood samples for radioactivity and chemical composition.

EURIPIDES made significant generic methodological advances with respect to modelling of the recorded image data to obtain quantitative values of the transporter's function. These were also successfully transferred for

analysing small animal derived imaging data at more than one pre-clinical centre within the consortium.

EURPIDES provided evidence of decreased function of the Pgp transporter in Alzheimer's Disease with the results published in several high impacting scientific journal. The result points to a possible underlying mechanism that causes Alzheimer's disease and is considered so important that it is inevitable that the studies will be repeated by others in the near future. Coupled with the Alzheimer's work was the study of the effects of age and gender on the function of the Pgp transporter. Of particular interest was the finding of decreased Pgp function in young females which may relate to Azheimer's Disease being more prevalent in women.

The effort required to obtain the data needed to validate the 'transporter hypothesis' in patients proved logistically challenging and will be a model for what is required for future cross Europe consortia addressing such complex, translational, molecular imaging based experimental medicine questions. The program fostered an important bridge between pre-clinical small animal and human imaging which is not commonly met. It demonstrated the added value of such linkage for the two investigative areas.

Much hindsight has been gained as to how to undergo a program of imaging biomarker discovery and development. This experience will be invaluable for future European consortia containing the need to develop new tracers for molecular imaging. An important and unsung service was provided by radio chemists in the systematic production of tracers radio labeled with short lived radio nuclides. This was provided not only for human but also for the many small animal studies undertaken within EURIPIDES.

The EURIPIDES program has realised a multidisciplinary approach to an important clinical question including clinician scientists, pathologists, pharmacologists, radio chemists, data processing scientists, data analysis scientists, human and small animal imagers, experts on the fundamental workings of the blood brain barrier transporter system and the Pgp system in particular as well as experts on pre-clinical models of disease. Not only has this been of fundamental importance in achieving the outcomes, but has been an enriching process for all of the participating partners.

EURIPIDES has developed new PET and SPECT tracers to assess ABC transporter function and expression in vivo. A considerable challenge in the development of radiotracers for ABC transporters is the lack of candidate molecules which show selectivity for only one ABC transporter type. However, by using radiolabelled transporter substrates in combination with unlabelled inhibitors and by carefully balancing the transporter selectivity profile of radiolabelled substrate against unlabelled inhibitor, the availability of purely selective radiotracers may not be mandatory.

The major focus for in vivo imaging of ABC transporters has so far been placed on Pgp located at the BBB and in solid tumors. A particularly promising application of an in vivo imaging method of Pgp could be in personalized medicine to identify epilepsy patients or cancer patients with increased Pgp function for applying altered treatment strategies, such as co-administration of a specific Pgp inhibitor.

The most widely used Pgp substrate radiotracers for PET imaging is (R)-[11C]verapamil. A drawback of these radiotracers is their low brain uptake, which hampers the mapping of regional differences in transporter function in the brain as they are expected to occur in epilepsy patients. Approaches to overcome this limitation include the performance of PET scans after half-maximum inhibition of Pgp with suitable inhibitors such as tariquidar, which has shown some promise in pilot studies 2,5, as well as the use of low-affinity transporter substrates, such as [18F]MPPF or [11C]phenytoin. Another important limitation of (R)-[11C]verapamil is the significant brain uptake of radiolabelled metabolites, which confounds the measurement of cerebral Pgp function. Therefore the availability of Pgp substrate probes with better metabolic stability and absence of brain-penetrating radiometabolites would be of considerable interest.

Many of the candidate tracer molecules did not prove an advance over the current validated imaging biomarker for Pgp [11C]-verapamil, despite its known short comings. The goal of providing a tracer with improved properties, labelled with a radioisotope whose radioactive half life is sufficiently long enough to allow delivery to clinical centres from points of its radio synthesis was unsuccessful. The most promising new tracer is [11C]-Tariquidar, a marker of Breast Cancer Resistance Protein (BCRP) which is known to play a role in multi drug resistance.

Another potentially powerful approach to overcome the problem of low brain PET signals obtained with Pgp substrate probes is the use of the metabolite extrusion method which has already been successfully applied to the imaging of cerebral MRP1. Following a report in March 2009 from a group in Japan on a new molecular imaging paradigm for measuring Pgp function, known as the metabolite extrusion method (MEM), an amendment was introduced into the EURIPIDES program to undertake similar research within the consortium. This has been confined to pre-clinical studies and is proving sufficiently promising that it will inevitably extend to human studies beyond the life of the EURIPIDES program. To this end collaboration has been established between scientists within EURIPIDES and the Japanese group. It was possible to introduce an important and productive amendment to the program. The case for this rested in part on a report from Japan on a new experimental paradigm for measuring the function of the Pgp transporter. While the work adopted within the Euripides program was not able in the time frame to progress further than the early pre-clinical stage, the new approach shows much promise and will inevitably make an impact in the future. The message here is that for any progressive area of research, it is inevitable that during a 4 year program, external reports will impact on a consortium's study portfolio. Hence from the outset this needs to be recognised, and contingency for this built into the research proposal.

The development of Pgp inhibitor based PET tracers, such as 11C-labelled laniquidar, elacridar and tariquidar, has so far been unsuccessful, as these radiotracers have failed to provide Pgp-specific brain PET signals, which was most likely caused by too high KD values of these molecules in relation to the Bmax of cerebral Pgp. In addition there is compelling evidence that 11C-labelled laniquidar, elacridar and tariquidar are avid substrates of BCRP and possibly Pgp at the BBB, which underlines the importance of assessing the affinity of potential Pgp PET probes to other ABC transporters expressed at the BBB as well. An effective Pgp inhibitor PET ligand remains to be identified and should ideally lack efflux activity by BCRP and the MRPs and possess subnanomolar binding affinity to Pgp.

Because in vitro transport experiments often fail to accurately predict the in vivo situation, researchers are encouraged to conduct in vivo experiments, for instance in wild-type and ABC transporter knockout mice, as early as possible in the rational design of ABC transporter imaging ligands. For other ABC transporters than Pgp, only very few PET and SPECT tracers have been described so far. It is hoped that the experience gained with the in vivo imaging of Pgp can be successfully applied in the future to the design of radioprobes to visualize other transport proteins such as BCRP or the MRPs.

List of Websites:

http://www.euripides-europe.com