In vivo quantification of proliferation and glucose metabolism in lung cancer patients

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7.1 GENERAL DISCUSSION

This thesis aims to contribute to the field of response monitoring in oncology and its need for quantitative imaging biomarkers. In this context, we focus on molecular imaging with positron emission tomography (PET) to quantify proliferation and glucose metabolism non-invasively in non-small cell lung cancer (NSCLC) patients. In this general discussion section the results described in this thesis are discussed and put in perspective of results published by others. Subsequently, we express the future directions and perspectives of molecular imaging research for response monitoring in oncology and its clinical implementation.

PET is a quantitative imaging modality in which molecular processes can be quantified. Full quantification with PET is obtained by kinetic analysis estimating rate constants of a pharmacokinetic compartmental model [1, 2]. This requires measurement of tracer activity concentrations in arterial blood and tissue as a function of time. The latter is provided by the PET scanner, the former (the arterial input function) is typically measured directly in arterial blood. Alternatively, the input function can be derived from arterial blood samples, venous blood samples, image derived input functions or population based input functions. Or, to avoid the need of an arterial input function, a reference region could be used. This latter is frequently used in brain PET studies, where e.g. cerebellum could serve as reference region for some tracers [3, 4]. However, for thoracic oncologic PET studies, arterial input functions are needed since no valid thoracic reference region has been determined for most oncological tracers so far. Fortunately, large blood pool structures such as aorta and left ventricular cavity are in the field of view (FOV), and these structures have shown to be suitable for arterial image derived input functions (IDIF) in [^{18}F]-fluorodeoxyglucose ([^{18}F]FDG) PET [5, 6] and [^{15}O]H_2O PET [7]. In **chapter two** we investigated the optimal input function for kinetic modeling of 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT) PET as proliferation imaging biomarker in NSCLC and we evaluated different arterial IDIF methods. We found a close correlation between pharmacokinetic modeling results obtained with arterial parent plasma IDIF and SUV (correlation coefficients 0.86-0.96). We found no significant differences in correlation between pharmacokinetic modeling results and SUV between the four different arterial structures used as IDIF (left ventricle, ascending aorta, aortic arch and descending aorta). This implies that an IDIF derived from any of these arterial structures and corrected for parent

fraction, plasma-to-blood ratio and calibrated with late venous blood samples could be used as input function for pharmacokinetic modeling of [^{18}F]FLT. In this study we did not evaluate arterial blood sampling since plasma-to-blood ratio or parent fraction are not significantly different between arterial and venous blood samples [8, 9] and arterial cannulation is preferably avoided whenever possible. Accordingly, no differences in kinetic analysis were observed between arterial and venous input functions for [^{18}F]FLT uptake quantification in colorectal cancer patients [9]. [^{18}F]FLT activity concentrations in venous blood samples have shown to correlate well with IDIFs, although area under the curve of aorta IDIFs were higher compared to venous samples [10]. This was also observed in our study and IDIFs were calibrated with late venous samples with calibration factors < 1.

An alternative to IDIF could be a (scaled) population based input function. Contractor et al. [11] evaluated limited arterial blood sampling to scale a population based input function for compartmental modeling of [^{18}F]FLT. Net influx, K_i, measured with arterial sampling and population derived input functions correlated well (r^2 = 0.85-0.98). However, although limited sampling was performed up to a minimum of 3 arterial blood samples during a 60 minute dynamic scan, arterial cannulation is still needed. Therefore, replacement of arterial blood samples with venous blood samples to scale population based input functions will be a great advantage, as was performed by Menda et al. [12]. Consistency in arterial [^{18}F]FLT concentrations were observed if normalized by administered dose and late venous samples. Therefore, IDIF might be replaced by a population based input function for pharmacokinetic modeling of [^{18}F]FLT uptake, although individual changes in the shape of the input function (caused by possible changes in blood flow, plasma clearance or tracer efflux from tissue) might be missed if a scaled population based input function is used.

Ultimately, the objective of performing [^{18}F]FLT PET studies is to use these for response monitoring to support clinical decision making and drug development. The potential of [^{18}F]FLT PET is likely limited for enhancing diagnostic evaluations, for which [^{18}F]FDG is already established. Moreover, [^{18}F]FLT uptake in tumors is usually lower compared to [^{18}F]FDG uptake and physiological [^{18}F]FLT uptake is high in bone marrow and liver [13, 14]. Only in brain tumors, where physiologic [^{18}F]FLT uptake is low, there might be a diagnostic benefit compared to [^{18}F]FDG [15, 16]. However, the strength of [^{18}F]FLT PET is the potentially predictive value in the detection of treatment mediated changes in proliferation at an early time point after start of treatment. In addition, besides a surrogate marker of proliferation, [^{18}F]FLT PET could determine

specific alterations in the "de novo" and salvage pathway of thymidine supply to the cell [14, 17, 18]. [^{18}F]FLT uptake is only regulated via the salvage pathway and therefore, treatment which inhibits the "de novo" pathway will result in increased [^{18}F]FLT uptake; a so called "flare" response. Such molecular changes could be observed with PET noninvasively and within hours after start of treatment. Specifically, thymidylate synthase (TS) inhibiting drugs like 5-fluorouracil, capecitabine and pemetrexed inhibit the "de novo" pathway and would cause increased [^{18}F]FLT uptake via the salvage pathway. This increased [^{18}F]FLT uptake has been confirmed in mice within two hours after treatment with 5-fluorouracil [19], within 24 hours after treatment with BGC 945 [20] and in breast cancer patients one hour after capecitabine treatment [21]. In NSCLC, pemetrexed is a first-line treatment option in combination with platinum chemotherapy in stage IV NSCLC [22, 23] and this "flare" response of [^{18}F]FLT uptake was expected early after start of treatment with pemetrexed. We performed a pilot study to evaluate changes in [^{18}F]FLT uptake 4 hours after start of pemetrexed infusion. Such a strict time interval between start of infusion of pemetrexed and start of [^{18}F]FLT PET scan is challenging, where the logistics in the clinical pulmonology department and timing of the [^{18}F]FLT PET scan had to be closely managed. The results of this study are described in **chapter three**. Unfortunately, this study did not show a systematic increase of [^{18}F]FLT uptake after the first therapeutic dose of pemetrexed as hypothesized. Several aspects could have influenced the results of this pilot study. First of all, timing of [^{18}F]FLT PET is crucial in this very early response monitoring examination and it might be possible that cellular changes caused by TS inhibition and shift of thymidine supply from the "de novo" pathway to the salvage pathway may vary between patients. In addition, the sensitivity of tumors to TS inhibition might be tumor specific [24, 25]. On the other hand, it might be argued whether the proposed TS inhibition by pemetrexed is the main mechanism of action and if the increased regulation of the salvage pathway is reached. Pemetrexed is known to inhibit dihydrofolate reductase and glycinamide ribonucleotide formyltransferase (GARFT) besides TS. GARFT is involved in the "de novo" pathway of purines [26] and this might explain the decrease of [^{18}F]FLT uptake in some of the tumors. Moreover, local thymidine levels in the microenvironment of the tumor might vary due to thymidine release from dying tumor cells [27]. Nevertheless, it will probably remain difficult to design a uniform time interval to detect the "flare" response caused by TS inhibition by pemetrexed in NSCLC patients. Therefore, we consider [^{18}F]FLT PET not feasible to evaluate early effects of TS inhibition by pemetrexed in NSCLC patients.

Yet, response monitoring with [^{18}F]FLT PET to evaluate changes in tumor proliferation within days after start of treatment is still very promising and [^{18}F]FLT uptake correlated well with proliferation rate of lung tumors, measured with immunohistochemistry ki-67 [28]. [^{18}F]FLT uptake is dependent on several biological factors such as:

- Expression of the equilibrative nucleoside transporter 1 (ENT1) on the cell membrane; ENT1 expression can be up- and down-regulated and this would affect the K_1 of the pharmacokinetic model [29, 19].
- Balance between salvage and "de novo" pathway for thymidine supply; this aspect is extensively described in the previous paragraph.
- Fraction of cells in S-phase; [^{18}F]FLT uptake is S-phase specific, which makes [^{18}F]FLT a surrogate marker of proliferation. However, if cells become quiescent after the S-phase, this would not be detected with [^{18}F]FLT PET [30].
- Levels of endogenous thymidine; endogenous thymidine competes with [^{18}F]FLT uptake, with higher affinity to thymidine kinase 1 than [^{18}F]FLT [31] so that thymidine levels might interfere with tracer uptake (similar as with serum glucose levels and [^{18}F]FDG uptake) [32, 33]. Fortunately, thymidine levels in blood have shown to be relatively low in humans [34] and would probably not affect [^{18}F]FLT uptake. In mice, however, thymidine levels are substantial, and therefore preclinical [^{18}F]FLT PET studies might not be translational to human.

So far, [^{18}F]FLT is the most promising tracer among the currently available proliferation tracers. Alternative tracers are [^{11}C]thymidine and 1-(2'-deoxy-2'-fluoro-1-β-d-arabinofuranosyl)-thymine (FMAU) but [^{11}C]thymidine has a high metabolite formations within the body and FMAU is not specific for thymidine kinase 1 as it is also a substrate of mithochondrial thymidine kinase 2 [13, 18]. Moreover, simplified [^{18}F]FLT synthesis has been developed (~90 minutes), [^{18}F]FLT administration has no side effects and radiation dosimetry is comparable to [^{18}F]FDG [35-37].

Dynamic PET acquisition to perform pharmacokinetic modeling is the reference method to quantify tracer uptake with PET as described above. However, pharmacokinetic modeling is relatively complex, time consuming, expensive and not feasible in most medical centers. For clinical use of [^{18}F]FLT PET, but also for research (e.g. (large) multicenter studies), there is a need for simplified methods to quantify molecular processes that are feasible and reproducible. Validation of simplified methods to quantify [^{18}F]FLT uptake should be performed before and after start of treatment, because treatment could alter the relationship between simplified methods and pharmacokinetic modeling as has been described for [^{18}F]FDG PET [38, 39].

In **chapter four** we validated the use of simplified measures of [^{18}F]FLT uptake in NSCLC patients with activating epidermal growth factor receptor (EGFR) mutations undergoing treatment with EGFR tyrosine kinase inhibitors (TKI). Activating EGFR mutations are present in ~10% of stage IV NSCLC patients and patients harboring such mutations have a benefit in progression free survival if treated with gefitinib or erlotinib [28, 40-43]. We evaluated changes in standardized uptake values (SUV), tumor-to-blood ratios (TBR) and pharmacokinetic modeling at baseline and 7 and 28 days after start of treatment. A reversible two tissue compartmental model fitted the kinetic data best, based on Akaike information criteria. This model fits 4 different rate constant together with a blood volume fraction parameter. However, the dephoshporylation step k_4 is relatively small and may only start after 50 minutes which renders k_4 difficult to estimate in scans of 60 minute duration and scans of 90 minutes may provide a more reliable estimate of k_4 [2, 8, 14]. The phosphorylation step by thymidine kinase is represented by microparameter k_3 and this is the rate limiting step which reflects tissue proliferation rate [17]. However, k_3 estimates are highly influenced by noise in the time activity curves and estimates of k_3 are often accompanied with high standard errors. Therefore, macroparameters like V_T or K_i, which are calculated with the individual kinetic rate constants, are more stable parameters. A mathematical study of kinetic modeling of [^{18}F]FLT confirmed this with a higher bias in estimates of k_3 and k_4 compared to K_i based on Monte Carlo analysis with simulated time activity curves with added Poisson noise (bias of 26, 49 and 4% respectively) [2]. In agreement with this, repeatability of K_i is superior to k_3 as shown in a clinical test-retest study (repeatability of 32 and 76%, respectively) [44]. Hence, macroparameters are more robust and less sensitive to detect changes in [^{18}F]FLT uptake caused by noise and these parameters were used as reference to validate simplified measures.

Our study showed that relative changes in V_T were not related to perfusion measured with [^{15}O]H_2O PET, indicating that [^{18}F]FLT uptake changes were not a function of perfusion. Moreover, differences in [^{18}F]FLT uptake measured with SUV or TBR correlated with V_T and these simplified parameters could replace pharmacokinetic modeling in NSCLC patients treated with EGFR TKI. This would eliminate the need of dynamic PET acquisition with scan durations up to 90 minutes and limited axial field-of-views of 18-25 cm. SUV and TBR can be measured with a static whole body acquisition protocol and these scans can be performed in ~20 minutes. Based on this validation study we propose the use of TBR parent plasma measured at 50-60 minutes post injection, if [^{18}F]FLT parent plasma concentration can be measured reliably. In this case, the uptake time interval should be strictly controlled since TBR increases

rapidly as function of uptake time. SUV could also be used, which eliminates the need of a blood sample and is fairly constant over a wide time interval at 30 minutes after injection. However, SUV possibly underestimates responses up to 28% compared to pharmacokinetic modeling. This underestimation of SUV for response measurement was also observed in [^{18}F]FLT PET in breast cancer patients after chemotherapy [45]. SUV changes in head and neck cancer patients 5 days after start of treatment with concomitant chemoradiotherapy correlated with pharmacokinetic modeling, indicating that static whole body scans could also replace dynamic scans in this setting [12]. Future biological validation studies should be performed to determine the accuracy of [^{18}F]FLT PET detected changes for prediction of clinical outcome. For such purpose a window study should be performed, in which [^{18}F]FLT uptake is measured at baseline and during and/or after completion of neo-adjuvant treatment and is compared with pathology after surgery. Subsequently, the predictive value of treatment effectuated changes of [^{18}F]FLT uptake could be investigated. Ideally, [^{18}F]FLT PET could function as imaging biomarker at early time points to evaluate treatment response and adapt individual treatment regimen. Moreover, [^{18}F]FLT PET could be used as outcome parameter in drug development trials in the future. Promising results of [^{18}F]FLT uptake changes at 1-6 weeks after start of treatment showed already good agreement with clinical outcome in various malignancies [46-50].

To use a quantitative imaging biomarker, the variability between scans should be determined to know when an observed effect is due to treatment effectuated changes and not due to measurement or physiological variation. Precision of a measurement can be determined in a "test-retest" study, where a measurement is repeated within a short time interval and without intervention. Repeatability of [^{18}F]FLT uptake derived from dynamic PET scans has been investigated in NSCLC by Langen et al. [44] and Shields et al. [51] with limits of agreement of 32% for K_i and 9-15% for mean SUV. Metabolically active tumor volume (MATV) is a parameter of tracer uptake volume instead of tracer uptake quantity. MATV is a relatively novel PET parameter and gained interest over the last decade, together with heterogeneity tracer uptake parameters, as additional quantitative parameters in the evaluation of PET scans [52-54]. MATV correlated with pathology assessed tumor diameter after surgery in NSCLC [55]. Repeatability of MATV has been described in **chapter five and six** for both [^{18}F]FLT and [^{18}F]FDG in order to determine the limits of agreement for interpretation of repeated MATV measures in future clinical trials. In **chapter five** repeatability of MATV in [^{18}F]FLT

and [^{18}F]FDG PET in NSCLC was investigated in a monocenter setting. We concluded that MATV with 50% threshold of the maximum pixel within the tumor and corrected for local contrast (MATV A50%) can be recommended based on its high feasibility and repeatability. Differences of > 37% for [^{18}F]FDG and > 73% for [^{18}F]FLT for lesions >4.2 mL measured with MATV A50% are beyond test retest variation and such changes are likely to represent real MATV changes. For smaller lesions evaluation of absolute changes might be preferred, because small absolute changes will result in high relative changes for small lesions at baseline. Limit of agreement was 1.0 and 0.9 mL for [^{18}F]FDG and [^{18}F]FLT, respectively, for lesions <4.2 mL. When all sizes were taken together, relative and absolute limits of agreement were 62 and 50%, and 4.2 and 6.3 mL for [^{18}F]FDG and [^{18}F]FLT, respectively. We did not evaluate manual MATV delineation in this study, because this would introduce an extra intra- and interobserver variability contribution which can be avoided with semiautomatic MATV delineation [56, 57]. Moreover, manual MATV segmentations are labor intensive and therefore less feasible in large studies or for routine clinical use. Repeatability of the fuzzy locally adaptive Bayesian (FLAB) methodology has been investigated in breast and esophageal cancer patients [58]. FLAB showed superior repeatability with limit of agreement of 20-35% compared to 51-65% for a fixed 50% threshold of the maximum voxel, uncorrected for local background uptake. Repeatability was dependent on lesion size, with larger variability for smaller lesions for the relative threshold MATV. However no separate analysis was performed to evaluate lesions with relative or absolute changes based on lesion size. In **chapter six**, MATV repeatability in [^{18}F]FDG PET was investigated in a multicenter study in gastrointestinal malignancies. In this study $SUV_{peak\ at\ max}$, $SUV_{highest\ peak}$ and SUV_{star} were included as reference value for relative threshold MATV segmentation methods besides the use of SUV_{max}. In addition, several other novel MATV segmentation methods were included; gradient based watershed segmentation, and iterative relative threshold level [59, 60]. MATV measured with 50% threshold of $SUV_{highest\ peak}$ had an improved repeatability compared to 50% threshold of SUV_{max}, both corrected for local contrast, with a limit of agreement of 36% compared to 59%. $SUV_{highest\ peak}$ suffers less from statistical noise within the image compared to SUV_{max} and this is directly reflected in improved repeatability for relative threshold levels for MATV delineation. Based on this MATV repeatability assessment we recommend the use of 50% of $SUV_{highest\ peak}$ corrected for local contrast in multicenter PET studies. Although this study was performed in [^{18}F]FDG PET in patients with gastrointestinal malignancies, this could be translated to other tracers and tumor types with similar tumor to background ratios. Heijmen et al. [61], evaluated MATV repeatability in

liver metastasis of colorectal cancer with limits of agreement of 46% for FLAB and 87% for source to background corrected relative threshold level of SUV_{max}. Use of $SUV_{highest\ peak}$ might improve the repeatability of the relative threshold MATV reported in this publication as well. Comparison of repeatability of FLAB and A50% $SUV_{highest\ peak}$ has, to the best of our knowledge, not yet been performed and would be very interesting to determine the optimal MATV method. Preferably, MATV delineation would be standardized to compare results between studies. Consensus of optimal MATV delineation should be based on software access, feasibility, repeatability and accuracy. Various MATV methods has already shown to have prognostic value [54, 62, 63] and response evaluation with PET might also benefit from incorporation of MATV measurements. In addition, the combination of MATV and SUV in total lesion glycolysis (TLG), MATV*SUV, might be a useful parameter to evaluate both quantities in one parameter for response monitoring [64-66]. For [^{18}F]FLT PET, such measure would represent total lesion proliferation (TLP) instead of TLG.

7.2 FUTURE PERSPECTIVES

[^{18}F]FLT PET is a promising imaging modality which could add to clinical response evaluation by quantifying changes in tumor proliferation rate non-invasively. In addition, [^{18}F]FLT PET provides spatial information of proliferation rate within the tumor and a whole body image of the patient could be assessed in limited time. Voxel-by-voxel analysis of [^{18}F]FLT PET could provide additional information in tracer uptake distribution within a tumor. Heterogeneity within a tumor could be used to stratify aggressive heterogeneous tumors and indicate high proliferation parts of the tumor [67]. Subsequently, treatment regimen and radiotherapy planning might be adjusted based on [^{18}F]FLT uptake quantity, volume and/or uptake pattern. In addition, response evaluation with [^{18}F]FLT PET early after start of treatment could detect changes in tumor proliferation rate at an early time point. Functional imaging to quantify proliferation would be a great benefit compared to repeated biopsies, since tumors are not always accessible for biopsy. Furthermore, information of only a small fraction of the complete lesion is provided by biopsies, and usually only one lesion instead of all tumor lesions within the patient can be evaluated by histology. [^{18}F]FLT PET might eliminate the need of repeated biopsies in the future.

The era of personalized and targeted treatment has started and PET might be a good candidate to evaluate effectiveness of such personalized medicine strategies. PET evaluation criteria in solid tumors (PERCIST) have been proposed for [^{18}F]FDG PET [54]. These criteria provide a guideline for response evaluation with PET. However, more research is needed to implement PERCIST in a clinical setting. [^{18}F]FLT PET measures proliferation instead of glucose metabolism and is therefore more specific for malignancies (no increased [^{18}F]FLT uptake in radiation pneumonitis or infection). Clinical trials with [^{18}F]FDG and [^{18}F]FLT PET using PERCIST for response evaluation should be performed to provide knowledge on the predictive value, and on the scientific and clinical use of PET for response evaluation. [^{18}F]FLT PET may be more sensitive to evaluate proliferation specific changes to evaluate cell survival compared to [^{18}F]FDG PET. However, [^{18}F]FLT PET is sometimes argued to be "too expensive" to be clinically relevant. But, if an ineffective (expensive) treatment is stopped at an early time point based on [^{18}F]FLT PET, imaging might save costs. It is the costeffectiveness of the diagnosis-treatment combination that counts, not the cost of individual components. With increasing costs of therapy (and its development) the costs of imaging and of other biomarkers to evaluate therapy should be seen in this context. Giving the wrong therapy to the wrong patient at the wrong time is the real cost-driver and, most importantly, at the expense of patient outcomes. Moreover, incorporation of [^{18}F]FLT PET in drug development trials might prevent large and expensive trials with ineffective drugs. Cost-effectiveness of [^{18}F]FLT PET in these settings should be determined in future studies [68].

It is important that PET is performed in a standardized method to make absolute and relative uptake parameters comparable within a patient, between patients and between centers. PET acquisitions and analysis have been shown to vary between centers [69-71]. Moreover, clinical PET trials usually comprise small sample sizes and standardization would make it possible to pool data and increase the power of statistical tests. Guidelines for [^{18}F]FDG PET have been developed to provide consistency of PET data acquisition and analysis worldwide [72-74], and these rules are not essentially different for [^{18}F]FLT.

Besides PET acquisition also PET evaluation should be standardized, including MATV delineation. Based on our multicenter evaluation of MATV repeatability we have suggested A50% of $SUV_{highest\ peak}$, but FLAB might be a good alternative. Head-to-head comparison should determine the optimal MATV strategy. In future research, standard measurements should be included together with new uptake or MATV parameters to place novel parameters in perspective.

Also, new tracers are under development to quantify other metabolic processes involved in malignant tumors non-invasively, such as angiogenesis, hypoxia, apoptosis and EGFR expression [75]. Apart from metabolic tumor characteristics, drug delivery could be studied with radiolabeled drugs [76, 77]. Radiolabeled drugs could determine mutation status, receptor affinity and individual drug pharmacokinetics [78, 79]. Such PET tracers are promising to aid drug development and individual treatment strategies for patients.

PET is a relatively new imaging modality and development is still ongoing. Progress at a technical level (instrumentation and quantification methods), biological level (tracers) and clinical level (time-interval of scanning, correlation with clinical outcome) is only achieved with dedicated research. Although options of PET seem to be unlimited, care should be taken that quantity and simplicity are not preferred above quality and accuracy. Technical and biological validation studies of new tracers, new quantitative parameters, or studied within different tumor types, should be thoroughly investigated before use.

Finally, an essential, but not yet achieved step is to ensure that published manuscripts include (at least electronically) the technical specifications required to assess which data can be pooled. As stated before, typical PET study sample size is relatively small, so that meta-analyses are essential to arrive at appropriate levels of evidence. At present, such analyses are frustrated by the lack of details provided in the manuscripts of such studies. We strongly suggest that existing generic guidelines of reporting (STARD for diagnostic accuracy, REMARK for prognostic biomarker research) [80, 81] incorporate specific add-ons for individual technologies. Furthermore, journals should encourage authors to publish individual patient data (again, in electronic supplements). Taken together, this would allow for individual patient data meta-analyses; we believe that this is the only way to generate appropriate evidence for biomarkers like [^{18}F]FLT and [^{18}F]FDG. With such an approach, one may investigate the impact of confounders and effect-modifiers of the [^{18}F]FLT biomarker (e.g. impact of tumor type, intervention, timing of PET, impact of image analysis methodology).

As stated in the introduction, biomarkers are used since the origin of medicine, and Hippocrates highlighted the importance of prognostic factors in one of his historic writings. Until today, efforts are made to design appropriate biomarkers for patient stratification and to predict clinical response. This thesis focused on the feasibility and precision of [^{18}F]FLT PET. Accuracy should be determined in upcoming trials. Hopefully, in the near future, the progress in metabolic imaging and quantification

of tracer uptake, including the development of new tracers, will establish PET as a recognized imaging biomarker for drug development and in clinical practice. PET has a broad spectrum of parameters for functional imaging already available (including metabolically active tumor volume, heterogeneity and uptake quantification) which could be applied to a range of tracers once thoroughly validated. Comparison with pathology and clinical outcome should be performed to establish these metabolic imaging parameters as valid clinical biomarkers.

7.3 REFERENCES

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