RESEARCH ARTICLE

Measuring Residual Estrogen Receptor Availability during Fulvestrant Therapy in Patients with Metastatic Breast Cancer

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Abbreviation list: BMI, body mass index; CT, computed tomography; ER, estrogen receptor; FES, [18F]fluoroestradiol; LC/MS/MS, liquid chromatography-tandem mass spectrometry; PD, progressive disease; PET, positron emission tomography; PFS, progression free survival; PR, partial response; ROC, receiver operating characteristic; SD, stable disease; SUV, standardized uptake value

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ABSTRACT

It is unknown whether the current dose of fulvestrant, an estrogen receptor (ER) downregulator, is sufficient for maximal ER downregulation in patients with metastatic breast cancer. We performed a feasibility study to assess ER availability before and during fulvestrant. Sixteen patients with ER-positive metastatic breast cancer underwent positron emission tomography/computed tomography (PET/CT) at baseline (Scan 1), day 28 (Scan 2) and day 84 (Scan 3) to monitor tumor $[^{18}\text{F}]$fluoroestradiol (FES) uptake. Incomplete reduction in ER availability was predefined as $< 75\%$ decrease in median tumor FES uptake and a residual standardized uptake value ($\text{SUV}_{\text{max}}$) $\geq 1.5$. In total 131 FES-positive lesions were identified (median $\text{SUV}_{\text{max}}$ of 2.9, range 1.7-6.5). The median change in patients during fulvestrant treatment was -85% at Scan 2, but varied widely (-99% to +60%). Fulvestrant reduced tumor FES uptake incompletely at Scan 2 in 6 (38%) of the 16 patients, which was associated with early progression.

SIGNIFICANCE

Serial imaging of tumor estrogen uptake by FES-PET can give insight into the dose needed for estrogen receptor antagonists to completely abolish ER. FES-PET showed significant residual ER availability in tumors during fulvestrant therapy in 38% of patients, which was associated with early progression.
INTRODUCTION

Fulvestrant, a pure estrogen receptor (ER) antagonist, is used as treatment for advanced and metastatic ER-positive breast cancer. Its method of action is unique in that it not only competitively binds to the ER, but also reduces its expression (1). In preclinical studies, fulvestrant was shown to completely down-regulate tumor ER expression and inhibit estrogen-mediated tumor growth (2). In early clinical studies, no dose-limiting toxicities were detected, and 250 mg intramuscularly every 4 weeks was approved as standard dose, given that higher doses cannot be administered as a single injection due to the solubility of the drug (3, 4). However, serial biopsy studies showed that ER expression is down regulated incompletely at the 250 mg dose (4). A loading-dose regimen was therefore explored more recently: 500 mg at day 1 and 250 mg at day 14, 28 and every 4 weeks thereafter, and a high-dose regimen consisting of 500 mg at day 1, 14, 28 and every 4 weeks thereafter. Due to a progression-free survival (PFS) gain in the high-dose regimen from 5.5 to 6.5 months in a phase III trial, the high-dose regimen ultimately became the current standard (5). High-dose fulvestrant as first line metastatic treatment also showed favorable outcome compared with anastrozole, with a median PFS of 23 versus 13 months (6). However, 38% of patients still do not obtain clinical benefit from fulvestrant and hence progress within 24 weeks of therapy (5). Reasons for therapy failure include lost ER expression and insufficient dosing of fulvestrant (7, 8).

Positron emission tomography/computed tomography (PET/CT) with the tracer \([^{18}\text{F}]\text{fluoroestradiol (FES)}\) can be used for serial whole body evaluation of tumor ER expression and blockage of ER binding by endocrine therapies (9). Previous studies have evaluated serial FES-PET during endocrine therapy, including one study with fulvestrant (10). In that retrospective study, the majority of patients used the lower (250 mg) dose of
fulvestrant, and only one follow-up scan was performed at various pharmacokinetic time points.

Our prospective study was designed to evaluate whether the current standard dose of 500 mg fulvestrant optimally abolishes ER availability in the tumor at two time points during treatment (day 28 and day 84), where the PET scans were scheduled just prior to the subsequent dose of fulvestrant.

**RESULTS**

**Patients and Treatment Outcome**

Between June 2011 and February 2013, 16 patients were included and assigned to fulvestrant treatment 500 mg intramuscularly on day 1, 14, 28 and every 4 weeks thereafter. All patients had received prior palliative tamoxifen and aromatase inhibitor therapy, and five had received prior palliative chemotherapy. Response to treatment was evaluated by serial CT scan and clinical assessment. Seven patients met the criteria for measurable disease according to response evaluation criteria in solid tumors (RECIST v1.1) at baseline (11), six patients had non-measurable nodal or visceral involvement and three patients had bone-only disease. Detailed patient characteristics at study entry are provided in Table 1.

According to RECIST 1.1 criteria, fulvestrant induced one partial response (PR). Stable disease (SD) was observed in eight patients ≥ 24 weeks; three of these had measurable disease at baseline. In the remaining five patients with SD, no evidence of radiologic or clinical progressive disease (PD) was detected, and all five had a decrease in tumor marker CA15.3 (range; -5% to -66%). Finally, four patients had radiologic PD, and two had clinical PD. Clinical PD was evident from deterioration of symptoms and a threelfold to twelwefold increase in CA15.3. Treatment response could not be determined in one patient who withdrew consent after 1 month of therapy.
The median follow-up was 6.5 months (range 1.9 to 15.9 months). Of the 16 patients, 15 discontinued fulvestrant with a median PFS of 6.2 months. The remaining patient had received fulvestrant for more than 16 months. Fulvestrant was well tolerated by nearly all patients. One grade 3 adverse event was observed, a urinary tract infection, after which the patient requested treatment withdrawal despite partial response on the CT scan.

**FES-PET/CT Analysis**

FES-PET was performed to evaluate tumor ER availability before and during treatment. All patients had at least one FES-positive lesion at Scan 1. A total of 131 FES-positive lesions were identified on FES-PET/CT (107 bone lesions, 17 lymph nodes, four lung metastases, one ovarian metastasis and one perineal metastasis). In addition, the CT scan revealed FES-negative metastases (n = 11 bone lesions and one lymph node) in five (31%) of 16 patients. Finally, seven liver metastases were detected. FES uptake could not be reliably measured in liver metastases due to high background FES uptake in healthy liver tissue. Baseline maximum standardized uptake value (SUV\textsubscript{max}) of FES varied greatly among lesions (median 3.4; range 1.4 to 17.4) and patients (median 2.9; 1.7 to 6.5).

A second scan was available for all patients; 12 patients received the second scan at day 28, two patients at day 56 (prior to 4\textsuperscript{th} fulvestrant administration), and two patients at day 84 (prior to 5\textsuperscript{th} fulvestrant administration). A third scan was available for nine patients, but seven patients did not receive the third scan due to early progression (n = 3), treatment withdrawal (n = 2) and logistic reasons (n = 2).

At the second scan, the median change in FES uptake for the 16 patients was -85% (-99 to +60%). Median residual tumor SUV\textsubscript{max} was 1.7 (1.1 to 3.8). A median reduction in FES tumor uptake of more than 75% was observed in nine of the 16 patients, while incomplete reduction (-58% to +60%) with a residual SUV\textsubscript{max} ≥ 1.5 was observed in six patients. One
patient had a relative decrease in FES uptake of less than 75%, but residual SUV$_{\text{max}}$ was below the threshold of 1.5.

Interestingly, even in the nine patients with ≥ 75% decrease in FES uptake, four patients had residual FES uptake (SUV$_{\text{max}}$) above 1.5. In the nine patients for whom three scans were available (in 8 of 9 patients on the predefined time points), residual tumor FES uptake did not decrease between Scan 2 and Scan 3 (median SUV$_{\text{max}}$ 1.6 v 1.7, $P = 0.23$), which suggests that the maximum effect of fulvestrant 500 mg can be measured after only two administrations. Larson-Fox-Gonen plots are provided for representative patients with complete (Fig. 1A) and incomplete (Fig. 1B) reduction in FES uptake at Scan 2. FES uptake characteristics before and during therapy are provided in Table 2.

Heterogeneity in the reduction in FES uptake was seen between lesions within individual patients. For example, one individual had six lesions with ≥ 90% reduction in FES uptake with a residual SUV$_{\text{max}}$ ≤ 1.5, while in seven other lesions a modest reduction of 50-70% was observed, with residual SUV$_{\text{max}}$ as high as 4.9 (Fig. 2). Based on our predefined cutoff point, at the second scan eight of 16 patients had lesions with complete reduction in FES uptake, but they also had lesions with incomplete reduction. Of all lesions quantified, 58 (44%) of 131 had incomplete reduction in FES uptake on the second FES-PET scan. The incomplete reduction in individual lesions shown at Scan 2 persisted during treatment. Paired analysis of the nine patients with three available scans showed incomplete reduction in FES uptake in 18 (26%) of 70 lesions at Scan 2 and 20 lesions (29%) at Scan 3.

**Correlation between Treatment Outcome and FES-PET Results**

Baseline tumor FES uptake in metastases of patients having clinical benefit from fulvestrant was similar to that in patients with PD (median SUV$_{\text{max}}$, 3.1 v 2.5, $P = 0.6$). In our study, a previously published (12, 13) threshold of baseline SUV$_{\text{max}}$ ≥ 2.0 did not help to
identify responding patients. Furthermore, the presence of FES-negative lesions at baseline did not predict therapy failure. PD developed in two of five patients with heterogeneous disease with at least one FES-negative site and four of 10 patients with only FES-positive sites. Consequently, baseline FES-PET was unable to differentiate between patients that would subsequently derive clinical benefit from fulvestrant treatment and those who would not.

However, the magnitude of changes in tumor FES uptake corrected for physiological background (SUV$_{cor}$) was significantly larger in patients having clinical benefit from fulvestrant compared to patients with PD (median change SUV$_{cor}$, -88% vs -58%, $P = 0.025$). Of nine patients with $\geq 75\%$ change in median FES uptake, 8 (89%) had clinical benefit from fulvestrant therapy, compared to only one of six with $< 75\%$ decrease. In addition, median PFS was 3.3 months for patients with $< 75\%$ decrease in FES uptake vs 11.7 months for patients with $\geq 75\%$ decrease ($P < 0.05$). FES-uptake at baseline and during treatment for all individual patients and its relation with response is depicted in Fig. 3. The relative decrease in FES uptake and its relationship with response are shown in Fig. 4. ROC analysis was performed to evaluate whether a threshold other than the predefined 75% would increase the predictive value. ROC analysis showed that the optimal cutoff point (-76%) was close to our pre-defined threshold of -75%. In a lesion-based analysis, no association between changes in FES uptake in the tumor and size changes on CT scan was detected for the small number of measurable lesions (n = 13).

**Correlation between Tumor FES Uptake and Plasma Fulvestrant Levels**

To evaluate whether changes in tumor FES uptake correspond to individual patient plasma fulvestrant levels, blood was drawn on the same day as the PET scan. Plasma fulvestrant levels were determined by liquid chromatography tandem mass spectrometry.
Median patient fulvestrant plasma levels were 33 nmol/L at day 28 and 27 nmol/L at day 84 of treatment (Supplementary Fig. S1A). Plasma fulvestrant levels varied between patients (16 to 53 nmol/L). Although median fulvestrant levels were slightly higher in patients that had clinical benefit from fulvestrant compared to patients with PD (39.6 $\text{v} 29.4$ nmol/L), plasma fulvestrant levels did not correlate with absolute or relative changes in either $\text{SUV}_{\text{max}}$ or $\text{SUV}_{\text{cor}}$ at day 28 or at day 84. Apparent serum estradiol levels increased by 0.19 $\pm$ 0.05 nmol/L due to cross-reactivity with fulvestrant. The actual serum estradiol levels, corrected for effects of fulvestrant, were 0.06 $\pm$ 0.02 nmol/L at day 28 and 0.06 $\pm$ 0.03 nmol/L at day 84, and hence remained in the postmenopausal range (Supplementary Fig. S1B).

**Tamoxifen Effect on Baseline PET Measures**

Although it was not pre-specified as a study endpoint, we did observe an impact of recent therapies on baseline scan results. This information could allow further optimization of FES-PET/CT study protocols (9). All four patients that withdrew from tamoxifen treatment shortly (5-6 weeks) before baseline FES-PET/CT had lower FES uptake when compared with patients that did not recently use tamoxifen (median $\text{SUV}_{\text{max}}$ 1.7 $\text{v}$ 4.1, $P = 0.004$). Coincidentally, an earlier FES-PET that was performed during treatment with an aromatase inhibitor was available for two of these four patients (14).

One patient had clinical benefit despite a reduction in FES uptake of only 32%. In this patient, baseline $\text{SUV}_{\text{max}}$ was 1.7 in the current study, while previous FES uptake (during aromatase inhibitor therapy) was much higher ($\text{SUV}_{\text{max}}$ 3.1) and more tumor lesions were visible. If the scan while on aromatase inhibitor was used as the baseline measure, a median 100% decrease in FES uptake would have been observed during fulvestrant therapy. This would have correctly identified this patient as a responder. The second patient who used tamoxifen until 5 weeks prior to baseline PET had similarly low uptake at baseline ($\text{SUV}_{\text{max}}$...
1.8). Paradoxically, fulvestrant induced a median 60% increase in FES uptake in this patient. FES uptake at the earlier scan during aromatase inhibitor therapy was higher (median SUV$_{\text{max}}$ 2.8). If this scan was used as baseline scan, FES uptake would have decreased by 26% during fulvestrant treatment, hence correctly identifying this patient as non-responder. Together, these results suggest that tamoxifen and its metabolites partly block FES uptake in the tumor, even after a 5-week drug-free period.

If patients with residual tamoxifen effects were excluded from our analysis, fulvestrant would have decreased median FES uptake in all patients by 86%, by 91% in patients with clinical benefit (n = 7 patients), and -58% in non-responding patients (n = 4 patients). Four of 12 patients (33%) would have had incomplete reduction in ER availability. Seven of eight patients (88%) with >75% decrease in FES uptake would have had clinical benefit from fulvestrant therapy, compared to none of four patients with incomplete reduction in FES-uptake. When data adjusted for previous tamoxifen use were included, the positive predictive value increased to 89% and negative predictive value remained at 100%.

**DISCUSSION**

This is the first serial PET imaging study evaluating the effects of fulvestrant 500 mg on FES uptake in metastatic breast cancer patients. The fulvestrant-induced reduction in FES uptake in the tumor varied widely and was inadequate in some patients: incomplete reduction in FES uptake after 4 weeks was observed in 38% of the patients.

In contrast to tamoxifen, fulvestrant is a pure ER antagonist, which can down-regulate ER expression in a dose-dependent fashion without any agonistic effects. Therefore FES-PET is a very suitable technique to study the ability of fulvestrant to reduce ER availability and to correlate this parameter with treatment response.
Previous studies provided FES-PET data for patients treated with tamoxifen (12), (10). For example, in a study in 40 patients, after 7-10 days of tamoxifen therapy FES uptake decreased by 55% in patients having clinical benefit compared to 19% in non-responding patients (12). However, this incomplete reduction in FES uptake should not lead to the conclusion that tamoxifen dosing is suboptimal, as it can take several weeks for tamoxifen to reach steady-state levels.

A retrospective study in 11 patients who underwent fulvestrant therapy showed a mean decrease of 49% in FES uptake in tumors (10). This study differed from our current study in several respects. Patients had received lower fulvestrant doses, i.e. 250 mg or 500 mg at day 1, followed by 250 mg on day 14, day 28 and every 4 weeks thereafter. Moreover, only one follow-up scan was performed, between 1 and 18 weeks after therapy initiation, and the FES-PET scans were not synchronized with fulvestrant injections. This could have affected the results since plasma fulvestrant levels can vary 10-fold between two doses (15).

We therefore performed FES-PET at stringent time points in all patients, concurrent with determination of plasma fulvestrant levels.

In our study, like in other FES-PET studies, patients were required to discontinue tamoxifen at least 5 weeks prior to baseline FES-PET to prevent competitive binding (14). However, the lowest median FES uptake was recorded in the four patients that used tamoxifen until 5 weeks prior to FES-PET. In two of these patients, an earlier FES-PET scan was available while they were still on aromatase inhibitor treatment. These PET scans showed more tumor lesions and a higher median SUV$_{\text{max}}$. It is likely that a generally used 5-week stopping period for tamoxifen is too short to reliably measure baseline ER expression by FES-PET. The reported half life for tamoxifen is 4 days, but this can be longer for its metabolites with a half life of even 400 days for $N$-desmethyltamoxifen (16). Therefore, after discontinuation of tamoxifen there may still be residual effects of tamoxifen metabolites in
the tumor for over 5 weeks. Interestingly, we did not observe a correlation between plasma
drug levels in individual patients and effects on tumor FES uptake. Thus, measuring plasma
fulvestrant levels does not provide information on whether the dose is sufficient for optimal
effect at the tumor site. Fulvestrant effects at the tumor site could possibly depend not only
on plasma levels, but also on various other factors (e.g. tumor size, vascularization, ER
levels, and presence of mutations in the ligand binding domain of the ER), which could
explain the lack of correlation.

The effect of fulvestrant on tumor ER availability could be visualized after only two
doses of fulvestrant. In our feasibility study with a small number of patients, a reduction in
FES uptake larger than 75% was significantly associated with clinical benefit. Because it
takes several months before therapy effects can be reliably measured by anatomical imaging
techniques, such as CT scan (17), earlier prediction of treatment response would be valuable.
Moreover, ER-positive breast cancer is characterized by bone-dominant disease (18), as was
also evident in this study. It is notoriously difficult to evaluate treatment response in bone
lesions, and therefore they are considered non-measurable by RECIST v1.1 criteria.
Therefore, future studies should address whether serial FES-PET can offer an early response
measurement in patients with bone-dominant disease.

Our study has some limitations. First, given the character of the study the sample size
was relatively modest. Due to progressive disease and treatment withdrawal a third scan was
available in only nine patients. In four patients, the second scan was delayed for logistic
reasons. However, the scans were still performed on the same pharmacokinetic trough, i.e.
just prior to dosing of fulvestrant. Given the fact that tumor FES-uptake remained stable
between Scan 2 and Scan 3 in the eight patients with three serial scans available on the exact
predetermined scan times, this delay likely did not affect our results. Second, from our data it
is difficult to address the effects of FES-negative lesions on fulvestrant efficacy. Given the
heavy pre-treatment of the patients in our study, CT scan may show bone lesions that are no
longer active. This might potentially lead to an overestimation of FES-negative sites (SUV$_{\text{max}}$
$<1.5$) at baseline. On the other hand, CT is relatively insensitive for bone metastases, and
therefore some FES-negative sites may have been missed. Others have used FDG-PET
together with FES-PET for the identification of FES-negative lesions (19). However, FDG-
PET can fail to visualize osteoblastic bone metastases (20). Third, correction of FES uptake
for recent tamoxifen treatment using an earlier FES-PET should be regarded exploratory as
temporal changes in ER expression cannot be excluded. Finally, we did not have access to
serial tumor biopsies, which could have enabled us to discriminate between decrease in FES
uptake due to downregulation of ER expression and occupancy of ERs with preserved
expression. However, ER might also be heterogeneously expressed across tumor lesions
within a patient.

We observed that patients with incomplete reduction in FES uptake were more likely
to develop PD within 24 weeks of therapy initiation. A recent phase I study on the anti-
androgen ARN-509 showed that increasing doses resulted in a plateau in which > 90% of
tumor androgen uptake was blocked as measured by [$^{18}$F]fluordihydrotestosterone (FDHT)
PET (21). If we had applied the -90% threshold, only four (25%) of 16 patients would have
obtained complete reduction in tumor ER uptake. Given the resemblance between fulvestrant
and ARN-509 – both are nuclear hormone receptor antagonists – and between FES-PET and
FDHT-PET, it may be possible that >90% reduction in FES uptake can be obtained with
higher doses of fulvestrant. This may also be clinically feasible. In a randomized phase III
study comparing fulvestrant 500 mg to 250 mg, toxicity was equally mild in both groups (5,
22). In addition, in a neoadjuvant study in premenopausal patients, a single injection with
fulvestrant 750 mg was well tolerated (23). As an alternative to an increased dose, a higher
frequency of administration (e.g. every 2 or 3 weeks) might also be an option. However, our
results suggest that not all patients need a higher dose, since fulvestrant reduced ER availability sufficiently in 62% of the patients. Conversely, not all patients with complete reduction in ER availability will experience clinical benefit, since other mechanisms aside from inadequate dosing can be responsible for therapy failure. Among these potential mechanisms are ESR1 mutation and up-regulation of growth factor receptor pathways (24).

The optimal drug dose that leads to complete reduction in ER availability could be verified with FES-PET. This approach would clearly differ from the current approach for endocrine drugs, where the principle ‘one-dose-fits-all’ is applied, despite the fact that serum and tissue drug levels can vary considerably between patients and at different doses (25). Some authors have therefore suggested therapeutic drug monitoring and dose escalation based on serum or plasma drug levels (26). In addition, FES-PET could prove useful during drug development, to evaluate the dose required for optimal ER downregulation for new compounds that block or degrade ER.

METHODS

The study was conducted at the University Medical Center Groningen (UMCG), the Netherlands, in accordance with the principles of Good Clinical Practice and the Declaration of Helsinki. The UMCG Institutional Review Board approved the protocol, and patients provided written informed consent before participation. The study was registered in the clinicaltrials.gov database (NCT01377324).

Study Population

Medical oncologists in our center and our referring non-academic hospitals identified patients for the imaging study. They get regular updates about ongoing trials in our center and have a web-based access to summaries of ongoing trials. Postmenopausal patients with ER-
positive metastatic breast cancer were eligible for the study when they had progressive
disease after 2 or 3 lines of palliative hormonal therapy. Other eligibility criteria were ECOG
performance ≤ 2, life expectancy > 3 months, and a creatinine clearance > 30 mL/min.
Exclusion criteria were previous fulvestrant therapy, the presence of life-threatening visceral
metastases, central nervous system metastases, and more than 2 lines of palliative
chemotherapy. To prevent competition between FES and drugs from previous therapies, the
patients were required to discontinue drugs known to bind ER for at least 5 weeks prior to
baseline PET imaging (14).

Study Design

We performed an imaging trial in patients on salvage endocrine therapy with standard
fulvestrant dosing. The patients were imaged just prior to each fulvestrant administration. The
aim of this feasibility study was to evaluate the effects of fulvestrant on ER availability. The
primary end point was to evaluate the number of patients and lesions in which fulvestrant
incompletely abolished tumor ER availability. Secondary end points were the following: 1)
the correlation between FES-PET results, plasma fulvestrant levels and treatment outcome; 2)
the heterogeneity of FES uptake among lesions and between individuals, and 3) the feasibility
of quantifying liver lesions by FES-PET.

Study measurements

All patients were treated uniformly with fulvestrant 500 mg intramuscularly on day 1,
14, 28, and every 4 weeks thereafter. Baseline measurements included recording of
symptoms, performance status, physical examination (including size and weight), laboratory
tests (including hormonal status and tumor markers) and a diagnostic CT scan. Clinical
follow-up included clinical history, performance status, physical examination and laboratory
tests, and was performed every 4 weeks. Follow-up of serum tumor marker (CA15.3) and
diagnostic CT scans was performed after 84 days (concurrently with FES-PET), after 6
months, and thereafter when progression was suspected based on clinical assessment or
biochemistry. Response assessment was performed by radiologic imaging using RECIST
v1.1 criteria, as well as clinical and biochemical parameters. Patients were considered to have
radiologic PD when they had >20% increase in measurable lesions, when new lesions were
detected during follow-up, or when there was unequivocal progression of existing lesions
(11). Clinical PD could also develop before radiologic response assessment or in the setting of
radiographic SD. In these instances PD was defined as an overall level of substantial
worsening such that the overall tumor burden, biochemistry (e.g. tumor markers, liver
function), and/ or complaints increased sufficiently to merit discontinuation of therapy (27).
Final response classification was done by the treating medical oncologist while blinded for
FES-PET results. All patients without PD for at least 24 weeks were classified as having
clinical benefit derived from fulvestrant (5). Treatment was continued until PD, withdrawal of
consent or severe toxicity. Adverse events were documented and graded during the first 6
months of therapy, according to the Common Terminology Criteria of Adverse Events
(version 3.0).

Pharmacodynamic Biomarker

In vivo ER availability was assessed by FES-PET/CT on a hybrid PET/CT camera
with a 64-slice CT and high definition and time-of-flight PET (Siemens Medical Systems,
Knoxville, TN). FES was produced and administered to the patient as described earlier (14,
28). The mean radiochemical yield FES was 30 ± 17%, with a radiochemical purity of 99.9 ±
0.2%, and a specific activity of 209 ± 112 GBq/µmol. The mean injected dose of $^{18}$F-FES
was 204 MBq ± 23 MBq. A few hours prior to the next fulvestrant dose, FES-PET/CT was
performed at baseline (Scan 1), day 28 ± 2 (Scan 2) and 84 ± 2 (Scan 3). The first and third PET scans were combined with a diagnostic CT scan as part of the staging. FES-PET scan 2 was combined with a low-dose CT for attenuation correction.

FES uptake was quantified at the three time points for tumor lesions detected by PET/CT at baseline, according to the guidelines of the European Association of Nuclear Medicine (EANM) (29). FES uptake for individual lesions was expressed as SUV\text{max}. We used the previously published threshold of $SUV_{\text{max}} \geq 1.5$ to define FES-positive lesions (9, 27). When calculating the relative change in FES uptake, only FES-positive lesions were included. FES uptake was also expressed as $SUV_{\text{max}}$ corrected for physiological background ($SUV_{\text{cor}}$) (30). Background correction was applied by using the unaffected contra-lateral site whenever available, or surrounding tissue of the same origin. Liver lesions were excluded from quantitative analysis given the high physiological background of FES uptake in healthy liver tissue.

We observed that patients who had used tamoxifen until 5 weeks prior to FES-PET had a very low tumor FES uptake. Therefore, for an explorative analysis, we used an earlier FES-PET scan while the patients were on aromatase inhibitor therapy as baseline measure to correct for tamoxifen effects in two patients. Since this resulted in not only higher FES uptake, but also the identification of more FES-avid lesions, we calculated the decline in FES-uptake for all lesions.

For patient-based analysis, the median SUV\text{max} and median SUV\text{cor} were calculated up to an arbitrary maximum of 20 lesions. Incomplete reduction in ER availability in a patient was defined as a relative decrease in FES uptake of less than 75% in median tumor lesion $SUV_{\text{cor}}$ and an absolute median tumor lesion SUV\text{max} > 1.5. The 75% cutoff point was chosen based on an earlier ~50% decrease with the lower (250 mg) dose of fulvestrant in a retrospective study (10).
**PK Assessments**

Plasma fulvestrant levels were determined by liquid chromatography-tandem mass spectrometry (LC/MS/MS) on the same days as scan 2 and scan 3. Serum estradiol levels were determined by fluorescent-immuno-assay concurrently with scan 1, scan 2 and scan 3. Since fulvestrant is known to cause cross-reactivity with the estradiol assay, calibration curves were obtained to assess the increase in apparent serum estradiol levels for increasing doses of fulvestrant (Supplementary data, Supplementary Fig. S2). This calibration curve was used to correct the measured estradiol levels in patient serum for the measured fulvestrant plasma levels on LC/MS/MS. Plasma fulvestrant levels and corrected serum estradiol levels were correlated with findings on FES-PET.

**Statistical Analysis**

We aimed to include 15 patients to provide an estimate of the proportion of patients with incomplete reduction in FES tumor uptake defined as described previously (relative decrease < 75% in median lesion SUV\textsubscript{cor} and absolute median tumor SUV\textsubscript{max} > 1.5). Statistical analysis was performed in IBM SPSS Statistics version 20.0. Tumor FES uptake was tested for normality. Wilcoxon signed-rank test was used to evaluate changes in FES uptake between scan 2 and scan 3, and Mann Whitney U for changes between patients having clinical benefit from fulvestrant versus patients with PD. Receiver operating characteristics (ROC) analysis was performed to evaluate the optimal threshold to predict therapy outcome by FES-PET.

**Disclosure of Potential Conflicts of Interest**
The authors declare no potential conflicts of interest. Dr. Hospers received a research grant made available to the UMCG from AstraZeneca.

**Authors’ Contributions**

**Conception and design:** G.A.P. Hospers, M. van Kruchten, C.P. Schröder, E.F.J. de Vries, E.G.E. de Vries.

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**Analysis and interpretation of data:** all authors

**Writing, review and/or revision of the manuscript:** all authors

**Final approval of the manuscript:** all authors

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**Tables**

**Table 1. Patient Characteristics**

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<td>Site of disease</td>
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</tr>
<tr>
<td>Bone only</td>
<td>3</td>
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<tr>
<td>Bone + visceral (± nodes)</td>
<td>8</td>
</tr>
<tr>
<td>Bone + nodes</td>
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</tr>
<tr>
<td>Visceral only</td>
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<tr>
<td>Prior lines of palliative endocrine therapies</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen, aromatase inhibitor</td>
<td>14</td>
</tr>
<tr>
<td>Tamoxifen, aromatase inhibitor, megestrol</td>
<td>2</td>
</tr>
<tr>
<td>Prior lines of chemotherapy for metastatic disease</td>
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</tr>
<tr>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.
Table 2. FES-PET results in individual patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor sites</th>
<th>FES+ lesions (n)</th>
<th>Baseline SUV(_{\text{max}})</th>
<th>Second SUV(_{\text{max}})</th>
<th>% Change SUV(_{\text{cor}})</th>
<th>Progression (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bone, lung</td>
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<td>14.7</td>
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<tr>
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<td>6.0</td>
<td>1.4</td>
<td>-96</td>
<td>21.9</td>
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<tr>
<td>3</td>
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<td>1.9</td>
<td>-85</td>
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<tr>
<td>4</td>
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<td>4.7</td>
<td>3.1</td>
<td>-88</td>
<td>11.7</td>
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<tr>
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<td>-32</td>
<td>13.3</td>
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<tr>
<td>5*</td>
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<td>1.0</td>
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<td>2.4</td>
<td>+5</td>
<td>1.9</td>
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<td>1.1</td>
<td>-91</td>
<td>16+ (ongoing)</td>
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<tr>
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<td>2.5</td>
<td>+60</td>
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<tr>
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<td>1.8</td>
<td>-92</td>
<td>PR (W)</td>
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<tr>
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<td>6.8</td>
</tr>
<tr>
<td>12§</td>
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<td>1.9</td>
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<tr>
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<td>2.1</td>
<td>-58</td>
<td>3.7</td>
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<tr>
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<td>NE (W)</td>
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<td>2.7</td>
<td>1.2</td>
<td>-99</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Abbreviations: SUV\(_{\text{max}}\), maximum standardized uptake value; SUV\(_{\text{cor}}\), background-corrected standardized uptake value; PR, partial response; NE, not evaluable; W, withdrew. Data presented are median values of all quantified lesions.

* Two patients had recent tamoxifen treatment and very low baseline uptake. An earlier FES-PET scan while using an aromatase inhibitor provided additional insights (see Results section: Tamoxifen Effects on Baseline PET Measures).

§ Patients with clinical PD.
FIGURE LEGENDS

Figure 1. Change in FES uptake in the tumor during fulvestrant treatment. (A) A representative patient with incomplete reduction in FES uptake, and (B) a patient with extensive reduction in FES uptake. Blue dots indicate individual lesions; the red square represents the median of all lesions used for patient-based analysis. At the right side of the plot the corresponding PET images are shown. The line indicates the 75% reduction threshold.

Figure 2. Heterogeneous effects of fulvestrant were observed between lesions within individuals. In this patient spinal bone metastases showed > 90% reduction in FES uptake (arrows), while the right femoral lesion had < 50% decrease (arrowhead). Physiological tracer distribution was observed in liver, intestines, bladder and at the injection site.

Figure 3. Pre-treatment (squares) and post-treatment (diamonds) background-corrected tumor FES-uptake ($SUV_{cor}$) for all individual patients. Patients are grouped according to their response, in blue patients with partial response and stable disease, and in red patients with progressive disease. In two patients with recent tamoxifen therapy, a previous baseline PET was available while on aromatase inhibitor therapy. The green squares and diamonds represent the values if the previous PET would have been used as baseline measure.

Figure 4. Waterfall plot showing the relative change in median tumor FES uptake ($SUV_{cor}$) in individual patients at the second scan compared to baseline. The predefined 75% reduction threshold is indicated. Patients that used tamoxifen until 5 weeks prior to baseline PET are indicated (*). In two patients with recent tamoxifen therapy, a previous baseline PET was available while on aromatase inhibitor therapy. The shaded bars represent the values if the previous PET would have been used as baseline measure.
Figure 2.

Baseline FES-PET

During Fulvestrant
CANCER DISCOVERY

Measuring Residual Estrogen Receptor Availability during Fulvestrant Therapy in Patients with Metastatic Breast Cancer

Michel van Kruchten, Elisabeth G de Vries, Andor W Glaudemans, et al.

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