Consolino, Lorena; Longo, Dario Livio; Dastrù, Walter; Cutrin, Juan Carlos; Dettori, Daniela; Lanzardo, Stefania; Oliviero, Salvatore; Cavallo, Federica; Aime, Silvio. Functional imaging of the angiogenic switch in a transgenic mouse model of human breast cancer by dynamic contrast enhanced magnetic resonance imaging. INTERNATIONAL JOURNAL OF CANCER. 139 (2) pp: 404-413.
DOI: 10.1002/ijc.30073

Functional imaging of the angiogenic switch in a transgenic mouse model of human breast cancer by dynamic contrast enhanced magnetic resonance imaging

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Abstract

Tumour progression depends on several sequential events that include the microenvironment remodelling processes and the switch to the angiogenic phenotype, leading to new blood vessels recruitment. Non-invasive imaging techniques allow the monitoring of functional alterations in tumour vascularity and cellularity. The aim of this work was to detect functional changes in vascularisation and cellularity through Dynamic Contrast Enhanced (DCE) and Diffusion Weighted (DW) Magnetic Resonance Imaging (MRI) modalities during breast cancer initiation and progression of a transgenic mouse model (BALB-neuT mice). Histological examination showed that BALB-neuT mammary glands undergo a slow neoplastic progression from simple hyperplasia to invasive carcinoma, still

preserving normal parts of mammary glands. DCE-MRI results highlighted marked functional changes in terms of vessel permeability (K_{trans}, volume transfer constant) and vascularisation (v_p, vascular volume fraction) in BALB-neuT hyperplastic mammary glands if compared to BALB/c ones. When breast tissue progressed from simple to atypical hyperplasia, a strong increase in DCE-MRI biomarkers was observed in BALB-neuT in comparison to BALB/c mice (K_{trans} = 5.3 ± 0.7E-4 and 3.1 ± 0.5E-4; v_p = 7.4 ± 0.8E-2 and 4.7 ± 0.6E-2 for BALB-neuT and BALB/c, respectively) that remained constant during the successive steps of the neoplastic transformation. Consistent with DCE-MRI observations, microvessel counting revealed a significant increase in tumour vessels. Our study showed that DCE-MRI estimates can accurately detect the angiogenic switch at early step of breast cancer carcinogenesis. These results support the view that this imaging approach is an excellent tool to characterize microvasculature changes, despite only small portions of the mammary glands developed neoplastic lesions in a transgenic mouse model.

Introduction

Breast cancer typically evolves through a multistep progression process, starting from epithelial hyperplasia and progressing to carcinoma *in situ* (CIS) and finally to metastatic carcinomas.[1] Tumour microenvironment is often claimed as a key player in tumour biological transformations into consequent more aggressive steps. To ensure tumour growth and proliferation from early stages of progression, tumour cells induce the formation of new blood vessels through a vascular remodelling process termed “angiogenic switch.” This event represents the turn-on step that allows cancer cells to invade and growth out their primary niche and it is becoming a key target for novel anti-cancer therapies.[2, 3] In particular, tumour associated neo-blood vessels display structural and functional

abnormalities, mainly due to the imbalanced regulation between pro- and anti-angiogenic factors that make them tortuous and leaky. This chaotic and unstructured vasculature is responsible of irregular perfusion that determines areas characterized by different pO_2.[4, 5] Since tumour microvessels exhibit such unique properties, the real time monitoring of alterations in vascularity using non-invasive imaging techniques appears to be the most suitable approach to succeed in early breast cancer detection and characterization. Imaging may report on tumour heterogeneity and identify more aggressive regions to directly guide biopsies and radiotherapy. Among the imaging techniques, Magnetic Resonance Imaging (MRI) is the only modality that combines high spatial resolution and excellent tissue contrast with functional information.[6, 7] The dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) technique involves the injection of a paramagnetic contrast agent (CA) and evaluates the differential enhancement produced upon time by its extravasation through leaky tumour vessels.[7, 8] Maps of tumour tissue parameters such as vascular permeability and perfusion (K_{trans}), extracellular volume fraction (v_e), and blood plasma volume fraction (v_p) can be obtained by using pharmacokinetic compartment models.[9] Besides DCE-MRI, the diffusion weighted imaging technique (DWI) provides additional information on tumour microenvironment. The apparent diffusion coefficient (ADC) is inversely dependent on water movements across the cell membrane and reflects tissue changes at cellular level. Increases in ADC correspond to decreases in cellular size or number of cells, due to apoptosis or necrosis, whereas lower ADC values correspond to a state of tumour progression, increased fibrosis or interstitial oedema.[10] Much attention is currently devoted to the combined use of DCE- and DW-MRI; most of the reported studies have shown the ability of MRI functional parameters to assess early response to neoadjuvant chemotherapy and antiangiogenic treatments at clinical and preclinical settings.[11-17]

Moreover, there is an increase interest in using DCE- and DW-MRI in breast cancer characterization and subtyping. Malignant and benign

breast tumours have been clearly differentiated by DWI, with lower ADC values recorded in malignant tumours compared to benign lesions.[18, 19] In addition, pharmacokinetic parameters obtained by DCE-MRI represent potential candidates for luminal and basal breast cancer identification.[20] For longitudinal studies of breast carcinogenesis, the use of transgenic mouse models might provide evident advantages at preclinical settings, compared to xenograft ones. Xenograft models are only representative of advanced stages of cancer, whereas transgenic mice recapitulate the stepwise progression and typical features of several human cancers, preserving the interaction between tumours and the surrounding microenvironment.[21] In the current study, we used the BALB-neuT transgenic murine model that shows a human-like breast cancer development, including mammary hyperplasia, atypical hyperplasia, CIS and invasive breast cancer.[22] The BALB-neuT mice overexpress the activated form of the rat ErbB2 (Her/2-neu) oncogene, whose amplification is observed in 20–30% of human breast cancer. The aim of the present study was to evaluate whether DCE- and DW-MRI modalities could detect functional tumour microenvironment changes along the multistep carcinogenesis process observed in the BALB-neuT model, before palpable mass formation, compared to normal mammary glands of BALB/c mice. Immunohistochemistry (IHC) analysis of the tumor microvasculature and cellularity was also performed to assess a relationship between the MRI data and the *ex vivo* characterization.

Material and Methods

Mice

BALB/c mice were purchased from Charles River Laboratories (Calco, Italy), whereas BALB-neuT mice[22] were bred at the Department of Molecular Biotechnology and Health Science, University of Torino, Italy. Mice were maintained in specific pathogen-free conditions (Allentown Caging Equipment, Allentown) and treated in accordance

with the University Ethical Committee and European guidelines under directive 2010/63. In BALB-neuT transgenic mice model mammary gland cells transform progressively into well distinctly morphological stages, namely simple hyperplasia, atypical hyperplasia, CIS and invasive carcinoma.[23, 24]

DCE-MR and DW-MR imaging experiments

MRI experiments were performed by imaging the left and right fourth mammary glands of female mice. Mammary glands at different histological stages of breast cancer progression (between 5 and 28 weeks of age) from BALB-neuT mice ($n = 156$) and glands from age-matched control littermates ($n = 74$) were observed. Before MRI analysis, mice were anesthetized by injecting a mixture of tiletamine/zolazepam (Zoletil 100; Virbac, Milan, Italy) 20 mg/kg and xylazine (Rompun; Bayer, Milan, Italy) 5 mg/kg and a 27-gauge catheter was introduced into the tail vein for CA injection. Mice were then placed supine in a solenoid Tx/Rx coil with an inner diameter of 3.5 cm. The breath rate was monitored throughout *in vivo* MRI experiments using a respiratory probe (SAII Instruments, Stony Brook, NY). A phantom filled with diluted ProHance® (Bracco Imaging SpA, Milan, Italy) was included in the field of view (FOV), close to each animal, to allow correction for changes in the instrument performance.

MR images were acquired on the 1 Tesla Aspect M2 MRI scanner (Aspect Magnet Technologies, Israel). After the scout image acquisition, a T_2-weighted (T_{2w}) anatomical image was acquired with a Fast Spin Echo sequence (TR = 2500 sec; TE = 44 msec; number of slices = 10; slice thickness = 1.5 mm; FOV = 40 mm; matrix = 152 × 160; four averages; acquisition time = 3 m 20 sec). The DCE-MRI dynamic protocol consisted of the application of an axial T_1-weighted (T_{1w}) 3D spoiled Gradient Echo sequence with three initial pre-contrast images acquisition that were followed by the injection through the catheter of 0.05 mmolGd/kg gadolinium binding serum albumin CA (Gd-AAZTA Madec, Cage Chemicals, Novara, Italy).[25] After injection, 47 dynamic post-contrast images were acquired with the following parameters: TR = 40; TE = 2.1 ms, flip angle = 60°,

number of slices = 10, slice thickness = 1.5 mm, FOV = 40 mm, matrix = 128 × 128, acquisition time = 58 sec. DW-MRI was performed using a Spin-Echo sequence with seven different *b*-values (0, 50, 100, 150, 200, 400, 600) with the following parameters: TR = 500 ms; TE = 15.4 ms; number of slices = 10; slice thickness = 1.5 mm; FOV = 40 mm; matrix = 128 × 128; 1 = average; acquisition time = 7 m 28 sec.

Image analyses

All the DCE-MRI images were analysed using an in-house developed software in C++ code implementing MITK (http://www.mitk.org/MITK), ITK and VTK libraries for the quantification of pharmacokinetic parameters and in MatLab (MathWorks, Natick, MA).

A rigid-ITK co-registration was applied for motion correction on VFA and DCE image by taking the anatomical T_{2w} image as reference. Pre-contrast T_1 has been determined using a variable flip angle fast gradient echo technique.[**26**] Dynamic post-contrast T_1 relaxation was calculated from the signal intensity (SI) curves after conversion into longitudinal relaxation rate R_1 ($1/T_1$) assuming a linear relationship between R_1 and CA concentration according to the following equation:

(1)

where $1/T_{10}$ is the pre contrast longitudinal relaxation rate, $1/T_1$ is the post contrast longitudinal relaxation rate and r_{1p} is the longitudinal relaxivity of the CA that was assumed to be equal to the value (40 mM^{-1}/sec) measured in blood serum. The extended Tofts' model with an individually measured arterial input function (AIF) has been used.[**9**] This model assumes a bidirectional exchange between two compartments, that is, the intravascular and the extravascular extracellular space (EES). To extract the kinetic parameters (K_{trans}, v_p, and k_{ep}) on a voxel-by-voxel basis, the concentration curve in the tissue has been fitted against the solution of the differential equation:

(2)

where $C_t(t)$ is the CA concentration in the tissue at time t, v_p is the fractional blood plasma volume, $C_p(t)$ is the CA blood plasma concentration at time t (AIF), K_{trans} is the volume transfer constant between the intravascular and the EES ($K_{trans} = k_{ep}\ Ve$), k_{ep} is the rate constant from EES to blood plasma and $\otimes$ is the convolution operator. The AIF and the injection time were automatically determined as previously described by the software using a three-dimensional region growing algorithm with an artery seed point automatically determined from the maximum increase of signal in the dynamic series. The accuracy of the automatic AIF identification was evaluated as previously described.[**16**] A region of interest (ROI) was manually drawn for both left and right IV mammary glands encompassing all the mammary gland tissue as visible in both T_{2w} and T_{1w} images as shown in Supporting Information Figure S1. K_{trans} and v_p mean values were extracted by an in house developed MATLAB script (The MathWorks, Natick, MA).

Apparent diffusion constant (ADC) maps were calculated fitting DW-MRI signal intensity as a function of *b*-values in MATLAB, working on a pixel by pixel basis. ADC mean values were calculated superimposing ADC maps on the selected ROIs.

Histological analysis and microvessels counting

After MRI images acquisition, BALB-neuT and BALB/c mice were euthanized and their skin was fixed in 4% formalin in phosphate saline buffer (PBS, Sigma Aldrich, Milano, Italy) at 4°C; 1 hr later mammary glands were gently scraped from the skin, fixed overnight in 4% buffered formalin solution and then embedded in paraffin. Tissue sections (5 µm thick) were cut from paraffin embedded blocks and prepared for immunohistochemistry staining. Briefly, sections were deparaffinized in xylol, rehydrated in graded alcohol series (Sigma Aldrich, Milano, Italy), then washed in PBS-Tween 0.1% (PBS-T) and heated in a microwave oven in citrate buffer 10 mM, pH 6 (Bioptica, Milano, Italy) for 10 min for antigen retrieval step. Afterwards, endogenous peroxidase activity was inhibited using 6% H_2O_2 in bidistilled water for 8 min and incubated with 10% normal

goat serum (Sigma-Aldrich, Milano, Italy) for 1 hr at room temperature (RT). Slices were then incubated overnight at 4°C with primary rabbit polyclonal antibody against von Willebrand factor (vWF, 1:100, Abcam, Cambridge, UK), then with secondary goat anti-rabbit HRP antibody (1:200, Abcam, Cambridge, UK) for 1 hr and 30 min at RT. The immune reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride hydrate (Sigma Aldrich, Milano, Italy). After washing in bidistilled water, counterstaining was performed with hematoxylin. For the immune-staining analysis, optical fields of the whole mammary glands were acquired with a 20× magnification by using a Leica 3000 microscope. Microvessel density (MVD) was assessed by counting the total vessel number and then calculating the average of vessels per area. Positively stained vessels were expressed per area examined (counts/mm^2) by using a commercially available software (Leica Application Suite, Milano, Italy).

Histopathological analysis

Formalin-fixed and paraffin-embedded sections (5 µm) of mammary glands were stained with hematoxylin-eosin (H&E) technique. Progressive morphological changes to invasive cancer were assessed by a pathologist blinded to the age of the animals and MR imaging findings. The diagnosis of simple hyperplasia, atypical hyperplasia, CIS and invasive carcinoma were performed according to previous publications.[**27, 28**] Glands were semi-quantitatively evaluated by microscopy and the presence of different histological states was expressed as percentage of the total mammary gland area affected. Cell density was determined by counting the number of nuclei cells on histologic specimen and divided for the FOV that consistently measured 713 × 532 µm by using ImageJ.

Statistical analysis

Statistical analysis of imaging data and microvessels counting was performed using GraphPad Prism 5 software (GraphPadInc, San Diego, California). All data are shown as mean ± SEM. One-wayANOVA analysis and Dunn's multiple comparison test was used to compare (i) K_{trans} and v_p mean values of mammary glands in BALB-

neuT mice *versus* wild-type BALB/c mice at the same week of age and (ii) K_{trans} and v_p mean values in different histological findings within the same murine model. For all tests, a p values < 0.05 was considered statistically significant.

One-way ANOVA analysis and Tukey's honestly significance difference test were performed to examine whether MVD and cellular density was significantly different in hyperplasia, atypical hyperplasia, CIS and invasive carcinoma of BALB-neuT mice.

Results

Vascular functional changes were observed by DCE-MRI when BALB-neuT mammary glands progressed from simple hyperplasia to atypical hyperplasia

Figure 1a summarizes the distribution of the four histological stages (simple hyperplasia, atypical hyperplasia, CIS, invasive cancer) in mammary glands from BALB-neuT mice at different weeks of age. The neoplastic transformation process proceeded slowly, with the typical overlapping of the histological phenotypes along ageing. At weeks 5–7, >60% of mammary glands tissue still showed normal histological features, whereas 30% of the area was occupied by simple hyperplasia. From 8th to 13th weeks of age, atypical hyperplasia was present in 41% of mammary glands section and highly persisted at week 14–21 (45%). Histological features of CIS were firstly detected from week 14 (9%) and progressively increased to 15% at weeks 22–28. Invasive breast cancer appeared from week 22 and occupied the 30% of the mammary glands examined. Figure 1b shows representative H&E images of whole mammary glands for each morphological stage at different age of BALB-neuT mice.

Table 1 and Figures 2a and 2b show DCE-MRI derived estimates for K_{trans} and v_p obtained for each step of BALB-neuT breast tumour progression and age-matched mammary glands of control BALB/c mice. In BALB-neuT mice, K_{trans} and v_p values markedly increased when breast tissue progressed from simple to atypical hyperplasia, with statistical significance for v_p ($p < 0.05$). Further stages of tumour

progression showed K_{trans} values comparable to values obtained in atypical hyperplasia, whereas v_p significantly increased in invasive breast when compared to simple hyperplasia lesions ($p < 0.05$). Signal intensity enhancement curves displayed a slow accumulation of the blood-pool agent, with a slight lower enhancement for the mammary glands at the simple hyperplasia stage (Supporting Information Fig. S2). Semi-quantitative estimates were calculated from the dynamic signal enhancement curves. Only AUC30 and AUC50 showed slightly lower values for simple hyperplasia in comparison to the other stages, despite not statistically significant (Supporting Information Fig. S3). BALB/c mice showed a slight decrease in K_{trans} values along ageing of the mice that became significant between weeks 5–7 and weeks 22–28 ($p < 0.001$). Comparing K_{trans} and v_p estimates between BALB-neuT and BALB/c mice, similar values of both parameters were obtained in hyperplastic and normal mammary glands, respectively. At the time of atypical hyperplasia transition in BALB-neuT mice, markedly increased values of K_{trans} and v_p were observed in comparison with BALB/c ones. This trend is preserved along the further stages of BALB-neuT tumour progression. When BALB-neuT mice showed invasive carcinoma, significant differences for both parameters were observed as compared to age-related BALB/c mice ($p < 0.05$ for both K_{trans} and v_p). Representative parametric maps of K_{trans} and v_p superimposed on T_2-weighted anatomical images for hyperplasia to atypical hyperplasia transition are shown in Figures 3a and 3b, respectively. V_e values were similar among simple hyperplasia, atypical hyperplasia and CIS of BALB-neuT histological stages (Supporting Information Fig. S4 and Table S1). An increase of Ve estimate was observed when mammary glands progressed to invasive carcinoma, however without statistical significance.

DW-MRI revealed age-related cellularity changes in BALB/c mammary glands

Figure 4a shows the mean apparent diffusion coefficient (ADC) values along BALB-neuT tumour progression stages and age-matched BALB/c mice. A slightly decrease in ADC values was progressively

observed for ageing BALB/c mice starting from week 15 that resulted in a significant difference when comparing results between weeks 5–7 and 22–28 ($p < 0.05$). When BALB-neuT mammary glands progressed to invasive carcinoma, ADC values resulted significantly smaller in age-related BALB/c mice ($p < 0.05$). Figure 4b reports an increase in cellularity along BALB-neuT tumour progression. The average cell density was similar comparing BALB-neuT mammary glands at simple hyperplasia to atypical hyperplasia (535 ± 52 for simple hyperplasia and 548 ± 72 for atypical hyperplasia). When mammary glands progressed to CIS and invasive carcinoma, cellular density increased (775 ± 48 and 1009 ± 109 for CIS and invasive carcinoma), with statistical significant difference when comparing invasive carcinoma with simple hyperplasia and atypical hyperplasia ($p < 0.001$).

Microvessel density (MVD) markedly increased with breast tumour progression from simple to atypical hyperplasia in BALB-neuT mice

MVD significantly increased ($p < 0.005$) progressing from simple to atypical hyperplasia in BALB-neuT mice (MVD = 36 ± 6 and 149 ± 28 for simple and atypical hyperplasia, respectively; Fig. 5a). This difference was maintained along the further stages of tumour progression (MVD = 151 ± 28 in CIS and 139 ± 15 in invasive carcinoma, $p < 0.05$, respectively). Similarly, percentage of total vessels per area showed an increased vascularization from simple to atypical hyperplasia, CIS and invasive carcinoma (Fig. 5b). Representative images of vWF staining are shown in Figure 5c.

Discussion

The goal of our work was to evaluate whether vascular and cellular changes along mammary tumour progression stages in a transgenic mouse model of breast cancer could be assessed by applying different functional MRI techniques. We found that DCE-MRI was able to detect differences in vascularity when mammary glands progressed from the histological stage of simple hyperplasia to

atypical hyperplasia. The observed differences were maintained between atypical hyperplasia, CIS and invasive breast cancer of BALB-neuT and normal mammary glands of control mice at the same week of age. These findings demonstrated the great potential of functional MRI methods for the detection of early changes associated to breast cancer and characterization of tumour progression stages *in vivo*, providing new insights into tumour angiogenesis and microenvironment changes.

Many efforts are currently made to improve breast cancer screening. Although mammography is widely used in clinical settings for breast cancer detection, it suffers for poor sensitivity and low accuracy, in particular in presence of dense breast tissue.[**29**] Among the several imaging technique, MRI provides a great potential for the visualization of early breast cancer. Jansen *et al*.[**30**] demonstrated MRI capability to anatomically detect left inguinal mammary glands progression from *in situ* neoplasia to invasive carcinoma predicting the tumour invasiveness in the SV40 T-antigen transgenic breast cancer mouse model, whereas more recently Fan and colleagues[**31**] extended the study to all mammary glands by using a whole-body MRI operating at 9.4 Tesla. We deemed of interest to seek for functional information in addition to anatomical characterization by getting more insights into the angiogenesis process that goes along tumour growth and progression. Bachawal and colleagues[**32**] recently showed that targeted ultrasound contrast microbubbles for VEGFR2 allows detection and staging in a breast cancer transgenic mouse model. MRI-based approaches have been successfully exploited to monitor neovascularity or antivascular treatments.[**33, 34**] In our study, we used Dynamic Contrast Enhanced (DCE)-MRI technique which reports about vascular volume and vessels leakiness of tumour vasculature thanks to the contrast enhancement generated by the injected blood pool MRI CAs. To the best of our knowledge, this is the first study able to detect functional vascular changes at early step of breast carcinogenesis in transgenic breast cancer models by means of the DCE-MRI technique.

In our study we used the BALB-neuT transgenic mouse model, which mimics the slow step by step progression of human ductal breast adenocarcinoma from normal to simple hyperplasia, atypical hyperplasia, carcinoma *in situ* and invasive breast cancer (Fig. 1). Compared to other transgenic mouse models (*e.g.*, SV40 T-antigen, that recapitulates the same progression in a shorter time-frame) mammary glands of the BALB-neuT mice progress along the different histological stages with a slow rate, showing the first palpable mass at 20 weeks of age.[**22**] Since this slow transformation closely reflects breast cancer development in patients, the angiogenic vasculature proceeds along with tumour progression in a more natural evolution path. Our results showed microvasculature changes in terms of permeability and vascularization when mammary glands progressed from simple hyperplasia to atypical hyperplasia. In particular, the volume transfer constant K_{trans} and the plasmatic volume v_p obtained by DCE-MRI considerably increased in BALB-neuT mice from the hyperplasia step, whereas their values remained stable in age-matched BALB/c control mice. In the BALB-neuT mouse model, the angiogenic switch occurs at 8–10 weeks of age, when mammary glands show atypical hyperplasia.[**35, 36**] The formation of new blood vessels is a crucial step for tumour growth and proliferation, since it provides oxygen and nutrients and offers a route for tumour cells to disseminate through blood stream for invasion of distant organs. The angiogenic switch ensures this transition from pre-vascular hyperplasia to highly vascularised and outgrowing tumours. Our finding highlighted the ability of the MRI approach to detect *in vivo* such angiogenic switch, showing a marked increase in the DCE-MRI pharmacokinetic parameters in correspondence to the transition step from simple to atypical hyperplasia. A significant increase of MVD was observed when mammary glands progressed from simple hyperplasia to atypical hyperplasia, confirming the *in vivo* results. Our data are in accordance with a recent work of Conti and colleagues[**37**] that, by

exploiting a new optical imaging probe targeting the angiogenesis marker $\alpha_v\beta_3$ integrin, showed early detection of mammary lesions at 12 weeks of age in BALB-neuT mice. In our study, the combination of a blood pool CA with the DCE-MRI approach for functional microvasculature characterization of the mammary glands allowed the assessment of the angiogenic switch already at the 8–10 weeks of age. The microvasculature estimates obtained by DCE-MRI generally remained higher in the further stages of breast tumour progression, such as CIS and invasive breast cancer. A similar trend was observed from *ex vivo* quantification of mammary glands vessels, confirming DCE-MRI findings (Figs. 5a and 5b). Taken together, our results were in accordance with recent studies in human breast cancer reporting that the angiogenic initiation starts at the hyperplasia stage with a further increase of angiogenesis upon the transition from carcinoma *in situ* to invasive carcinoma.[**38, 39**] By contrast, the calculated semi-quantitative AUC estimates did not detect significant differences among the histological stages of tumour progression. Our results demonstrated that quantitative pharmacokinetic estimates could provide robust assessment of early vascular changes. Therefore, this approach represents a reliable tool to investigate functional vessel remodelling processes.

Wild type BALB/c mice, used as controls in the present study, showed a decrease of both K_{trans} and v_p values at the age corresponding to CIS and invasive breast cancer in BALB-neuT mice. We surmise that the decrease in the functional parameters could be influenced by the loss of parenchyma as a consequence of the atrophy process of mammary glands in adult mice. The loss of parenchyma is generally followed by a loss of vascularization, likely resulting in a decrease of the MRI functional estimates.[**40**] A decrease of ADC values was observed in normal mammary glands along ageing of BALB/c mice (Fig. 3). Since the ageing of normal mammary glands is characterized by a loss of parenchyma and vascularization, we hypothesize that the reduced perfusion of the microcapillary network at later stages could contribute to the decrease of ADC in normal mammary glands.

During BALB-neuT mammary tumour progression, a high percentage of normal tissue was still present in the mammary gland (Fig. 1). This is likely due to the slow progression rate of the neoplastic transformation process as well as to the fact that it does not affect synchronically all the acinar-lobular units. However, changes in vascularization accompanying breast cancer progression were successfully detected by our approach. Therefore, our results highlighted DCE-MRI ability to detect pathological breast tissue even if breast lesions represented only a small fraction of the whole mammary gland. This particular stromal morphological feature may explain the lack of correlation between DW-MRI with histologically determined cellularity along tumour progression stages of BALB-neuT mammary glands. Moreover, we cannot exclude that ADC value might also depend on tumour stromal features. In addition to cell density, ADC values can be influenced by tissue perfusion, extracellular space and cell membrane integrity.[**41**]

In this study we used a new Gd-based CA able to reversibly bind to serum albumin by forming a macromolecular adduct.[**25**] It is known that macromolecular tracers, in comparison to small size ones, allow for better assessment of the tumour vasculature permeability[**42**]; their accumulation in the tumour tissue is due to the hyperpermeability of tumour vasculature and the retention effect, resulting in a more effective tumour delineation.[**43**] The use of supramolecular paramagnetic adducts results in dramatic contrast efficiency at low magnetic fields (0.5–1.5 T).[**44-46**] Therefore, the high sensitivity of blood pool CA for the tumour vasculature characterization combined with the use of a 1 T magnetic field scanner may facilitate the translation of our approach to the clinical settings, in comparison to previous studies where high field scanners were employed. Previous studies from Jansen and colleagues[**47**] combined DCE-MRI with X-ray fluorescence microscopy to quantify the spatial distribution of CA in mammary glands of transgenic mice. Although gadolinium uptake in ducts allowed carcinoma *in situ* identification, no significant changes in pharmacokinetic parameters were reported mainly due to the small size of the employed CA.

We acknowledge some limitations of our study. First, MRI acquisitions were limited to the forth right and left mammary glands of transgenic and control mice. BALB-neuT mice displayed a complete mammary tumour penetrance in all ten mammary glands, even if tumour progression is not simultaneous for all of them. We investigated only these mammary glands to avoid undesired artefacts flow related to movement during DCE-MRI acquisition. Second, DW-MRI images were acquired without fat signal suppression, which may lead to underestimated ADC values.[**48**] However, we suppose that this underestimation likely affected in a similar amount ADC values for both normal and neoplastic mammary glands. Third, DCE-MRI data could have been subjected to partial volume effect that occurs due to motion artefacts and to slice thickness; however, this is a much smaller effect in mice compared with human data.

In summary, the current study highlighted the potential of functional MRI as a sensitive modality for tumour microenvironment characterization. In particular, our results outlined the ability of DCE-MRI to detect tumour angiogenesis initiation in a transgenic breast cancer mouse model. K_{trans} and v_p values significantly increased when mammary glands evolved from hyperplasia to atypical hyperplasia; this transition was confirmed by an increase of MVD, which is a clinical surrogate marker for assessing tumour angiogenesis. In addition, our approach was able to detect the angiogenic switch in spite of the fact that only a small portion of the mammary gland tissue progressed to a neoplastic stage, thus providing a real non-invasive approach for early breast cancer detection. Considering that the BALB-neuT model shares genetic and phenotypic similarities with human breast cancer, evaluation of vascularization/permeability in this model could have strong impact for understanding the pathophysiology of breast cancer during carcinogenesis.