DCE-MRI quantification of leukemia-induced changes in bone marrow vascular function

by Ana L. Gomes, John Gribben, Bernard Siow, Diana Passaro, and Dominique Bonnet

Haematologica 2021 [Epub ahead of print]

Citation: Ana L. Gomes, John Gribben, Bernard Siow, Diana Passaro, and Dominique Bonnet. DCE-MRI quantification of leukemia-induced changes in bone marrow vascular function. Haematologica. 2021; 106:xxx

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
DCE-MRI quantification of leukemia-induced changes in bone marrow vascular function

Ana L. Gomes¹, John Gribben², Bernard Siow ³, Diana Passaro¹,⁴* and Dominique Bonnet¹*

* These authors contributed equally.

¹ Hematopoietic Stem Cell Laboratory, The Francis Crick Institute, 1 Midland Road, London, NW1 1AT, United Kingdom; ² Department of Haemato-Oncology, Barts Cancer Institute, Queen Mary University of London, London, EC1M 6BQ, United Kingdom; ³ In Vivo Imaging, The Francis Crick Institute, 1 Midland Road, London, NW1 1AT, United Kingdom; ⁴ Present address: Leukemia and Niche Dynamics Laboratory, Université de Paris, Institut Cochin, INSERM, CNRS, F-75014 PARIS, France

Running head: Quantification of leukemia induced vascular dysfunction

Correspondence to dominique.bonnet@crick.ac.uk, diana.passaro@inserm.fr, bernard.siow@crick.ac.uk

Data sharing statement: scan data available upon request. Please contact corresponding author.

Acknowledgments

ALG was supported by an i2i translational grant scheme from the Francis Crick Institute. DP was supported by a non-clinical junior research fellowship from EHA. This work was supported by The Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001045), The UK Medical Research Council (FC001045), and
the Welcome Trust (FC001045). For the purpose of Open Access, the author has applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission.

The authors would like to thank Biological Research Facility, Flow Cytometry and In Vivo Imaging core facilities at the Francis Crick Institute for their valuable help. The authors are grateful to Prof. John Gribben (St Bartholomew’s Hospital, London, UK) for providing human AML samples.

Disclosure of Conflicts of Interest

The authors have no conflict to declare.

Contributions

ALG/DP/DB: wrote the manuscript; ALG/DP/DB: designed the experiments; ALG/DP: performed experiments and analyzed data; DB/DP: secured funding; ALG/BS: developed and optimized MRI protocol; JG: provide human AML samples.
In this study we show that DCE-MRI quantifies the impact of human AML on BM vasculature by optimizing an in vivo DCE-MRI method and assess its diagnostic and prognostic potential.

Acute myeloid leukemia (AML), the most common acute leukemia in adults\(^1\). AML is a very heterogeneous disease both genetically and therapeutically, and the importance of the BM microenvironment lies with the fact that leukemia cells are able to change the normal tissue functions to favor their proliferation and protect them from external harm\(^2-4\). BM vascular remodeling induced by AML cells, favors a more hypoxic microenvironment\(^4\), and is thought to protect leukemia stem cells from therapy, being a poor prognostic factor in AML\(^5, 6\). Most preclinical studies use microscopy-based methods to assess environmental changes, namely vascular leakiness and perfusion\(^7, 8\), leaving little space for direct translation of the optimized methodology. In the clinic, perfusion imaging is an important tool in the diagnosis and management of various diseases. The advantage of using magnetic resonance imaging (MRI) for perfusion imaging is its superiority in terms of soft tissue contrast, absence of ionizing radiation (compared to computerized tomography and nuclear imaging such as PET and SPECT) and the possibility of imaging larger body areas of even full body. Dynamic contrast enhanced MRI (DCE-MRI) is a common method to measure perfusion\(^9\). DCE-MRI quantifies signal changes due to the passing of a contrast agent through the imaging area. By modeling the signal kinetics, it is possible to quantify vascular parameters such as perfusion, leakiness, and blood volume. Even though it has been around for over 20 years, DCE-MRI has not yet been fully implemented in many more clinical pathways due to the lack of standardization in image acquisition and analysis methods, rendering it mostly a research-based tool.

In-house MatLab scripts were used to quantify non model-based parameters taken directly from the contrast agent kinetic curves (see below). The parameters quantified are illustrated in Figure 1A. Changes in the first part of the DCE curve reflect blood flow and blood volume, while changes in the second part of the DCE curve reflect changes in vascular permeability and extravascular space (Figure 1A)\(^9\).
We have previously reported that leukemia induces BM vascular leakiness, using intra-vital imaging of the calvaria \(^4\), which was not observed in cord blood injected mice (CB, Figure S1A-B), when comparing to non-injected (NI) healthy age matched mice. To test DCE-MRI’s sensitivity in quantifying leukemia-induced BM vascular dysfunction, three different leukemia cell lines were used (U937, HL60, and ML1), and mice were scanned at different stages of the disease (Figure S1C). When compared to healthy non-injected (NI) age-matched controls, leukemia mice showed altered BM DCE kinetics (Figure1B), with reduced CE (BM vascular density), reduced WiR (blood flow), and increased iWoR and WoR (vascular permeability and extravascular space) (Figure 1C). BM vascular dysfunction is not dependent on high leukemia burden, as even low levels of leukemia engraftment (<20% mCD45\(^+\)hCD33\(^+\) cells present in the BM) significantly alter BM vascular function (Figure1C, open circles). Leukemia is known to promote angiogenesis, but with disorganized and leaky vessels \(^2\,4\,10\). The reduced CE in our leukemia cohort is in agreement with this, as CE relates to the proportion of functional blood vessels per pixel. With deregulated BM vascular parameters correlating with aging in healthy mice (Figure S1D), we compared younger (12-16 weeks) versus older (19-32 weeks) mice to understand if age was affecting leukemia-induced BM vascular dysfunction. As in Figure S1D, NI older mice showed significantly reduced BM vascular density and increased BM vascular permeability, but we observed no effect of age in leukemia injected mice (Figure 1C). This indicates that aging and leukemia seem to alter BM vascular permeability (WoR) and functional density (CE) to a similar extent. However, decreased BM blood flow/perfusion (WiR) seems to be a leukemia-specific effect, as it was the only parameter unaltered by aging (Figure S1E). In fact, receiver operating characteristic analysis highlighted WiR as the best parameter to distinguishing healthy from leukemic BM (Figure 1D, AUC\(_{WiR}\) = 1.000).

Differences in vascular density and function between different areas of the bone have been reported \(^3\). Using intravital two-photon imaging of the calvaria, we confirmed that at early stages of the disease the areas of leukemia engraftment (GFP\(^+\) cells) coincided
with areas of reduced perfusion and irregular vessel structure, confirming a heterogeneous vascular dysfunction linked with leukemia burden (Fig S1F). For this reason, we hypothesize that averaged DCE-MRI parameters could have some limitations with an inhomogeneous and low-engrafted mice. To obtain a detailed picture of DCE-MRI-measured vascular aberration and evaluate its spatial distribution along the femur, we performed pixel-by-pixel (pbp, with each pixel corresponding to an area of ~200 μm) analysis for three DCE-MRI parameters: CE, WiR, and WoR. NI mice present a homogeneous distribution of functional vessels in the epiphysis and along the endosteum of the diaphysis (Figure 1E, CE); high vascular perfusion in the central marrow of the diaphysis (Figure 1E, WIR), and higher vascular permeability at the epiphysis and in proximity with the endosteum of the diaphysis. In the presence of AML, there is an aberrant heterogeneous distribution of these parameters along the femur. In HL60 and ML1 groups, pbp analysis highlighted pocket areas with severe vascular dysfunction (Figure 1E, white rectangles), surrounded by less affected vasculature. Importantly, areas that showed lower functional vascular density (low CE) corresponded to areas in which both the vascular perfusion (WiR) and permeability (WoR) were mostly affected (Figure 1E, white rectangles). In the U937 group, there were no obvious pocket areas of vascular dysfunction, being present throughout the femur. Thus, our pbp analysis approach was successful to resolve the spatial distribution of vascular parameters along the femur and unveil the heterogeneity of vascular disfunction in AML xenografts.

To further test the clinical potential of BM DCE-MRI, we scanned mice engrafted with samples from AML patients (PDX). Different AML samples had different engraftment capacity (Figure S2A) and DCE kinetics (Figure 2A), echoing the heterogeneous nature of this disease. Mice injected with AML1 presented the highest degree of vascular dysfunction (Figure 2A-B), which can also be seen in the pbp analysis (Figure 2C, AML1). Mice from remaining AML PDX showed no significant changes in CE or WoR, when compared to age-matched controls (old NI), similarly to our AML cell lines results (Figure S1E). However, all the AML patients induced a significant deregulation of the four DCE-MRI
parameters compared to young NI controls (Figure 2B). As hypothesized, averaged DCE-MRI vascular parameters were not sensitive enough to detect altered BM vasculature in AML PDX model of low disease burden (<20% hCD33+ cells present in the BM) (Figure 2B, open circles), a limitation which we could overcome with pbp analysis (detailed below). Our data highlights the heterogeneous nature of the primary disease compared to cell line models. However, regardless of the wide spectrum of vascular dysfunction, reduction in BM vascular perfusion (WiR) was again the most significantly affected parameter (Figure 2B) in AML PDX, with ROC analysis showing that WiR is of clinical relevance in distinguishing normal from leukemic BM (Figure S2B).

To determine whether DCE-MRI could be used to monitor the BM vascular response to chemotherapy, PDX cohorts were treated with a standard chemotherapy protocol and analyzed during the remission phase. Although significantly reducing leukemia burden (Figure S2C), cytarabine did not rescue the altered BM vascular phenotype. BM vascular response to cytarabine treatment was diverse, with some groups showing partial rescue (AML1, AML2), and other groups showing no effect at all (Figure S2D).

Pbp analysis showed that vascular dysfunction in some AML samples located to the whole or most of the diaphysis (AML1, AML3), while in others was located in small pockets (AML2). Mice injected with sample AML1 exhibited severe BM vascular dysfunction throughout the whole diaphysis, with hardly any perfused pixel (Figure 2C). Cytarabine treatment partly rescued the lack of vascularity in the diaphysis but could not rescue the vascular perfusion nor permeability (Figure 2C, AML1). Even though it did respond to cytarabine treatment (Figure S2C), AML3 showed great similarities with AML1 in terms of most of the vasculature in the diaphysis being compromised, with little to no perfused pixels corresponding to high vascular permeability areas (Figure 2C, AML3). AML4 and AML5 showed similar pattern of pixel distribution. Before therapy, both AML samples showed a modest effect on BM vasculature, and for both samples, AraC treatment
seemed to worsen the phenotype for both perfusion and permeability (Figure 2C, AML4 and AML5).

Imaging the microenvironment can provide important insights and have diagnostic power \(^{11, 12}\). In leukemia, clinical imaging is not part of the routine follow-up of patients, although clinical studies have shown the utility of DCE-MRI \(^{5, 13}\). Our preclinical imaging data, focusing on the femoral BM, sheds some lights on the nature of the vascular microenvironment involvement in AML at diagnosis and remission phase, and our in-house image analysis pipeline maximizes the translational potential of the technique, without the need of complex modelling analysis. This strategy resolves the caveats encountered with standard model-based quantification of the parameters, whose clinical relevance highly depends on the mathematical model chosen \(^{14}\). Our results pave the way for the implementation of a novel human BM tailored DCE-MRI model which would help to obtain absolute or correlative biomarker in the clinical settings. Amongst the analyzed parameters, the WiR has the best diagnostic potential as measured by ROC analysis. This could potentially be helpful in the scenario of long-term disease, patient refusal to have a bone marrow puncture, or high-risk and/or older patients in which frequent and thorough follow-up monitoring might be a value. Future clinical investigations will be needed to formally address the utility of this technique.

We were also able to show that vascular dysfunction occurs in healthy aging. Recent reports suggest that a damaged BM niche could have a role in the aging process of the hematopoietic system \(^{15}\). High vascular permeability being associated with increased HSC activation and egress from the niche \(^{8}\), our data are in line with these findings and point to a possible therapeutic benefit in restoring normal vascular functionality.

All animal experiments were performed under the project license (PPL 70/8904) approved by the Home Office of UK and in accordance with the Francis Crick Institute AWERB guidelines (Animal Welfare and Ethics Review Board). NOD.Cg-Prkdc\(^{scid}\)Il2rg\(^{tm1Wjl}\).
(NSG) strains were obtained from Jackson Laboratory (Bar Harbor, Maine, USA) and were bred in-house.

Cell lines (HL60, ML1, U937) came originally ATCC (distributor LGC standards, UK) and were grown by our cell service at the Institute. Before use these lines, they were authenticated using the Short Tandem Repeat (SRF) profiling. 2x10^6 per mouse were injected IV in NSG mice of different ages. BM engraftment was assessed by FACS analysis of BM aspirate 2-4 weeks post-injection. For specific experiments, cell lines were transfected with GFP-Luciferase lentivirus vector, as previously described 4.

The collection and use of all human samples were approved by the East London Research Ethical Committee (REC:06/Q0604/110) and in accordance with the Declaration of Helsinki. Umbilical Cord Blood (CB) samples were obtained from normal full-term deliveries after signed informed consent.

Acute Myeloid Leukaemia (AML) samples were obtained after informed consent at St Bartholomew’s Hospital (London, UK). AML samples T cell depleted (2x10^6 cells per mouse) were injected intravenously into 8- to 12-weeks old unconditioned NSG mice.

Ten to fourteen weeks after injection, mice were treated with a daily subcutaneous injection of AraC (10mg/Kg) for 7 consecutive days. Assessment of treatment response was done 2 weeks after start of treatment, by flow cytometry analysis of BM aspirates.

At the end of each experiment, animals were euthanized and bone processed as already reported for FACS analysis 4. Antibodies used: anti-mouse CD45-PerCPCy5 (1:200, eBioscience, #45045182); anti-human CD33-PE (1:100, BD Pharmingen, #555450); anti-human CD45- APC-eFluor 780 (1:100, eBioscience, #47045942). Flow cytometry analysis was performed using a Fortessa flow cytometer (BD Biosciences, Oxford, UK).

For magnetic resonance imaging, mice injected with leukaemia cell lines, were scanned 2-4 weeks after post-injection. Mice injected with AML patient samples were scanned before and after AraC treatment. MRI was performed on a 9.4T horizontal bore system (Bruker
GMBH) equipped with a B-GA12SH gradient coil system. RF transmission and reception were performed with a 40mm ID quadrature birdcage coil (Bruker GMBH). A series of Fast Low Angle Shot (FLASH) scans were used for femur localization and for slice positioning.

DCE scans were performed using a FLASH with the following parameters: \( TR = 17.639 \text{ms}; \ TE = 1.859 \text{ ms}; \ FA = 10^\circ; \) Repetition = 1100; FOV 30x30x0.5mm\(^3\); matrix 128x128, and resolution of 234\( \mu \)m. Dotarem (0.4mL/Kg, Guerbet, France) was injected 4 mins after start of scan. Total scan duration was 41 mins. All mice were placed in a head-first prone position for imaging. Anaesthesia was induced and maintained using isoflurane (1–4\%) in room air supplemented with oxygen (80%/20%). Temperature and respiration rate were monitored using SA Instruments system (Bayshore, NY, USA).

To assess vascular perfusion in BM vessels by two photon microscopy, we used previously described protocol \(^4\). Images were obtained on a Zeiss 710 NLO laser scanning multiphoton microscope with a 20x 1.0 NA water immersion lens. Bone signal (Second Harmonic Generation, SHG) was collected at 380-485nm; GFP signals from AML cells was collected at 500-550nm; signal from Qtracker\(^\circledR\) 655 Vascular Label was collected at 640-690nm by not descanned detectors. Each z stack of images (100-150 \( \mu \)M) was rendered in 3D using Imaris software (Bitplane).

For DCE-MRI analysis we used Matlab 2019a for all image analysis. One region-of-interest (ROI) was drawn for the bone marrow and another for the muscle, per scan, per mouse. The signal in each ROI was then averaged and normalized to its baseline, so that it reflected percentage change from baseline. Signal from frames 3-90 were used as baseline. The muscle ROI was used as internal control to rule out problems with the injection and/or systemic issues with blood circulation. Mice that showed abnormal DCE muscle kinetics were excluded from the analysis.

Parameters quantified from DCE time intensity curves: contrast enhancement (CE); wash-in rate (WiR); wash-out rate (WoR); and initial wash-out rate (iWoR). The parameters were quantified as follows:
1. CE: percentage signal difference between baseline and maximum value from frames 95-300;

2. WiR: slope of the linear fit between frame at which CE was reached and frame at which signal starts to increase;

3. WoR: slope of the linear fit between frame at which CE was reached and the end of the scan;

4. iWoR: slope of the linear fit between frame at which CE was reached the frame 5 mins later;

Pixel by pixel analysis was done using the same scripts but applying them to every pixel inside of the bone marrow ROI.

Lastly, Statistical difference in parameters between mice groups was calculated using two-tailed unpaired t-tests. Correlation analysis was done using two-tailed Pearson correlation analysis. Receiver-operating characteristics (ROC) curves were calculated using the Wilson/Brown method.
References

Figure legends

**Figure 1. BM Vascular changes in aging and leukemia cell line models, measured by DCE-MRI.** (A) Schematic representation of a DCE-MRI time intensity curve and the parameters measured. Insert table explains what each parameter relates to. (B) Bone marrow DCE-MRI time intensity curves of non-injected mice (black), mice injected with HL60 (red), ML1 (blue) or U937 (olive green). (C) Quantification of BM DCE-MRI parameters CE, WiR, WoR, and iWoR in non-injected mice (NI) and mice injected with either HL60, ML1 and U937. White circles represent mice with leukemia burden less than 20%. Each dot represents one mouse. White columns represent average of group and error bars represent standard deviation of the mean. (D) Receiver operating characteristic analysis of the diagnostic capabilities of the BM DCE-MRI parameters to distinguish malignant from non-malignant BM in the cell line model. (E) Pixel by pixel analysis of BM DCE-MRI parameters showing leukemia induced changes to the BM tissue. For HL60 and ML1 group, white rectangles represent same area in the bone marrow. BM: Bone Marrow; DCE-MRI: Dynamic Contrast Enhanced Magnetic Resonance Imaging; CE: Contrast Enhancement; WiR: wash-in Rate; WoR: Wash-out Rate; iWoR: initial Wash-out Rate; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

**Figure 2. DCE-MRI can help distinguish normal from leukemic BM, in AML PDX models.** (A) Bone marrow DCE-MRI time intensity curves of non-injected young mice (NI, black), non-injected age-matched mice (NI, dash black) and mice injected with AML samples 1-5 (AML1-AML5). (B) Quantification of BM DCE-MRI parameters CE, WiR, WoR, and iWoR for the mice represented in A. White circles represent mice with leukemia burden less than 20%. Each dot represents one mouse. White columns represent average of group and error bars represent standard deviation of the mean. (C) Pixel by pixel analysis of BM DCE-MRI parameters from mice in Fig S1F, showing leukemia induced changes to the BM tissue. White rectangles represent same area in the bone marrow for that specific
mouse. BM: Bone Marrow; AML: Acute Myeloid Leukemia; DCE-MRI: Dynamic Contrast Enhanced Magnetic Resonance Imaging; CE: Contrast Enhancement; WiR: wash-in Rate; WoR: Wash-out Rate; iWoR: initial Wash-out Rate; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001
A

MR Signal (% baseline)

Parameters | Related to
--- | ---
CE: Contrast Enhancement | Functional vascular density
WiR: Wash-in Rate | Blood inflow/velocity; perfusion
WoR: Wash-out Rate | Tissue clearance capacity; permeability
iWoR: initial Wash-out Rate | Same as above, but shorter time frame

B

MR Signal (% baseline)

D

True Positive Rate

E

Vascular density (CE)

Perfusion (WiR)

Permeability (WoR)

1 pixel = 234 x 234 μm
Supplementary Figures and legends

Figure S1. BM Vascular changes in aging and leukemia cell line models, measured by DCE-MRI.

(A) Age, in weeks, of non-injected young mice (young NI), non-injected old mice (old NI), and mice injected with human CD34 positive cells derived from cord blood (CB).

(B) Bone marrow DCE-MRI time intensity curves of non-injected young mice (young NI, grey), non-injected old mice (old NI, black), and mice injected with human CD34 positive cells derived from cord blood (CB, red), showing absence of vascular changes between non-injected and CB injected mice.

(C) Disease burden quantified by percentage of human CD33 positive cells in the BM of mice injected with either HL60, ML1 or U937 cell lines. Each dot represents one mouse. White columns represent average of group and error bars represent standard deviation of the mean.

(D) Effect of aging on DCE-MRI bone marrow parameters CE and WoR in a population of control mice. Each dot represents one mouse.

(E) Quantification of DCE-MRI parameters CE, WIR, WoR, and iWoR for the BM of non-injected mice (NI) and HL60 mice, by age group with young being <16 weeks of age and old being >19 weeks of age.

(F) Representative z stacks of BM vasculature in the calvarium of mice engrafted with ML1, HL60 or U937 cells, as depicted. Scale bars represent 100 μm. Green dotted areas represent leukemia engrafted vasculature showing reduced perfusion and irregular vessels, white dotted areas represent non engrafted vasculature with normal vasculature.

BM: Bone Marrow; AML: Acute Myeloid Leukemia; DCE-MRI: Dynamic Contrast Enhanced Magnetic Resonance Imaging; CE: Contrast Enhancement; WIR: wash-in Rate; WoR: Wash-out Rate; iWoR: initial Wash-out Rate; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns: not significant.
Figure S2. BM Vascular changes in PDX, before and after Ara-C treatment measured by DCE-MRI. (A) Disease burden quantified by percentage of human CD33 positive cells in the bone marrow of mice injected with AML samples 1-5 (AML1-AML5). Each dot represents one mouse. White columns represent average of group and error bars represent standard deviation of the mean. (B) Receiver operating characteristic analysis of the diagnostic capabilities of the BM DCE-MRI parameters to distinguish malignant from non-malignant BM using the data from the AML patient samples. (C) Disease burden quantified by percentage of human CD33 positive cells in the bone marrow of mice injected with AML samples 1-5 (AML1-AML5), before (BT) and post (PT) AraC therapy. (D) Quantification of BM DCE-MRI parameters CE, WiR, WoR, and iWoR for mice from C. DCE-MRI: Dynamic Contrast Enhanced Magnetic Resonance Imaging; CE: Contrast Enhancement; WiR: wash-in Rate; WoR: Wash-out Rate; iWoR: initial Wash-out Rate; *p<0.05; ***p<0.001; ****p<0.0001; ns: not significant.
A

% hCD33+ cells (in the BM)

AML1 AML2 AML3 AML4 AML5

B

True Positive Rate

False Positive Rate

AUC\textsubscript{CE}: 0.7276

AUC\textsubscript{WiR}: 0.9441

C

% hCD33+ cells (in the BM)

Before therapy Post\textsubscript{AraC}

D

CE (% baseline/\text{sec})

WIR (% baseline/sec)

WoR (% baseline/sec)

iWoR (% baseline/sec)

NI AML1 AML2 AML3 AML4 AML5