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On the Relationship between Dynamic Contrast-Enhanced Ultrasound Parameters and the Underlying Vascular Architecture Extracted from Acoustic Angiography / Panfilova, A., Shelton, S.E., Caresio, C., van Sloun, R.J.G., Molinari, F., Wijkstra, H., Dayton, P.A., Misch, M.. - In: ULTRASOUND IN MEDICINE AND BIOLOGY. - ISSN 0301-5629. - ELETTRONICO. - 45:2(2019), pp. 539-548. [10.1016/j.ultrasmedbio.2018.08.018]

Availability:

This version is available at: 11583/2954677 since: 2022-02-04T11:36:57Z

Publisher:

Elsevier USA

Published

DOI:10.1016/j.ultrasmedbio.2018.08.018

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<http://dx.doi.org/10.1016/j.ultrasmedbio.2018.08.018>

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On the relationship between dynamic contrast - enhanced ultrasound parameters and the underlying vascular architecture extracted from acoustic angiography

Anastasiia Panfilova^{a,*}, Sarah E. Shelton^b, Cristina Caresio^c, Ruud JG van Sloun^a, Filippo Molinari^c, Hessel Wijkstra^{a,d}, Paul A. Dayton^b, Massimo Mischi^a

^aDepartment of Electrical Engineering, Technical University of Eindhoven, Eindhoven, P.O.Box 513, 5600 MB Eindhoven, SPS Group, The Netherlands

^bJoint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, 152 MacNider Hall, Campus Box 7575, Chapel Hill, NC 27599, United States of America

^cPolitecnico di Torino, Torino Italy, Corso Duca degli Abruzzi 24, 10129 Torino, Italy

^dAMC University Hospital, Urology Dept, Amsterdam, Postbus 22660, 1100 DD Amsterdam, the Netherlands

Abstract

Dynamic contrast-enhanced ultrasound (DCE-US) has been proposed as a powerful tool for cancer diagnosis by estimation of perfusion and dispersion parameters reflecting angiogenic vascular changes. This work aims at identifying which vascular features are mainly reflected by the estimated perfusion and dispersion parameters through comparison with Acoustic Angiography (AA). AA is a high resolution technique that allows quantification of vascular morphology. 3D AA and 2D DCE-US bolus acquisitions monitored growth of fibrosarcoma tumors in 9 rats. AA-derived vascular properties were analyzed

*Corresponding Author: Anastasiia Panfilova, Department of Electrical Engineering, Technical University of Eindhoven, De Groene Loper 19, Eindhoven, The Netherlands; Email, A.P.Panfilova@tue.nl; Phone, +31616070943

along with DCE-US perfusion and dispersion in order to investigate the differences between tumor and control, and their evolution in time. AA-derived microvascular density and DCE-US perfusion showed good agreement, confirmed by their spatial distributions. No vascular feature was correlated with dispersion. Yet, dispersion provided better cancer classification than perfusion. We therefore hypothesize that dispersion characterizes vessels that are smaller than those visible with AA.

Keywords: Acoustic angiography, Dynamic contrast-enhanced ultrasound, Cancer, Dispersion, Perfusion, Ultrasound contrast agents

1 **Introduction**

2 Malignant tissue shows a set of alterations from benign tissue that can be
3 used as markers to detect it (Koumoutsakos et al., 2013). Of particular in-
4 terest for cancer imaging are the altered vascular architecture and the conse-
5 quent changes in blood supply. Angiogenic vessels grow to nourish the tumor
6 and support its proliferation. These vessels have been found to be tortuous,
7 to grow chaotically, without the typical vessel hierarchy, and with a high oc-
8 currence of arteriovenous shunts. Many of these properties can be recognized
9 with contrast-enhanced ultrasound techniques, which have shown promising
10 results for distinguishing malignant tissue from benign (Brock et al., 2013;
11 Gessner et al., 2013; Kuenen et al., 2013b, 2011; Mischi et al., 2012; Quaia,
12 2011; Shelton et al., 2015).

13 Dynamic contrast-enhanced ultrasound (DCE-US) captures the contrast-
14 agent passage through the vascular bed after its injection in the patient’s
15 bloodstream. Specifically, it registers the local evolution of gray-level inten-
16 sity at each pixel, referred to as the time intensity curve (TIC), which reflects
17 the varying ultrasound contrast agent (UCA) concentration. The recorded
18 intensities are then converted into UCA concentration with a linearization
19 function specific to the employed ultrasound scanner (Rognin et al., 2008),
20 yielding an indicator dilution curve (IDC) for every pixel in the video. Vari-
21 ous characteristics of IDCs have been proven to be useful for distinguishing
22 malignant from benign tissue (Mischi et al., 2012).

23 Several approaches have been adopted to extract information from IDCs
24 derived from DCE-US bolus acquisitions. Some heuristic features of the
25 IDCs, such as the wash-in time and the peak intensity, are related to cancer

26 (Mischi et al., 2012; Zhao et al., 2010). Multiple other techniques employ
27 IDC fitting by analytical models, such as the lognormal, gamma, and local
28 density random walk (LDRW) model (Strouthos et al., 2010). Functional
29 parameters of the curves (e.g. area-under-the-curve) are extracted and dis-
30 played in colormaps, aiming to obtain a clearly distinguishable malignant
31 region. All these approaches mainly attempt to quantify perfusion, which
32 is motivated by the presence of ample arteries feeding the tumor, increased
33 microvascular density (MVD), and presence of arteriovenous shunts. Despite
34 this, clinical evidence has shown that cancerous lesions in the prostate can
35 also be iso- or hypo-perfused (Brock et al., 2013). Indeed, it is known that
36 tumor tissue has higher resistance to blood flow (Narang and Varia, 2011).
37 This induces a counterbalancing factor that complicates predictions about
38 the level of blood supply within the tumor, as compared to surrounding tis-
39 sue (Cosgrove and Lassau, 2010). Furthermore, the MVD inside the tumor
40 can be strongly heterogeneous, creating highly perfused regions as well as
41 hypoxic, avascular regions. Therefore, assessment of perfusion alone is in-
42 sufficient for reliable cancer diagnostics. These findings have motivated the
43 development of contrast ultrasound dispersion imaging (CUDI), a method
44 which enables assessment of UCA dispersion, in addition to quantification of
45 perfusion (Kuenen et al., 2011; Mischi et al., 2012).

46 CUDI aims at quantifying the UCA dispersion due to the architecture
47 of the vascular tree and complex multipath trajectories available for UCA
48 transport. The main hypothesis that lies in the foundation of the method
49 states that dispersion reflects structural vascular changes induced by angio-
50 genesis. The first CUDI approach involved modelling of the IDCs in time

51 domain with a LDRW model and extraction of a dispersion-related parame-
52 ter from the fitted model (Kuenen et al., 2011). An important complication
53 associated with this approach was poor signal to noise ratio, hindering the
54 fitting procedure and decreasing its reliability. This problem has been mit-
55 igated by spatiotemporal similarity analysis (Kuenen et al., 2013a; Mischi
56 et al., 2012). In a promising implementation, this approach involves calcu-
57 lation of an average correlation coefficient measuring the similarity of a TIC
58 at a pixel and its surrounding pixels (Kuenen et al., 2013b). A theoretical
59 description of the problem within the framework of the LDRW model has
60 shown that the correlation coefficient between IDCs is monotonically related
61 to the dispersion coefficient (Kuenen et al., 2013a). Moreover, this approach
62 has demonstrated its superior performance compared to perfusion-related
63 parameters at localizing prostate cancer in a clinical setting (Kuenen et al.,
64 2013b). This method has been validated against cell differentiation reflected
65 with the Gleason score for prostate cancer (Schalk, 2017). Another study
66 identified that regions of low dispersion correlated with those of high MVD,
67 quantified by immunohistology (Saidov et al., 2016). However, in this study
68 detailed characterization of the vascular architecture (e.g. tortuosity and
69 vessel size) was not available.

70 Acoustic angiography (AA) can provide accurate characterization of the
71 vascular architecture: it is a high-resolution technique, capable of imaging
72 individual microvessels (Gessner et al., 2013; Shelton et al., 2015). AA per-
73 mits imaging vessels at a high resolution of 100-200 μm at 2 cm depth with
74 minimal signal from tissue. While transmitting ultrasonic waves at frequen-
75 cies in the order of a few MHz, close to the UCA bubble resonance frequency,

76 it records the nonlinear response of the contrast agents in a high frequency
77 range centered at 30 MHz. This technique grants the possibility to quantify
78 vessel density and morphology measures such as the sum of angles metric
79 (SOAM) and distance metric (DM) (Rao et al., 2016; Shelton et al., 2015).
80 These parameters have been reported to be significantly different for malig-
81 nant and benign tissue (Gessner et al., 2013; Shelton et al., 2015). Thereby,
82 AA gives the opportunity to validate whether these features are reflected in
83 DCE-US due to the different character of UCA perfusion and dispersion in
84 these vessels.

85 The aim of this work is to determine whether DCE-US is able to char-
86 acterize the underlying vascular architecture. It involves DCE-US and AA
87 imaging of fibrosarcoma tumors and control regions in a longitudinal study of
88 9 rats. AA and DCE-US acquisitions were performed every 3 days, at 4 time
89 points, starting with the day when the tumors could be palpated. An overall
90 comparison of the tumor's and control's vascular properties was performed.
91 Additionally, a longitudinal study of these properties was conducted, aiming
92 to find similar trends in features extracted from the two different techniques
93 of DCE-US and AA.

94 **Materials and Methods**

95 *Rat Models*

96 Fibrosarcoma tumor implantation was performed in rats according to a
97 previously applied protocol (Streeter et al., 2011). The tumor models were
98 established from propagated tumor tissue provided by the Dewhirst Lab at
99 Duke University. Before surgery the (Fischer 344) rats were anesthetized

100 with isoflurane; their left flank was then shaved and disinfected. An incision
101 (~ 2 mm) was made above the quadriceps muscle, and a sample of tumor
102 tissue (~ 1 mm^3) was positioned under the skin. The incision was closed
103 with 1-2 staples. This procedure was performed at 3 different time points
104 with 9 rats in total. Rats belonging to the same series were operated on the
105 same day.

106 On day 8 after implantation, the first ultrasound acquisition was per-
107 formed if the tumors were palpable. Otherwise, we waited for 2-3 days for
108 subsequent assessment. When the tumors were palpable, UCA was injected
109 in the rats' tail vein through a 24 gauge catheter while the animals were anes-
110 thetized with vaporized isoflurane in oxygen. DCE-US was performed on the
111 tumor-bearing flank for assessment of perfusion and dispersion. The AA ac-
112 quisition protocol immediately followed the DCE-US acquisition to minimize
113 the amount of time each animal spent under anesthesia. The beginning of
114 the DCE-US and AA acquisitions were different between the series, start-
115 ing with day 8, day 11, and day 13, respectively. For all but one animal,
116 subsequent imaging acquisitions were performed with an interval of 3 days,
117 amounting to 4 time points in total. One rat was an exception since we
118 were not able to inject the contrast (for both modalities) in its tail vein, and
119 managed to image only at the first and third time points. All experiments
120 were performed at the University of North Carolina at Chapel Hill, approved
121 by the Institutional Animal Care and Use Committee at the University of
122 North Carolina at Chapel Hill.

123 *Image acquisition*

124 *DCE-US bolus injection protocol*

125 A UCA bolus of 2×10^8 microbubbles was injected in the rats' tail vein.
126 The contrast agent used in this study was made in-house; it has a lipid
127 shell and perfluorocarbon core, similar to Definity[®] (Latheus Medical Imag-
128 ing/U.S.A, N. Billerica). A 15L8-S probe was utilized with a Siemens Se-
129 quoia scanner in Cadence Pulse Sequencing mode at an insonifying central
130 frequency of 7 MHz. The acquired DCE-US recordings were stored in DI-
131 COM format.

132 *AA continuous infusion protocol*

133 A continuous infusion of microbubbles was administered using a syringe
134 pump (PHD 2000, Harvard Apparatus) at a rate of 1.5×10^8 microbubbles
135 per minute. AA imaging was performed with a dual-frequency single-element
136 transducer transmitting at 4 MHz, and receiving around 30 MHz. The 3D
137 AA images were acquired plane by plane, with a step size of 100 μm .

138 *DCE-US bolus data processing*

139 *Preprocessing*

140 All the bolus recordings were filtered with a Gaussian filter, as previously
141 performed in (Mischi et al., 2012), using a kernel of 0.13 mm equal to 1.6
142 pixels. This value improved the signal-to-noise-ratio at the cost of additional
143 spatial correlation between TICs at neighbouring pixels. The TIC power
144 of every pixel was evaluated as the root mean square of the TIC after the
145 baseline was removed. Regions with a level of TIC power below -22 dBs of
146 the maximum TIC power over all images were excluded from further analysis

147 (shown in black in Fig. 1 a.). This limited the effect of random noise on the
148 parameters of interest (Kuenen et al., 2014). Characteristic of DCE-US is
149 multiplicative noise: noise proportional in its power to the signal amplitude.
150 By eliminating regions with low TIC power, we avoided erroneous parameter
151 estimation from regions with low signal power where random noise dominates.
152 After this, the intensity values of the remaining regions were linearized by
153 inverting the logarithmic compression function implemented in the adopted
154 scanner, yielding the IDCs.

155 *Assessment of dispersion*

156 An average correlation coefficient was calculated for every pixel between
157 its own IDC and those at its surrounding pixels within a ring-shaped kernel
158 (Mischi et al., 2012) with an inner radius of 0.6 mm and an outer radius
159 of 2 mm. The inner radius was chosen equal to the lateral resolution of
160 the preprocessed bolus recordings at ~ 2 cm depth as identified with local
161 autocorrelation analysis. Details about the latter procedure can be found in
162 (Mischi et al., 2012). The lateral resolution was taken as a reference since
163 it was worse than the axial resolution. The outer radius of the kernel was
164 set equal to the size of 2 mm, which a tumor can usually reach without
165 neovascularization (Folkman, 1971). The time window over which the IDCs
166 were correlated to each other was selected to maximize the area under the
167 receiver operating characteristic curve for tumor classification, resulting in a
168 value of 17 seconds as proposed in previous work (Panfilova et al., 2016). This
169 is the only informative segment of the IDC (Fig. 2) due to early recirculation,
170 as often observed in small animals (Stapleton et al., 2009). In this work, the
171 beginning of the analyzed time window was set with 3 seconds before the

172 appearance time, ensuring the wash-in phase to be entirely captured.

173 *Assessment of perfusion*

174 Wash-in-rate was adopted to assess perfusion and computed as the slope
175 of a line fitted to the IDC in the 2-second interval after appearance time, as
176 illustrated in Fig. 2. The value of 2 seconds was chosen to reflect the rise of
177 UCA concentration in the initial part of the IDCs in all acquired clips.

178 *AA data processing*

179 The AA volumes were interpolated to reduce the inter-plane distance to
180 50 microns and make the pixels isotropic. Visible vessels were manually seg-
181 mented and characterized in terms of vessel dimensions: vessel length (VL)
182 and mean radius (MR). VL was computed as the length of the vessel segment
183 identified between successive branching points, and MR was computed as the
184 mean radius of this vessel segment along its length. Vessel tortuosity was as-
185 sessed with the distance metric and the sum of angles metric (Bullitt et al.,
186 2003). The DM was computed as the ratio of vessel length to the Euclidian
187 distance between its beginning and end. The SOAM was calculated as the
188 sum of angles between successive points on the vessel centerline divided by
189 VL, using the same formula as in (Bullitt et al., 2003), but excluding the
190 torsional angle. Besides these individual vessel properties, MVD was calcu-
191 lated as a global characteristic of the tumor at a given timepoint, defined as
192 the number of visible vessel segments divided by tumor volume. The volume
193 vascular density (VVD) was computed with a moving 3D isotropic kernel in
194 the central slice of the tumor (~ 1 mm in thickness). Otsu’s method (Vala
195 and Baxi, 2013) was used to select a threshold to separate noise from vessel

196 signal within the central slices; the percentage of pixels with vessel signal
197 from the overall number of pixels in the 3D kernel was calculated.

198 *Statistical analysis*

199 The DCE-US parameters were spatially downsampled by a factor 7 in
200 both directions, equal to the resolution of the preprocessed images. This
201 was performed to exclude spatial correlation and prepare the data for the
202 statistical tests that require sample independence.

203 *Comparison between tumor and control*

204 Dispersion and perfusion values were divided into two groups. The tumor
205 group was composed of the manually selected tumor regions (inside the red
206 contour, Fig. 1 a.) from all rats at all time points binned together. The
207 control group was taken from pixels outside the tumor contour, dilated by
208 ~ 1 mm (in blue, Fig. 1 a.). The region between the red and blue contour
209 was excluded from analysis to avoid erroneous pixel assignment to tumor
210 or control, since DCE-US information was not considered sufficiently com-
211 prehensive for such accurate tumor delineation, as required by e.g. ablation
212 therapy and surgery. The AA parameters were extracted in a similar fashion:
213 vessels were taken from within the tumor region and outside it in the same
214 flank (Fig. 3). Vessel segments on the border of the selected contour, whose
215 belonging to a tumor or control group was debatable were disregarded from
216 analysis.

217 An Anderson-Darling goodness of fit hypothesis test was performed on
218 all the parameter distributions to check for data normality. Since all the dis-
219 tributions were identified as non-Gaussian, a Mann Whitney non-parametric

220 test was performed to establish the significance (p-value) of the difference
221 between tumor and control. No additional subsampling or upsampling was
222 performed to make the control and tumor data sets balanced, since the Mann
223 Whitney test can be applied to data sets with distributions of different size
224 (Mann and Whitney, 1947).

225 The Cohen’s d was used as a measure of the ‘effect size’ (Sullivan and
226 Feinn, 2012) that the tumor has on the underlying vasculature, calculated as
227 the difference between the means of two distributions divided by the standard
228 deviation of the control. The values of the Cohen’s d term allow to classify
229 the difference between two distributions according to 4 categories: small,
230 medium, large, and very large for values of 0.2, 0.5, 0.8, 1.3, respectively.

231 *Longitudinal study of tumor and control*

232 A longitudinal study of the tumor evolution was performed with the
233 Kruskal-Wallis post hoc test, evaluating the differences among the distribu-
234 tions of dispersion and perfusion, and vascular features of tumor and control
235 at 4 time points. The Kruskal-Wallis test (Kruskall and Wallis, 1952) does
236 not require equal sample sizes, which is an advantage considering that our
237 data set is unbalanced and incomplete: data is missing for one tumor at two
238 time points as well as control at several time points for the large tumors.
239 Moreover, the number of visible vessels is different for every image acqui-
240 sition. For all rats, all parameter values were binned together according to the
241 time point of the acquisition.

242 The statistic test calculation is influenced by the number of observations
243 and can result in different outcomes for different sample sizes (Kruskall and
244 Wallis, 1952). Since the number of pixels provided more samples for disper-

245 sion and perfusion compared to the number of vessels extracted with AA,
246 these pixels were randomly subsampled to yield the same number of samples
247 as vessels per each representative dataset of tumor and control at each time
248 point. The only parameter that remained different in terms of group size is
249 the MVD; being a global parameter that characterizes the entire tumor and
250 control at a specific time point.

251 After the post-hoc tests were performed, the Pearson correlation coeffi-
252 cient was computed between the medians of the parameters showing similar
253 longitudinal trends.

254 *Mapping of vascular properties on the bolus acquisition plane*

255 During the DCE-US bolus acquisitions the operator always tried to im-
256 age the largest cross-section of the tumor, and keep the same orientation of
257 the probe as used for AA. However, it was noticed that these precautions
258 were not sufficient to reliably identify the DCE-US plane within AA: even a
259 movement of the order of ~ 1 mm alters the imaged vascular pattern of a tu-
260 mor. It was noticed that the perfusion maps highlight larger vessels, clearly
261 visible in the AA (Fig. 2 b. and d.). These vessels were used as markers
262 to locate the bolus recording plane in the AA volume. For this, a dedicated
263 tool was developed, allowing to freely scroll through the AA volume planes
264 and change their orientation.

265 The selection of the plane was performed by visual inspection, choosing an
266 image containing as many as possible vessel markers present in the perfusion
267 maps. A slice in the AA volume of ~ 1 mm thickness was selected and
268 an extension of the skeletonization algorithm described in (Meiburger et al.,
269 2016) was applied to extract MVD (Fig. 2 e.), MR (Fig. 2 f.), VL, and

270 SOAM. This slice thickness was chosen to be of the order of the elevational
271 resolution in the bolus recordings and sufficiently large to register vessel
272 segments. This allowed a qualitative comparison of the spatial distribution
273 of the vascular features with those of dispersion and perfusion in the same
274 plane.

275 All the image processing and statistical analysis was performed with Mat-
276 lab software (the MathWorks, Natick, MA).

277 **Results**

278 *Statistical analysis*

279 *Comparison between tumor and control*

280 For all the extracted parameters, tumor and control have significantly dif-
281 ferent distributions ($p < 0.001$). However, the magnitude of the differences,
282 expressed in Cohen's d , spans a wide range (Fig. 4), showing a marginal
283 effect size for the DM (Fig. 4 c.), and small to very large differences for the
284 rest of the parameters.

285 *Longitudinal study of tumor and control*

286 Since the DM showed almost no difference between tumor and control,
287 it was excluded from the longitudinal analysis. Boxplots with all parame-
288 ter values binned according to the time points are shown in Fig. 5, while
289 Fig. 6 illustrates the results of the post hoc Kruskal-Wallis test, color-coded
290 according to the significance level of the intra-distribution differences.

291 The dispersion median is relatively constant in time for both tumor and
292 control, showing a significant difference for control and tumor distributions

293 (Fig. 5 a., Fig. 6 a.). Tumor perfusion is significantly different from the
294 control at all time points (Fig. 5 b., Fig. 6 b.), peaking for the tumors at
295 the second time point. Interestingly, the longitudinal trend of the control's
296 perfusion seems to mimic the tumor's trend in time, however, at a smaller
297 magnitude, not identified as significant with the post hoc test.

298 The VVD is stably higher for tumor, while the MVD seems to follow a
299 similar trend to that of perfusion, peaking for tumors at the second time
300 point. However, the result of the MVD post hoc test is difficult to compare
301 to others since the number of samples is different: only one value of MVD
302 per time point is available, while the other parameters were subsampled
303 according to the number of segmented vessels in the AA volume at a given
304 time point.

305 The post hoc results, illustrated by colormaps in Fig. 6, are comparable
306 for dispersion, the VVD, the VL, and the SOAM. However, no significant
307 correlation between the medians of the dispersion levels and the mentioned
308 AA parameters has been identified. As for perfusion, the mean perfusion in
309 tumors and their MVD showed a significant correlation coefficient of 0.572
310 ($p < 0.001$) and inclusion of both control and tumor values resulted in a
311 Pearson correlation coefficient of 0.67 ($p < 0.001$).

312 *Mapping of vascular properties on the bolus acquisition plane*

313 The spatial parametric maps of the AA skeleton confirmed our observa-
314 tion that there is a correlation between regions of high perfusion and elevated
315 MVD (Fig. 1 b. and f.). No spatial correspondance was found between dis-
316 persion and the other AA - derived parameters.

317 **Discussion**

318 Dispersion shows a large difference (Cohen’s $d = 1.68$) between tumor
319 and control, exhibiting stable performance at tumor detection as it develops.
320 Perfusion shows a lower discrimination power than dispersion, that is high for
321 younger tumors, peaking at time point 2, and decreases with tumor growth.
322 Interestingly, the perfusion level in the control around the tumor is also
323 elevated (Fig. 5 b.), showing a similar trend as in the tumor itself. This
324 may reflect that the overall perfusion of tissue around the tumor is increased
325 and influenced by the tumor. This effect has been shown for the SOAM,
326 which exhibits intermediate values between that of tumor and control in
327 tissue adjacent to the tumor (Rao et al., 2016). Moreover, it has been shown
328 for the fibrosarcoma model that the vascular source is often located in the
329 periphery of the tumor (Ponce et al., 2007; Tozer et al., 1990; Viglianti et al.,
330 2004).

331 Dispersion of the control stays stable over time, indicating that dispersion-
332 related changes mainly occur within the tumor itself, and not in the sur-
333 roundings. The spatial perfusion and dispersion maps are complementary,
334 showing different patterns of highlighted regions (Fig. 1 b. and c.). Perfusion
335 highlights large vessels, as well as regions with high MVD.

336 The SOAM indicates that the tumor has more tortuous vessels, exhibiting
337 a similar trend to that of dispersion (Fig. 5 a., g.) and comparable results
338 for the post-hoc test (Fig. 6 a., g.). Nevertheless, the effect size difference, as
339 indicated by Cohen’s d , is much lower for the SOAM than for dispersion. In
340 general, the control regions in this experiment show a higher tortuosity than
341 we previously observed for these rats, expressed by the DM in (Shelton et al.,

2015). Direct comparison of the SOAM in this work and in (Shelton et al., 2015) is not available since the calculation of the SOAM has been adjusted since then. The unusually high tortuosity for control may be caused by the presence of the bowel region in some of the AA images, which was excluded from analysis in earlier studies, and may have elevated tortuosity. Previous data also shows that the SOAM exhibited an intermediate level of tortuosity in tissue up to 1 cm away from a tumor, with a mean tortuosity between that of tumors and non tumor-bearing animals (Rao et al., 2016). The discrimination power of the SOAM in our data set increases for smaller vessels (Cohen’s $d=0.14$ for vessels with a radius >0.11 mm, 0.28 with an intermediate radius, and 0.43 with a radius <0.09 mm). Therefore, its relation with the extracted DCE-US features can not be fully appreciated due to the finite resolution of AA. Similarly, a previous study has shown that the difference in MVD between tumor and control increases for smaller vessels (Sedelaar et al., 2001). Therefore, it may be that the SOAM, MVD, and other metrics extracted in this study are related to dispersion; however, mainly smaller vessels’ properties have a significant influence on it. Supporting this hypothesis is the former observation that regions with increased MVD correspond to those with low dispersion (Saidov et al., 2016), as derived from immunohistology. The immunohistology derived MVD was based on evaluation of tomato lectin binding to the endothelial cells and therefore characterized the presence of vessels of all sizes.

Spots of increased vascular density or large vessels were detected with perfusion colormaps. The correlation between median perfusion level and MVD is the only significant inter-parameter agreement found in this work.

367 The Kruskal-Wallis test is ideally constructed for a study design when
368 subjects are randomly assigned to different groups, so that each subject ap-
369 pears in one group only (Kruskall and Wallis, 1952). Moreover, the subjects
370 within the group must be independent. We realize that these assumptions
371 are not strictly valid in this study, since we observe the tumor evolution in
372 the same rats over time and since the vessels selected from the same rat are,
373 strictly speaking, not independent. However, we do not expect these limita-
374 tions to be crucial for deriving a meaningful conclusion about the significant
375 trends in time.

376 Imaging initialization was different among 3 series of experiments, start-
377 ing with day 8, 11, and 13 after tumor implantation, as explained before. We
378 consider that combining all the rats together according to the number of the
379 acquisition is justified as the imaging was initialized according to the same
380 strategy: when the tumors became palpable. However, since we waited for
381 2-3 days for subsequent assessment if tumors were not palpable on day 8, in
382 future work it may be beneficial to assess the tumors every day or evaluate
383 all tumors in a single cohort. This would ensure that the development of the
384 imaged tumors is more consistent.

385 It is often observed that the wash-out phase is masked by recirculation in
386 small animals. (Stapleton et al., 2009) shows that for a range of administered
387 UCA doses the wash-out phase is more prominent in mice. Different UCA
388 doses should therefore be investigated in our future work, since a prominent
389 wash-out phase, in our experience, enhances the performance of CUDI (Kue-
390 nen et al., 2013b). A clear wash-out would also allow evaluating the wash-out
391 as a complementary perfusion parameter.

392 An important limitation of this study is the 2D character of the extracted
393 parameters of dispersion and perfusion. The results of the post hoc tests,
394 therefore, must still be taken with caution since it was performed for 3D
395 vascular features evaluated in the whole tumor volume and 2D dispersion
396 and perfusion that leave us blind to out of plane information and restrict us
397 to the central tumor slice, which is not always representative of the whole
398 tumor (Streeter et al., 2011). We mitigated this limitation by performing
399 an additional spatial comparison of the parameter maps in the same plane,
400 matched with the help of large vessels identified in the perfusion maps. The
401 agreement between perfusion and MVD, noticed in the longitudinal trends,
402 was also identified in the spatial distribution of these parameters in the same
403 plane, raising more confidence to the finding that perfusion and MVD are
404 correlated.

405 An improved study design should either include 3D DCE-US (Schalk
406 et al., 2015), giving more accurate overall tumor characteristics, or a regis-
407 tration procedure, allowing to fix the orientation of the probes and identify
408 the location of the DCE-US plane within the AA volume. The finding that
409 perfusion highlights large vessels can be used to further improve registration.

410 The absence of any parameters correlated with dispersion may pinpoint
411 to the limitation of AA as a validation method for CUDI: while enabling very
412 high resolution ultrasound imaging, it may not be sufficient to find out which
413 vascular properties substantially influence dispersion, since dispersion may
414 be mainly defined by properties of subresolution vessels. In this respect, it is
415 possible to direct our attention to superlocalization methods that overcome
416 the limit of diffraction: they are able to track single bubbles and determine

417 their exact positions by finding the centers of their point spread functions
418 (Cox and Beard, 2015; Errico et al., 2015). Another possible reason for the
419 absence of vascular parameters that correlate with dispersion is that the
420 adopted dispersion parameter, is in fact related to both dispersion and flow
421 velocity (Kuenen et al., 2013a). Different vascular parameters may contribute
422 to the separate terms of dispersion and flow velocity, while we assessed their
423 combination. In this regard, it would also be of interest to apply another
424 analysis to the DCE-US bolus recordings that allows to separate dispersion
425 and velocity contributions (van Sloun et al., 2017).

426 **Conclusions**

427 In this work, dispersion demonstrated its superior performance at tumor
428 classification compared to perfusion, as previously found for prostate cancer
429 (Kuenen et al., 2013a,b; Mischi et al., 2012). Perfusion colormaps highlight
430 large vessels and regions of elevated MVD. The vascular factors that deter-
431 mine the dispersion level remain yet to be found, as well as the role of vessels
432 with a diameter below 100-200 μ in defining perfusion levels.

433 **Acknowledgements**

434 This work was supported by the European Research Council Starting
435 Grant (#280209) and the Impulse2 programme within TU/e and Philips.
436 This work was also supported by R01CA170665, R43CA165621, and R01CA189479
437 from the National Institutes of Health. Disclosure: P.A. Dayton declares that
438 he is an inventor on a patent enabling the acoustic angiography technology

439 and a co-founder of SonoVol, Inc, a company which has licensed this tech-
440 nology.

441 **Abbreviations**

442 **AA** Acoustic angiography

443 **CUDI** Contrast ultrasound dispersion imaging

444 **DCE-US** Dynamic contrast-enhanced ultrasound

445 **DM** Distance metric

446 **IDC** Indicator dilution curve

447 **LDRW** Local density random walk

448 **MR** Mean radius

449 **MVD** Microvascular density

450 **SOAM** Sum of angles metric

451 **TIC** Time intensity curve

452 **UCA** Ultrasound contrast agent

453 **VL** Vessel length

454 **VVD** Volume vascular density

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559 **Figure Captions**

560 **Figure 1:** DCE-US and AA images of the same plane, and maps of the
561 extracted features. a: maximum intensity projection of the DCE-US
562 video. The tumor is encircled by a red contour, while the region out-
563 side the blue contour belongs to the control, separated by a margin
564 which was not included in the analysis. Regions with power below the
565 threshold of -22 dBs of the maximum intensity are displayed in black.
566 b-c: perfusion and dispersion colormaps, respectively. Regions with
567 power below -22 dBs of the maximum intensity are displayed in white.
568 d: Selected AA slice. e-f: vascular skeleton, colorcoded according to
569 the values of the microvascular desity, and mean radius, respectively
570 (yellow indicates low values, while red inicates high values). The num-
571 bers in b and d illustate the vessels identified in the perfusion maps,
572 used as markers to locate the right plane in AA volumes.

573 **Figure 2:** A typical preprocessed indicator dilution curve. T1 shows the
574 appearance time, T0 is taken 3 seconds before appearance time. The
575 interval from T0 to T2 shows the interval of the IDC used for disper-
576 sion analysis. The tangens of the angle alpha of the line fitted to the
577 indicator dilution curve in the 2 seconds after appearance time is the
578 wash-in-rate.

579 **Figure 3:** AA maximum intensity projection. The tumor region is indicated
580 by the red contour, surrounded by the control region.

581 **Figure 4:** Boxplots of tumor and control parameters, binned together from
582 all time points. a: dispersion, b: perfusion, c: distance metric, d: sum

583 of angles metric, e: vessel length, f: vessel radius, g: microvascular
584 density, h: volume vascular density. Cohen's d measure is indicated
585 above the plots.

586 **Figure 5:** Boxplots of tumor (T1, T2, T3, T4) and control (C1, C2, C3, C4)
587 parameters, binned together at different time points. a: dispersion,
588 b: perfusion, c: volume vascular density, d: microvascular density, e:
589 vessel radius, f: vessel length, g: sum of angles metric.

590 **Figure 6:** Results of the post hoc Kruskal-Wallis test performed on tumor
591 and control parameters at four time points (indicated by T1, T2, T3,
592 T4 and by C1, C2, C3, C4, respectively). The colors of the rows indi-
593 cate whether the distribution is significantly different from the others,
594 green and yellow representing different significance levels. a: disper-
595 sion, b: perfusion, c: volume vascular density, d: microvascular density,
596 e: vessel radius, f: vessel length, g: sum of angles metric.