On the assessment of angiogenesis: its time to change (go further) from an estimate to a measurement

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It has been reported that hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver [1], representing the second leading cause of cancer-related deaths worldwide [2], its hypervascular nature underlines the importance of angiogenesis in the pathophysiology of this tumour [3]. Amongst its clinical management, the main non-surgical method is image-guided percutaneous radiofrequency ablation (RFA), which causes tumour necrosis through inducing high intra-tumoral temperatures [1]. We read the manuscript entitled "Angiogenesis in residual cancer and roles of HIF-1α, VEGF, and MMP-9 in the development of residual cancer after radiofrequency ablation and surgical resection in rabbits with liver cancer" by Li *et al* with great interest. [4]. The authors investigated the blood flow signal changes in residual cancer after ultrasound-guided RFA of rabbit liver cancer, and analysed the correlation between changes in blood flow signal and changes in Hypoxia-inducible factor 1-alpha (HIF-1α), vascular endothelial growth factor (VEGF), and matrix metallopeptidase 9 (MMP-9). Additionally, the potential link between blood flow signals and angiogenesis in residual cancer, after RFA and surgical resections in rabbits with liver cancer was investigated [4]. Tissue fragments were collected from each rabbit and included in the control, RFA, and surgical resection groups, which were subsequently stained and used to determine the microvascular density (MVD). They found that the blood flow signal was positively correlated with the VEGF expression, MMP-9 expression, and the MVD in both the RFA and surgical resection groups. In particular, the higher the blood flow signal grade, the higher the VEGF and MMP-9 expression and MVD. At later time points (days 7 and 14),

the VEGF expression, MMP-9 expression and the MVD, were found to be higher in RFA samples than in surgical resection samples. These findings have led the authors to conclude that in the control, RFA, and surgical resection groups, the ultrasound blood flow signal is associated with the expression of the two angiogenesis-related factors, VEGF and MMP-9, and the MVD [4].

As reported by the authors, micro-angiogenesis was assessed according to the MVD staining method proposed by Wendy. In particular, *a)* brownish yellow staining of the interstitial substance indicated positive cells; *b)* cell masses near the positive staining were attributed as vessels, and *c)* vessels were counted under low magnification in three selected fields with a relatively dense distribution. The expression of VEGF and MMP-9 in liver cancer was, instead, measured using the S-P method. In the present manuscript however, the authors do not mention important methodological details or useful references (for example explaining the meaning of "MVD staining method proposed by Wendy" or "VEGF and MMP-9 in liver cancer was measured using the S-P method") limiting the reproducibility of their findings. Furthermore, general observations need to be discussed with the aim of enhancing the scientific value of the study. In particular, it has been demonstrated that angiogenesis is regulated by a dynamic balance of pro- and anti-angiogenic factors secreted from cancer cells, endothelial cells and stromal cells [5]. It is also ascertained that angiogenic vessels have a "disorganized" and "irregular" structure, and that the blood flow is abnormal and characterized by a non-linear behaviour [6, 7]. Sprouting angiogenesis, one of the seven distinct ways (i.e. vasculogenesis, intussusceptive angiogenesis, vascular co-option, mosaic vessels, vasculogenic mimicry and trans-differentiation of cancer stem-like cells into tumour endothelial cells) in which malignant tumours can generate their vasculature, can be represented as a dynamic process that is discontinuous in *space* and *time*, but advances through differnet consecutive "states". In geometrical terms, the continuous generation of these states determines a complex ramified structure that irregularly fills the surrounding environment. The main feature of the newly generated vasculature is the multifarious diversity of the vessel sizes, shapes, and connecting patterns. Despite its potential importance as a prognostic indicator in untreated tumours, MVD has not yet been revealed to be an appropriate measure for determining local micro-angiogenesis [8]. MVD does not appear to be predictive of tumour response under anti-angiogenic treatment and therefore may not be useful for stratifying patients for clinical trials [8]. Low MVD does not portend a poor response to anti-angiogenic therapy and tumour MVD may not vary in accordance with the tissue or blood levels of any single pro-angiogenic factor. Moreover, rapid growth does not imply high MVD [8]. The MVD of a tumour need not be higher, and is often lower, than that of its corresponding natural counterpart, which is experiencing no net growth [9]. The efficacy of anti-angiogenic agents cannot be simply visualized by alterations in MVD during

treatment. In addition, the MVD is substantially limited by the complex biology characterizing tumour vasculature [10] and the highly irregular geometry that the vascular system assumes in "real space" which cannot be measured (i.e. MVD is an estimate not a measure) using the principles of Euclidean geometry because it is only capable of interpreting regular and smooth objects that are almost impossible to find in nature [7]. Despite this, the authors have stated that: "MVD is a relatively accurate indicator for determining local microangiogenesis" and that "It is commonly used for local detection of tumours to determine the nature and recurrence of tumours". It remains indubitable that scientific knowledge develops through the introduction of new concepts, and this process is usually driven by new and more appropriate methodologies that provide previously unavailable observations. It is without a doubt that the broad applicability of "quantitative methods" and not mere subjective qualitative or semi-quantitative indexes, makes it possible to explore the range of the morphological variability of neo-vasculature that can be produced in nature, thus increasing its importance in pre-clinical as well as clinical cancer research.

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