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AGE-RELATED PROPERTIES OF THE TUMOR VASCULATURE IN RENAL CELL CARCINOMA

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Abstract:	OBJECTIVE To assess whether ageing processes influence angiogenesis in renal cell carcinoma (RCC) we carried out a pilot study of vascular properties in a series of archival primary kidney tumors in patients of different ages. PATIENTS AND METHODS A cohort of patients with RCC was identified restrospectively, where the age spectrum varied between 35 and 84 years. Paraffin embedded, formalin fixed sections of surgical tumor specimes were stained for endothelial (CD31, vWF), pericyte (SMA) and leukocytic (CD45) markers, as well as for proliferative (Ki67) and angiogenic activity (TEMs, Dll4, Dll1, eNOS). Vascular properties were compared between patients older and younger than 65 years of age. RESULTS Microvascular density (MVD) within capillary hot spots was generally higher in patients with non-metastatic clear cell renal cell carcinoma (CCRCC; n = 21) than in those with the metastatic disease (MRCC; n = 9). CCRCC patients who were older than 65 years exhibited significantly higher MVD than their younger (< 65) counterparts. Dividing (Ki67-positive) endothelial and mural cells were observed in both small (<20um) capillary and large (>20um), precapillary vessels, suggesting the involvement of both angiogenic

and remodeling/arteriogenic processes. Tumor Endothelial Markers (TEM1, TEM7, TEM8), Notch ligands (Dll1, Dll4), and other molecular characteristics (endothelial nitric oxide synthase - eNOS) were analysed. Age related differences were observed in the frequency of precapillary vessels expressing delta like 1 (Dll1), which was significantly higher in tumors of younger patients (< 65 years), while eNOS was more prevalent amongst capillaries associated with CCRCC in older patients (> 65 years).

CONCLUSIONS
These results suggest that age influences the structural and molecular properties of the tumor vasculature in CCRCC. We postulate that vascular ageing could also be relevant in the context of antiangiogenic therapy

KEY WORDS
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AGE-RELATED PROPERTIES OF THE TUMOR VASCULATURE IN RENAL CELL CARCINOMA

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Abstract

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INTRODUCTION

Neovascularization is critical for the progressive growth of solid tumors, but the mechanisms responsible for driving this process are still poorly understood, multifactorial and variable, especially between different tumor types [1]. The practical relevance of these processes is underscored by the growing interest in, and recent approval of several novel anticancer therapeutics designed to target the tumor vasculature [1-3]. In this regard, primary kidney tumors, especially clear cell renal cell carcinoma (CCRCC) in its metastatic presentation (MCRCC), constitute a particularly compelling case due to their unique pathogenesis. CCRCC is frequently driven by loss of the von Hippel-Lindau (VHL) tumor suppressor gene and this mutation results in constitutive activation of the hypoxia response pathway [4]. This in turn, drives a uniquely high expression of vascular endothelial growth factor (VEGF) [5]. This is believed to result in both the high vascularity and therapeutic responsiveness of these kidney tumours to agents targeting VEGF-driven angiogenesis [6,7], of which 4 (sunitinib, sorafenib, bevacizumab, pazopanib) are currently approved for clinical use in MCRCC [6,8].

Moderate levels of VEGF expression are normally present in renal parenchyma and are required for normal kidney homeostasis in the absence of angiogenesis [9]. However, the exuberant upregulation of this growth factor in CCRCC qualitatively changes the responses of resident endothelial cells and provokes fulminant blood vessel growth [10], along with several molecular changes [11]. In this regard, Notch and its ligands, especially delta like 1 and 4 (Dll1 and Dll4), are of particular interest as important regulators of precapillary “large” [12] and capillary “small” blood vessel growth [13],

respectively. The growing blood vessel network is also equipped with feedback mechanisms to control blood volume distribution, through processes of vessel remodeling and regulation of vascular tone. The latter is executed, at least in part, by endothelial nitric oxide synthase (eNOS), which acts as the sensor of the intravascular sheer stress [14], and a mechanism of the reactive vasodilatation through local production of nitric oxide (NO)[15,16]. While these mechanisms have been detected in various cancer settings and linked to VEGF-driven angiogenesis [17], their role in CCRCC remains relatively unexplored [18,19].

In several epithelial cancers, high microvascular density (MVD) may predict increased disease aggressiveness [20]. In CCRCC, however, this linkage appears to be more complex, controversial and largely unexplained. Clearly, methodological factors, such as different blood vessel detection and quantification protocols, heterogeneity of tissue samples (e.g. tumor stage, grade, size or levels of hypoxia [21]), as well as random variablility in clinical and pathological characteristics of the respective patient populations could have contributed to the aforementioned inconsistencies.

Alternatively, however, the variable results of MVD analyses could also be a reflection of more biologically meaningful properties of the CCRCC patient populations [22,23]. In this regard, one largely overlooked aspect of the inter-individual diversity amongst CCRCC patients is their age. Indeed, patients included in various CCRCC studies straddle the age spectrum of nearly 6 decades [24] and such a long period of time could be consequential for the status of the kidney and tumor microcirculation [25]. Age related

effects could be inferred from studies on the role of ageing in the function of the vascular system as a whole [26] and the changing functionality of the VEGF pathway [27]. Ageing is also known to modulate tumor angiogenesis in experimental settings [28-30] and may affect both tumor responses and side effects of antiangiogenic therapies [31]. These effects are either a direct consequence of ageing and exhaustion within the endothelial and bone marrow compartments [26,28,31], or are related to age-dependent vascular comorbidities such as atherosclerosis, thrombosis and inflammatory conditions [31]. Analyses of these processes in CCRCC are presently lacking.

In this pilot study aimed at exploting some of the aforementioned questions we report the results of vascular analysis performed in a cohort of 39 kidney tumor patients, of whom 21 were diagnosed with CCRCC and represented the age spectrum between 36 to 79 years. We show that age-related differences exist between CCRCC tumors removed from younger (< 65 years) *versus* older patients (> 65 years). For instance, higher MVD counts, lower prevalence of Dll1-positive precapillary vessels, and higher numbers of eNOS-positive microvessels were found in older patients. We postulate that age-specific vascular features (both architectural and molecular) could be of translational value in malignant kidney tumours, especially with respect to the further development of antiangiogenic agents for this disease.

PATIENTS, MATERIALS AND METHODS

Patients and samples

The clinical, pathological and other relevant characteristics of the patient cohort included in this study are summarized in Table 1. Overall, 44 samples were analyzed in detail, including 21 cases of CCRCC, 9 cases of metastatic RCC (MRCC), 5 cases of non-clear cell RCC, 4 cases of oncocytoma and 5 specimens of normal kidney. Amongst CCRCC patients 11 were older than 65, and 10 were younger (individual specimens were occasionally found inadequate for specific analyses, as indicated). All tumors included in this study were diagnosed, and patients cared for at the Department of Urology, Sunnybrook Hospital, University of Toronto (L.K). Preliminary analysis was conducted using an independent small sample of 6 CCRCC tumors obtained from the Provincial Specialist Hospital, Research and Development Center in Wroclaw, Poland (KP). Normal human kidney slides were purchased from Biochain (Cat # T2234142), Harvard, California, USA and Abcam (Cat # ab4347) Cambridge, Massachusetts, USA.

Antibodies

Mouse anti-PECAM (NCL-CD31-1A10) was purchased from Novocastra (United Kingdom). Rabbit polyclonal anti-TEM-1 (#67275) was purchased from Abcam. Mouse anti-TEM7 and TEM8 were generated in the laboratory of Dr. Brad St-Croix (NCI Frederick, USA). Monoclonal mouse anti-human CD45 and anti-human von Willebrand Factor (A0082) antibodies were purchased from Dako (Missisauga, ON, Canada). Ki-67 and anti-Smooth Muscle Actin (A-2547) antibodies were purchased from SIGMA (St. Louis). Rabbit polyclonal anti-eNOS antibody (PA1-37624) was purchased from Affinity Bio Reagents, Golden, CO, USA. Rabbit anti-mouse polyclonal antibody against Dll1 (LS-B72) was purchased from Lifespan Biosciences Inc., Seattle, WA USA.

Rabbit polyclonal antibody to Dll4 was purchased from Abcam (Cat # ab7280). Antibodies recognizing angiopoietin-1/2 and phospho-VEGFR-2 were purchased from R&D Systems (Minneapolis, MN).

Immunohistochemical analysis

Paraffin embedded formalin fixed tissue samples were sectioned at 5 um. All tissues were dewaxed in xylene and rehydrated through a graded series of ethanol, as described elsewhere [32]. Microwave antigen retrieval was performed using Vector Antigen Unmasking Solution (H-3300, Vector Laboratories, Burlingame, CA, USA) at pH = 6.0. Primary antibodies were incubated with the samples overnight at 4 C. For bright field visualization slides processed using the Vector AEC kit were mounted using Vectamount (H-5000) media (Vector Laboratories, Burlingame, CA, USA). For immunoflourescent staining the appropriate secondary antibodies conjugated with AlexaFlour 488, 594 and 350 fluorophores (Molecular Probes, CA) were used accordingly. Slides were mounted using Vectashield with DAPI (H-1500, Burlingame, CA, USA), viewed and analysed using a Zeiss Axiophot microscope.

MVD and PVD analysis

All samples were analysed by a blinded observer using precoded samples. Upon staining of tissues with panendothelial markers (CD31, vWF) microvascular density (MVD) was evaluated by counting capillary sized vessles (≥ 20 μm in diameter). The density of larger vessels (> 20 μm) was designated as precapillary vessel density (PVD), irrespective of their arterial or venous identity. For MVD counts, tumor sections stained for CD31 were

first surveyed under low magnification to locate microvascular 'hot spots' defined as areas of the highest capillary density within a given slide, as described [20]. All microvessels within each of the three independent 'hot spot' areas per specimen were counted under 400x magnification and their average MDV was expressed as vessel density per high power field (hpf) [20]. Similar procedures were applied to determine PVD using vWF immunofluorescence to highlight endothelial cells of larger vessels. Again, three areas containing clusters of the largest precapillary vessels were selected for each tissue specimen under low power (50x). The number of blood vessels in each such region (precapillary 'hot spot') was subsequently counted under 400x magnification and expressed as the vessel number per hpf, or 'hot spot PVD'. We also independently evaluated tumors for global PVD, using CD-31 stained sections. In this case the entire section area was divided into 9 equivalent regions (3 x 3 fields). Photographs of identical fields defined by the intersecting vertices were taken at 100x magnification and the numbers of precapillary vessels in these regions were calculated and expressed as the average global PVD. The sizes (longest width) of all precapillary vessels in these regions were measured and their numbers in each specimen plotted as indicated.

Cell proliferation of and necrosis

Triple staining for von Willebrand Factor (vWF/Factor VIII related antigen), alpha smooth muscle actin (∀SMA) and Ki67 was used to determine the mitogenic activity of the respective vascular (endothelial and mural) cells. Tumor cell proliferation rate was also assessed by Ki-67 staining. Positivity of various cells for Ki67 was evaluated at 400x magnification and the number of cells with positive nuclei were counted in 10

independent fields per specimen and averaged. Tumor necrosis was evaluated by examining at least two different hematoxylin and eosin (H & E) stained slides from each specimen, followed by morphometric measurements of the respective viable and necrotic regions. Necrotic area was calculation as percentage of the entire tumor section area occupied by necrotic masses.

Expression of functional vascular markers

The functional state of tumor microvessels was assessed by immunofluorescent staining for eNOS and Dll1 and other markers, as indicated. For eNOS immunostaining, slides were exposed to the respective antibody and co-stained for CD31. The number of capillary and precapillary blood vessels positive for eNOS was assessed in 10 random fields per section under 400x and 200x magnifications, respectively. The average number of eNOS-positive vessels per hpf was used for comparisons between patients. For Dll1 analysis, the entire slide was viewed under 400x and 200x magnifocations. All vessels that stained positive for Dll1 were measured and classified as either capillaries or pre-capillaries (as above). The respective vessel categories were enumerated and expressed as the number of Dll1 positive vessels per slide. Slides were also stained for CD45 and vWF to evaluate the extent and perivascular location of the infiltrating leukocytic cells.

Statistical analyses

Statistical analysis was conducted by T-test analysis between two groups by using SPSS version 12 for Windows.

RESULTS

Heterogeneous vascular patterns in primary renal tumors.

Immunostaining for panendothelial markers, including CD31 and vWF, reveals a rich network of blood vessel capillaries in both normal and neoplastic kidneys (Fig. 1AB and data not shown). However, while the cortical vasculature of the normal kidney is well organized, with defined glomeruli and larger supplying vessels, tumor-associated microcirculation is profoundly disorganized with capillary, precapillary and larger vascular structures present throughout the parenchyma at variable densities, often clustering into microvascular 'hot spots' (Fig. 1B). The microvascular density (MVD) within these latter regions is often viewed as informative, as to the intensity of the angiogenic process and the related disease aggressiveness [20]. Therefore, we assessed 'hot spot' MVD in a diverse cohort of archival tissue specimens including normal kidney, benign kidney tumors (onocytoma) and aggressive renal cell carcinomas (RCC), of either clear cell (CCRCC) or non-CCRCC (NCCRCC) morphology. The CCRCC samples also included material from patients who eventually progressed to develop metastatic disease (MRCC) (Table 1).

Vascular densities revealed by CD31 staining in these respective tissues were vastly different. While MVD in oncocytoma resembled that of the normal renal cortex, some RCC lesions were highly vascular, but as a whole tumor vasculature exhibited considerable numerical variation between individual patients (Fig. 1C). Even when tumors were stratified into non-CCRCC, CCRCC and MRCC subsets, the hot spot

capillary vessel densities varied markedly within each of these patient groupings (Fig. 1D). Interestingly, tumor sections representative of MRCC exhibited significantly lower MVD values then those of CCRCC patients, who did not progress to MRCC after surgery. Though counterintuitive, this finding is consistent with a prior report [33].

Age-related changes in the vascularity of CCRCC

Even upon restriction of our MVD analysis to CCRCC, the largest subset within our cohort, we observed a marked patient-to-patient variability. We sought to understand the nature of this variability by plotting the data as a function of several different paramaters, including: tumor linear length, tumor grade, mitogenic activity and necrotic region, and other features, of which none correlated with the tumor MVD counts (data not shown).

Since tumor angiogenesis was previously shown to be modulated by vascular ageing [26,28,31], we also plotted MVD values as a continuous function of age, or upon stratification into groups of patients: either < 65 or >65 years old (Fig. 2AB). Interestingly, this stratification revealed notable differences, in that tumors resected from patients older than 65 years exhibited significantly higher MVD than those from their younger counterparts (≤65). These changes were not linked to noticeable differences in mitotic indices of cancer cells, or to the extent of tumor necrosis (data not shown).

Since blood vessel growth and remodeling implicitly involves mitogenesis of endothelial and mural cells [31], samples of CCRCC were subjected to triple immunofluorescent staining for markers of proliferation (Ki67), endothelium (vWF) and mural cells

(∀SMA). As might be expected, tumor blood vessels in both younger and older patients contained dividing endothelial cells (Ki67/vWF-positive) and pericytes (Ki67/∀SMA-positive), but their corresponding vascular cells in the normal kidney remained completely Ki67 negative (data not shown). Overall, the low numbers of endothelial nuclei unequivocally positive for Ki67 in standard histological sections did not permit an accurate quantification and comparison of their mitogenic activity between different patients subsets.

Of note, features of endothelial and smooth muscle cell proliferation were apparent in both capillary and larger, precapillary tumor blood vessels (Fig. 3), an observation suggestive of a coexistence of angiogenic and an arteriogenic-like vascular growth in these tumors [34]. To assess whether the related ('neo')arteriogenesis [34] exibited age-dependent properties, we assessed the precapillary vascular density (PVD) in CCRCC tumors of patients older and younger than 65 years. PVD counts were collected either within the preselected clusters of large vessels ('PVD hot spots'), or in random fields across the entire cross-section of each tumor specimen ('PVD global count'; Fig. 4A). While there was a trend towards higher density of intermediately sized vessels (20-50 μm in diameter) in older patients, this difference (unlike MVD) did not reach statistical significance, and the densities of larger vessels (50-500 μm) were relatively similar in both age groups (Fig. 4BC). Overall, this analysis suggests that certain characteristics of tumor related vascular structures, especially the 'hot spots' of capillary microvessels, correlate with ages of individuals affected with CCRCC.

Age-related molecular features of CCRCC-related blood vessels

We reasoned that the aforementioned structural differences (MVD) between tumor blood vessels in younger and older CCRCC patients must be related to some underlying molecular processes and characteristics [31]. In this regard several molecular hallmarks of tumor-related blood vessels have been revealed in recent years and those include tumor endothelial markers (TEMs) [31,35], upregulation/activation of VEGF receptors, and changes in other angiogenses regulating molecules [2], such as elements of the angiopoietin system, Notch ligands and several other entities [36]. Some of these molecules have already been evaluated as a function of vascular ageing (e.g. TEM1/glycosialin, VEGFR-2/flk-1), but on a very limited scale and only in experimental studies [31].

To extend this analysis to human CCRCC, we surveyed the levels of several angiogenesis related molecules by immunofluorescence. This included staining for several human TEMs (TEM1, TEM7 and TEM8), Dll1, Dll4 and endothelial nitric oxide synthase, along with the presence of myelocytic (CD45-positive) cells in the perivascular tumor regions (Fig. 5 and data not shown). Staining patterns for TEM1, TEM7, TEM8, Dll4, and CD45 were comparable between the CCRCC specimens from younger and older patients (data not shown). In the case of TEM1 and TEM8 the staining was found in both endothelial and in some perivascular cells, while TEM7 signal was primarily detected in a subset of endothelial cells (Fig. 5A-C). This pattern could reflect the emerging and differential association of some of these molecules (TEM1) with endothelial, mural [37] and endothelial progenitor cell populations [38]. CCRCC tissues stained poorly for the Notch

ligand, Dll4 (Fig. 5E), even though the same reagents yielded a robust and endothelial-specific signal of human glioblastoma specimens (data not shown).

Interestingly, age-related differences were noted in the case of another Notch ligand, Dll1, which has been implicated in both angiogenesis and arteriogenesis [12]. Thus, the number of Dll1-positive capillaries did not differ significantly between tumors in younger and older CCRCC patients. However, similar analysis of precapillary vessels (> 20 μm) revealed that Dll1 antibody was able to decorate a greater fraction of tumor vessels in patients who were younger than 65, as compared to their older counterparts (> 65; Fig. 6AB). In both instances Dll1 was present mainly on the surface of endothelial cells (Fig. 5).

Age-related differences were also noted in the case of eNOS expression (Figs. 5 and 7). This marker of increased vascular shear stress [14] was elevated more frequently in CCRCC-associated capillaries of older patients ($p < 0.02$), then in those that were younger than 65 years. Similar trend was observed in the case of larger vessels, but in this case it did not reach statistical significance. Taken as a whole, these observations illustrate both structural and molecular, age-related differences in the vasculature of CCRCC.

DISCUSSION

Our exploratory analysis of CCRCC suggests that kidney tumors may possess age-related alterations in their vascular characteristics. It it striking that such differences were noticeable even in very small cohort of patients, and larger studies are warranted to investigate this pattern more fully. Thus, we observed a higher capillary density (MVD) and higher frequency of microvessels positive for eNOS in patients older than 65, relative to tumors that originated in younger patients. Conversely, vascular Dll1 expression was more prevalent in the latter group, but only in larger, precapillary tumor vessels. The specific reasons for this pattern are presently unknown. Nonetheless, Dll1 has been implicated in postnatal arteriogenesis [12], a process that is regulated by shear stress, of which the elevated expression of eNOS is a frequently found indicator [14]. It is conceivable, that with age the coupling between formation of larger 'feeding' vessels ('neoarteriogenesis')[34] and their corresponding tumor capillaries (angiogenesis) is altered and leads to greater sheer stress and remodeling within the microcirculation.

We observed an increased mitogenic activity in both endothelial and mural cell compartments of blood vessels within CCRCC tumors. Because this occurred in both capillary and precapillary vessels one can surmise that CCRCC progression triggers both angiogenic (capillary) and arteriogenic (precapillary) vascular growth, as we suggested earlier [34]. While much attention is devoted to tumor angiogenesis, the concomitant circumferential/arteriogenic remodeling of larger tumor vessels ('neoarteriogenesis') is relatively unstudied and poorly understood. In the context of tissue ischemia, formation of larger vessels (collaterals) is attributed to retrograde shear stress, endothelial activation and influx of monocytic cells [15]. Other contributing changes include the expression of

inflammatory chemokines, adhesion molecules [11], Dll1 [12], nitric oxide signaling [39]. Thus, unlike angiogenesis, the growth of larger vessels may be independent of hypoxia [40]. Instead, these vessels may be influenced by bone marrow cells which, in turn, could be affected by an age-dependent exhaustion of bone marrow [31,41,42]. It remains to be established whether these processes are relevant in the context of CCRCC.

Irrespectively of mechanistic considerations, the linkage between vascularity and progression of kidney cancers has long been perplexing. Thus, while some studies found a positive correlation between high MVD and poor patient survival [21,43,44], others concluded the opposite to be true [21,24,33,45,46], or questioned the prognostic significance of MVD in CCRCC altogether [47,48]. These discrepancies motivated several methodological refinements to capture the heterogeneity of the tumor-related vasculature with a greater precision, for instance, by pointing to the preponderance of larger vessels in more aggressive lesions [33], or by analysis of more subtle features of the vascular architecture [49] including its fractal properties [46]. Interestingly, some of this work also revealed the existence of both cellular and molecular heterogeneity within the blood vessel networks of CCRCC tumors including the presence of distinct CD34-positive and CD34-negative subsets of tumor capillaries [50]. Variable levels of circulating endothelial progenitor cells (EPCs) [51], and differences in the expression of various angiogenesis-related genes other than VEGF have also been reported [52]. It remains to be seen whether these processes are affected by vascular aging.

It is of considerable interest whether the age-related vascular properties of CCRCC, and especially MRCC, have therapeutic relevance [6]. Nearly 50% of all patients with RCC are older than 65 years, although the disease can emerge as early as in the third decade of life [8]. While age is recognized as a factor relevant for the cardiovascular safety of antiangiogenic agents [8], its influence on therapeutic efficacy and mechanisms of biological response have received much less attention. Nonetheless, it is intriguing that in one preliminary study, which addressed age-related responses to therapy, sorafenib appeared most efficatious in RCC patients over the age of 65 [7].

In conclusion, our study suggests that ageing may influence structural, molecular and possibly functional characteristics of kidney tumor blood vessels. Indeed, processes related to vascular ageing could potentially affect multiple characteristics of the disease, including tumor growth and metastasis [53], aggressiveness [54] and the effects of various therapeutics [55], including those with antiangiogenic and non-angiogenic mechanisms of action. Our exploratory study, which is based on a small patient cohort, represents an essential first step towards understanding how aging affects vascular architecture and molecular profiles in RCC. Follow-up studies are required to determine if these vascular parameters can aid in evaluation of tumor vascular responses to anti-angiogenic agents. Age-related alterations in the vasculature could also help explain some of the inconsistent results obtained from independent patient cohorts regarding the prognostic value of vascular density counts.

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CONFLICT OF INTEREST

No conflict of interest is declaired.

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LEGENDS TO ILLUSTRATIONS

Figure 1. Microvascular density in primary kidney tumors. A-B. Representative patterns of capillary blood vessels revealed by staining for the panendothelial marker CD31. Well organized glomerulal structures in cortical sections of the normal kidney (A) contrast with chaotic blood vessels in renal cell carcinoma (RCC; B). **C.** Hot spot microvascular/capillary density (MVD) of benign (oncocytoma) and malignant (RCC) kidney tumors. Notably, MVD in oncocytoma resembles that of normal kidney cortex (NK, dotted line), while an extremely wide MVD distribution is observed in RCC. **D.** Separation of the RCC cohort into RCC subsets reveals higher MVD in clear cell RCC (CCRCC), than in non-clear cell (NCCRCC) and metastatic (MRCC) tumors. The latter difference is statistically significant ($p < 0.05$), even though the values for individual patients continue to exhibit wide heterogeneity. Median values are denoted by thick horizontal lines.

Figure 2. Age-related distribution of microvascular density in clear cell renal cell carcinoma. A. Gradual increase in MVD values with the age of CCRCC patients. **B**. Markedly higher MVD of CCRCC patients older than 65, relative to younger patients ($p < 0.001$). Median values are denoted by thick horizontal lines.

Figure 3. Evidence of vascular cell proliferation in capillary and precapillary vessels in CCRCC. Triple immunofluorescence for endothelial (vWF/blue), mural (SMA/green)

and proliferation markers (Ki67/red and red arrows) reveals evidence for proliferation of pericytic/smoth muscle (A, B, D) and endothelial cells (C). Proliferating vascular cells were found throughout CCRCC-associated vascular structures, including capillary (A-B), medium size (D) and larger, precapillary vessels (C, red dotted line indicated the vessel diameter of approximately 100 μm).

Figure 4. Analysis of age-related changes in CCRCC-associated precapillary blood vessels. A. Cartoon depicting the criteria used to evaluate capillary (MVD) and precapillary (PVD) vascular densities in this study. Counts were performed either within the hot spots (clusters) of capillary (> 20 μm) or larger (20 – 500 μm) blood vessels, or the respective vascular structures were enumerated in random fields across the entire tissue specimen (global count). **B.** Global count of precapillary blood vessel (PVD) in CCRCC tumors of patients younger (triangle) or older (filled circle) than 65 years. A trend toward greater density of intermediate size (20 – 50 μm) vessels in older patients was observed, consistent with the results of the MVD count (Fig. 2B). However, with increasing vessel sizes their densities were lower and indistinguishable between both groups of patients. **C.** Vascular density count (PVD) in hot spots of precapillary vessels reveals a trend similar to that of a global count (B). Median values are denoted by short horizontal lines.

Figure 5. Immunofluorescent staining for vascular markers associated with CCRCC. A. Expression of tumor endothelial marker 1 (TEM-1/green) by endothelial and extravascular cells infiltrating CCRCC. Endothelial cells are highlighted by co-

staining for vWF (red). **B.** TEM7 staining (green) colocalized with vessels but was predominantly non-overlapping with vWF (red) in CCRCC tumors. **C.** Staining of CCRCC for TEM-8 (green) was predominantly perivascular; vWF staining is shown in red. **D.** Massive perivascular infiltration of the tumor mass with CD45-positive (green) leukocytic cells. Blood vessels are highlighted by staining for vWF (red). **E.** Endothelial pattern of CCRCC staining for delta like 4 (Dll4/green) and vwf (red)– large precapillary vessel. **F-G.** Staining of precapillary CCRCC-associated vessels for Dll1. **H-I.** Specific immunoreactivity of endothelial cells in small- and large caliber blood vessels with the anti-eNOS antibody. In H, the unspecific intravascular signal is associated with autofluorescence of red blood cells.

Figure 6. Age-related expression of Dll1 in the vasculatures of CCRCC tumors. A. Comparable frequency of Dll1 expressing capillary blood vessels in patients younger or older than 65 years. **B.** Reduced number of Dll1-positive precapillary vessels (< 20 μm) per high power field (hpf) in CCRCC patients older than 65 years, relative to their younger counterparts ($p < 0.05$). Median values are denoted by thick horizontal lines.

Figure 7. Age-related expression of eNOS in the vasculatures of CCRCC tumors. A. Marked increase in frequency of eNOS expressing capillary blood vessels in patients older than 65 years, compared to those younger than 65 ($p < 0.02$). B. Frequencies of eNOS positive precapillary vessels (< 20 μm) in CCRCC patients younger and older than 65 years. Interestingly, a virtual absence of eNOS in most younger patients contrasts with the positivity for this shear stress-related marker in approximately 50% of older patients.

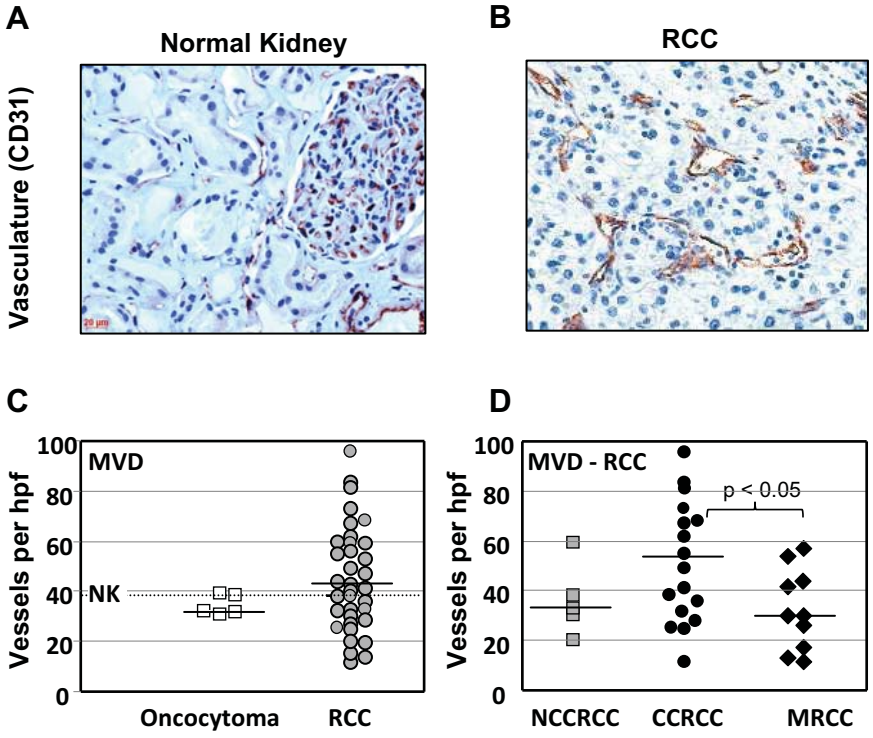
However this trend has not reached statistical significance. Median values are denoted by thick horizontal lines.

TABLES

Table 1. Clinical and Pathological Characteristics

Age (median)	N	Gender (m/f)	Tumor Size (median)	Grade (median)
Normal Human Kidney (Control)				
50 – 60 yrs (NA)	5	4/1	None	N/A
Oncocytoma				
63 - 81 yrs (78.5)	4	2/2	2.0 – 7.5 cm (3.75)	N/A
Clear Cell Renal Cell Carcinoma (CCRCC)				
36 - 79 yrs (60.5)	21	9/12	1.3 – 13 cm (5.5)	1 - 4 (2.5)
*Non-Clear Cell Renal Cell Carcinoma**				
51 - 70 yrs (62)	5	3/2	2.1 -17 cm (5.0)	1 - 3 (2)
Metastatic Renal Cell Carcinoma				
35 - 84 yrs (63)	9	5/4	2.5 – 12 cm (5.0)	2 - 4 (3)

* Non-Clear Cell Renal Cell Carcinoma cohort consists of Papillary RCC (2), Chromophobe Eosinophilia RCC (1), Leiomyosarcoma (1) and Hemangiopericytoma (1).



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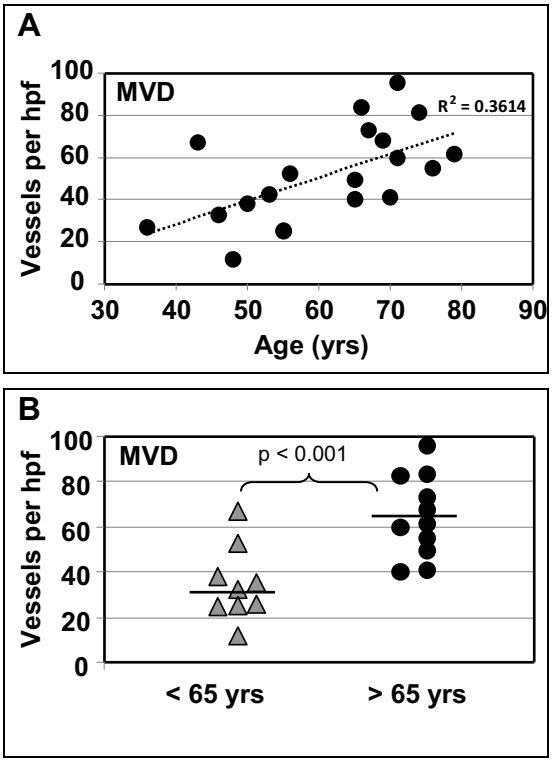

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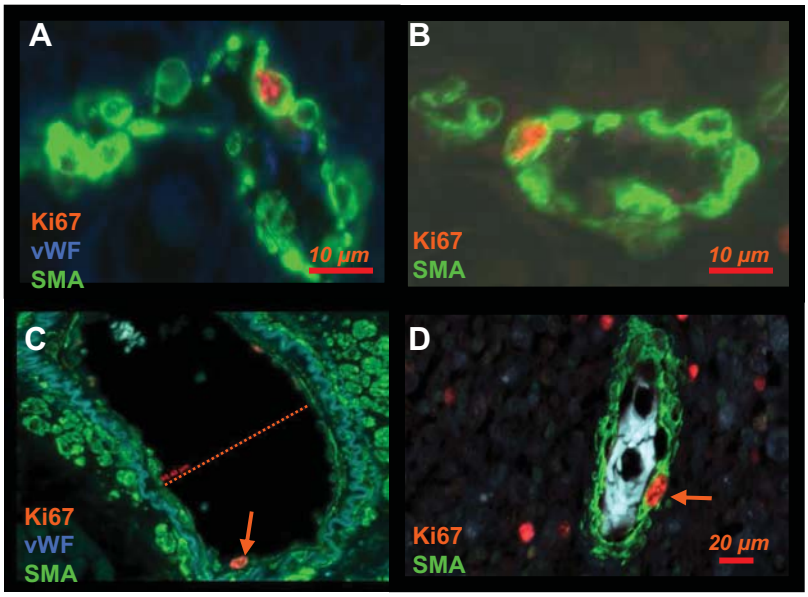

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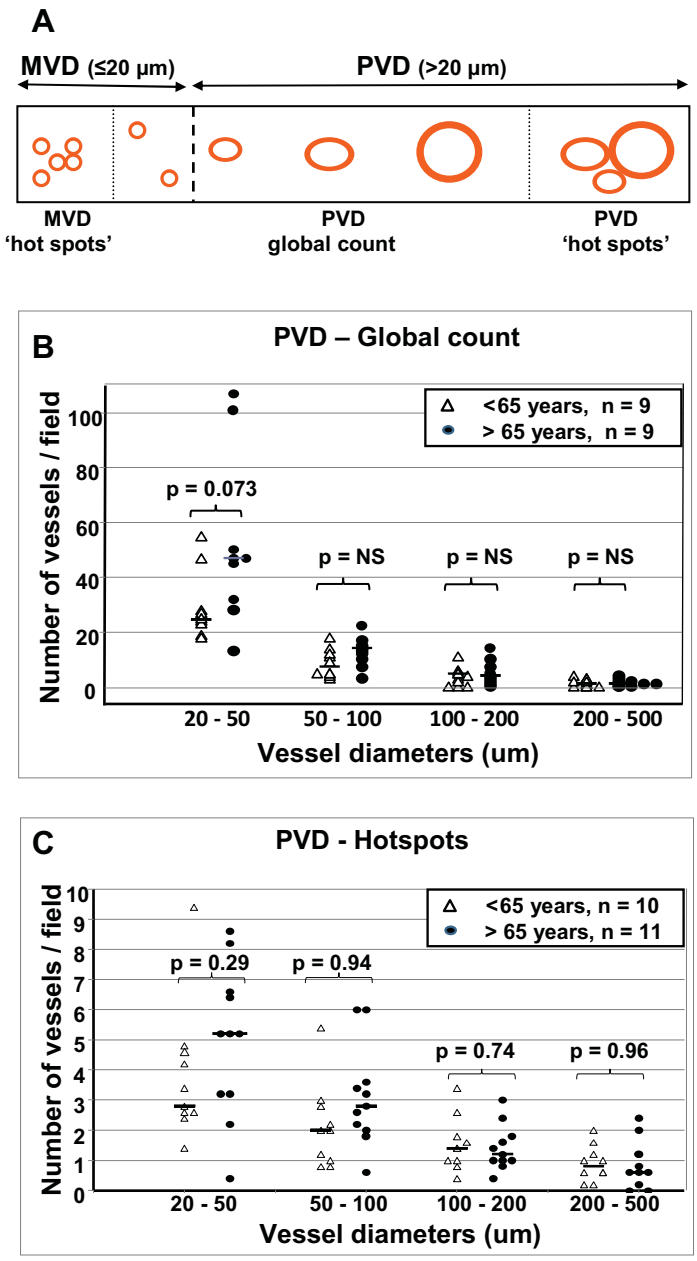

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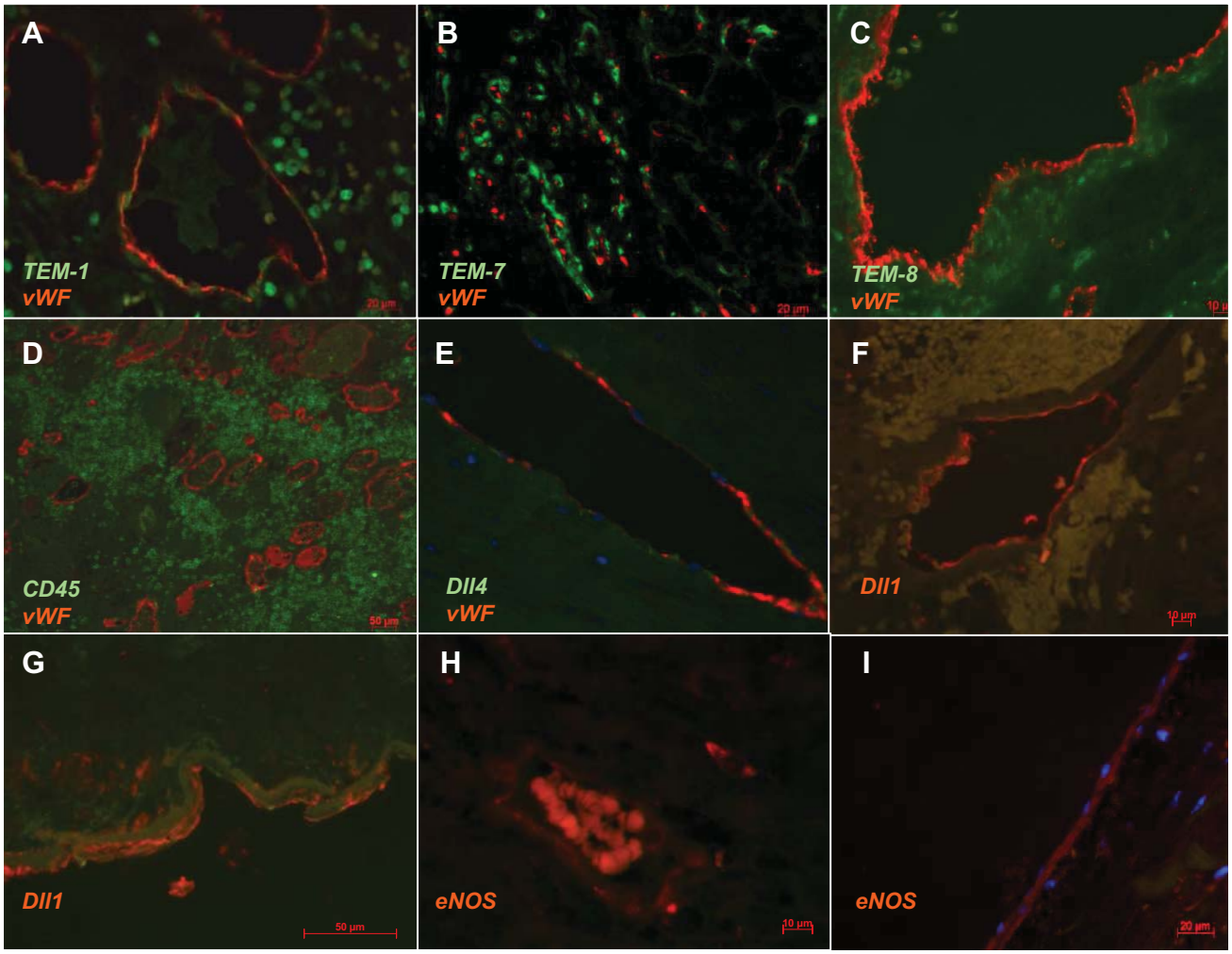

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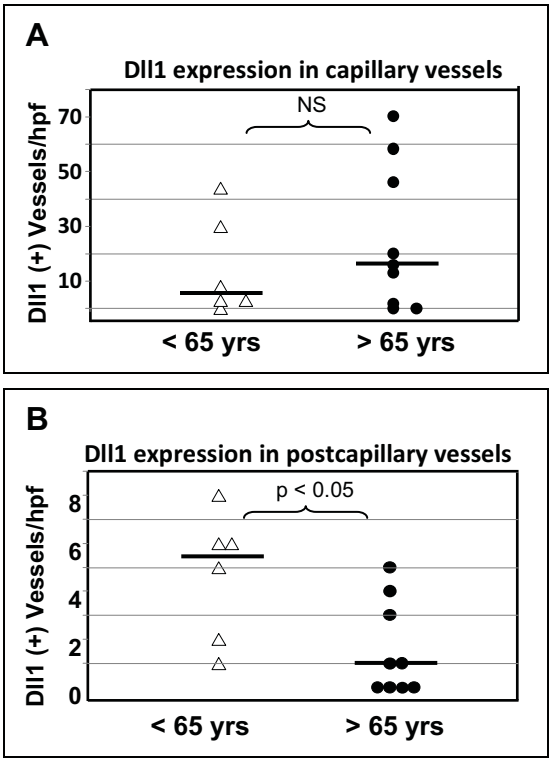

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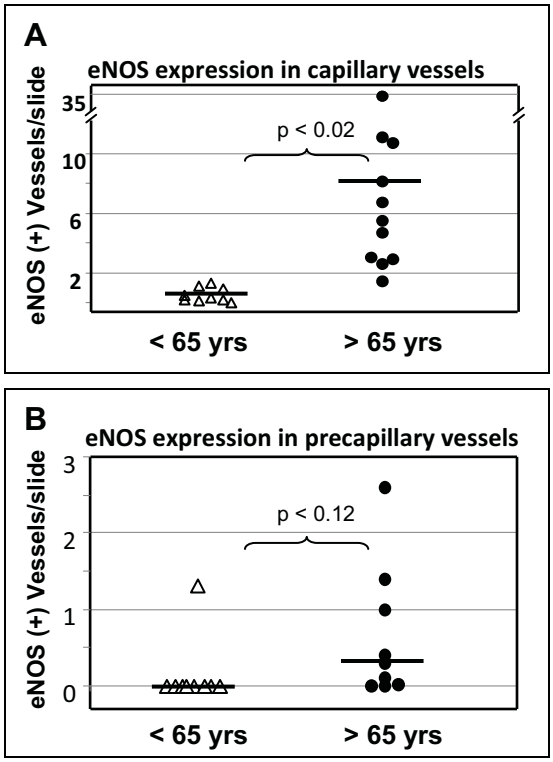

Meehan et al Figure 7