Ageing-related responses to antiangiogenic effects of sunitinib in atherosclerosis-prone mice

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Abbreviations: LLC – Lewis lung carcinoma; MVD – microvascular density; CAIX – carbonyl anhydrase IX

Abstract

Antiangiogenic therapies in cancer exert their effects in the context of age-related comorbidities, which affect the entirety of the vascular system. Among those conditions, the impact of atherosclerosis is especially prevalent, but poorly understood, and not reflected in mouse models routinely used for testing antiangiogenic therapeutics. Our earlier work suggested that these obstacles can be overcome with the use of atherosclerosis-prone ApoE-/- mice harbouring syngeneic transplantable Lewis Lung Carcinoma (LLC). Here we report that, sunitinib, the clinically approved, antiangiogenic inhibitor impedes global tumor growth to a greater extent in aged then in young mice. This activity was coupled with changes in the tumor microenvironment, which in aged mice was characterized by pronounced hypoxia, reduction in microvascular density (MVD) and lower pericyte coverage, relative to young controls. We also detected soluble VEGR2 in plasma of sunitinib treated mice. Interestingly, sunitinib modulated tumor infiltration with bone marrow-derived cells (CD45+), recruitment of M2-like macrophages (CD163+) and activation of inflammatory pathways (phospho-STAT3) in a manner that was age-dependent. We suggest that age and atherosclerosis may alter the effects of sunitinib on the tumor microenvironment, and that these considerations may also apply more broadly to other forms of antiangiogenic treatment in cancer.

Keywords: ageing, atherosclerosis, sunitinib, anti-angiogenesis, inflammation.

1. Introduction

Cancers emerge across the entire age spectrum and their nature differs at many levels between affected children, adolescents, adults and elderly (Balducci and Ershler 2005;Orjalo et al. 2009). In many instances these differences are attributed to intrinsic molecular events associated with the cellular transformation process itself (Gilheeney and Kieran 2012). Indeed, similar morphology may often mask more profound differences in genetic underpinnings, biology, clinical course and therapeutic responses between malignancies occurring decades apart in the brain, kidney, hematopoietic system and other disease sites (Balducci and Ershler 2005).

However, age-related changes and co-morbidities also occur within host tissues and at the organismal level (extrinsically to cancer cells). Their influences may intersect with intrinsic aspects of malignant transformation in many complex and consequential ways that are still poorly understood (Rak et al. 2008). In this regard the vascular system is of special interest, as a crucial element of the tissue (and tumor) microenvironment, regulator of cellular growth, interface for oxygen supply and metabolic homeostasis, and the main conduit for regulatory molecules, extracellular vesicles, cells and drugs. Indeed, vasculature is central to bidirectional trafficking of bone marrow-derived, inflammatory cells and metastatic cells in and out of the tumor mass, respectively (Fidler2003; Folkman2007;Mantovani and Locati 2013), and these processes may change with organismal agenig. In support of this notion, we and others have documented age-related alterations in tumor angiogenesis (Pili et al. 1994;Franco et al. 2002;Reed and Edelberg 2004;Shimada et al. 2004;Klement et al. 2007) and metastasis (Ershler et al. 1983;Meehan et al. 2013), in both experimental and clinical settings (Meehan et al. 2011).

Ageing processes affect the vasculature at several important levels, as reflected by prevalent and progressive development of cardiovascular pathologies leading to inflammatory, thrombotic and ischemic syndromes culminating among the elderly (Lusis2012). These conditions encompass macro- and microvascular endothelial cell decline, as well as diminution of bone marrow and vascular repair functions (Xu2006) (Rauscher et al. 2003). Ageing also affects the inflammatory system and mechanisms of pathological neovascularization (Pili, Guo, Chang, Nakanishi, Martin, and Passaniti 1994;Rivard et al. 1999;Zhu et al. 2003;Reed and Edelberg 2004;Reed et al. 2007).

In the human population ageing coincides with a cluster of progressive, diffuse, inflammatory and degenerative alterations that surround the process of atherosclerosis (Lusis2012). It is noteworthy that while studies on atherosclerosis usually focus on altered lipid metabolism and damage to the large vessel wall, sources of known medical emergencies, the disease process affects the entirety of the vascular system and is mechanistically linked to bone marrow-derived cell functions (Linton and Fazio 2003). Indeed, while atherosclerosis begins silently early in life it progressively engages both endothelial and inflammatory cells, cytokine networks and the coagulation system in a manner that leads to a degree of vascular damage apparent at the middle/late age, and affecting virtually the entire human population (Joakimsen et al. 1999; Lusis 2012). Since cancer incidence also increases with age it follows that atherosclerosis represents a highly prevalent comorbidity in elderly cancer patients (Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007;Rak, Milsom, and Yu 2008).

The overwhelming majority of studies on cancer biology and therapy are currently conducted with the use of mouse models (Francia et al. 2011), usually with animals that are within the first 10 - 50% of their average life expectancy (Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007;Rak, Milsom, and Yu 2008). Moreover, mice are genetically resistant to atherosclerosis, unless their lipid metabolism pathways are genetically disrupted, for example by introducing null mutations into the *ApoE* gene required for the lipid uptake by macrophages (Linton and Fazio 2003). We have previously documented that while young ApoE-/- mice are hyperlipidemic, their angiogenic proficiency and ability to support growth of transplantable tumors are virtually indistinguishable from those of their syngeneic young C57BL6 wild-type counterparts (Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007). This functionality declines dramatically with age of ApoE-/- mice, and in parallel with the appearance of aortic plaques and other signs of macrovascular, microvascular and bone marrow deterioration, all of which resemble the vascular ageing processes in the human population (Linton and Fazio 2003;Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007; Lusis2012). Thus, we suggested that aged tumor-bearing ApoE-/- mice may represent a particularly realistic model for studies on vascular processes in human cancer.

This is relevant for blood vessel-directed (antiangiogenic) therapy in cancer which has been among the most dynamic areas of exploration during the past decade. The related progress is marked by the approval (since 2004) of several new agents mostly directed at the vascular endothelial growth factor (VEGF) pathway (Kerbel2008;Ellis and Hicklin 2009;Phan et al.

2013), including an array of small molecule, oral receptor tyrosine kinase (RTK), or multikinase, inhibitors that have entered clinical practice for several cancer indications (Rini2009;Tie and Desai 2012). In this regard sunitinib malate (Sutent ™) is among the best characterized agents in terms of both preclinical and clinical activity, especially in metastatic clear cell renal cell carcinoma (mccRCC), but also in gastrointensitinal stromal tumors (GIST), pancreatic neuroendocrine tumors (PanNET), hepatocellular carcinoma (HCC) and other cancers, including the emerging evidence for activity in non-small cell lung cancer (NSCLC) (Huang et al. 2010;Vazquez et al. 2012;Escudier et al. 2013;Sampat and O'Neil 2013).

Sunitinib inhibits several vascular-related RTKs, including VEGF receptors 1-3 (VEGFR1-3), platelet derived growth factor receptor (PDGFR), colony stimulating factor receptor 1 (CSFR1), as well as KIT, FLT3 and RET (Faivre et al. 2007), some of which play important roles in tumor angiogenesis (Carmeliet and Jain 2011). While these are encouraging developments the individual variation in response to sunitinib are not uncommon and the biological responses to the drug in vivo are still poorly understood (Ebos and Kerbel 2011). While functionality of the VEGF pathway changes markedly with age (Rivard, Fabre, Silver, Chen, Murohara, Kearney, Magner, Asahara, and Isner 1999), systematic studies in this regard involving sunitinib effects are largely lacking. Moreover, the efficacy of this agent is predominantly compared between older groups of cancer patients (Chowdhury et al. 2013;Pal et al. 2013; Killock2014), while the biological effects in more distant age groups (elderly and children) are scarcely characterized.

Here we present experimental data describing age-related differences in biological responses to sunitinib in experimental tumors growing in young and old atherosclerosis-prone mice. We observed that older mice sustained more pronounced antitumor effects coupled with increased hypoxia, reduced vascularisation and diminished inflammatory infiltration. These results are consistent with the notion that age modulates tumour responses to targeted antiangiogenic agents in subtle, but biologically consequential ways, and suggest that vascular ageing should be considered as a possible modulator of biological activity of specific antiangiogenics.

2. Materials and Methods

2.1 Cell Lines and Mouse Models;

The low metastasic Lewis Lung Carcinoma cells (LLC-LM or LLC) were obtained from American Type Culture Collection (ATCC; Manassas, VA), while their derivative LL/2-luc-M38 cells were purchased from Perkin-Elmer (USA). Cells were grown in Dulbecco modified Eagle's medium (DMEM; Cat. 11995-065, Life Technologies), 10% fetal bovine serum (FBS; Cat. 080150, Wisent, USA) with Penicillin-Streptomycin (Cat. 15140-122, Life Technologies) on 0.1% gelatin coated petri dishes (Cat. 172958, Nunc, Denmark). C57Bl/6 wild type and C57Bl/6 ApoE-/- female mice were purchased from Harlan Laboratories (USA) and Taconic Farms (USA), respectively. Mice designated as "young" were purchased at 6-8 weeks of age and used as close to their arrival and acclimatization date as possible. Mice designated as "old" were purchased as retired breeders at 8 months of age. Subsequently, old mice were aged to at least 12-18 months of age while fed normal chow. Under these conditions the ApoE-/- mutation

leads to severe hyperlipidemia and progressive atherosclerosis, which can be macroscopically observed as plaques in large vessels of old (but not young) mice, as described earlier (Linton and Fazio 2003;Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007).

2.2. Tumor Models and Treatment;

Tumors were generated in young or old (atherosclerotic) mice by subcutaneous (s.c.) flank injection of LLC or LL/2-luc-M38 cells, as indicated, at 1 x 10^6 and 5 x 10^5 cells per inoculum, respectively Since the latter cell line exhibited only weak bioluminescence all tumors were monitored by vernier caliper measurements three times per week, during which time mice were weighed. Tumors were grown for 14 days before 53.6 mg/kg of Sunitinib malate (equivalent to 40 mg/kg of active compound; Pfizer) was given orally, as gavage once per day. The treatment was initiated after tumors became established and reached the volume of at least 200-300 mm^3, and dosing continued at least until the experimental endpoint (termination of the control group), or clinical endpoint (as per animal care guidelines), which in different experiments ranged between 5 and 10 days of continuous exposure. Upon reaching the clinical, ethically approved, endpoint mice were euthanized, sacrificed and necropsied, to collect samples of tissues and blood. At least 30 minutes before euthanasia, the mice received an intraperitoneal injection of the hypoxyprobe reagent (pimonidazole; Hypoxyprobe, Burlington, MA) to label in vivo the hypoxic tissues. Blood was collected by cardiac puncture with a heparin coated syringe. Plasma was isolated by centrifugation at 2000 rpm for 10 minutes, aliquotted and frozen at –80^{o}C. Tumor tissue and organs were collected in 4% paraformaldehyde (PF; Cat. P6148, SIGMA, USA) in PBS.

2.3 Immunohistochemistry;

PF-fixed tumor tissue specimens were processed in ethanol, xylene, and paraffin embedded before 5 um sections were cut and mounted on slides. Sections were stained with antibodies against pimonidazole adducts (HP2-200KIT, HypoxyProbe Inc., Burlington, MA), carbonic anhydrase IX (CAIX; AF2344, R&D Systems, Minneapolis, MN), CD105 (AF1320, R&D Systems), alpha smooth muscle actin (aSMA; ab5694, Abcam, Cedarlene, Burlington, ON), CD45 (557235, BD/Pharmingen, Mississauga, ON), Gr-1 (553128, BD/Pharmingen), CD163 (3659-1, Epitomics/Cedarlane, Burlington, ON) and pSTAT3 (9145P, Cell Signalling Technology, Danvers, MA). AlexaFlour 488 was used as a secondary antibody to visualize CD105 staining and AlexaFlour 594 was similarly used to detect CAIX and aSMA (Life Technologies, Burlington, ON). Antibody concentrations were as recommended by the suppliers, or as otherwise indicated. Vector ABC kits for Rat (PK-6104, Vector Labs, Burlington, ON) were used for secondary detection of Gr-1 and CD45 antibodies whereas Vector ABC kit for Rabbit (PK-6101, Vector Labs) was used to detect p-STAT3 with 3,3' diaminobenzidine (DAB) as a colorimetric substrate (SK-4105, Vector Labs).

2.4 Morphometric Quantification of Changes in the Tumor Microenvironment

Each quantification was performed identically for control and sunitinib treated tumors, and in each age group. Thus, microvascular density (MVD) was determined by the survey of the entire cross section (slide) of every tumor under high magnification upon staining endothelial cells for CD105. Once the most highly vascularized area ('hot spot') in the section was established the total number of CD105-stained vascular structures was counted within one microscopic field at

the 100X magnification to obtain the initial MVD. For each tumor MVD was established for four independent tissue regions and the average MVD count was calculated and presented as a measure of vascular density, a surrogate of angiogenic activity. Blood vessel maturation was estimated by establishing endothelial-to-pericyte ratio (E:P). For this purpose sections were double stained for CD105 and aSMA and the entire slide was enumerated for CD105 positive vessels, excluding necrotic regions. Subsequently, vessel cross-sections that contained at least one adjacent aSMA-labeled cell (pericyte) were enumerated in the same field under 100X magnification. The total number of vessels to the number of vessels with adjacent aSMA-positive pericytes was calculated as E:P ratio. For CD45 and Gr-1 immunohistochemistry, the total tumor cross-sectional area of each tissue section was divided into equal fields fitting into a grid. The number of squares occupied by CD45 or Gr-1 positive cells was determined, as a percentage of the total tissue area. CD163 macrophages were evaluated in areas devoid of necrosis and calculated CD163+ cell number per surface area (cells/mm^2) in the entire cross-sections of viable tumor regions. For phospho-STAT3 (p-STAT3) and carbonic anhydrase IX (CAIX) staining the percentage of tissue stained positive for these markers was estimated in each microscopic field under 200X magnification. For p-STAT3 the whole tissue was scanned for positivity and the average area was calculated from all fields of the entire tumor tissue cross-section. The signal was corrected by subtracting minimal area of coloration observed in unstained control slides.

2.5 VEGFR2 ELISA

To explore whether ageing or sunitinib treatment provokes emission of VEGFR2, its main target, we performed the VEGFR2 Elisa (R&D Systems) using plasma samples, according to the protocol provided by the supplier.

2.6. Data analysis.

All assays were reproduced at least twice, with similar results. Whenever applicable, the statistical analyses were performed using Student t-test with the $p < 0.05$ value taken as significant.

2.7. Experiments involving animals.

The aforementioned experimentation with mice was conducted in accordance with the guidelines of the Canadian Council of Animal Care (CCAC) and while following the Animal Utilization Protocol (AUP) approved by the Faculty Animal Care Committee (FACC) at our Institution.

3. Results

3.1. Age-related responses of tumors to sunitinib.

Our previous study documented that the same types of cancer cells, including LLC, exhibit different growth and metastatic capacities, and different responses to metronomic chemotherapy when transplanted to aged and atherosclerotic mice, compared to their young counterparts used routinely in these kinds of studies (Klement, St Croix, Milsom, May, Guo, Yu, Klement, and Rak 2007;Meehan, Dombrovsky, Lau, Lai, Magnus, Montermini, and Rak 2013). This prompted us to examine whether an approved, clinically relevant and molecularly targeted antiangiogenic agent, such as sunitinib malate, would exhibit similar or differential activity against well characterized tumors in young versus aged mice.

As shown in Fig. 1 AB either LLC or related LL/2-luc2-M38 tumor cells were injected to young C57BL/6 wild type, or C57BL/6/ApoE-/- mice (4-12 weeks of age), or to their respective aged counterparts (age of 52 weeks or older). The drug was administered within a previously validated dose range (40 mg/kg, (Ebos et al. 2009), our unpublished observations) and the impact on tumor volume was calculated as a treated versus control ratio (T/C%). In this setting, we observed that while sunitinib was active in all experimental groups, the effect of the drug was somewhat more pronounced in old (vs young) mice whether wild type (ApoE+/+), or atherosclerosic (ApoE-/-) (Fig. 1AB). While these changes were not quantitatively dramatic they prompted us to examine the nature of sunitinib effects on tumor vascular milieu, especially in the human-like contexts of atherosclerosis-prone (ApoE-/-) mice.

3.2.Sunitinib exerts age-dependent effects on the tumor microvasculature.

Sunitinib exerts its effects mainly by targeting angiogenesis-regulating receptors (VEGFR1-3, PDGFR) expressed on endothelial cells and pericytes (Carmeliet and Jain 2011). Therefore, we first assessed the status of these respective cells in the tumor (LL/2-luc-M38 s.c.) microenvironment following drug exposure, and as a function of vascular ageing on the ApoE-/- background (Fig. 2). As expected a network of CD105+ endothelialized capillaries was readily detected in all tumor sections, and the density of these structures (MVD) was clearly reduced in both young and aged mice treated with the drug (Fig. 2AC). A somewhat greater ($p = 0.003$ vs $p = 0.04$) extent of this effect was observed in the old group, whose median MVD was lower than that of untreated controls and sunitinib treated young mice.

Interestingly, co-staining for endothelial cells and pericytes (CD105/aSMA) revealed that while in younger mice sunitinib exerted an expected effect by slightly reducing the pericyte coverage of endothelial structures (Mancuso et al. 2006;Bergers and Hanahan 2008), the opposite effect of the drug was noted in older mice. In this case the extent of pericyte association with endothelial cells was *a priori* lower than in young mice, and sunitinib exposure lead to an increase in this association (Fig. 2AC), as might be expected in the case of pronounced vessel normalization (Jain2001;Carmeliet and Jain 2011). Overall these observations point to quantitative and qualitative, age-dependent rearrangements in the vascular architecture under the influence of sunitinib therapy in this model.

3.3. Aging-related exacerbation of hypoxic response and VEGFR2 release induced by sunitinib.

In order to understand the functional consequences of the aforementioned responses we evaluated necrosis and hypoxia in tumor tissues of sunitinib-treated young and old mice (Fig. 3). As expected, antiangiogenic therapy resulted in the increase in necrotic area of tumors in both age groups, albeit somewhat more strongly in older mice (Fig. 3AB). We also used Hypoxyprobe assay that measures immunohistochemically the protein-pimonidazole adducts in cells that have experienced hypoxic conditions, and sunitinib exposure markedly increased this staining in both groups of mice. However, the median percentage of Hypoxyprobe reactive cells per field was notably greater in tumors of sunitinib-treated old ApoE-/- mice then in their younger controls (Fig. 3C). Similarly, the hypoxia-dependent expression of carbonyl anhydrase IX (CAIX) was induced by sunitinib therapy in all tumors (data not shown). Finally, we assessed the levels of soluble VEGFR2 in plasma and found it to be somewhat higher in old ApoE-/- mice. Interestingly, sunitinib triggered further VEGFR2 release which was also age related, but without statistical significance under conditions of this treatment (after several days of treatment; Fig. 3D).

3.4. Sunitinib therapy provokes recruitment of bone marrow derived cells to the tumor site.

Bone marrow-derived cells (BMDCs) are an integral part of angiogenesis regulation (De Palma M. and Naldini 2006), vascular repair, and resistance to antiangiogenic therapy (Phan, Wu, Cheng, Sheng, Chung, Zhuang, Tran, Song, Kowanetz, Sambrone, Tan, Meng, Jackson, Peale, Junttila, and Ferrara 2013). Since we previously reported a decline of such cells in the circulation of aged-atherosclerotic mice harbouring LLC tumors (Klement, St, Milsom, May, Guo, Yu,

Klement, and Rak 2007), we asked whether sunitinib-induced changes in the tumor microenvironment also include some of these cells. Indeed, LLC tumor sections can be readily decorated with antibodies against pan-leukocytic (CD45), myeloid (CD11b) and granulocytic (Gr1) markers (Figs. 4-6 and data not shown).

Notably, antiangiogenic therapy increases the tumor infiltration with both CD45+ (Fig. 4) and Gr1+ (Fig. 5) cellular populations resulting not only in their elevated presence, but also in a spatial shift from the periphery to the tumor centre (Figs. 4 and 5). These effects were somewhat less pronounced in aged mice versus their younger counterparts (Fig 4C and Fig. 5C), which is not surprising, as these mice would be more likely to exhibit bone marrow exhaustion and possibly reduced ability to counteract drug-related microvascular damage (Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007;Phan, Wu, Cheng, Sheng, Chung, Zhuang, Tran, Song, Kowanetz, Sambrone, Tan, Meng, Jackson, Peale, Junttila, and Ferrara 2013).

3.5. Age-related functional effects of sunitinib on the inflammatory tumor microenvironment.

The mere presence of BMDCs within the microenvironment of LLC tumors, especially upon sunitinib treatment, does not define their functional state. To gain more insights into this question we examined the prevalence of tumor-promoting M2 phenotype among macrophage-like tumor infiltrating cells (Mantovani and Locati 2013). While the full characterization of these cells is relatively complex (Pollard2004) the expression of CD163 marker is often used as a simplified measure to detect such macrophage polarization (Komohara et al. 2013). In this regard quantification of the intensity of CD163+ cellular infiltration per high power field (in areas

where such cells were found) resulted in counts that were markedly lower in aged mice relative to young ApoE-/- controls, in both sunitinib-treated and untreated groups (Fig. 6AB). Interestingly, unlike in young mice sunitinib exposure was unable to efficiently trigger increases in CD163+ counts in old ApoE-/- mice, a circumstance that may relate to more pronounced tumor growth suppression in these animals (Fig 1).

As a surrogate of the pro-inflammatory microenvironment we have also examined the status of STAT3 phosphorylation in tumor tissues, as a function of age and sunitinib treatment (Fig. 6CD). Cells expressing phosphorylated STAT3 (pSTAT3) were more abundant in areas surrounding necrosis, and their densities understandably increased upon sunitinib treatment, but only in young mice. In contrast, tumors in old mice exhibited markedly lower numbers of pSTAT3 positive cells and sunitinib treatment paradoxically decreased this signal even further. Overall these observations suggest that ageing of atherosclerosis-prone mice results in decline in their ability to mobilize inflammatory features, such as M2 phenotype and pSTAT expression in the context of antiangiogenic therapy. It could be speculated that interference with this pathway (or its cytokine inducers) could increase the tumor sensitivity to VEGF blockade in young hosts.

4. Discussion

In humans, vascular ageing is inseparable from diffuse vascular changes associated with atherosclerosis (Lusis2012). This condition is not recapitulated in standard mouse experiments and we have previously characterized C57BL/6 ApoE-/- mouse strain as an informative, human-like model of age-related vascular changes, which impact tumor progression (Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007). Using this approach we interrogated the effects of sunitinib, an antiangiogenic agent given to patients with metastatic cancers, essentially irrespectively of their vascular ageing status (Vazquez, Leon, Fernandez, Lazaro, Grande, and Aparicio 2012;Escudier, Albiges, and Sonpavde 2013;Hutson et al. 2014).

In this regard we made several new observations. First, we noted that old and atherosclerotic mice exhibit a somewhat more pronounced response to a standardized course of sunitinib therapy in terms of the overall changes in tumor volume. Second, this effect was associated with age-dependent differences in the tumor microenvironment, especially responses of the tumor vasculature to the drug, and the resulting extent of hypoxia. Third, we observed that sunitinib-induced inflammatory infiltration occurred in both young and aged mice, but their age status influenced the activity and phenotype of infiltrating cells, as indicated by differences in the content of CD163+ macrophages and pSTAT3 staining.

Prior clinical and experimental studies provided multiple indications as to the role of ageing in cancer biology and progression (Ershler, Socinski, and Greene 1983;Ershler et al. 1984;Pili, Guo, Chang, Nakanishi, Martin, and Passaniti 1994;Rivard, Fabre, Silver, Chen, Murohara,

Kearney, Magner, Asahara, and Isner 1999;Zhu, Iurlaro, MacIntyre, Fogel, and Nicosia 2003;Balducci and Ershler 2005;Kaptzan et al. 2006), including our own efforts (Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007;Rak, Milsom, and Yu 2008;Meehan, Appu, St, Rak-Poznanska, Klotz, and Rak 2011;Meehan, Dombrovsky, Lau, Lai, Magnus, Montermini, and Rak 2013). One of the outstanding challenges in this regard is to understand the implications of these events for the effects of specific types of anticancer therapies. Indeed, it is possible that the very biology of drug interactions with their cellular targets, biodistribution, evasive resistance, tumor repopulation, stemness and other relevant processes may be affected by ageing in ways that are presently obscured, or inaccessible experimentally, or unexplored in the available clinical datasets (Rak, Milsom, and Yu 2008).

Sunitinib is a powerful and effective drug (Vazquez, Leon, Fernandez, Lazaro, Grande, and Aparicio 2012), currently approved for several indications, but also controversial. Experimentally, the exposure to this and other VEGF inhibitors is thought to carry a risk of increased tumor invasiveness and micrometastasis, while its effects in the clinic are usually transient and exhibit some inter-individual variability (Paez-Ribes et al. 2009;Ebos and Kerbel 2011;Escudier, Albiges, and Sonpavde 2013). In spite of substantial experience with these agents in the clinic, their biology is still poorly understood, and the effects of vascular ageing have not been studied in mechanistic detail.

Ageing is presently seen merely as a source of additional side effects and limitations in the context of antiangiogenic therapeutics, and the clinical activity of VEGF inhibitors is thought to

be retained in both younger and older patients (Pal, Hsu, Hsu, Hu, Bergerot, Carmichael, Saikia, Liu, Lau, Twardowski, Figlin, and Yuh 2013;Escudier, Albiges, and Sonpavde 2013; Killock2014). This is enforced by comparable clinical efficacy of these drugs at the late age cut-off, typical for major indications such as in mccRCC patients (Killock2014). However, this perception does not account for several age-related changes already noted in the tumor microcirculation. For example, we observed that tumor tissues in the case of older ccRCC patients exhibit higher MVD scores and changes in expression of important molecular markers such as nitric oxide synthase (eNOS), in spite of comparable tumor sizes between both groups (Meehan, Appu, St, Rak-Poznanska, Klotz, and Rak 2011). Similarly, we have documented that age may change the nature, but not necessarily the magnitude of experimental metastasis (Meehan, Dombrovsky, Lau, Lai, Magnus, Montermini, and Rak 2013). Finally, in the present study, multiple and dramatic qualitative changes in the tumor interior accompany a somewhat greater clinical response of experimental LCC to sunitinib therapy. This is important as the nature of biological changes occurring in the tumor microenvironment may have bearing on mechanisms of therapeutic resistance and ways to circumvent them in the long run (Rak and Kerbel 1996;Rak, Milsom, and Yu 2008;Bergers and Hanahan 2008).

Arguably, ageing is not a factor that can be accurately captured by the calendar age, but instead occurs heterogeneously in the human population. It is of note that co-morbidities traditionally associated with ageing such as hyperlipidemia, hypertension, obesity, metabolic syndrome or diabetes are increasingly recognized for their impact on the vascular system and tumor microcirculation. These conditions also occur earlier in life (accelerated vascular ageing) with conceivable consequences for cancer incidence, biology and treatment (Klement, St Croix,

Milsom, May, Guo, Yu, Klement, and Rak 2007; Avraham-Davidi et al. 2012; Okwan-Duodu et al. 2013; Sharma et al. 2014). Indeed, the existence of time-dependent, but individually variable and complex contributions of biological processes associated with ageing may be important to take into account in the context of precision (individualized) cancer care, which is currently dominated by genetic considerations. We postulate that atherosclerosis and other manifestations of the vascular ageing could impact cancer progression and therapy in multiple ways, even through selection of aggressive cellular populations (Meehan, Dombrovsky, Lau, Lai, Magnus, Montermini, and Rak 2013;Magnus et al. 2014). It should also be noted that vascular ageing, comorbidities and cancer may also be affected by gender and other factors that were not tested in the present manuscript, but deserve future attention.

Given the prevalence, mechanisms and age-dependence of atherosclerosis surprisingly little is known about the role of this process in cancer, which (implicitly) coincides with atherosclerosis in the elderly and adult patient population. It is also surprising that the effects of anticancer therapies, especially those targeting vascular and inflammatory pathways (antiangiogenic, anti-vascular and anti-inflammatory agents), are rarely considered in the context of ageing and age-related vascular comorbidities, such as atherosclerosis or thrombosis (Rak, Milsom, and Yu 2008;Emmerechts et al. 2012). This disconnect is especially striking since inflammatory cytokines, such as interleukins 6 and 8 (IL-6, IL-8), are among the most consistent predictors of resistance to antiangiogenic agents and poor prognosis (Jain et al. 2009;Tran et al. 2012). These factors are also hallmarks of atherosclerosis (Kleemann et al. 2008;Garrido-Urbani et al. 2014).

Thus far, only a handful of studies explored the biological effects of antiangiogenic agents in cancer, as a function of vascular ageing (Kaptzan, Skutelsky, Itzhaki, Sinai, Huszar, Siegal, Ben-Zvi, Jossiphov, Michowitz, Schiby, and Leibovici 2006;Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007). In this regard, our present study intended to explore this question in relation to a molecularly targeted, well characterized and clinically approved drug. Our results suggest that even modest, age-related differences in tumor volume following therapy with sunitinib, may mask disproportionately greater, quantitative and qualitative rearrangements within tumor stroma, microcirculation, perfusion and inflammatory infiltration. While the mechanisms of these changes are not entirely clear, we propose that they may reflect ageing processes occurring at multiple levels. Those may include peripheral vasculature, but also bone marrow, cytokine networks, cellular recruitment mechanisms and other systemic responses to antiangiogenesis (De Palma M. and Naldini 2006;Shaked et al. 2006;Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007).

Overall, our study provides novel experimental insights into the role of vascular ageing and atherosclerosis in relation to tumor responses to sunitinib. If the changes in tumor microenvironment we describe are also found in patients receiving similar treatments new adjunctive therapies could be envisaged to maximize the efficacy and avoid undesirable effects of blood vessel targeting agents.

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Conflict of Interest. The authors declare no conflict of interest

Figure legends.

Figure 1. Differential anti-tumor effects of sunitinib in young and old mice. Analysis of two different strains of mice and two different sublines of the LLC-related transplantable cancer model document a preferential tumor growth inhibition in old mice upon treatment with the antiangiogenic agent, sunitinib malate. T/C% reflects the average relative size ratio between tumors in treated (T) and control (C) groups at the endpoints indicated, respectively. In both cases mice were treated orally with 40 mg/kg/day of sunitinib for durations indicated. **A**. Non-atherosclerotic C57Bl/6 mice were used as LLC tumor recipients either at the young age ("young") of 6-12 weeks or when older (over 52 weeks; "old"). The T/C% score was calculated at day 14 of the experiment; the numbers of mice with tumors in the respective groups were young-C (5), young-T (3), old-C (4) and old-T (5), and the SD was calculated for tumor volumes in individual treated mice per average tumor size in control; * - statistically significant at $p < 0.05$. **B.** Atherosclerosis-prone C57Bl/6/ApoE-/- mice were used as recipients of the LL/2-luc-M38 tumor cell line and used either at the younger age ("young"; 4-12 weeks, hyperlimidemia without pronounced atherosclerosis) or when older ("old" 52-78 weeks, with high penetrance of vascular atherosclerosis (Klement, St, Milsom, May, Guo, Yu, Klement, and Rak 2007)). Sunitinib was administered for 7 days and T/C% score was calculated at day 21 of the experiment; the numbers of mice with tumors in the respective groups were young-C (7), young-T (8), old-C (7) and old-T (9), and the SD and p values were calculated as above.

Figure 2. Impact of sunitinib on the density and pericyte coverage of the tumor microvasculature. A-B. Staining for tumor blood vessel markers in sections of LL/2-luc-M38 tumors: top panels: endothelial cells - CD105 (green), pericytes – aSMA (red), cell nuclei – DAPI (blue), Bar = 50 μm. **C-D.** Quantification of tumor blood vessel responses to sunitinib treatment: microvascular density (MVD) counts per high power field (C) and endothelial cell to pericyte ratio E:P (D), details in methods; p values as indicated.

Figure 3. Age-related exacerbation of tumor hypoxia and VEGFR2 release in sunitinib-treated mice. A/C. Pimonidazole staining reveals differential extent of tumor (LL/2-luc-M38) hypoxia in sunitinib treated and control, young and old ApoE-/- mice. **B.** Tumor necrosis evaluated upon DAPI staining (as in A) staining and under fluorescent microscopy at 100 X magnification (percentage area of necrosis in the whole tumor tissue section). **C.** Tumor hypoxia - Hypoxyprobe reagent (pimonidazole) was injected intraperitoneally 30 minutes before euthanasia and tumor sections were stained with the antibody directed at pimonidazole adducts, to establish the percentage of positive (hypoxic) cells per tissue section (A and C); bar = 50 μm. **D.** Circulating soluble mouse VEGF2 in plasma of tumor bearing young and old ApoE-/- mice.

Figure 4. Tumor infiltration with CD45+ bone marrow derived cells following the exposure to sunitinib. A-D. Overview of CD45 staining of LL/2-luc-M38 tumors viewed under 12.5 X magnification in young (A/C), and old (B/D) ApoE-/- mice under control conditions (PBS; A-B) or following treatment with sunitinib (C-D); *Bar = 100 μm (A-D).* **E.** Quantification of CD45 positive cells (% in total tissue); **F.** High power image of CD45 staining; *Bar = 20μm.*

Figure 5. Tumor infiltration with GR1+ granulocytic cells following the exposure to sunitinib. A-D. Overview of GR1 staining of LL/2-luc-M38 tumors viewed under 12.5 X magnification in young (A/C), and old (B/D) ApoE-/- mice under control conditions (PBS; A-B) or following treatment with sunitinib (C-D). **E.** Quantification of GR1 positive cells (% in total tissue); **F.** High power image of CD45 staining; *Size bars as indicated: A-C - 200μm; D - 500μm; F - 50μm.*

Figure 6. Age-related pro-inflammatory changes in the tumor microenvironment following treatment with suninib. A-B. Differential influx of CD163 (M2-like) cells into control and sunitinib treated tumors (LL/2-luc-M38) in either young or old ApoE-/- mice. **C-D**. Changes in phospho-STAT3 staining in young correlate with age and sunitinib treatment in LL/2-luc-M38 tumor recipients.

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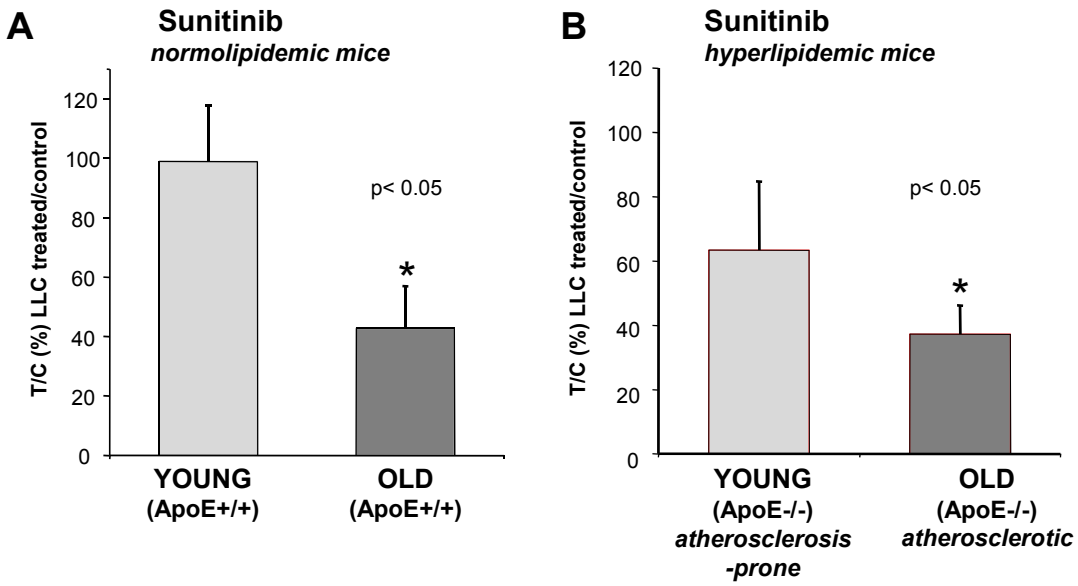

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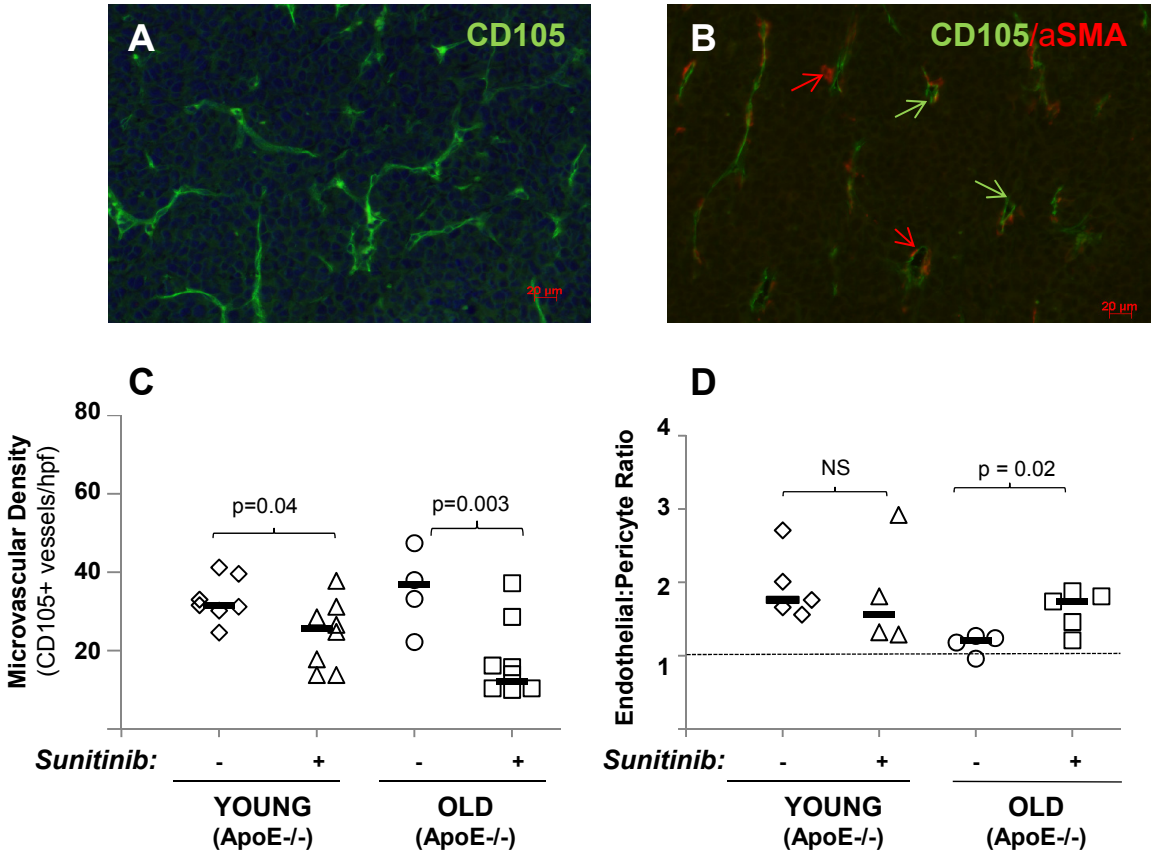

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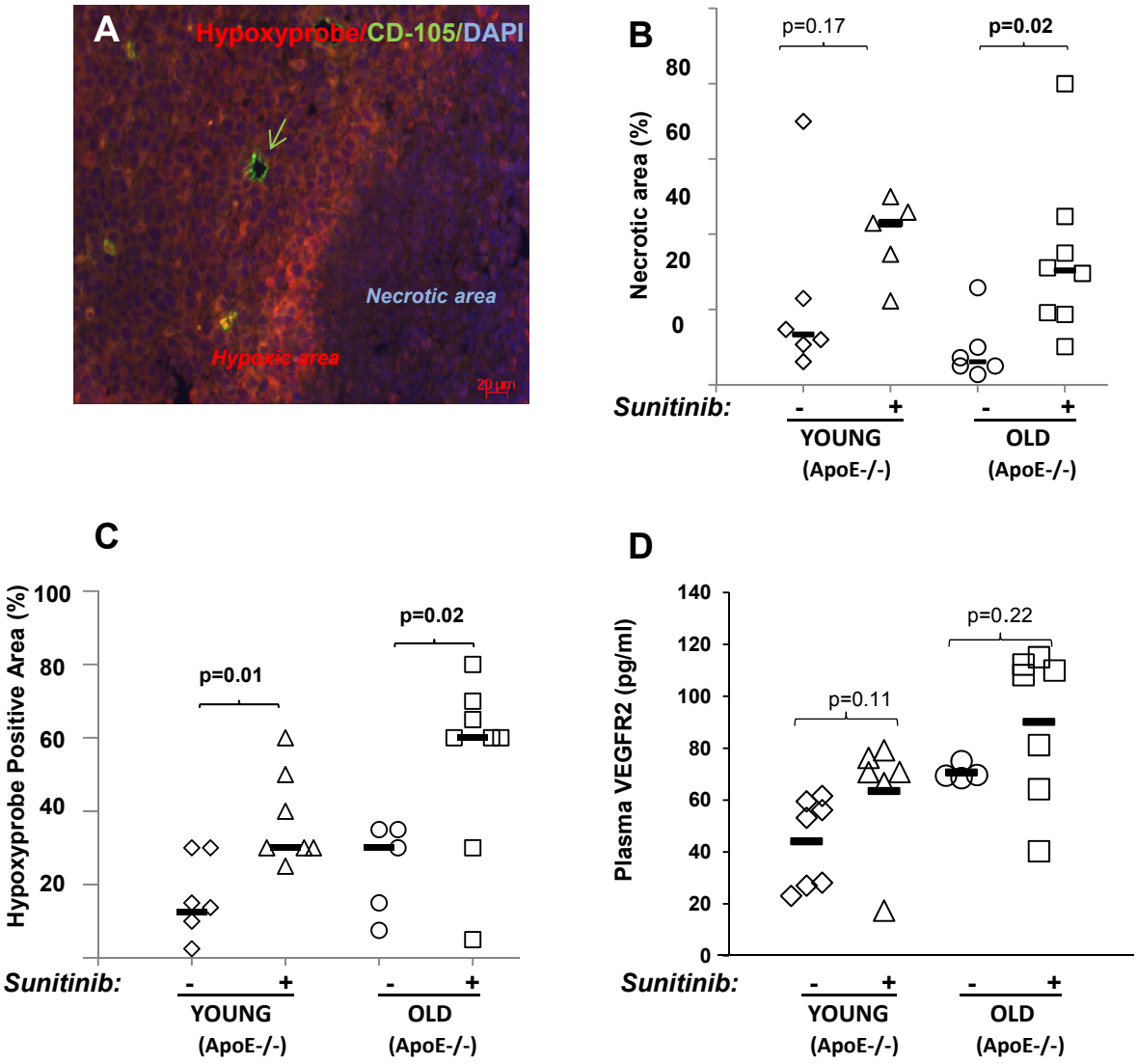

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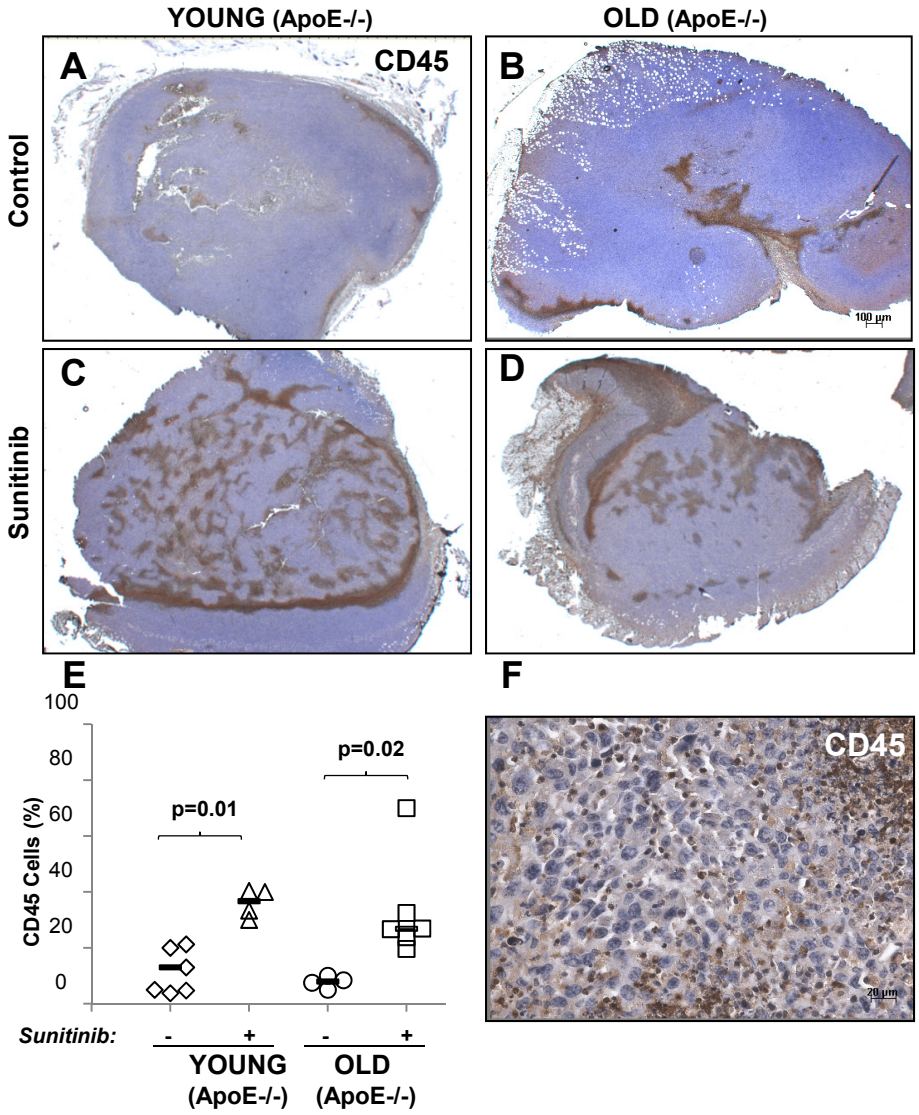

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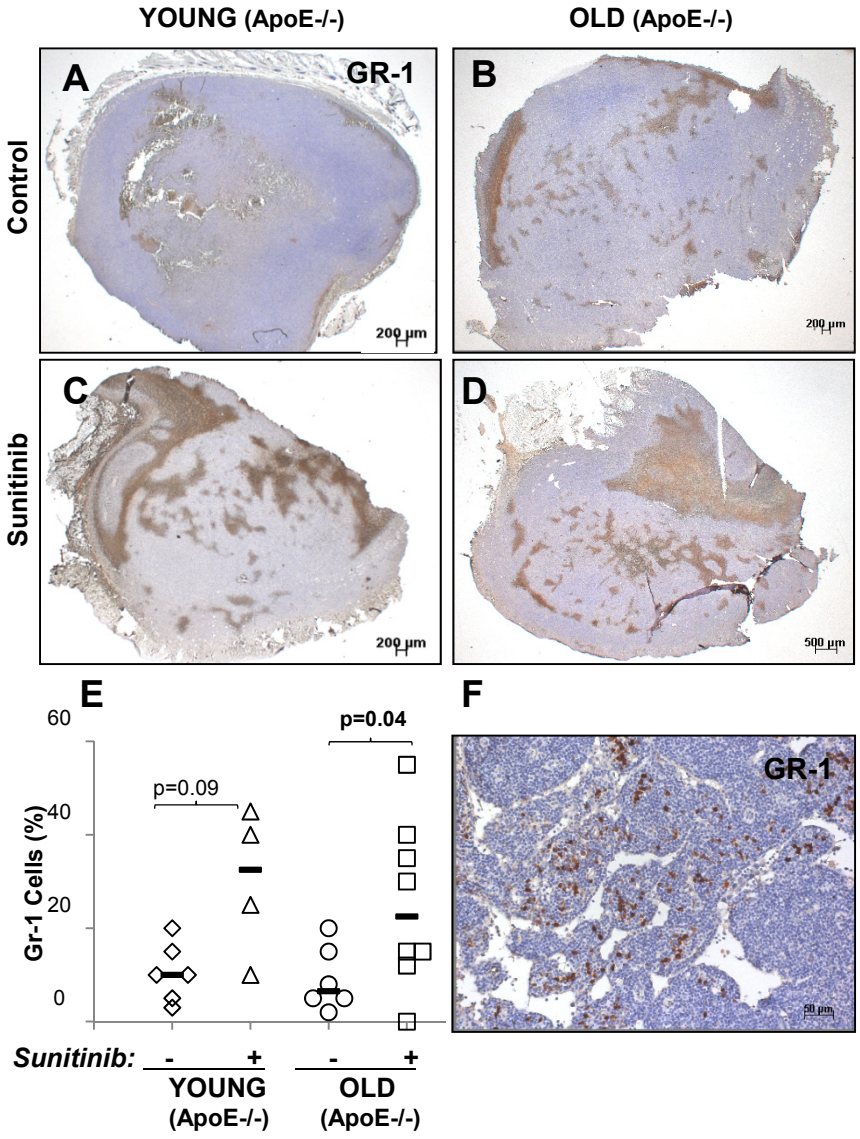

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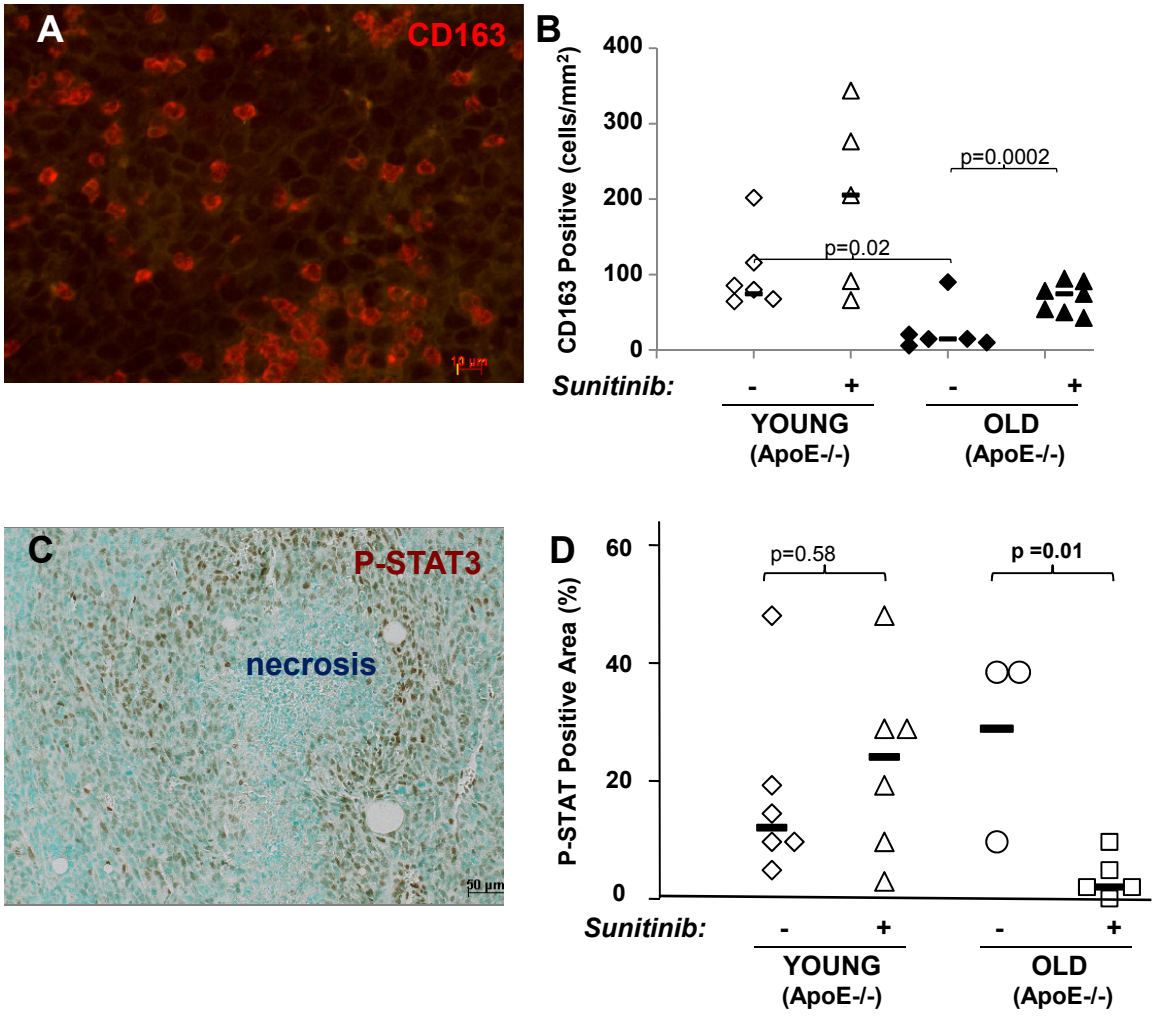

Meehan et al - Figure 6