

## CLINICAL SCIENCE

# Vascular endothelial growth factor is elevated in ocular fluids of eyes harbouring uveal melanoma: identification of a potential therapeutic window

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**Background:** Improved local treatment of uveal melanoma makes it possible for many patients to retain the affected eye, but a proportion will develop secondary complications such as neovascularisation of the iris (NVI) and require enucleation. Although vascular endothelial growth factor A (VEGF-A) is known to correlate with NVI and can cause NVI in experimental models, this pro-angiogenic cytokine is consistently reported to be absent in uveal melanoma. Novel anti-VEGF therapies are now in clinical trial, and the authors therefore wished to determine whether VEGF-A was indeed elevated in melanoma bearing eyes.

**Methods:** VEGF-A concentrations were measured in aqueous and vitreous from 19 and 30 enucleated eyes respectively.

**Results:** Elevated VEGF-A concentrations (up to 21.6 ng/ml) were found in melanoma bearing eyes compared with samples from patients undergoing routine cataract extraction (all had values below 0.96 ng/ml). Immunohistochemistry showed VEGF-A protein in the iris and/or ciliary body of 54% and basic fibroblast growth factor (bFGF) in 82% of the eyes examined. VEGF was found to a limited extent and at very low levels in only 9% of these tumours. Aqueous or vitreous VEGF levels showed no apparent correlation with retinal detachment, tumour size, vascularity, or immunohistochemistry. Though limited in number, the highest VEGF levels correlated with previous radiation therapy, and with the presence neovascularisation of the iris or optic nerve head. bFGF was not significantly elevated in ocular fluids: it is known to be a pro-angiogenic agent and was detected in the majority of primary uveal melanomas.

**Conclusion:** Based on this study, though the source of VEGF within eyes harbouring uveal melanoma is not clear, these data suggest that anti-VEGF therapy might prove useful in the management of some patients with NVI secondary to uveal melanoma.

Uveal melanoma is a rare tumour with a poor prognosis both in terms of local morbidity and high rates of mortality following systemic spread.<sup>1,2</sup> Enucleation of the eye is recommended to reduce the likelihood of metastases in the presence of large tumour mass and is often additionally required owing to the development of iris neovascularisation (NVI) and painful neovascular glaucoma (NVG).<sup>3–5</sup> Enucleation rates caused by NVI are reported to increase following brachytherapy or external beam radiation.<sup>6,7</sup>

Angiogenesis, the growth of new blood vessels from a pre-existent vascular bed, underlies both tumour growth and iris neovascularisation and is believed to result from an imbalance of pro-angiogenic and anti-angiogenic stimuli. Neovascular eye diseases such as retinopathy of prematurity, diabetes mellitus, and age related macular degeneration are associated with pro-angiogenic growth factors such as vascular endothelial growth factor A (VEGF-A) and basic fibroblast growth factor (bFGF). VEGF-A is generally reported to be absent within uveal melanoma,<sup>8–10</sup> while bFGF has only recently been described (Boyd *et al*, see accompanying paper).<sup>10</sup> We asked whether these two growth factors could be found in the ocular fluid of patients with uveal melanoma and whether these growth factors could be detected within other (that is, non-tumour) structures of the eye in the presence of uveal melanoma. Identification of such pro-angiogenic growth factors in the presence of uveal melanoma may provide an opportunity for therapeutic intervention, particularly in the period immediately following radiation therapy, which could reduce the incidence of enucleation.

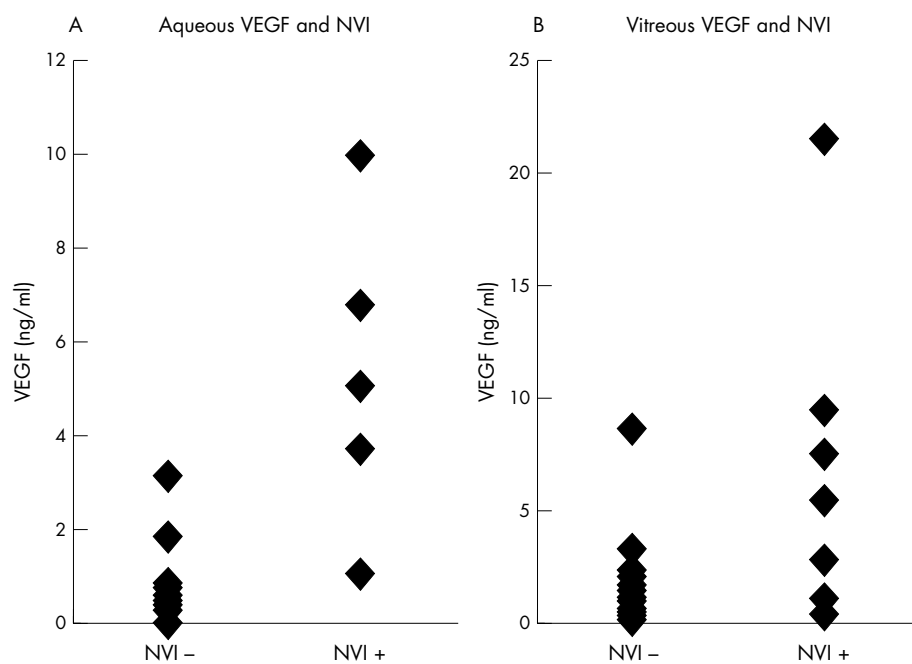
## MATERIALS AND METHODS

### Melanoma eyes

Vitreous and/or aqueous samples were obtained from 30 eyes with uveal melanoma. Patients (n=30) had a median age of 63 (range 39–85) years with a male:female ratio of 14:16. Six patients had received previous radiation therapy (two proton beam, four plaque irradiation). The largest tumour diameter (LTD) varied from 4 to 24 mm (median 13 mm). Twenty four tumours exclusively involved the choroid, three had both ciliary body and choroidal involvement, one had ciliary body and iris involvement, one was located exclusively within the ciliary body, and one entirely within the iris. Eight patients had NVI and 21 had retinal detachment with subretinal fluid.

Following surgical enucleation, whole eyes were placed in DMEM cell culture medium with antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin (Sigma Chemical Co, Dorset, UK) and processed under sterile conditions by an ophthalmic pathologist within 15–90 minutes. Eyes were oriented on a sterile metal eye cup and the position of the tumour localised by transillumination. Up to 600 µl of vitreous and

**Abbreviations:** bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; NVG, neovascular glaucoma; LTD, largest tumour diameter; NVI, neovascularisation of the iris; PCR, polymerase chain reaction; PEDF, pigment epithelium derived factor; RT-PCR, reverse transcriptase polymerase chain reaction; TBS, TRIS buffered saline; VEGF, vascular endothelial growth factor



**Figure 1** VEGF levels in (A) aqueous and (B) vitreous, showing high levels in most patients with NVI.

aqueous were removed by 19 and 23 gauge needles respectively and eyes then cut anteroposteriorly through the tumour. The main block containing the bulk of the tumour, cornea, and optic nerve was placed immediately into buffered 4% formaldehyde. Material not required for diagnostic histopathology was used for analysis. Ethics committee approval was granted for the experimental use of tissue not required for diagnosis, in accordance with the consent form signed by each patient prior to enucleation. The vitreous and aqueous samples were used for ELISA studies, while spare sections were cut from the main AP block following the completion of the histopathology report for immunohistochemistry. The results of immunohistochemistry on the tumour itself are reported elsewhere (Boyd *et al*, see accompanying paper, p 440).

### ELISA

ELISAs for VEGF-A and bFGF were performed using commercially available kits (R&D Systems, Abingdon, Oxford, UK) according to the manufacturer's instructions. Vitreous fluid was analysed from 30 specimens, and paired aqueous/vitreous samples from 19. The limit of detection for VEGF-A and bFGF was 5 pg/ml and 4 pg/ml respectively. In order to analyse samples in triplicate and simultaneously preserve sample, ocular fluids were diluted at ratios of 1:4 to 1:100 as necessary. Owing to small specimen volumes, only eight were examined for bFGF. Control specimens were analysed from patients undergoing uncomplicated cataract extraction (n = 16).

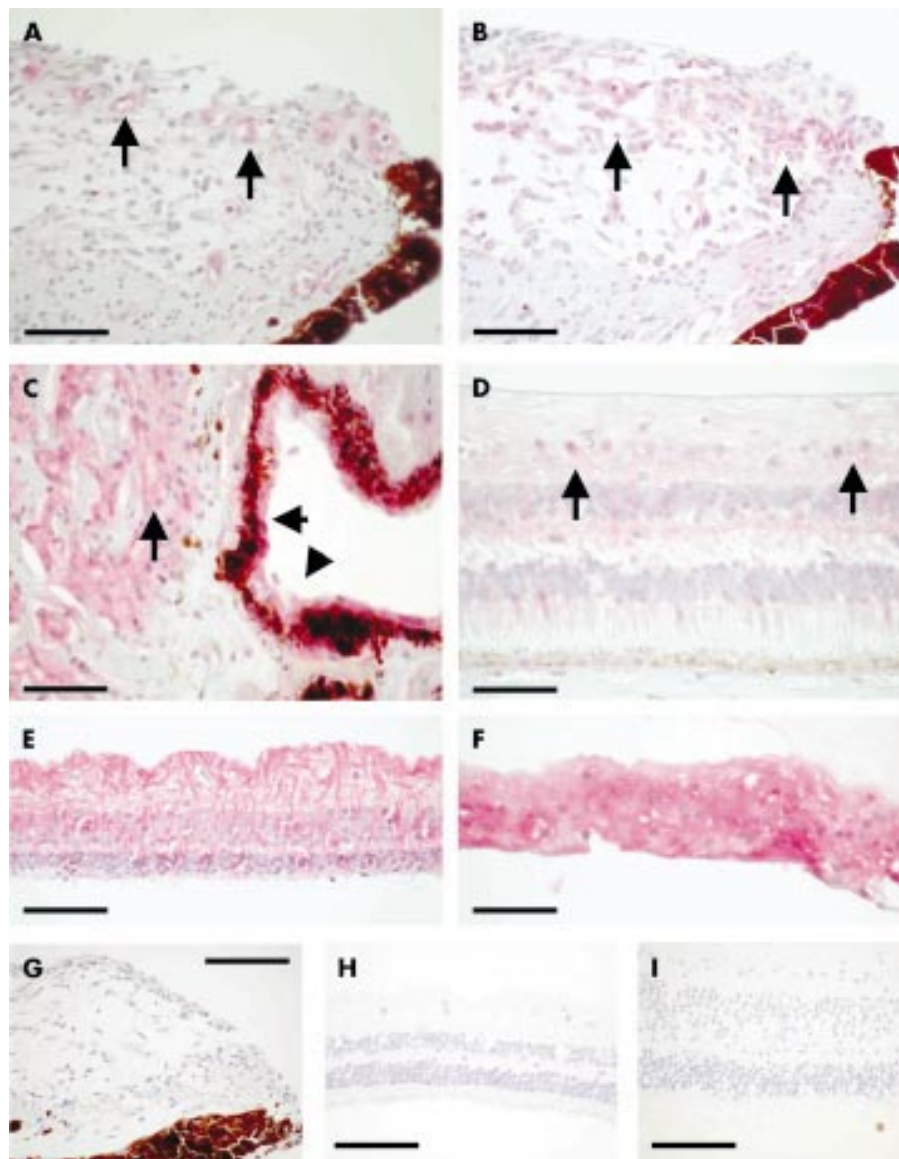
### Immunohistochemistry

Paraffin processed sections (6 µm) of the eye were assessed for VEGF-A and bFGF by immunohistochemistry using an avidin-biotin immuno-alkaline phosphatase method as previously published.<sup>11</sup> Briefly, sections were de-waxed in xylene, rehydrated through a series of alcohols, and washed in water. Sections were immunostained for VEGF-A using an affinity purified rabbit IgG polyclonal antibody (SC-152, Santa Cruz, Autogen Bioclear UK Ltd, Calne, Wilts, UK) at a 1:75 dilution. For this antibody, antigen retrieval was performed as described by Sheidow *et al*.<sup>9</sup> Sections were placed in a microwave in citrate buffer pH 6.0 for 3.5 minutes on high power and 10 minutes on medium power, then 0.015% trypsin at 37°C for 45 minutes. Polyclonal anti-bFGF was obtained

from Calbiochem/Oncogene Research Products (PC194L, Nottingham, UK) and used at a 1:20 dilution following trypsinisation of the sections for 15 minutes. A polyclonal antibody to factor VIII related antigen (von Willebrand factor, M616, Dako Ltd, High Wycombe, UK) was used at 1:500 following 30 minutes of trypsinisation. Following repeated washing in TRIS buffered saline, pH 7.6, (TBS) for 10 minutes, non-specific antibody binding was blocked by 0.1% bovine serum albumin (BSA) in TBS for 25 minutes. Primary incubations were performed for 1 hour at room temperature in a humidified chamber. Slides were washed three times in TBS and the second antibody added. In all cases, this was a biotinylated multilink antibody (Dako) used at 1 in 300 dilution in TBS for 45 minutes. After washing, sections were incubated with a tertiary streptavidin-alkaline phosphatase reagent (Dako). The sections were again washed in TBS and incubated in Vector Red for 15 minutes according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK), washed, and lightly counterstained with Mayer's haematoxylin for 5 seconds. Sections were viewed by direct microscopy and the positivity of the iris and ciliary body assessed qualitatively after ranking the specimens in order of greatest staining.<sup>12, 13</sup> Immunohistochemical staining was graded as 0 (no staining), 1 (weak staining of >50% cells), 2 (intermediate staining of >50% cells), and 3 (strong staining of >50% of cells). Specificity of staining was determined by competition with soluble recombinant cytokines (R&D Systems, each at up to 1 µg/ml) in three positive tumours with each antibody (data not shown).

### Data analysis

Clinical and histological data for each patient were derived from the clinical notes and pathological description of the enucleated eye. The Wilcoxon signed rank test was used to compare aqueous VEGF with vitreous VEGF levels in the same eyes (n = 19). The rank sum test was used to compare aqueous VEGF in the melanoma eyes with the control eyes. Spearman's rank correlation coefficient was used to assess correlation between aqueous VEGF and LTD. Subsequent analysis was conducted by dividing VEGF levels into high (>1 ng/ml) and low. This was based on statistical calculation of the mean (+SD) (0.97 pg/ml) and also on the physiological threshold for NVI previously determined in CRVO: both these values are



**Figure 2** (A) New vessels (arrows) close to the anterior surface of the pupillary margin of the iris in an eye with NVI (von Willebrand factor), and (B) the same eye showing bFGF expression in some of the stromal cells (arrows). (C) Positivity of the ciliary body epithelium (arrowheads) and smooth muscle (arrow) for VEGF-A. (D) Attached retina adjacent to uveal melanoma showing mild positivity for VEGF-A, with strong positivity in ganglion cells. (E) Strong VEGF-A positivity in detached retina with considerable atrophy. (F) bFGF expression in atrophic retina. Artefactual detachment of the retina is easily determined histologically by the presence of pigment granules in retinal pigment epithelium processes stripped with the retina forming a line along the base of the photoreceptor outer segments: the detachments shown in both (E) and (F) were tumour related. (G–I) Negative controls for von Willebrand factor, VEGF-A, and bFGF respectively. (All original magnifications  $\times 400$  unless otherwise stated: scale bars = 100  $\mu\text{m}$ .)

approximately 1 ng/ml. Fisher's exact test was then used to assess association between the dichotomised VEGF levels and other categorical study factors, such as the presence or absence of NVI or retinal detachment.

## RESULTS

### Vitreous and aqueous ELISAs

Aqueous VEGF-A concentrations ranged from undetectable to 10 ng/ml (median 0.80 ng/ml); vitreous levels ranged from undetectable to 21.6 ng/ml (median 0.75 ng/ml) (Fig 1). Although aqueous concentrations of VEGF were lower than vitreous concentrations in paired samples, median values of vitreous and aqueous VEGF-A levels were not statistically different ( $p = 0.13$ ); bFGF levels were low, detectable only in five of eight samples tested (median 37 pg/ml, range 0–137 pg/ml).

Aqueous samples from a series of 16 eyes undergoing cataract surgery (median 0.17 ng/ml, range 0.05–0.96 ng/ml), were

compared for VEGF-A levels with melanoma containing eyes and were found to be statistically significantly higher in the melanoma eyes (rank sum test,  $p < 0.05$ ). Samples from melanoma containing eyes were above the threshold of 1 ng/ml in seven cases (Fisher's exact test:  $p = 0.002$ ), and six out of seven of which showed neovascularisation (five of the iris, one of the optic disc).

### Immunohistochemistry

VEGF-A was detected within anterior structures of the eye, particularly the ciliary body (CB) and iris (Fig 2) in 15/28 (54%) eyes. This staining was found diffusely within the stroma or, often, in a perivascular distribution and was not limited to regions of NVI. Specific staining was competitively eliminated with excess recombinant cytokine (R&D Systems), performed in a subset of specimens. bFGF staining was found in ciliary body and iris in 23/28 (82%) of these eyes, in a more

diffuse pattern than VEGF-A (Fig 2). The retina was often detached, with overlying subretinal fluid, and atrophic. Grading of immunostaining was difficult because of patchy and sometimes non-specific staining of the atrophic tissue. Nevertheless, there was convincing evidence in some eyes that retinal tissue could express both VEGF and bFGF (Fig 2). Retinal immunohistochemistry was deemed assessable in 24 tumours, with nine (38%) showing VEGF positivity and 21 (88%) showing bFGF positivity. Immunohistochemistry for 28 tumours out of the 30 eyes investigated in this series are reported elsewhere as part of a larger series (Boyd *et al*, see accompanying paper). For the subset studied here, two tumours (7%) showed weak VEGF positivity, while 25 (92%) were positive for bFGF.

### Correlation of vitreous and aqueous VEGF-A levels with clinicopathological parameters

We compared aqueous and vitreous level of VEGF against the following clinical or pathological parameters: presence or absence of neovascularisation, previous tumour treatment, extent of retinal detachment, tumour size, tumour vessel density, and anterior segment immunohistochemistry. Owing to the small numbers, similar analysis was not possible for bFGF. Among those eyes with the seven highest VEGF concentrations, six had neovascularisation (five with NVI, one with neovascularisation of the disc). The single eye with high VEGF but no neovascularisation had no unique features we could determine. By contrast, a single eye was determined to have NVI but a low level of VEGF (0.3 ng/ml). This eye had a large tumour located directly adjacent to the iris in the ciliary body. Of the 30 tumours included in this study, 24 were treated by primary enucleation alone and six had received previous irradiation before enucleation. The numbers in the irradiated and non-irradiated groups are too small for statistical analysis, but it is interesting to note that the VEGF-A levels in vitreous were among the highest values (1.6–21.6 ng/ml) in five of six irradiated samples, and include the highest value measured. This compares with a median VEGF-A level in the non-irradiated specimens of 0.4 ng/ml. Though retinal detachment was reported to be present either clinically or histopathologically in 21 patients, the VEGF-A values in aqueous or vitreous were not statistically higher than those without detachment; however, most detached retinas were atrophic. No relation was noted between the level or pattern of immunostaining for VEGF-A in the iris/CB and VEGF-A levels in aqueous and vitreous, or with NVI.

No correlation was observed between VEGF-A levels and the largest tumour diameter or vessel density. There was also no correlation between fluid levels of VEGF and immunostaining in the anterior segment. There were too few tumours involving the ciliary body to determine whether tumour proximity influenced iris neovascularisation.

## DISCUSSION

Enucleation of the globe is a psychologically stressful event for a significant proportion of patients with uveal melanoma. Though often recommended to reduce the risk of metastases, enucleation is frequently also performed in response to the development of iris neovascularisation and consequent glaucoma, which is reported to occur at higher frequency following radiation therapy.<sup>3-7</sup> The pro-angiogenic stimulus for NVI in the context of treated and untreated uveal melanoma is not known, although VEGF-A is known to be of particular importance in NVI due to other causes.<sup>14</sup> VEGF-A is a major pro-angiogenic cytokine found in association with numerous tumour types,<sup>15</sup> but uveal melanoma is generally described as being devoid or showing low expression of VEGF-A.<sup>8 9 16-18</sup>

Our data show that VEGF-A concentrations are elevated in the ocular fluids of eyes harbouring treated and untreated

uveal melanoma compared with eyes undergoing uncomplicated cataract surgery. In a study of human ischaemic central retinal vein occlusion (CRVO),<sup>14</sup> we demonstrated that the threshold concentration at which NVI occurs is approximately 1 ng/ml in aqueous fluid. In this study, 7/19 (37%) eyes had aqueous VEGF-A concentrations in excess of this value. It is interesting to speculate that the threshold for NVI determined in ischaemic CRVO is due mainly or exclusively to the ischaemic drive, whereas in the presence of a tumour, the driving force may be more complex. In the oncological setting, in addition to a hypoxic component, there may be a group of pro-angiogenic and anti-angiogenic molecules produced by the tumour. It follows that the threshold for neovascularisation may not be the same. None the less, on reviewing our data we determined that a "statistical" threshold could be set by using the mean VEGF-A level (+2 SD) and that this gave a threshold value of just under 1 ng/ml, and is similar to the threshold described in ischaemic CRVO.<sup>14</sup>

In the majority of studies, immunohistochemistry and *in situ* hybridisation fail to demonstrate VEGF-A in uveal melanoma, though recent reports, including one of our own, suggest that it may be expressed at low level.<sup>8-10</sup> None the less, the infrequency of detection suggests that intratumoral VEGF-A is unlikely to be the major source of VEGF-A. A second possibility is that extensive tumour growth or tumour associated serous retinal detachment leads to separation of the outer retina from its choroidal oxygen supply; subsequent outer retinal ischaemia would lead to upregulation of VEGF gene expression<sup>18</sup> and its accumulation within ocular fluid. Our current immunostaining data suggest that the ciliary body and iris may also be potential contributors to the pool of angiogenic substances within tumour bearing eyes. Immunohistochemical staining of VEGF-A protein was detected in 54% of the iris and ciliary body and may result from the anterior diffusion of VEGF-A from the tumour or detached retina. In the absence of *in situ* hybridisation data, we cannot currently determine whether these anterior structures are themselves a source of pro-angiogenic cytokines or are merely bathed in these cytokines.

Accumulations of subretinal fluid in the presence of uveal melanoma may indicate increased VEGF-A production, as VEGF-A is a potent vasopermeability factor. Eyes bearing uveal melanoma are known to have discrete regions of vascular leakage remote from the tumour site which are coordinately positive for both VEGF-A and serum albumin,<sup>16</sup> the latter being a marker for breakdown of the blood-brain barrier. However, in this study, eyes with retinal detachment and accumulation of subretinal fluid did not show a statistically significant increase in soluble VEGF-A.

We also found low but detectable levels of bFGF in 5/8 (62.5%) of aqueous and vitreous samples. Although these levels are low, it is not known if they are of clinical relevance as the threshold for NVI in the presence of bFGF has not been determined. We have recently determined that bFGF is found diffusely throughout the parenchyma of the majority of uveal melanomas (Boyd *et al*, see accompanying paper). bFGF, like VEGF-A, is a potent direct acting pro-angiogenic agent, and can act synergistically with VEGF-A to promote angiogenesis.<sup>19 20</sup> Whether tumour derived bFGF could interact with intratumoral or other sources of VEGF-A to promote angiogenesis is not yet known.

VEGF-A values of up to 21.6 ng/ml were observed in this series following radiation. The role of irradiation in the development of NVI is not understood, but clinical observation of aqueous flare preceding irradiation, an indicator of increased ocular vasopermeability, is a poor prognosticator for NVI following radiation therapy (J L Hungerford, personal communication). Radiation is known to induce VEGF-A in other systems.<sup>21</sup> In this study, the highest levels of aqueous VEGF-A were found in association with proton irradiation therapy.

Why NVI was not detected in all eyes with elevated aqueous VEGF-A concentration is not known but may reflect the presence of anti-angiogenic factors such as endostatin or pigment epithelium derived factor (PEDF) secreted by the tumour, or from sites other than the tumour. Expression of PEDF has been reported from cultured uveal melanoma cells, from native pigment epithelium, and from retina.<sup>22-24</sup> In addition, we have also observed that eyes with radiation damage to anterior structures such as the iris are less able to generate a robust local neovascular response (Boyd *et al*, unpublished). There is currently intense interest in the use of anti-angiogenic therapy in both oncology and ophthalmology. Numerous clinical trials are proposed or under way, and include the use of anti-VEGF antibodies (RhuVAF), small molecule antagonists of VEGF or its receptors, and anti-sense or gene therapy.<sup>25, 26</sup> Our finding of high concentrations of VEGF-A in the ocular fluids of eyes harbouring uveal melanoma suggests that an anti-VEGF-A strategy might be reasonable, particularly in patients with secondary neovascularisation following radiation. Although likely to produce less than complete anti-angiogenesis effect, such VEGF blockade could permit the conservative management of patients as an alternative to enucleation, particularly in the period immediately following radiation therapy.

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